INVESTIGATIONS INTO THE GASTROINTESTINAL CONTROL OF APPETITE AND NUTRITIONAL FRAILTY

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Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Kamilia Tai

March 2008

Dedication

I dedicate this thesis to my dearest husband Payman, and to my dear parents Faezeh and Manouchehr Tai, for their unfailing support, love, and encouragement.

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Thesis Summary

The research presented in this thesis relates to the gastrointestinal control of appetite and some of the consequences of nutritional frailty, namely postprandial hypotension and vitamin D insufficiency. Undernutrition and its consequences are increasingly common problems in an ageing population, and improved management is dependent on an understanding of the factors which are involved in the control of appetite, and the physiological decline of appetite with increasing age termed 'the anorexia of ageing'. The role of the gastrointestinal hormone ghrelin was specifically evaluated, in relation to the effects of age and nutrient digestion on circulating ghrelin concentrations (Chapters 6 and 7). The effect of fat digestion on the postprandial blood pressure response in healthy older subjects was evaluated in the study reported in Chapter 8. In addition, the results of some intervention studies are described in Chapters 9 and 10, the former study relating to nutritional supplementation as a strategy to increase energy intake, and the latter study to the effects of vitamin D replacement therapy on glucose and insulin metabolism.

Whilst plasma ghrelin concentrations are less in older than young rodents, the consequences of healthy ageing on circulating plasma ghrelin concentrations in humans are unclear. The variations in fasting ghrelin concentrations over a sixty year age range were evaluated in healthy young and older subjects (Chapter 6). Plasma ghrelin concentrations were higher in females than males, but did not correlate with age, and were inversely related to body mass index. Ghrelin was independently, and inversely, related to total body skeletal muscle mass, but not to any other body composition

variable. Strategies for increasing muscle mass, through resistance exercises, may, accordingly, aid in abolishing the compensatory rise in ghrelin concentrations seen with undernutrition and weight loss.

Plasma ghrelin concentrations increase before, and decrease to trough levels within one hour of ingestion of a meal. Macronutrients differ in their ability to suppress ghrelin, being earlier and more pronounced after carbohydrate, and relatively delayed after fat or protein, ingestion. The role of carbohydrate and fat digestion in the suppression of plasma ghrelin concentrations was investigated in healthy young adults (Chapter 7). The suppression of ghrelin concentrations following a sucrose drink was attenuated by acarbose, which slows small intestinal carbohydrate absorption. Ghrelin concentrations were also suppressed after consumption of a fat-enriched drink, however addition of orlistat, which reduces fat digestion and absorption, attenuated the fall in plasma ghrelin. Thus, nutrient digestion is required, in addition to exposure of the small intestine to nutrients, for suppression of ghrelin.

Postprandial hypotension describes a significant fall in blood pressure occurring up to two hours after a meal. The magnitude of the fall in postprandial blood pressure depends, in part, on the macronutrient composition of a meal, and the effects are particularly discernable in older adults. Although carbohydrates are particularly potent in reducing postprandial blood pressure in older adults, fat ingestion appears to have comparable, but delayed effects. The role of fat digestion in modifying the blood pressure responses was evaluated in healthy older adults (Chapter 8). There was a fall in blood pressure after ingestion of a high-fat drink. Orlistat, a lipase inhibitor which

reduces intestinal fat absorption, potentiated the fall in postprandial blood pressure after a fat-enriched drink.

Gastrointestinal function and appetite can be modulated by dietary manipulation of the macronutrient composition of an individual's diet. The intervention study described in Chapter 9 evaluated the effects of two weeks of dietary fat supplementation on the sensitivity to the satiating effects of intravenous cholecystokinin-8 in healthy older subjects. No differences were observed in fasting, or postprandial plasma cholecystokinin concentrations after the dietary supplementation period compared to regular diet. There were also no differences in spontaneous energy intake at a buffet meal in response to exogenously administered cholecystokinin between the two diet periods.

Vitamin D deficiency is common, as is type 2 diabetes, and the two conditions may be linked. There is mounting evidence linking vitamin D deficiency with abnormalities of glucose and insulin metabolism. The effects of vitamin D therapy in healthy young and older adults with low vitamin D concentrations in the setting of normal or impaired glucose tolerance were evaluated (Chapter 10). Vitamin D therapy, which normalised serum 25-hydroxyvitamin D concentrations in these individuals, did not alter glucose or insulin concentrations or insulin sensitivity during an oral glucose tolerance test.

Chapter 1

GASTROINTESTINAL CONTROL OF APPETITE AND NUTRITIONAL FRAILTY

1.1 Introduction

Undernutrition and nutritional frailty represent common and significant problems in older persons aged 65 years and over. Ageing is associated with an increased prevalence of many disorders, including vitamin D deficiency and postprandial hypotension, which are both established risk factors for falls, which in turn are associated with increased morbidity and mortality (Aronow and Ahn 1997; Flicker et al. 2003).

The control of appetite and food intake is determined by a complex interplay of central and peripheral mechanisms. The studies presented in this thesis will focus on the gastrointestinal control of appetite and food intake, with an emphasis on the role of ghrelin. They will also deal with some of the sequelae of undernutrition in older persons, in particular with regards to vitamin D deficiency and postprandial hypotension. This chapter reviews the effects of ageing on appetite and food intake, and presents an overview of some of the major central and peripheral factors in the regulation of appetite in humans.

1.2 Epidemiology and effects of ageing on appetite

As in many other developed and developing countries, the proportion of older people (defined as those aged 65 years and over) in the Australian population is projected to increase steadily. Census data in 2003 showed that 12.8% of the Australian population was aged 65 years and over (Australian Bureau of Statistics 2003). This is estimated to almost triple or quadruple from 2.3 million in 1999 to between 6.2 million and 7.9 million by 2051 (Australian Bureau of Statistics 2003). The proportion of those aged over 85 years is also expected to increase from 1.3% in 2003 to around 5% by 2051. In view of these demographic changes, any adverse effects of ageing will become more prevalent and important over the next few decades.

Healthy ageing is associated with a physiological decline in appetite and energy intake (Figure 1.1) , which has been termed the 'anorexia of ageing' (Morley 1990), and may predispose at-risk individuals to malnutrition, and increased morbidity and mortality (MacIntosh et al. 2000). Energy intake decreases by approximately 30% between 20 and 80 years of age (Wurtman et al. 1988; Chapman 2004). In the cross-sectional National Health and Nutrition Examination Survey (NHANES III, 1988-1991) from the United States of America, average energy intake decreased between the ages of 20 and 79 years, by 4761 kJ/day (1134 kcal/day) in men and 2182 kJ/day (520 kcal/day) in women (Briefel et al. 1995). A recent study also confirmed the reduction in energy intake from younger to older age, by 5040 kJ in men and 3360 kJ in women (Wakimoto and Block 2001). Furthermore, a longitudinal study in 156 adults aged 64 - 91 years, reported a decrease of 105 kJ/day/year in men and 81 kJ/day/year in women (Koehler 1994).

Changes in body composition occur with healthy ageing (Figure 1.2). In many individuals, the decline in total energy expenditure that accompanies normal ageing (Morley 1997) is less than the reduction in energy intake, so body weight is lost in older adults aged over 70 years (Morley and Thomas 1999). There is a gradual increase in total body fat with increasing age, as well as a reduction in lean or fat-free body mass (Dawson-Hughes and Harris 1992; Morley and Thomas 1999; Guo et al. 1999; Nair 2000). In a longitudinal study of 210 adults aged 40-66 years, with an average follow-up of 9 years, body weight increased by approximately 0.3 kg/year in men and 0.55 kg/year in women, with a corresponding increase in total body fat in both sexes and a decrease in lean body mass in women (Guo et al. 1999). In another longitudinal study of 131 adults aged 46-80 years, with a mean follow up of 9.4 years, average body weight did not change with increasing age, and this may reflect the inclusion of subjects ≥65 years in this study (Hughes et al. 2002). In this same study, fat-free mass decreased at a rate equivalent to 2.0% per decade in men, but not in women, and body fat mass increased by 7.5% per decade in both men and women (Hughes et al. 2002).

The reduction in energy intake with ageing, and decrease in physical activity which leads to muscle disuse and atrophy, results in an age-related disproportionate decline in muscle mass, i.e. sarcopaenia (Evans and Campbell 1993; Morley 2001). Sarcopaenia is one of the major contributing factors to physical frailty in older persons, with an associated increased risk of falls, functional disability, vulnerability to illness, and mortality (Vanitallie 2003).

1.3 Central mechanisms regulating appetite and energy intake

There is considerable redundancy in the regulation of feeding in humans, which involves complex interactions between central and peripheral inputs (MacIntosh et al. 2000). Several chemical mediators, including neurotransmitters, have been implicated in the central satiety system, and include orexigenic signals, such as opioids and neuropeptide Y (Morley 1987; MacIntosh et al. 2000), and anorexigenic signals such as melanocortin (Neary et al. 2004a). A number of key factors in the peripheral satiety system, such as the gastrointestinal hormones cholecystokinin and insulin, can also act centrally (Morley 1987; MacIntosh et al. 2000). The pleasurable aspects of eating, including the sight, taste and smell of food, also influence the central feeding system, and influence the type and amount of food consumed (MacIntosh et al. 2000).

The hypothalamus is a key region in the central nervous system that regulates feeding and energy homeostasis (Wilding 2002). A series of neuronal circuits connect the hypothalamus to the brain stem, which, in itself, receives satiety signals from the vagus nerve (Williams et al. 2001).

The endogenous opioids, dynorphin and β-endorphin, are produced by neurones in the hypothalamus (Kalra et al. 1999). Both these and exogenous opioid agonists have a stimulatory effect on appetite and food intake, and are thought to play a role in reward sensations to food (Gosnell et al. 1983; Morley 1987).

Neuropeptide Y (NPY) is a 36-amino-acid peptide hormone synthesised and secreted from nuclei located in the hypothalamus (Wilding 2002). Neuropeptide Y stimulates

appetite and food intake in rodents (Clark et al. 1984), and its synthesis is negatively regulated by leptin (Schwartz et al. 1996).

The melanocortin α-melanocyte-stimulating hormone (α-MSH) is an anorexigenic peptide found in the hypothalamus (Kalra et al. 1999). Its administration reduces food intake in rodents (Kalra et al. 1999; Neary et al. 2004a), and, when given to humans, melanocortin agonists (Fehm et al. 2001) reduce body weight. The appetite suppressant effects of α-MSH are blocked by another hypothalamic peptide Agouti-related protein (AgRP), which thus has the opposite effect of increasing food intake (Rossi et al. 1998a; Neary et al. 2004a).

Although several other neurohormonal signals are involved in the central regulation of appetite and body weight, and play a role in both short- and long-term energy homeostasis, they are not presented in detail here. There is a complex interplay and overlap between these peptides and neurotransmitters, and current knowledge is evolving, particularly in the area of research into treatments of obesity.

1.4 Peripheral mechanisms regulating appetite and energy intake

The peripheral satiety system is influenced by both mechanical and hormonal factors. The effects of gastric distension and of some important gastrointestinal peptide hormones on appetite, food intake, and satiety, will be discussed, particularly those of relevance to studies in this thesis.

1.4.1 Gastric motor function and physiology of normal gastric emptying

As food passes from the oesophagus into the stomach, the proximal stomach relaxes to accommodate the swallowed food, and there is grinding and chemical breakdown of solid food matter in the distal (antral) region of the stomach into liquid chyme (Azpiroz 1994; Horowitz et al. 1994; Horowitz and Camilleri 1997). Gastric emptying occurs from the antrum through the pylorus into the duodenum, predominantly in a pulsatile manner (Horowitz and Camilleri 1997; Rayner and Horowitz 2005) (Figure 1.3). Coordinated gastric contractions are determined by the electrical activity originating from the gastric pacemaker, which is located along the greater curvature of the stomach (Koch 1999). The timely coordination, rather than amplitude, of motor activity in the proximal stomach, antrum, pylorus, and duodenum appears to be most important in determining the emptying of gastric contents (Horowitz et al. 1994).

Gastric distension results in feelings of fullness via stimulation of local stretch or mechano-receptors (Read et al. 1994; Havel 2001). Chemoreceptors in the small intestine then respond to partially-digested nutrients (sugars, amino acids, fatty acids) in the meal, and there is feedback from both chemoreceptors and mechanoreceptors via the vagus nerve to the central nervous system (Havel 2001). Vagal afferent endings in the stomach and duodenum can detect the presence of food by several means - through gastric distension and contractions, the chemical composition of nutrients, and via gastrointestinal hormones, particularly cholecystokinin (Schwartz 2000). The afferent signals from the gastrointestinal tract to the brain may be mediated directly via neuronal circuits or indirectly via humoral (hormonal) mechanisms (Schwartz 2000).

1.4.2 Relationship between gastric distension, satiation, and satiety

Sensations of fullness and satiety following ingestion of a meal are determined by the composition of the meal and the duration of direct exposure of the small intestine to nutrients (Read et al. 1994). Furthermore, antral distension is closely related to postprandial satiation (Jones et al. 1997), and is also likely to be an important negative determinant of subsequent energy intake (Sturm et al. 2004).

1.5 Endocrine role of the stomach and gastrointestinal peptide hormones

The entry of food into the small intestine results in release of peptide hormones from the small intestine, including cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptide-1 (GLP-1), and amylin, and suppression of the release of other peptide hormones, typically ghrelin. Peripheral signals, including leptin, and possibly tumour necrosis factor-α, also come from body fat stores.

Two of these hormones, CCK and ghrelin, are the main focus of the studies presented in this thesis, and plasma concentrations of these hormones were measured in the studies presented in Chapters 6, 7, and 9.

1.5.1 Cholecystokinin (CCK)

CCK is found widely in the gastrointestinal tract and central nervous system, and possesses the ability to act both on the peripheral and central satiety systems. CCK is predominantly produced by mucosal I-cells in the upper small intestine, the duodenum and jejunum. It is also found in smaller concentrations in enteric nerves, vagal afferent

nerves, the lower gastrointestinal tract, and released from nerve terminals in the central nervous system (Baldwin et al. 1998). The sulphated form of the carboxy-terminal octapeptide of CCK, CCK-8S, is thought to be one of the main forms of CCK in the circulation, and particularly in the central nervous system (Baldwin et al. 1998). Two distinct subtypes of CCK receptors, CCK-1 and CCK-2 (formerly known as CCK-A and CCK-B), have been identified. CCK-1 receptors are found predominantly in the periphery- on the cells of the pancreas, gall bladder, smooth muscle within the pyloric sphincter, and vagal afferent nerves in the pylorus, while CCK-2 receptors are found mainly in the central nervous system, with both receptor subtypes demonstrating a high affinity for the CCK-8S form (Baldwin et al. 1998; Moran 2000).

CCK release is triggered by certain food constituents in the duodenum, specifically fat and protein (MacIntosh et al. 2000; de Graaf et al. 2004). In animal studies, CCK administered, either intraperitoneally or intravenously, slows gastric emptying (Moran and McHugh 1982). In humans, the rise in plasma CCK concentrations after a meal is strongly inversely correlated with the rate of gastric emptying and also with hunger sensations (French et al. 1993). Intravenous infusion of CCK slows gastric emptying of a mixed meal, in a dose-dependent fashion (Liddle et al. 1986; Kleibeuker et al. 1988; Konturek et al. 1990). It appears that the effect of CCK to slow gastric emptying is the result of several mechanisms- increased pyloric contractility, decreased antral motility, reduced intragastric pressure, and increased relaxation of the proximal stomach (Melton et al. 1992; Rayner et al. 2000; van der Schaar et al. 2001).

Exogenous CCK administration affects energy intake. In some of the earliest studies in humans, intravenous CCK administered rapidly, but not via infusion, was capable of decreasing energy intake, however, the CCK preparation used was not a pure extract (Sturdevant and Goetz 1976). Subsequently, it was demonstrated that intravenous infusion of purified CCK-8, at various doses, suppressed energy intake in both lean (Kissileff et al. 1981; Stacher et al. 1982) and obese (Pi-Sunyer et al. 1982) individuals. Plasma concentrations of CCK, however, were not determined in these studies. There was, therefore, some debate as to whether the suppressive effect of CCK on appetite and energy intake was a physiological, or pharmacological effect. However, there is evidence that CCK infusions, which produce a physiological rise in blood CCK concentrations also reduce food intake. In one study, CCK-33 infusion at a dose which produced plasma concentrations of CCK comparable to those following a meal, significantly reduced food intake by approximately 18.5% compared to saline infusion, and reduced subjective ratings of preference for fat-enriched food items (Lieverse et al. 1995a). Subsequent studies where an intravenous CCK-8 or CCK-33 infusion was administered confirmed its inhibitory effect on energy intake in humans (Muurahainen et al. 1991; Greenough et al. 1998; Rayner et al. 2000; Gutzwiller et al. 2000; MacIntosh et al. 2001; Gutzwiller et al. 2004; Brennan et al. 2005). In addition, CCK-8 reduces hunger and increases fullness sensations (Melton et al. 1992; Greenough et al. 1998; Brennan et al. 2005). The results of a weighted analysis of ten studies in 214 young and older subjects, aged 18-83 years, showed that intravenous exogenous CCK reduced food intake by 22.5% compared to saline infusion, with dose-dependent effects (de Graaf et al. 2004). Plasma CCK concentrations generally increase from fasting levels of approximately 1-2 pmol/L to 5-6 pmol/L after a meal, and intravenous

infusion of CCK-8 at doses of 0.23 $pmol.kg^{-1}.min^{-1}$ results in comparable plasma CCK concentrations of approximately 5 pmol/L (Liddle et al. 1985). Some of the doses of CCK-8 used in the above studies were higher than that expected to reproduce physiological CCK concentrations (Muurahainen et al. 1991; Geary et al. 1992; Greenough et al. 1998; Rayner et al. 2000), or resulted in measured plasma CCK concentrations in the supraphysiological range (Gutzwiller et al. 2000; MacIntosh et al. 2001; Brennan et al. 2005). However, higher physiological plasma CCK concentrations above 10 pmol/L, as measured by radioimmunoassay, after a mixed meal (MacIntosh et al. 2001) or mixed nutrient preload (Sturm et al. 2003), have been demonstrated. It may thus be that, under certain conditions, actions of CCK-8 are physiological (Lieverse et al. 1995a), and it has been suggested that satiety effects of endogenous CCK may be predominantly paracrine, rather than endocrine (Geary et al. 1992), such that the high plasma CCK concentrations seen with exogenous CCK administration may not necessarily reflect a supraphysiological action of CCK in the gastrointestinal tract.

Consistent with findings in monkeys (Moran and McHugh 1982), the suppressive effects of CCK on food consumption in humans is enhanced in the presence of concurrent gastric distension (Muurahainen et al. 1991; Kissileff et al. 2003), highlighting the important role of the stomach in potentiating the response to hormonal satiety signals. It is likely that endogenous CCK plays an important role in suppression of appetite and is a physiological satiety hormone (Morley 1990; MacIntosh et al. 2000).

If the satiating effect of CCK is physiological, it should be inhibited by the use of specific CCK receptor antagonists. Earlier studies, using the oral CCK-1 receptor antagonist MK-329 in young healthy males, showed that hunger sensations in the fasted state were higher with MK-329 compared to placebo (Wolkowitz et al. 1990). In contrast, other studies showed that, in healthy young adults, an intravenous infusion of the CCK-1 receptor antagonist loxiglumide, in combination with an intraduodenal lipid infusion, did not significantly increase energy intake, compared to intraduodenal lipid given with placebo infusion (Lieverse et al. 1994). Similarly, in another study, an intravenous infusion of loxiglumide together with intrajejunal administration of lipid did not affect spontaneous energy intake (Drewe et al. 1992).

It is known that a nutrient preload of approximately 400-500 mL, which fills the stomach, enhances the satiety effects of exogenous CCK (de Graaf et al. 2004). Similarly, it is likely that a nutrient preload also enhances the appetite-stimulating effects of CCK receptor antagonists. Furthermore, the composition of an *ad libitum* meal, in particular the fat content, may affect the response to CCK receptor antagonists. In one study in healthy young males, a low-fat, carbohydrate-rich (banana and whey), preload given in combination with an intraduodenal fat infusion raised plasma CCK concentrations and reduced energy intake, the effects of which were blocked by loxiglumide (Matzinger et al. 1999). This was in contrast to earlier studies which failed to show an effect of loxiglumide on food intake (Drewe et al. 1992; Lieverse et al. 1994). Thus, the use of an oral nutrient preload appears to enhance the CCK-mediated, appetite-suppressive, effects of an intraduodenal lipid infusion, and the antagonistic actions of loxiglumide.

In healthy young adults, an intravenous loxiglumide infusion increased energy intake at a mixed meal, by 10% ($P = 0.004$) compared to saline infusion (Beglinger et al. 2001), or by 18% ($P = 0.015$) compared to a CCK-8 infusion (Gutzwiller et al. 2000), supporting a physiologic role for endogenous CCK in satiation. In contrast, in another study in both healthy lean and obese females, a carbohydrate-rich *ad libitum* meal was offered, which would not be expected to markedly increase endogenous plasma CCK concentrations, and intravenous infusion of loxiglumide did not significantly increase food intake, when compared with saline infusion (Lieverse et al. 1995b). Hence, the appetite-enhancing actions of CCK receptor antagonists are more potent when a fat-enriched or mixed *ad libitum* meal is offered, rather than a carbohydrate-rich meal. The above observations indicate a physiologic satiety effect of CCK.

There are differences between circulating CCK concentrations in older and young adults, and also between feeding responses to exogenously administered CCK in older and young adults, suggesting possible age-related differences in sensitivity to endogenous CCK. Baseline fasting plasma CCK concentrations have been consistently shown to be higher in healthy older compared to young subjects (Khalil et al. 1985; MacIntosh et al. 1999; MacIntosh et al. 2001; Sturm et al. 2003), with mean plasma CCK concentrations between 47% to 406% higher (MacIntosh et al. 1999; MacIntosh et al. 2001; Sturm et al. 2003). Plasma CCK concentrations are also higher in older compared to younger subjects after oral fat ingestion (Khalil et al. 1985) and also following intraduodenal lipid or glucose infusions (MacIntosh et al. 1999; MacIntosh et al. 2000). In one study, after ingestion of a standardised liquid meal, maximal

postprandial concentrations of CCK were 137% higher in malnourished elderly than in older healthy adults, and 152% higher than in young healthy adults (Berthelemy et al. 1992). In another study, fasting plasma CCK concentrations were 254% higher (P = 0.02) in undernourished older than in healthy young adults, and remained elevated in the pre-meal period (Sturm et al. 2003) . In that same study, however, CCK concentrations were not significantly different between undernourished and healthy older adults (Sturm et al. 2003). The above studies indicate that fasting plasma CCK concentrations are increased in older individuals, regardless of nutritional status, and that postprandial CCK concentrations are higher in undernourished, than in healthy, older individuals.

Sensitivity to the appetite-suppressive effects of CCK may alter with age. In animal studies, older mice and rats have been found to be more sensitive to the satiating effects of intraperitoneal administration of CCK-8 than younger animals (Silver et al. 1988; Voigt et al. 1996). In one study in humans comparing the effects of low-dose and high-dose intravenous CCK in 12 young and 12 older subjects, CCK suppressed energy intake significantly more in older than in younger subjects (mean suppression of 32% versus 15.5%; effect of age $F = 5.70$, $P < 0.05$) (MacIntosh et al. 2001).

Thus, older adults have higher circulating baseline CCK concentrations and are apparently more sensitive to the satiating effects of CCK than young adults. These combined effects may, at least in part, account for the reduction in food intake in older persons, and indicate a contribution of CCK to the anorexia of ageing. The effects of loxiglumide or other CCK-1 receptor antagonists in stimulating appetite and food intake

in older people remain to be investigated, and these agents may have therapeutic potential in undernourished older persons.

1.5.2 Ghrelin

Ghrelin is a hormone first identified in 1999, which acts as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) to stimulate growth hormone release (Kojima et al. 1999). It was subsequently found to also play an important role in hunger and appetite stimulation (Zigman and Elmquist 2003; Wynne et al. 2004). Most ghrelin is produced in the stomach by enteroendocrine X/A-like cells in the oxyntic glands located in the gastric fundus (Gualillo et al. 2003; van der Lely et al. 2004). Ghrelin is also expressed in many other tissues, including kidneys, testis, pituitary, pancreas, small intestine, brain, lung, ovary, and placenta (Gualillo et al. 2003). The overall structure of ghrelin is closely conserved among different species, with a difference of only two amino acid substitutions between rat and human ghrelin, suggesting a significant physiological role for this hormone (Wren et al. 2000; Druce et al. 2004).

Post-translational modification is required for most of the biological activity of ghrelin (Kojima et al. 1999; van der Lely et al. 2004). Most active ghrelin is in the form of a 28-amino acid peptide, after acylation with N-octanoic acid of a hydroxyl group on one of its serine residues. The acylated form is capable of binding to the growth hormone secretagogue receptor type 1a, and has strong growth hormone-releasing activity, comparable to that of growth hormone releasing hormone (GHRH) (Kojima et al. 1999; van der Lely et al. 2004). However, 80-90% of ghrelin in human serum is in the non-

acylated form, and lacks the known endocrine and other biological effects of ghrelin (van der Lely et al. 2004). Growth hormone secretagogue receptors are found mainly in the hypothalamus-pituitary axis, including the arcuate nucleus, and brain stem nuclei, and also expressed in other central and peripheral tissues (Druce et al. 2004; van der Lely et al. 2004).

Ghrelin expression and secretion is increased in negative energy balance states (Gualillo et al. 2003). Consumption of food suppresses ghrelin release, and plasma ghrelin concentrations increase in the fasted state, reaching a peak prior to a meal (Druce et al. 2004).

In animal studies, intravenous ghrelin administration results in increased food intake in rodents (Wren et al. 2000; Wren et al. 2001b). In addition, intracerebroventricular administration of ghrelin has a more prolonged and potent stimulatory effect on *ad libitum* food intake in rodents compared with the intraperitoneal route (Wren et al. 2000). Exogenous ghrelin administration is thus effective both centrally, and systemically, altering energy intake (Wren et al. 2000).

In humans, subcutaneous, or intravenous ghrelin administration increases appetite and energy intake (Wren et al. 2001a; Wynne et al. 2005; Schmid et al. 2005; Druce et al. 2005; Druce et al. 2006). Initial studies showed that intravenous ghrelin infusion in healthy young adults resulted in a 28 ± 3.9% increase in the mean energy intake at a buffet meal (Wren et al. 2001a). Ghrelin also accelerates the rate of gastric emptying (Asakawa et al. 2001; Dornonville de la Cour et al. 2004). Gastric emptying slows

slightly as part of normal ageing (Evans et al. 1981; Horowitz et al. 1984), and the rate of gastric emptying is probably inversely related to food intake in older people (Clarkston et al. 1997). Delayed gastric emptying in older people may possibly relate to reduced ghrelin action (either due to reduced concentrations or reduced sensitivity to its effects), and could contribute to reduced voluntary food intake compared to young individuals. Although intravenous ghrelin administration increases energy intake in older subjects with metastatic cancer and chronic heart failure (Nagaya et al. 2004; Neary et al. 2004b), the comparative appetite-stimulating effects of ghrelin administration in healthy young and older adults have not been evaluated.

Ghrelin interacts with other gastrointestinal hormones, and animal studies have shown that intraperitoneal CCK-8 co-administration with ghrelin completely inhibits the stimulatory effects of ghrelin on food intake in rats (Kobelt et al. 2005). Furthermore, a recent study in humans has shown that intravenous CCK-8 infusion suppresses secretion of ghrelin (Brennan et al. 2007).

The determinants of ghrelin secretion, specifically in relation to the effects of ageing and macronutrient composition on ghrelin concentrations, are discussed in greater detail in Chapter 2.

1.5.3 Glucagon-like peptide-1 (GLP-1)

GLP-1 is a 33-amino acid peptide, produced in the ileum, and its release is stimulated by ingestion of fat and carbohydrate (de Graaf et al. 2004; Beglinger and Degen 2006). Proglucagon, the translational product of the glucagon gene, undergoes post-

translational modification to form two peptides, GLP-1 and glucagon-like peptide-2 (GLP-2). GLP-1 is released from enteroendocrine L-cells in the distal small intestine (Gutzwiller et al. 1999b), and GLP-1 receptors have been found in the stomach and pancreas, as well as in the brain (Hellstrom and Naslund 1999). GLP-$1_{(7\text{-}36\ \text{amide})}$, the biologically active form of GLP-1, is rapidly degraded to its inactive form GLP-$1_{(9\text{-}36)}$ in human serum by the enzyme dipeptidyl peptidase IV (DPP-IV) (de Graaf et al. 2004).

In early studies in animals, central (intracerebroventricular), but not peripheral (intraperitoneal), infusion of GLP-1 markedly reduced food intake in fasted rodents (Turton et al. 1996; Gutzwiller et al. 1999b). In subsequent studies in rats, however, subcutaneous injection of either GLP-1 or exendin-4, a GLP-1 receptor agonist, given acutely, reduced food intake compared to control injection (Rodriquez de Fonseca et al. 2000). Furthermore, both central (intracerebroventricular) and peripheral (subcutaneous) administration of exendin-4 reduced food intake and weight gain in obese rats compared to control treatment (Rodriquez de Fonseca et al. 2000). Other studies showed that once-daily intraperitoneal injections of exendin-4, in diabetic and non-diabetic mice, reduced spontaneous food intake and body weight compared to control injections, however, these differences were only noticeable in the short-term, and not beyond 6 days (Greig et al. 1999). On the other hand, twice-daily intraperitoneal injections of exendin-4 given to obese Zucker fatty rats resulted in a sustained reduction in food intake and less weight gain compared to saline-injected rats (Szayna et al. 2000). Furthermore, an antagonist of the GLP-1 receptor, exendin 9-39, resulted in more than a doubling of food intake in satiated rodents (Turton et al. 1996).

Overall, these results support a physiological satiety effect of GLP-1, however the effects of exendin 9-39 on energy intake in humans have not been evaluated.

In humans, most studies have shown that intravenous infusion of GLP-1 decreases energy intake in a dose-dependent manner in healthy individuals (Flint et al. 1998; Gutzwiller et al. 1999b; Verdich et al. 2001), although some studies showed no significant effect on energy intake in healthy normal-weight (Long et al. 1999; Brennan et al. 2005) and obese (Naslund et al. 1998) individuals. In both normal-weight and overweight individuals, GLP-1 infusion induces feelings of fullness and satiety, and decreases hunger sensations, particularly at high doses of GLP-1 (Flint et al. 1998; Naslund et al. 1998; Gutzwiller et al. 1999b).

It is well-established that infusion of GLP-1 delays gastric emptying in both normal-weight and obese subjects (Nauck et al. 1997; Naslund et al. 1998; Long et al. 1999; Naslund et al. 1999; Flint et al. 2001; Little et al. 2006b). The magnitude of the slowing of gastric emptying by exogenous GLP-1 is substantial, and the postprandial glucose-lowering effect is probably due to the slowing of gastric emptying more than the stimulation of insulin secretion (Nauck et al. 1997; Little et al. 2006b). These effects are comparable to those of CCK, which slows gastric emptying in a dose-dependent fashion (Liddle et al. 1986; Kleibeuker et al. 1988; Konturek et al. 1990), and the gastric emptying effects may partly explain the satiating properties of GLP-1. Interestingly, whilst gastric distension alone augments the suppressive effects of CCK infusion on energy intake, the suppression of energy intake by GLP-1 infusion is enhanced by a preceding nutrient preload but not by gastric distension alone (Degen et al. 2006).

Intravenous GLP-1 infusion reduces spontaneous energy intake by 12 - 32% compared to saline (Flint et al. 1998; Gutzwiller et al. 1999b). Similarly, a meta-analysis of 115 individuals from nine trials, including overweight subjects and those with type 2 diabetes, showed that intravenous GLP-1 infusion reduced mean energy intake by 11.7% compared to placebo (Verdich et al. 2001). The suppressive effects of GLP-1 on food intake are seen at physiological concentrations of GLP-1 (Gutzwiller et al. 1999b), comparable to those seen after a meal, and also at higher, so-called 'supraphysiological' concentrations of GLP-1 (Flint et al. 1998; Gutzwiller et al. 1999b). In one study of obese subjects, GLP-1 infusion which reproduced physiological plasma concentrations of GLP-1, failed to show a reduction in food intake, although gastric emptying was delayed and subjective hunger sensations were reduced (Flint et al. 2001).

In individuals with type 2 diabetes, GLP-1 infusion increases feelings of satiety and reduces energy intake, compared to control infusion (Gutzwiller et al. 1999a). Overall effects in overweight individuals with type 2 diabetes appear to be greater when compared with overweight and lean healthy subjects (Gutzwiller et al. 1999a; Verdich et al. 2001). Intravenous administration of GLP-1 in combination with CCK-33 or CCK-8 did not appear to potentiate the suppressive effect of either peptide hormone on energy intake (Gutzwiller et al. 2004; Brennan et al. 2005). Exenatide, an incretin mimetic which mimics the antihyperglycaemic actions of GLP-1 and enhances glucose-dependent insulin secretion, when administered subcutaneously twice-daily over 26-30 weeks, produces a progressive dose-dependent reduction in body weight in patients

with type 2 diabetes mellitus (Buse et al. 2004; DeFronzo et al. 2005; Kendall et al. 2005; Heine et al. 2005).

Available evidence indicates that plasma GLP-1 concentrations do not change with increasing age, while no studies to date have compared the sensitivity to GLP-1 administration in young and older individuals. Fasting plasma GLP-1 concentrations are not significantly different between young and older adults (MacIntosh et al. 1999; Sturm et al. 2003), and there were no differences between young and older adults in plasma GLP-1 concentrations following an oral mixed nutrient preload (Sturm et al. 2003) or intraduodenal infusions of lipid or glucose (MacIntosh et al. 1999). Whilst the comparative satiety effects of GLP-1 administration in young and older adults have not been evaluated, in a study of 16 healthy young and 16 older subjects, administration of subcutaneous liraglutide, a long-acting GLP-1 analogue, resulted in a similar pharmacokinetic profile (distribution and clearance) of this drug in both age groups (Damholt et al. 2006).

1.5.4 Peptide YY (PYY)

Peptide tyrosine-tyrosine (PYY) is a 36-amino-acid peptide, which belongs to the neuropeptide Y family of pancreatic polypeptides (Corp et al. 1990; Druce et al. 2004). It is released from the gastrointestinal mucosa of the small intestine, colon, and rectum, in response to fat and carbohydrates in the duodenum and ileum (MacIntosh et al. 2000). PYY is secreted as PYY_{1-36}, and is rapidly degraded by the enzyme dipeptidyl peptidase IV to PYY_{3-36} (Grandt et al. 1994).

In animal studies, central, but not peripheral, administration of PYY increases *ad libitum* food intake (Corp et al. 1990). In humans, intravenous infusion of PYY_{3-36} delayed gastric emptying and intestinal transit time, in a dose-dependent manner (Savage et al. 1987). PYY_{3-36} infusion also reduces energy intake, but only at the high doses, which result in supraphysiological plasma concentrations (Degen et al. 2005; Beglinger and Degen 2006). The role of PYY in the regulation of appetite and energy intake in humans remains unclear.

There appear to be no significant differences in plasma PYY levels between young and older subjects, either fasting or following intraduodenal lipid or glucose infusions (MacIntosh et al. 1999), but some gender differences may be present, with higher concentrations of PYY reported in females (Kim et al. 2005).

Other gastrointestinal hormones interact with PYY to influence its release. GLP-1 infusion suppresses plasma PYY concentrations (Naslund et al. 1999). On the other hand, CCK-8 infusion stimulates PYY (Brennan et al. 2007), and the fat-induced release of PYY was partially prevented by a CCK-A antagonist in animal studies (Lin et al. 2000), suggesting that CCK is required for the release of PYY.

1.5.5 Amylin

Amylin, previously named islet amyloid polypeptide, is a 37-amino acid peptide hormone. It is co-released with insulin from the beta-cells of the pancreatic islets of Langerhans following meals (Brunetti et al. 2002). In fasted animals, food intake decreased after administration of intraperitoneal amylin, and this effect was maximal

during the first two hours after amylin injection (Lutz et al. 1994). The doses of amylin required to suppress appetite in animal studies are similar to those of cholecystokinin (Lutz et al. 1994). In animal studies, peripheral (intraperitoneal) amylin administration reduces energy intake, with no significant differences between young and old rats, or obese versus lean mice (Morley et al. 1993; Morley et al. 1994).

In humans, a synthetic amylin analogue, pramlintide, given to individuals with type 2 diabetes for four weeks resulted in minor weight loss of up to 0.9 kg in the highest dose group, although this weight loss was not significantly different compared to placebo (Thompson et al. 1998). Pramlintide also reduces hunger sensations and spontaneous energy intake in individuals with type 2 diabetes (Chapman et al. 2005), and, similarly, in healthy young males, reduces energy intake at a buffet meal (Chapman et al. 2007).

The changes in circulating amylin concentrations with ageing are unclear. Plasma amylin concentrations, following glucose stimulation, have been found to increase between middle age and old age (Edwards et al. 1996; MacIntosh et al. 2000), with an overall U-shaped distribution in amylin levels, being highest in young (20 - 40 years) and older (61 - 90 years) adults, and lowest in the middle-aged group (41 - 60 years) (Edwards et al. 1996). However, both fasting and glucose-stimulated plasma amylin concentrations, were not significantly different between a group of 22 healthy young (21 – 37 years) and 20 healthy older (51 – 77 years) adults (Mitsukawa et al. 1992).

1.5.6 Leptin

Leptin is a 16 kDa protein, encoded in the *ob* (obese) gene, which is produced primarily in white adipose tissue. It is a peripheral adiposity signal, in that serum leptin concentrations reflect fat stores (MacIntosh et al. 2000). Leptin acts on the brain at hypothalamic receptors to decrease food intake and increase energy expenditure, thus decreasing body weight (Pico et al. 2003). Serum leptin levels can increase up to three-fold after sustained overfeeding, but only moderately after a single large meal (Kolaczynski et al. 1996b). One of the physiological responses to fasting is a marked decrease in leptin concentrations within 12 hours, and prompt return to baseline levels after re-feeding with a normal diet (Kolaczynski et al. 1996a; Boden et al. 1996).

In animal and human studies, leptin administration reduces food intake and increases energy expenditure (Ahima and Flier 2000). While leptin deficiency might, therefore, be a cause of increased weight and obesity, congenital leptin deficiency is a very rare cause of human obesity (Montague et al. 1997). Indeed, most obese individuals have high, not low, serum leptin concentrations and are resistant to exogenous administration of leptin (Ahima and Flier 2000), with only minor weight loss occurring in obese individuals given daily subcutaneous leptin injections for four weeks (Heymsfield et al. 1999). Leptin resistance is, therefore, a feature of most human obesity.

Circulating leptin concentrations have been shown to increase with healthy ageing, and, even after adjustment for body fat, age was significantly inversely related to serum leptin concentrations in a group of healthy women athletes aged between 18 to 69 years ($P < 0.04$) (Ryan and Elahi 1996). Serum leptin concentrations have been shown to be

higher in older women than men, even after adjustment for fat mass, and, in men, serum testosterone concentrations were inversely related to leptin levels (Baumgartner et al. 1999a). In men, some studies have indicated that there is an increase in leptin concentrations with age, despite adjusting for fat mass (Baumgartner et al. 1999b).

There appears to be no consistent relationship between leptin and ghrelin concentrations, with some animal studies showing an increase (Toshinai et al. 2001; Ariyasu et al. 2002), and others showing a decrease (Barazzoni et al. 2003), in ghrelin concentrations in the face of increasing leptin concentrations (Cummings and Foster 2003).

1.6 Conclusions

Some of the satiating effects of gastrointestinal hormones are, at least in part, mediated by their effects on gastric emptying. Both CCK and GLP-1 delay gastric emptying and reduce hunger and energy intake, whilst ghrelin has the opposite effect. Gastric distension enhances the suppressive effects of CCK on food consumption (Muurahainen et al. 1991; Kissileff et al. 2003).

Circulating ghrelin concentrations increase in negative energy balance states, and may play a role in meal initiation. It has been proposed that CCK acts shortly after a meal to induce its satiety effects and result in meal termination, and GLP-1 has a subsequent role in a cascade of events prolonging the duration of satiety and reducing prospective food consumption in the short-term (Blundell and Naslund 1999). Leptin, which reflects

the body's fat stores, is likely to be more important as a long-term energy regulator (Ahima and Flier 2000), and does not appear to influence the actions of ghrelin.

NOTE: This figure is included on page 26 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1: Mean energy intake for U.S. males and females by age. Within the same age group, females have a lower mean energy intake than males. Adapted from Briefel et al. (1995).

NOTE: This figure is included on page 27 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2: Changes in body fat mass and fat-free mass with ageing, determined by hydrostatic weighing, in men (○) and women (▽). Men have a higher initial level of fat-free mass than women. With advancing age, the rate of progressive decline in fat-free mass and increase in fat mass is similar in men and women. Adapted from Holloszy (2000).

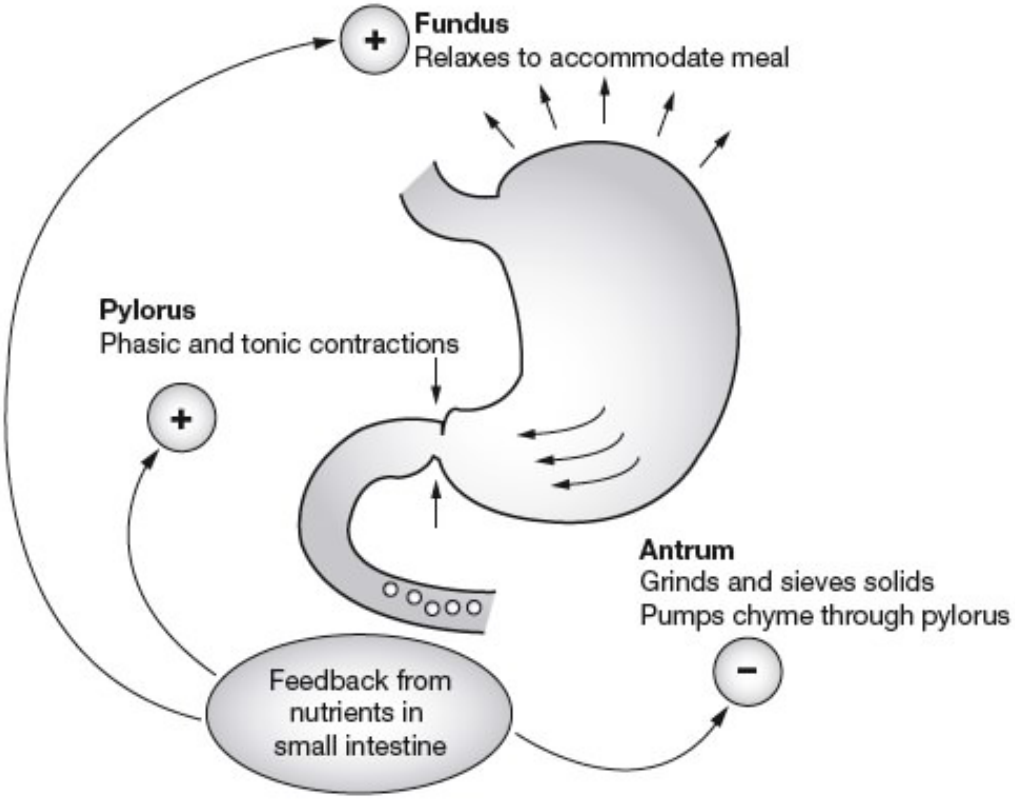


Figure 1.3: Schematic diagram of gastric emptying. The fundus relaxes to accommodate the meal, while the antrum grinds and sieves solids, pumping the resultant chyme into the duodenum against resistance generated by phasic and tonic pyloric contractions. The presence of nutrients in the small intestine generates neurohumoral feedback on gastric motor function, enhancing fundic relaxation and pyloric contraction, while suppressing antral motility, with the net effect of slowing further emptying to a closely regulated rate. Adapted from Rayner and Horowitz (2005).

Chapter 2

MACRONUTRIENTS, NUTRIENT COMPOSITION AND DIETARY EFFECTS ON APPETITE, GHRELIN, AND CHOLECYSTOKININ

2.1 Introduction

The major macronutrients (carbohydrate, fat, and protein) found in food have differential effects on appetite, satiation, and gastrointestinal hormone release. Ghrelin may play a role in meal initiation, and plasma ghrelin concentrations fall in response to food ingestion. Other determinants of circulating ghrelin concentrations may include glucose and insulin, and the effect of healthy ageing on plasma ghrelin levels is controversial. CCK plays an important role in meal-induced satiety, and circulating plasma CCK concentrations are also influenced by food ingestion. Furthermore, the effects of CCK are modulated by short-, and long-term, dietary manipulation, in particular when the diet is composed predominantly of fat.

This chapter will review the major determinants of ghrelin secretion, particularly the effects of ageing and nutrient ingestion, and the effects of manipulations in the macronutrient composition of diet on the appetite-suppressive effects of CCK.

2.2 Determinants of ghrelin secretion

Ingestion of a meal results in increases in insulin and glucose levels, and suppression of ghrelin concentrations for at least four hours after the meal, with a return to baseline by six hours (Caixas et al. 2002).

Oral glucose has been shown to be a potent inhibitor of ghrelin secretion, in both animals and humans (Tschop et al. 2000; Caixas et al. 2002; Shiiya et al. 2002). In normal healthy subjects, ghrelin concentrations rapidly decrease after oral and intravenous glucose, but not after an oral water load (Shiiya et al. 2002). These effects of glucose on ghrelin may be mediated by insulin (Saad et al. 2002). In fact, fasting ghrelin concentrations have been shown to be negatively correlated with insulin levels (Rigamonti et al. 2002). An increase in insulin after meal ingestion could result in decreased ghrelin levels, thereby reducing food intake (Saad et al. 2002). Furthermore, intravenous infusion of insulin, given together with a glucose infusion to maintain euglycaemia, resulted in a rapid decrease in plasma ghrelin concentrations within 30 minutes, with a maximal mean suppression of 32% (Saad et al. 2002). Similarly, in another study, insulin infusion decreased ghrelin concentrations by 22% ($P < 0.01$) in healthy young subjects, with a nadir at 30 minutes (Lucidi et al. 2002). The suppression of ghrelin by a bolus intravenous dose of glucose (Shiiya et al. 2002), and the observation that hyperinsulinaemia suppresses ghrelin independently of glucose (Flanagan et al. 2003) support a role for insulin. However, other studies have demonstrated that parenteral insulin administration, either by subcutaneous injection or intravenous infusion, does not suppress circulating ghrelin concentrations (Caixas et al.

2002; Schaller et al. 2003), except at supraphysiological insulin concentrations (Schaller et al. 2003).

The mechanisms by which insulin may inhibit ghrelin synthesis or secretion have not been fully elucidated. It has been suggested that insulin may act directly on ghrelin-producing cells either via the systemic circulation or in a paracrine manner, or possibly via humoral or neural mechanisms (Saad et al. 2002; Date et al. 2002). Cells with ghrelin immunoreactivity have been found in rat and human pancreatic islets, in a similar distribution to glucagon-producing alpha-cells, and GH secretagogue receptors are also found in the pancreas (Date et al. 2002). Furthermore, in vitro studies on mouse pancreatic islet cells and in-vivo studies in mice have shown that ghrelin administration decreases insulin secretion in response to glucose infusion (Date et al. 2002; Reimer et al. 2003).

Glucagon administration also results in suppression of ghrelin, but does not appear to play a role in determining fasting ghrelin concentrations (Hirsh et al. 2005; Soule et al. 2005; Arafat et al. 2005; Arafat et al. 2006).

2.2.1 Effects of food intake and nutrient composition on circulating ghrelin concentrations

Plasma ghrelin concentrations increase before the start of a meal, and decrease to trough levels within one hour of meal ingestion (Cummings et al. 2001). Ghrelin concentrations then gradually increase again between meals, to reach a peak just before

the next meal (Cummings et al. 2001). There are differences between macronutrients in their ability to suppress ghrelin after a meal (see below).

The mechanisms by which the ingestion of nutrients modulate ghrelin release have not been fully established. For example, it is not known whether the presence of nutrients in the small intestine is sufficient to alter plasma ghrelin levels or whether digestion and absorption is required. The aim of the study described in Chapter 7 is to investigate the role of carbohydrate and fat digestion in the meal-induced suppression of plasma ghrelin concentrations.

2.2.1.1 Effects of carbohydrate on ghrelin secretion

Ghrelin suppression appears to be more pronounced after consumption of carbohydrates compared to any other macronutrient (Erdmann et al. 2003; Erdmann et al. 2004).

In fasted rats, circulating ghrelin concentrations decrease following an orally-administered 50% dextrose solution, but not after ingestion of an equal volume of water (Tschop et al. 2000), establishing that 'nutrient' factors are of greater importance than gastric distension in determining ghrelin secretion. In humans, a 75 g oral glucose load in healthy adults resulted in suppression of plasma ghrelin concentrations which reached its lowest level one hour after administration, with a subsequent increase in ghrelin levels (Shiiya et al. 2002). Furthermore, intraduodenal infusion of glucose suppressed ghrelin concentrations slightly earlier than intragastric glucose infusion, but the extent of suppression was not significantly different between the two routes (Parker et al. 2005). These studies indicate the importance of exposure of the small intestine to

nutrients in the control of ghrelin secretion, although it is not known whether the suppression of ghrelin is load and/or concentration-dependent. The length of small intestine exposed to nutrient is also important, and the postprandial suppression of ghrelin requires the exposure of > 60 cm of small intestine to glucose (Little et al. 2006a).

Incorporation of carbohydrates into a mixed meal has a similar effect on ghrelin secretion. Ingestion of a carbohydrate-rich meal decreases plasma ghrelin concentrations within one hour of having the meal (Cummings et al. 2001; Erdmann et al. 2003), and in some studies ghrelin levels remain suppressed for up to four hours after the meal (Erdmann et al. 2003).

2.2.1.2 Effects of fat on ghrelin secretion

The response to consumption of a fat-rich meal is different to that of a carbohydrate enriched meal. One of the earliest studies showed that ingestion of a fat-enriched meal (85.5% fat) resulted in an initial slight rise in ghrelin concentrations up to 19% above baseline 45 minutes after the meal, and subsequent fall towards baseline levels (Figure 2.1) (Erdmann et al. 2004). A subsequent study showed that ingestion of a fat-enriched test meal (200 mL cream, 85% of energy as fat) resulted in suppression of plasma ghrelin concentrations from 30 to 180 minutes postprandially, to a nadir of 21% below baseline levels at 180 minutes (Erdmann et al. 2003). Another study in healthy young women showed that postprandial acylated ghrelin concentrations remained suppressed for one hour after consumption of a high-fat meal (Al Awar et al. 2005).

The suppression of ghrelin secretion by an intraduodenal infusion of a triglyceride emulsion has been shown to be dependent on digestion of fat to free fatty acids (Feinle-Bisset et al. 2005). The effects of fatty acids on gastrointestinal hormone release, appetite, energy intake, and gastric emptying are dependent upon their acyl chain length (Hunt and Knox 1968; McLaughlin et al. 1999; Feltrin et al. 2004; Feltrin et al. 2006). The increase in GLP-1, PYY, pancreatic polypeptide, and suppression of ghrelin, in response to an intraduodenal infusion of fatty acids is dependent on an acyl chain length of at least 12 carbon atoms (Feltrin et al. 2004; Feltrin et al. 2006). In addition, the magnitude of the stimulation of CCK in response to an intraduodenal infusion of fatty acids is more marked with lauric acid, a fatty acid with an acyl chain length of 12 carbon atoms, than with decanoic acid (chain length of 10 carbon atoms) (McLaughlin et al. 1999; Feltrin et al. 2004). In addition, an intragastric infusion of fatty acids with an acyl chain length of $\geq$ 12, but not $\leq$ 10, carbon atoms has been shown to slow gastric emptying (Hunt and Knox 1968). Therefore, the effects of fatty acids with an acyl chain length of $\geq$ 12 carbon atoms on gastrointestinal function and hormone release are more potent than those with $\leq$ 10 carbon atoms.

2.2.1.3 Effects of protein on ghrelin secretion

The role of protein in altering ghrelin secretion has not been clarified. Initial studies in healthy young adults showed that ingestion of a protein-rich meal results in a sustained rise in plasma ghrelin concentrations (Erdmann et al. 2003; Erdmann et al. 2004). In fact, plasma ghrelin concentrations remained approximately 15.8% higher than baseline levels for 150 minutes after a protein-rich meal, compared with suppression of ghrelin concentrations for the first 120 minutes following a carbohydrate-rich meal (Erdmann

et al. 2003). In contrast, a subsequent study, in healthy young women showed that postprandial acylated ghrelin concentrations remained suppressed for one hour after consumption of a carbohydrate-rich meal, and for three hours after an iso-energetic high-protein meal (Al Awar et al. 2005), however, the discrepant results may be explained by the differences in protein content of the test meals in these two studies. In the earlier studies, a 99% protein meal (turkey meat) (Erdmann et al. 2003) and 83% protein-rich meat meal (Erdmann et al. 2004) were used, whereas in the latter study, the high-protein test meal consisted of 35% protein, 45% carbohydrates, 20% fat (Al Awar et al. 2005). Thus, the high carbohydrate content of the meal used in the study by Al Awar et al. (2005) may have contributed to the suppression in ghrelin concentrations (Al Awar et al. 2005). In another study, postprandial ghrelin concentrations were suppressed for up to 120 minutes following a liquid protein (whey or casein) preload (containing 1000kJ; 83% energy as protein), and this suppression was more prolonged than that following an isoenergetic glucose drink (Bowen et al. 2006). However, that study was performed in overweight men, and the results cannot be directly extrapolated to healthy-weight individuals. A recent calorie-restricted weight-loss study in overweight adults showed that subjects on a high-protein, low-fat, diet (40% protein, 30% fat, 30% carbohydrates) over 16 weeks had reduced appetite ratings for prospective consumption, compared with those on a high-fat, standard protein, diet (20% protein, 50% fat, 30% carbohydrates), however, there were no effects of diet type on postprandial ghrelin suppression (Moran et al. 2005). Interestingly, in that same study, weight loss was associated with a greater suppression of postprandial ghrelin concentrations (14%) over the first 60 minutes following ingestion of a meal, than before weight loss (3%, $P = 0.017$) (Moran et al. 2005). Overall, protein, by itself,

probably has little influence on postprandial ghrelin suppression, and the discrepant results from various studies are likely to be related to differences in the nutrient composition of the test meal, the weight range of the subjects, and the difficulties in discriminating the potential ghrelin-suppressive effects of protein from those of carbohydrate.

2.3 Effects of ageing on circulating ghrelin concentrations

Because appetite and food intake decrease with healthy ageing (as discussed in Chapter 1), and this can have harmful effects, it is of interest to determine whether reduced orexigenic effects of ghrelin contribute to this. There is some evidence from animal studies that this is the case. Older mice have been found to have lower circulating ghrelin levels compared to young mice, and to express the ghrelin gene in the stomach at only 5% of peak levels present in young mice (Liu et al. 2002). In a more recent study, freely-fed aged mice had increased gastric antral and fundal weights compared to those of young mice, however circulating ghrelin concentrations were not significantly different between young and aged mice (Yang et al. 2007). Therefore, the effects of ageing on circulating ghrelin concentrations in rodents remain uncertain.

2.3.1 Changes in circulating ghrelin concentrations with healthy ageing

There are conflicting data on the effects of healthy ageing on circulating ghrelin concentrations in humans (Cummings et al. 2001; Rigamonti et al. 2002; Sturm et al. 2003; Purnell et al. 2003; Marchesini et al. 2004; Langenberg et al. 2005; Makovey et al. 2007; Bauer et al. 2007; Schutte et al. 2007). In part this uncertainty probably arises from the confounding effects of age-related body composition changes on ghrelin

concentrations. Plasma ghrelin concentrations have been reported to be higher (Cummings et al. 2001; Purnell et al. 2003), lower (Rigamonti et al. 2002; Marchesini et al. 2004; Bauer et al. 2007), or no different (Sturm et al. 2003; Langenberg et al. 2005; Bertoli et al. 2006; Makovey et al. 2007) in healthy older, compared to young, adults. In some of these studies, age-related differences in ghrelin concentrations found on univariate analysis were no longer apparent when body composition measures were corrected for on multivariate analysis (Purnell et al. 2003; Marchesini et al. 2004). Postprandial suppression of ghrelin concentrations may also differ between young and older individuals. In one study of 8 healthy young and 8 older subjects, the extent of postprandial ghrelin suppression after an oral mixed nutrient preload was not significantly different between the two age groups, although the mean reduction in plasma ghrelin concentrations was slightly greater in healthy older (26 ± 6%) than young subjects (18 ± 4%; $P = 0.4$) (Sturm et al. 2003). In another study, when 21 young and 18 older subjects were given standard meals, pooled ghrelin concentrations over a 24-hour time period were not significantly different between young and older age groups (Yukawa et al. 2006). Similarly, in a study of 26 healthy older (mean age of 68.6 years) and 10 young subjects, a mixed meal suppressed plasma ghrelin concentrations by 17.1% and 13.3% respectively (P =0.4) (Bertoli et al. 2006). Thus, there is no clear evidence that healthy ageing alters the postprandial suppression in circulating ghrelin concentrations.

The study described in Chapter 6 examines the effects of healthy ageing on plasma ghrelin concentrations, across a broad spectrum of ages from young to older age, whilst accounting for changes in body composition and energy intake.

2.3.2 Circulating ghrelin concentrations in under-nourished younger and older persons

There is evidence that under-nourished humans have increased circulating ghrelin concentrations. In one study, older under-nourished subjects had fasting plasma ghrelin concentrations that were some 58% higher than those of healthy older and 50% higher than those of healthy young subjects ($P < 0.01$). Plasma ghrelin levels remained significantly higher in this under-nourished older group after an oral nutrient preload than the other groups ($P < 0.05$) (Sturm et al. 2003). In a study of young subjects with anorexia nervosa, mean ghrelin concentrations were 157% higher than in young healthy subjects (Rigamonti et al. 2002). As ghrelin would be expected to stimulate food intake, and these under-nourished individuals have reduced food intake, these findings suggest that there may be a compensatory rise in ghrelin concentrations in chronic negative energy balance states. In contrast, young and older malnourished adults, staying between 2 - 30 days in an Intensive Care Unit on admission to hospital, had lower fasting plasma ghrelin concentrations during their stay, than healthy control subjects, which gradually returned to normal during the recovery phase of their illness (Nematy et al. 2006). In this study, there was a strong positive correlation between food intake at 4 weeks after admission to hospital and the percentage increase in ghrelin concentrations over this time ($R = 0.9$, $P < 0.05$) (Nematy et al. 2006). Thus, the decrease in ghrelin concentrations in those with severe acute illnesses compared to healthy individuals may well contribute to the reduction in appetite and energy intake.

2.4 Effects of dietary manipulation on appetite

Gastrointestinal function and appetite can be modulated by the nutrient composition of a meal. There is evidence for differential satiating effects of fat, carbohydrate, and protein, and a hierarchy in the degree to which they influence energy intake.

The infusion of nutrients, particularly fat, directly into the duodenum is associated with a feedback response to the stomach, which results in increased intragastric volume and gastric relaxation, and also increases fullness and decreases appetite sensations (Feinle et al. 1997; Feinle et al. 2002). In healthy and obese young males, intraduodenal lipid suppresses appetite and energy intake to a greater extent than an equicaloric intraduodenal glucose infusion (Andrews et al. 1998; Chapman et al. 1999). In contrast, in older individuals, energy intake, but not appetite or hunger sensations, appear to be reduced comparably after both intraduodenal fat and glucose infusions (Cook et al. 1997).

The effects of a nutrient preload on appetite and food intake are influenced by the timing of the preload in relation to the test meal (Rolls et al. 1994). Initial studies in humans demonstrated comparable acute suppressive effects on appetite and food intake of equal volumes of pure macronutrients - fat, carbohydrate, and protein, all of which had an energy content of 1184 kJ, administered as a preload one hour before a test meal (Geliebter 1979). In another study, oral supplementation of carbohydrate, but not an equi-energetic oral fat load, given on one occasion at breakfast, suppressed appetite and energy intake 90 minutes after the meal, although the effects were short-lived, and not present at 4.5 hours (Blundell et al. 1993). In yet another study, a high-fat soup given

prior to a meal did not have a satiating effect or suppress spontaneous energy intake, when compared to a low-fat soup, at 20 minutes after the soup preload (Sepple and Read 1990). Furthermore, while a high-protein breakfast suppressed hunger sensations to a greater extent throughout a 24-hour period than an iso-energetic high-fat, or high-carbohydrate, breakfast, subsequent spontaneous energy intake 5 hours after breakfast was not affected by the type of breakfast consumed (Stubbs et al. 1996). In this same study, the suppressive effect of a protein-rich breakfast on hunger was sustained for a 24-hour period, whereas the carbohydrate-rich breakfast resulted in acute suppression of hunger, and the high-fat breakfast did not noticeably reduce hunger sensations until approximately 5 hours after ingestion (Stubbs et al. 1996). When administered at least 90 minutes prior to an *ad libitum* meal, a high-protein liquid or solid preload reduced energy intake compared to a carbohydrate (Porrini et al. 1995; Poppitt et al. 1998), or fat (Poppitt et al. 1998), preload. In contrast, in another study, there were no significant differences in the extent of suppression of food intake over a 7 hour period by high-fat, high-carbohydrate, and high-protein preloads, each containing 3000kJ, when subjects were free to eat when they wished (Vozzo et al. 2003). In addition, yoghurt preloads of high-energy density, either high-fat or high-carbohydrate, equally suppressed energy intake at a buffet meal (Rolls et al. 1991; Shide et al. 1995). Thus, although protein may suppress hunger to a greater extent and for a longer duration than either carbohydrate or fat, it does not appear to alter subsequent energy intake when subjects are free to eat when, as well as how much, they want.

Several factors, including the rate of gastric emptying and consequent delivery of nutrients into the small intestine, macronutrient composition, and hormonal signals, are

important in determining the acute gastrointestinal responses to nutrient ingestion. For example, in one study, intragastric, but not intravenous, infusion of an iso-energetic load of either lipid (fat) or dextrose (carbohydrate) reduced subsequent energy intake (Shide et al. 1995). While rapid intragastric infusions of either fat or carbohydrate over 15 minutes had comparable suppressive effects on subsequent energy intake, a 3.5 hour infusion of fat, but not carbohydrate, suppressed subsequent energy intake, although maximal concentrations of CCK were lower during the slow fat (4.5 pmol/L), than the rapid fat (12.6 pmol/L), infusion (Shide et al. 1995). The slower infusion of fat into the stomach was associated with a greater overall rise in plasma cholecystokinin concentrations compared with the carbohydrate infusion (Shide et al. 1995). Thus, it is not just the type of macronutrient that determines the appetite response, but also the site, mode, and rate of delivery of nutrients.

Studies of the longer term effects of dietary manipulation have also been performed. In healthy young males, dietary supplementation with a glucose polymer for 7 days attenuated the satiating effect of a subsequent intraduodenal lipid infusion compared to no supplementation (Andrews et al. 1998). This study thus suggests that ingestion of one macronutrient influences the gastrointestinal disposal of other macronutrients. In both young and older individuals, dietary glucose supplementation for 4 to 10 days has been shown to accelerate gastric emptying of an oral glucose load (Horowitz et al. 1996; Beckoff et al. 2001), and, in older subjects, does not appear to affect energy intake at a buffet meal (Beckoff et al. 2001). The effects of dietary supplementation on appetite and gastrointestinal function may possibly be mediated by changes in small intestinal feedback.

2.4.1 Effects of a high-fat diet on gastrointestinal hormones, appetite, and food intake

The gastrointestinal tract adapts to increased proportions of fat in the diet with adjustments to its morphology and function.

2.4.1.1 Animal studies

Adaptive changes in the intestinal mucosa are seen in rats fed a high-fat diet for four weeks, with mucosal hypertrophy of the proximal ileum (Balint et al. 1980), and increased jejunal and ileal absorption of oleic acid, a long-chain monounsaturated fatty acid (Singh et al. 1972; Balint et al. 1980). Other morphological changes in the small intestine of rats maintained on a high-fat (monounsaturated or polyunsaturated) diet include increased height of villi, which form the vast absorptive surface of the intestine (Sagher et al. 1991). The site of these changes (proximal and/or distal) is determined by the type of fat included in the diet (Sagher et al. 1991). The secretion and activity of pancreatic lipase, an enzyme which hydrolyses fat into fatty acids, is increased by a high-fat, compared to a low-fat, diet (Sabb et al. 1986; Spannagel et al. 1996).

Early studies in rats suggested that a pure nutrient load composed of oil (fat) was less satiating than either pure carbohydrate or protein (Geliebter 1979). Furthermore, the suppression of food intake and gastric emptying in rats by an intraduodenal oleic acid infusion is less pronounced on a high-fat diet compared with a low-fat diet (Covasa and Ritter 1999; Covasa and Ritter 2000), and a high-fat diet promotes hyperphagia in rats (Savastano and Covasa 2005).

Concordant with these observations, the inhibitory effects of exogenous CCK on both gastric emptying and food intake are attenuated in rats following a 2 - 3 week period on a high-fat, compared to an isoenergetic low-fat, diet (Covasa and Ritter 1998; Covasa and Ritter 2000; Savastano and Covasa 2005). In addition, plasma CCK concentrations in response to an intraduodenal infusion of fat in rats are higher on a high-fat diet than on a low-fat diet (Spannagel et al. 1996). Chronically elevated CCK concentrations in rodents, either through maintenance on a diet high in fat or protein, or through continuous intraperitoneal infusion of exogenous CCK-8, reduce the satiating effect of an additional dose of exogenous CCK-8 when compared with a low-fat diet (Covasa et al. 2001). It has been hypothesised that down-regulation of CCK receptors or changes in gastric emptying on a high-fat diet may explain the diminished satiating effect of exogenous CCK (Covasa and Ritter 1998; Covasa et al. 2001).

2.4.1.2 Human studies

Human studies have also shown that chronic dietary changes affect the acute intestinal responses to food ingestion which modulate gut function, appetite, and food intake.

In young, healthy individuals, a more rapid gastric emptying of a high-fat test meal was observed following a two-week period on a high-fat diet, which provided an extra 258g of fat daily compared to a low fat diet (Cunningham et al. 1991a). However, studies have shown no differences in spontaneous food intake after exposure to high-fat or low-fat diets (Cunningham et al. 1991a; French et al. 1995). Consistent with these findings, a comparison of medium- (40% of energy as fat) and high-fat (60% of energy as fat) diets with a low-fat (20% of energy as fat) diet over 7 days failed to show any

significant differences in spontaneous food intake (Stubbs et al. 1995). It has been suggested that high dietary fat exposure may render the small intestine less 'sensitive' to the inhibitory effects of fat on gastric emptying (Cunningham et al. 1991a).

As in experimental animals, a high-fat diet has also been shown in humans to increase plasma CCK concentrations in response to an orally ingested meal, consistent with 'down-regulation' or reduced sensitivity of CCK receptors responsible for feedback inhibition of CCK release (French et al. 1995). However, plasma CCK responses to a duodenal lipid infusion did not differ after a 14-day period on either a high-fat or low-fat diet (Boyd et al. 2003). Furthermore, in this same study, spontaneous energy intake after an intraduodenal lipid infusion was not significantly different after the high-fat, compared to the low-fat, diet (Boyd et al. 2003). A recent study in healthy young males has shown that fasting plasma CCK concentrations are slightly higher after a 3-week period on a high-fat (44% energy as fat) diet when compared with an isocaloric low-fat (9% energy as fat) diet, however spontaneous energy intake and appetite sensations were not influenced by the type of diet (Little et al. 2007). The above findings suggest that accelerated gastric emptying secondary to a high-fat diet and hence, increased exposure of the small intestine to nutrients, may be responsible for the increased postprandial CCK concentrations seen in the previous study (French et al. 1995).

A high-fat diet might act to decrease the satiating effect of CCK and, hence, increase appetite, in older adults, who are potentially at risk of undernutrition. The intervention study described in Chapter 9 compares the satiating effect of intravenous CCK-8 in

older persons with and without a preceding two-week period of dietary fat supplementation.

2.5 Conclusions

Although ingestion of carbohydrates has an immediate inhibitory effect on circulating ghrelin concentrations, the effects of fat are relatively delayed, and protein ingestion may stimulate ghrelin secretion. The effects of healthy ageing on ghrelin concentrations are controversial, and it, accordingly, remains uncertain whether ghrelin is an important determinant of the physiological decline in appetite with increasing age.

Variations in macronutrient composition of meals on a day-to-day basis can affect appetite and food intake, and also influence gastrointestinal hormone release. If maintenance on a high-fat diet with nutritional supplementation can reduce sensitivity to the satiating effects of CCK in older persons, this may prove beneficial in improving nutritional status.

NOTE: This figure is included on page 46 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.1: Effect of ingestion of (a) a high-fat meat meal and (b) a high carbohydrate meal, and a second standardised sandwich meal (S) on scores for hunger and satiety, and plasma ghrelin, glucose, and insulin, concentrations. Adapted from Erdmann et al. (2004).

Chapter 3

POSTPRANDIAL HYPOTENSION IN OLDER PERSONS

3.1 Introduction

Food ingestion is often followed by a decrease in blood pressure. When excessive, this is known as postprandial hypotension, and can result in syncope, falls, visual disturbances, cerebrovascular accidents, and angina (Jansen and Lipsitz 1995). Postprandial hypotension is defined as a 20 mmHg or greater fall in systolic blood pressure, or a decrease from a systolic blood pressure of > 100 mmHg to 90 mmHg or less, within two hours of a meal (Jansen and Lipsitz 1995). The fall in blood pressure is usually maximal within 30 to 60 minutes of ingestion of a meal (Jansen and Lipsitz 1995), but can occur as late as 75 minutes postprandially (Vaitkevicius et al. 1991; Aronow and Ahn 1994).

This chapter will review the pathophysiology of postprandial hypotension, in particular gastric and small intestinal mechanisms, and the therapeutic strategies which may be applied in the management of individuals with postprandial hypotension.

3.2 Normal cardiovascular responses to food ingestion

Increased splanchnic blood flow following ingestion of a meal, and a doubling of superior mesenteric arterial blood flow, results in 'pooling' of blood in the splanchnic veins (Mathias 1991; Waaler and Eriksen 1992; Jansen and Lipsitz 1995). Splanchnic

blood volume increases by approximately 20% after a meal, thus reducing systemic vascular resistance, and resulting in a reduction of blood volume returning to the heart. This is followed by a compensatory increase in heart rate and cardiac output, and a decrease in skeletal muscle blood flow (Mathias 1991; Waaler and Eriksen 1992; Jansen and Lipsitz 1995). Splanchnic blood volume increases to a similar degree in both young and older adults, however, the maximal increase is evident later in older than young subjects (Lipsitz et al. 1993).

The increase in heart rate results from normal baroreceptor reflexes, and the heart rate response in healthy young and older adults is generally comparable (Jansen and Lipsitz 1995). Activation of the sympathetic nervous system occurs, with an increase in plasma noradrenaline concentrations, and increased muscle sympathetic nerve activity (Mathias 1991; Jansen and Lipsitz 1995). In healthy young and older adults, all of the above compensatory autonomic and cardiovascular responses ensure that blood pressure remains essentially stable following ingestion of a meal (Jansen and Lipsitz 1995). However, the heart rate response to a meal in healthy older adults may be blunted compared to young adults, and may result in a fall in postprandial blood pressure (Sidery et al. 1993). Whilst mild reductions in blood pressure following ingestion of a meal are common in older adults, greater changes in blood pressure may be clinically significant, especially in the setting of comorbid illnesses (Jansen and Lipsitz 1995).

3.3 Pathophysiology of postprandial hypotension

Postprandial hypotension is more common in healthy older, compared to young, adults (Jansen and Lipsitz 1995; Smith et al. 2003). Splanchnic blood pooling following a meal is thought to be an important initiating event in the development of postprandial hypotension (Qamar and Read 1988; Moneta et al. 1988; Sidery et al. 1993; Jansen and Lipsitz 1995). Although the compensatory increase in heart rate after a meal appears to remain intact in healthy older persons (Westenend et al. 1985; Jansen et al. 1987b), baroreflex sensitivity is diminished, and a blunted response of the sympathetic nervous system in older age may partly explain the differences in postprandial blood pressure responses between young and older persons (Lipsitz et al. 1983; Jansen et al. 1987b; Jansen and Hoefnagels 1989).

The rate of gastric emptying may also influence the blood pressure response to a meal. Gastric emptying of liquid, or liquefied nutrients normally occurs at a rate of 1 – 3 kcal/min, and is regulated by feedback inhibition from small intestinal luminal receptors (Lin et al. 1989; Horowitz et al. 1996). In one study, the rate of gastric emptying of a 75 g glucose drink was shown to be related to the extent of the fall in postprandial blood pressure in patients with diet-controlled type 2 diabetes, but not in healthy young or older adults (Jones et al. 1998). In this same study, postprandial mean arterial blood pressure was up to 8 - 10 mmHg greater in healthy young and older adults compared with patients with type 2 diabetes, thus it may have been feasible to detect a relationship between the fall in blood pressure and gastric emptying (Jones et al. 1998). In healthy older subjects, with a mean age of 70.3 years, an intraduodenal glucose infusion at a rate of 3 kcal/min, but not 1 kcal/min, resulted in a substantial fall in systolic, diastolic,

and mean arterial blood pressures (Figure 3.1) (O'Donovan et al. 2002). In another study of 8 healthy older subjects, aged 65 - 78 years, intraduodenal glucose infusion was given at a constant rate of 3 kcal/min, and the fall in blood pressure did not differ at varying concentrations of glucose solution (Gentilcore et al. 2008). Therefore, the magnitude of the fall in blood pressure with glucose is increased by more rapid gastric emptying and by larger glucose loads in the small intestine, but does not appear to be influenced by the concentration of glucose in the small intestinal lumen.

3.3.1 Mechanisms of postprandial hypotension

The mechanisms of postprandial hypotension are poorly understood. It was initially suggested that the rise in circulating insulin concentrations following a meal, particularly carbohydrate ingestion, may mediate the blood pressure decrease, by blunting baroreflex sensitivity (Lipsitz et al. 1983). In support of a role of insulin, oral glucose ingestion was found to induce a larger increase in plasma glucose and insulin concentrations than an iso-energetic fructose load, and produce a significant fall in blood pressure, whereas fructose did not (Jansen et al. 1987b). In that same study, the increase in plasma noradrenaline concentrations and heart rate, which reflect activation of the sympathetic nervous system, were not significantly different between the glucose and fructose drinks, suggesting that differential activation of the sympathetic nervous system was not a factor (Jansen et al. 1987b). However, other studies did not support a role for insulin, by showing no correlation between the increase in plasma insulin concentrations and the fall in blood pressure after oral glucose loading (Jansen et al. 1987b; Jansen et al. 1990). In addition, postprandial hypotension has been previously described in individuals with type 1 diabetes, who are, by definition, insulin-deficient

(Stevens et al. 1991; Maule et al. 2004). This has led to the suggestion that the action of insulin may not be an important mechanism of postprandial hypotension, and that ingestion of glucose results in direct vasodilatation of splanchnic veins and increased pooling of blood in the splanchnic circulation (Jansen et al. 1987b; Jansen and Hoefnagels 1987).

In a recent study evaluating the effect of age on the mechanisms of postprandial blood pressure reduction, the magnitude of the fall in systolic blood pressure following an intraduodenal glucose infusion was significantly greater in 8 healthy older than in 8 healthy young subjects (mean maximal fall of 17.0 ± 4.1 mmHg vs 6.5 ± 1.6 mmHg, $P = 0.03$) (van Orshoven et al. 2008). Furthermore, the fall in systolic blood pressure was substantially greater in two patients with postprandial hypotension than in healthy subjects (van Orshoven et al. 2008). While there was a greater fall in blood pressure in healthy older compared to young subjects, the magnitude of the increase in muscle sympathethic nerve activity in response to the intraduodenal glucose infusion was comparable in young and older subjects, suggesting decreased sympathetic baroreflex response in older adults (van Orshoven et al. 2008).

As oral, but not intravenous, glucose administration lowers blood pressure, it has also been proposed that the gastrointestinal tract plays an important role in the pathogenesis of postprandial hypotension (Jansen et al. 1990; Jansen and Lipsitz 1995). Several vasoactive gastrointestinal peptides, including substance P and neurotensin (Jansen and Lipsitz 1995) which have powerful vasodilating properties, have been implicated in splanchnic vasodilatation after oral glucose loading (Jansen et al. 1990). In fact, the

blood pressure-lowering effects of oral glucose loading is prevented by somatostatin, a hormone produced by enteroendocrine cells in the gastrointestinal tract and pancreas and by neural cells particularly in the hypothalamus, which inhibits most gastrointestinal hormones including vasoactive intestinal peptides, and may therefore modulate splanchnic vasodilatation (Jansen et al. 1990; Jansen and Lipsitz 1995).

Gastric distension has been shown to increase blood pressure, and was first demonstrated in healthy young subjects, where proximal gastric distension with a barostat device increased blood pressure in proportion to the rise in intragastric pressure, increased heart rate and muscle sympathetic nerve activity, via a mechanism termed the "gastrovascular reflex" (Rossi et al. 1998b). Similarly, in a study of 19 patients with severe orthostatic hypotension secondary to autonomic failure, consumption of 480 mL of water increased mean systolic blood pressure by 11 ± 2.4 mmHg 35 minutes after ingestion ($P < 0.001$) (Jordan et al. 1999). In another study of 7 patients with autonomic failure, ingestion of 480 mL of water within 5 minutes, consumed just prior to a high-carbohydrate meal, increased systolic blood pressure to a maximum of 36 ± 23 mmHg after 20 minutes, compared with a fall in systolic blood pressure up to 43 ± 36 mmHg when water was not ingested before the meal (Shannon et al. 2002). Consumption of water has been shown to increase blood pressure in both healthy older adults (Jordan et al. 2000) and in patients with autonomic failure (Jordan et al. 1999; Jordan et al. 2000; Cariga and Mathias 2001).

3.4 Epidemiology and clinical significance of postprandial hypotension

An early study of the effects of a meal on blood pressure was undertaken in older persons living in institutional care in the United States of America, almost all of whom were not functionally independent (Lipsitz et al. 1983). In a subgroup of 20 older subjects in that study, aged 87 ± 1 years, mean systolic blood pressures fell significantly by 25 ± 5 mmHg in 10 older subjects with a history of syncope, and by 24 ± 9 mmHg in 10 older subjects without a history of syncope, at 35 minutes after the start of a standardised meal, compared with no significant changes in systolic blood pressure in a group of 11 healthy young control subjects ($P < 0.001$ compared with either group of older subjects) (Lipsitz et al. 1983). In that study, the proportion of older subjects with postprandial blood pressure reductions of ≥ 20 mmHg was not reported (Lipsitz et al. 1983). Subsequently, in 21 healthy community-dwelling older adults in the United States of America, aged 73 ± 6 years, postprandial mean maximal systolic blood pressure reductions of 11 ± 9 mmHg were reported (Lipsitz and Fullerton 1986). Studies of healthy young adults have consistently shown no significant changes in blood pressure following meal ingestion (Westenend et al. 1985; Heseltine et al. 1990; Sidery et al. 1990; Sidery et al. 1991; Jones et al. 1998).

As large population surveys have not been done, the prevalence of postprandial hypotension is not known accurately. Available data suggest, however, that it may be more common than orthostatic (or postural) hypotension, the latter occurring in up to 30% of asymptomatic, healthy, older adults residing independently in the community (Jansen and Lipsitz 1995; Carey and Potter 2001). For example, in one study,

postprandial hypotension, following ingestion of a liquid meal, was identified in 8 of 16 older adults with syncope of unknown aetiology, residing in the community or in residential care (Jansen et al. 1995). In another study, 96% of a population of 113 older adults (mean age 78 ± 9 years) living in a nursing home had a mean fall in systolic blood pressure of 17.9 ± 15.5 mmHg after a standardised carbohydrate meal, with a reduction in systolic blood pressure of ≥ 20 mmHg (or postprandial hypotension) in 36% of the cohort (Vaitkevicius et al. 1991). In another study, 24% of 499 older adults (mean age 80 ± 9 years) residing in a residential care facility had systolic blood pressure reductions of ≥ 20mmHg following consumption of the subject's usual lunchtime meal (Aronow and Ahn 1994).

Certain populations are at increased risk of postprandial hypotension, especially older adults, and those with autonomic dysfunction, such as patients with diabetes mellitus and Parkinson's disease (Bannister et al. 1987; Mathias 1991; Jansen and Lipsitz 1995). The mechanism of postprandial hypotension in autonomic dysfunction is likely to be related to impaired sympathetic tone and diminished heart rate response to splanchnic vasodilation (Mathias 1991). In one of the early studies in patients with primary chronic autonomic failure, all 12 subjects studied had profound postprandial hypotension after a meal, with a mean fall in systolic blood pressure of 44 mmHg (Mathias et al. 1989a). In a study of patients with type 2 diabetes, treated with oral medications, insulin, or with diet alone, 7 of 35 patients (20%) had evidence of postprandial hypotension after a 75 g oral glucose load, with no fall in blood pressure in any of the 15 non-diabetic subjects (Sasaki et al. 1992). Similarly, in another study, postprandial hypotension was seen in 7 of 16 patients (44%) with diet-controlled type 2 diabetes given a 75 g oral glucose load,

with no fall in blood pressure in any of the 10 young healthy control subjects (Jones et al. 1998). Postprandial hypotension has also been identified in 82% of older patients, aged 66 - 84 years, with Parkinson's disease (Mehagnoul-Schipper et al. 2001). Therefore, postprandial hypotension is a common condition in older adults, particularly those with autonomic dysfunction.

Normal ageing is associated with a gradual rise in blood pressure (Jansen and Lipsitz 1995; Cheitlin 2003), and hypertension is more common in older, than young, adults (Sagie et al. 1993). The prevalence of isolated systolic hypertension, defined as a systolic blood pressure of $\geq$ 160 mmHg and a diastolic blood pressure of $<$ 90 mmHg, gradually increases with age, from 1 – 4% at 30 years, to approximately 20% by 70 years of age (Sagie et al. 1993). Of note, older adults with hypertension, even in the absence of apparent cormorbid cardiovascular disease, have a greater propensity to postprandial reductions in blood pressure than normotensive older adults (Jansen et al. 1987a; Jansen et al. 1987b; Jansen and Lipsitz 1995). In the presence of hypertension, baroreflex sensitivity is diminished compared to normotensive individuals, and, as a result, it has been suggested that there is less effective activation of the sympathetic nervous system to counterbalance the increased splanchnic blood flow after a meal (Jansen et al. 1987b).

Morbidity and mortality in older adults may be influenced by the presence of postprandial hypotension. Postprandial hypotension is associated with an increased risk of falls and syncope (Aronow and Ahn 1994; Puisieux et al. 2000). In a study of 499 older adults in a nursing home, greater postprandial blood pressure reductions were

seen in those with, than without, a history of falls (21 ± 5 mmHg versus 13 ± 4 mmHg, $P < 0.0001$), after controlling for the effects of medications (Aronow and Ahn 1994). In another study, 23% of 120 hospitalised older adults admitted with falls or syncope had evidence of postprandial hypotension, significantly higher than the prevalence in a control group without a history of falls or syncope (9%, $P = 0.03$) (Puisieux et al. 2000).

A 29-month follow-up study in 499 nursing home residents aged 62 years and over showed that, the extent of the fall in systolic blood pressure after a meal is an independent risk factor for new falls, syncopal episodes, coronary events, stroke, and overall mortality (Aronow and Ahn 1997). In another study of 179 older adults aged ≥ 65 years in a low-level care residential facility, followed up for 4.7 years, the mortality rate was increased, at 145 per 1000 person-years in those with postprandial hypotension, compared with 98.5 per 1000 person-years in those without postprandial hypotension ($P = 0.007$) (Fisher et al. 2005).

3.5 Precipitating factors for postprandial hypotension

3.5.1 Effects of meal composition on blood pressure

The extent of the reduction in postprandial blood pressure is believed to depend, to a large part, on the macronutrient composition of the meal, and these differential effects are particularly discernable in older adults. Thus, modification of the type of food eaten may decrease the fall in blood pressure in older people and thus reduce the adverse effects of postprandial hypotension.

The size of a meal does not appear to affect postprandial blood pressure. In one study in healthy young adults, systolic and diastolic blood pressures did not change after ingestion of a high-carbohydrate meal of 1000, 2000, or 3000 kJ, although heart rate was higher after the 3000 kJ than 1000 kJ meal (Sidery and Macdonald 1994). Similarly, in another study of 4 healthy young subjects studied on four occasions, two after a large meal and two after a small meal, showed that the postprandial increase in heart rate was related to meal size, with a larger increase in heart rate seen after a bigger compared to a smaller meal (Waaler et al. 1991). In this same study, there were inconsistent effects on postprandial mean arterial pressure, with a sustained decrease in only half of the subject visits, after a large or small-sized meal, and no change in blood pressure in the other half (Waaler et al. 1991). Therefore, at least in healthy young adults, meal size does not significantly affect postprandial blood pressures. While the effects of meal size in healthy older subjects have not been evaluated, in another study of 7 subjects with primary chronic autonomic failure, aged 45 – 69 years, mean absolute supine systolic blood pressures were lower after consumption of three large mixed meals, compared to six small meals, of equal total energy (2500 kJ) throughout a 24-hour period (131 versus 151 mmHg, $P = 0.005$), with no significant effects of meal size on sitting or standing blood pressures (Puvi-Rajasingham and Mathias 1996). While the decrease in meal size in these subjects was effective in reducing the fall in postprandial blood pressure (Puvi-Rajasingham and Mathias 1996), this may have been a result of decreased carbohydrate intake with the smaller meals, however, the amount of carbohydrate in the mixed meals was not reported. The effect of meal size in attenuating postprandial blood pressure reductions in healthy older adults is unclear.

3.5.1.1 Effect of carbohydrates on postprandial blood pressure

In healthy young, non-hypertensive, adults, no significant changes in systolic or diastolic blood pressure have been demonstrated following ingestion of glucose or a carbohydrate-enriched meal (Westenend et al. 1985; Heseltine et al. 1990; Sidery et al. 1990; Sidery et al. 1991; Jones et al. 1998). In hypertensive young adults, systolic and diastolic blood pressures have been shown to fall by 5 ± 1 mmHg and 7 ± 2 mmHg respectively ($P < 0.01$), after a 75 g oral glucose load, and even more in older hypertensive adults, where systolic and diastolic blood pressures fell by 23 ± 4 mmHg and 14 ± 2 mmHg respectively ($P < 0.001$) after 75 g oral glucose (Jansen et al. 1987b). In another study of 10 hypertensive older adults, a 75 g oral glucose load also reduced systolic and diastolic blood pressures by up to 15 ± 3 mmHg and 13 ± 2 mmHg respectively ($P < 0.001$) (Jansen et al. 1990).

In healthy older adults, ingestion of carbohydrates, in the form of glucose, sucrose, or as a component of a mixed meal, results in a significant fall in blood pressure (Westenend et al. 1985; Jansen et al. 1987b; Potter et al. 1989; Jones et al. 1998; Visvanathan et al. 2005; Visvanathan et al. 2006). Studies in community-dwelling older adults have shown that supine systolic blood pressure decreases to a greater extent after a high-carbohydrate, compared to a high-fat or mixed, meal (mean maximal fall of 13, 0, and 7 mmHg respectively, $P < 0.05$) (Potter et al. 1989). In one of the earlier studies in healthy older adults, ingestion of a standard mixed meal resulted in a significant fall in systolic and diastolic blood pressures by 11.6 ± 2.5 mmHg and 10.5 ± 1.3 mmHg respectively (Westenend et al. 1985). Similarly, a 75 g oral glucose load significantly decreased mean arterial blood pressure by approximately 6 mmHg in healthy older

adults (Jansen et al. 1987b; Jones et al. 1998), and by up to 12 mmHg in patients with type 2 diabetes aged 39-79 years (Jones et al. 1998). In another study of 10 healthy older adults, ingestion of a high-carbohydrate meal resulted in a significant maximal fall in mean systolic blood pressure of 13 mmHg (Sidery et al. 1993). Thus both ageing and the presence of hypertension appear to enhance the fall in blood pressure induced by oral carbohydrate intake. Similarly, in healthy older adults, in the absence of gastric distension, a modest fall in systolic and diastolic blood pressure following intraduodenal infusion of glucose has been observed (O'Donovan et al. 2002; Gentilcore et al. 2006; Gentilcore et al. 2008).

The greater the amount of carbohydrate administered, the greater the blood pressure reduction. For example, in one study in healthy older adults, the fall in systolic blood pressure was substantially greater when glucose was infused intraduodenally at a rate of 3 kcal/min than when it was infused at 1 kcal/min (O'Donovan et al. 2002). Similarly, in another study of 10 healthy older adults, while a 75 g oral glucose load resulted in similar blood pressure reductions to a 25 g glucose load, in the first 60 minutes after ingestion, there was a greater reduction in systolic blood pressure from 105 minutes after ingestion (Jones et al. 2005). At similar glucose concentrations (12.5%), a 25 g glucose load in 200 mL resulted in a greater fall in postprandial blood pressure compared to 75 g glucose in 600 mL, suggesting that greater gastric distension, with the larger volume drink, attenuates the fall in blood pressure after ingestion of glucose (Jones et al. 2005). Varying the concentration of glucose solution infused into the small intestine has no apparent effect on postprandial blood pressure in healthy older adults (Gentilcore et al. 2006), whereas changing the volume of glucose solution ingested does

(Jones et al. 2005). The observations from the above studies support the concept that the postprandial fall in blood pressure after oral or intraduodenal glucose depends on the glucose load in the small intestine. When duodenal load is kept constant, the concentration of glucose does not affect postprandial blood pressure, and furthermore, gastric distension from higher volumes of solution attenuates the fall in blood pressure.

The effects of carbohydrates on blood pressure relate primarily to the glucose content, rather than other forms of carbohydrate. In healthy older adults, the ingestion of 50 grams of sucrose, a disaccharide which is hydrolysed by sucrase in the small intestine to glucose and fructose, decreased systolic blood pressure by a mean of 3 mmHg, which was comparable to the fall in blood pressure after 50 g of oral glucose (Visvanathan et al. 2005). However, an oral fructose drink did not affect blood pressure in healthy young (Jansen et al. 1987b) or older adults (Jansen et al. 1987b; Visvanathan et al. 2005). Similarly, oral fructose ingestion had no effect on blood pressure in hypertensive young and older adults (Jansen et al. 1987b). In patients with autonomic failure, the ingestion of xylose, a monosaccharide, decreased mean blood pressures up to a maximum of 8.9 ± 4 % from baseline, compared with a decrease of 34 ± 7 % following an oral glucose load (Mathias et al. 1989b). The small, and transient, effects of xylose on blood pressure may be secondary to osmotic effects of xylose within the small intestine, leading to a reduction in plasma volume (Mathias et al. 1989b). Therefore, ingestion of glucose and sucrose decreases blood pressure, whilst oral fructose and xylose have little, if any, effect on blood pressure.

3.5.1.2 Effect of fat on postprandial blood pressure

The effects of fat ingestion on blood pressure are controversial. In an early report of a 70 year old patient with a history of chronic alcohol abuse and autonomic neuropathy, ingestion of fat resulted in an 80 mmHg fall in mean arterial blood pressure which lasted for one hour after ingestion, compared with a 60 - 80 mmHg fall in blood pressure lasting 2 - 3 hours after ingestion of carbohydrate or a mixed meal (Hoeldtke et al. 1985). In a subsequent study of 5 patients with autonomic failure, ingestion of a lipid meal resulted in a maximal fall in mean arterial blood pressure of 20.5 ± 4.5% ($P <$ 0.01) from baseline, at 40 minutes after ingestion (Bannister et al. 1987). In another study, in hypertensive older adults, an oral fat load did not significantly change blood pressure (Jansen et al. 1990). The effects of fat on blood pressure appear to be different in healthy individuals. In healthy young adults, no significant changes in blood pressure have been demonstrated following a high-fat meal (Heseltine et al. 1990; Sidery et al. 1991). In a study of 10 healthy older individuals, aged 63 - 74 years, ingestion of a high-fat meal significantly decreased diastolic blood pressure by 5 mmHg 45 minutes after consumption, although there was no significant fall in systolic blood pressure (Sidery et al. 1993). In that study, the fall in diastolic blood pressure after the high-fat meal was smaller, and later than, the maximal fall after the high-carbohydrate meal (8 mmHg at 30 minutes) (Sidery et al. 1993). In another study of 7 healthy older adults, there were non-significant increases in systolic and diastolic blood pressures after a high-fat meal, over a 120 minute time period (Potter et al. 1989). In contrast, a recent study of 12 older adults, with a mean age of 72.2 years, showed a significant fall in systolic blood pressure (maximum of 15.6 ± 10.5 mmHg) following ingestion of a high-fat, which was not significantly different to a maximal fall of 13.4 ± 7.4 mmHg ($P <$

0.47) after an isoenergetic high-carbohydrate, drink (Visvanathan et al. 2006). In this same study, the onset of the fall in systolic blood pressure after the high-fat drink was evident later (26.5 minutes) than the high-carbohydrate drink (13.0 minutes, $P = 0.01$) (Visvanathan et al. 2006). Similar reductions in diastolic blood pressure were noted after the high-fat and glucose drinks (Visvanathan et al. 2006). In contrast to that study, where blood pressures were measured every 3 minutes over a 90 minute period (Visvanathan et al. 2006), the lack of a detectable effect of fat on systolic blood pressure in the previous study by Sidery et al. (1993) may have been due to the short duration of blood pressure measurements (60 minutes), and the frequency of measurements (every 15 minutes) (Sidery et al. 1993). Similarly, in the previous study by Potter et al. (1989), where blood pressure increased slightly after a high-fat meal, blood pressures were measured every 15 minutes up to one hour, then every 30 minutes up to two hours (Potter et al. 1989). Thus, it is possible that the effects of fat on blood pressure were not detected because of the frequency of haemodynamic measurements.

Infusion of fat directly into the small intestine also appears to have similar blood pressure-lowering effects to oral ingestion of fat. In a recent study of healthy older adults, intraduodenal infusion of fat resulted in comparable maximal reductions in systolic blood pressure (11.7 ± 4.8 mmHg) to those of intraduodenal glucose infused at the same rate (11.7 ± 2.8 mmHg) (Gentilcore et al. 2008). However, the maximal decrease in blood pressure occurred later after fat (46 ± 11 minutes) than glucose infusion (18 ± 3 minutes, $P = 0.02$) infusion (Gentilcore et al. 2008).

Thus, oral or intraduodenal fat appears to result in comparable reductions in blood pressure to carbohydrates, but the maximal effects of fat on blood pressure are seen later than with carbohydrate. The delayed hypotensive response may reflect the time taken to digest fat to free fatty acids in the small intestine.

3.5.1.3 Effect of protein on postprandial blood pressure

The effects of protein ingestion on blood pressure have not been extensively evaluated. In an early study in 7 healthy older adults (mean age 70.7 ± 1.9 years), ingestion of a high-protein meal resulted in lowering of systolic blood pressure (mean maximal fall in blood pressure of 13 mmHg), which was not significantly different to that of an iso-energetic high-carbohydrate meal (9 mmHg) (Potter et al. 1989). In the same study, when protein was ingested as a component of a mixed meal (24% protein, 49% carbohydrate, 27% fat), systolic blood pressure also fell by a maximum of 7 mmHg (Potter et al. 1989). In a recent study in 8 healthy older subjects (median age 74 years), intraduodenal infusion of glucose resulted in significantly lower systolic blood pressures between 0 and 90 minutes after the start of the infusion, than either intraduodenal fat, or protein infusions (P <0.05), given at the same rate of 3 kcal/min (Gentilcore et al. 2008). The maximum falls in systolic blood pressure after intraduodenal infusion of glucose, protein, and fat, did not, however, significantly differ (11.7 ± 2.8 mmHg, 11.0 ± 1.5 mmHg, and 11.7 ± 4.8 mmHg respectively; P = 0.97), although the maximal decrease in blood pressure occurred later with protein (33 ± 7 min) than with glucose (18 ± 3 min, P = 0.04) infusion (Gentilcore et al. 2008). Thus, oral or intraduodenal protein administration lowers blood pressure, apparently to a comparable extent to carbohydrate.

3.6 Therapeutic strategies for altering the postprandial response to nutrient ingestion

The hypotensive response to carbohydrate and fat may be mediated by their digestion products glucose and non-esterified free fatty acids respectively. Thus, therapeutic strategies which slow or inhibit absorption or digestion of carbohydrate or fat may ameliorate the reduction in postprandial blood pressure.

Guar gum, a viscous complex carbohydrate of vegetable origin, which slows gastric emptying and small intestinal glucose absorption, has been shown to attenuate postprandial reductions in systolic and diastolic blood pressure following glucose ingestion in older adults (Jones et al. 2001) and in patients with type 2 diabetes (Russo et al. 2003), and during intraduodenal glucose infusion (O'Donovan et al. 2005).

Acarbose, an alpha-glucosidase inhibitor, which slows the absorption of glucose in the small intestine, and delays gastric emptying of a sucrose drink (Ranganath et al. 1998) and both carbohydrate-containing and carbohydrate-free solid meals (Enc et al. 2001), also reduces the fall in blood pressure after sucrose ingestion (Sasaki et al. 2001; Gentilcore et al. 2005). In the earlier report of a 58 year old man with type 2 diabetes mellitus, the fall in postprandial systolic blood pressure was between 45 - 50 mmHg before, and 18 mmHg after, acarbose administration (Sasaki et al. 2001). In the more recent study by Gentilcore et al. (2005), acarbose was shown to attenuate the falls in systolic and diastolic blood pressure occurring from within 39 minutes of ingestion of an acarbose-containing sucrose drink, whilst the delayed gastric emptying effects of

acarbose were seen later, at approximately 90 minutes (Gentilcore et al. 2005). Thus, whilst postprandial blood pressure reduction is related to the rate of gastric emptying (Jones et al. 1998), the observed effects of acarbose on postprandial blood pressure are not fully accounted for by changes in the rate of gastric emptying (Gentilcore et al. 2005), and, presumably, reflect the reduced interaction of glucose with the small intestine.

Orlistat, a lipase inhibitor, reduces the absorption of fat in the small intestine, and also increases the rate of gastric emptying of a fat-containing drink or meal (Pilichiewicz et al. 2003; O'Donovan et al. 2004). In a study of 8 patients with diet-controlled type 2 diabetes mellitus, a single dose of orlistat added to a mixed carbohydrate/fat meal decreased systolic blood pressure by up to 11 mmHg compared to the mixed meal without orlistat, and the effects were significant in the first 30 minutes after ingestion (Figure 3.2) (O'Donovan et al. 2004). Previous weight-loss studies in overweight adults, which have included those with type 2 diabetes or hypertension, have shown a reduction in overall blood pressure and improvements in cardiovascular risk profile with the use of orlistat, and this may be, at least in part, related to the greater weight loss seen with orlistat compared to placebo (Bakris et al. 2002; Sharma and Golay 2002; Derosa et al. 2003; Derosa et al. 2005; Swinburn et al. 2005; Schneider et al. 2005; Zanella et al. 2006). However, in one randomised double-blind study, orlistat reduced blood pressure, whilst equivalent degrees of weight loss using sibutramine, a serotonin and noradrenaline reuptake inhibitor used in obesity management, failed to have an effect on blood pressure (Derosa et al. 2005). These findings may imply that the hypotensive effect of fat is accentuated, rather than attenuated, when fat digestion is

reduced by orlistat, thus the presence of fatty acids may not be mandatory to the hypotensive response. Some of the hypotensive effects of orlistat may be explained by the faster rate of gastric emptying of fat in the presence of orlistat found in previous studies in healthy adults (Schwizer et al. 1997; Borovicka et al. 2000; Chaikomin et al. 2006) and in patients with diet-controlled type 2 diabetes mellitus (Pilichiewicz et al. 2003; O'Donovan et al. 2004). It should be noted, however, that the gastric emptying rate of an entire fat-containing drink or meal is accelerated with orlistat (Borovicka et al. 2000; Pilichiewicz et al. 2003; O'Donovan et al. 2004; Chaikomin et al. 2006), thus the effect on gastric emptying may relate to carbohydrate or protein.

On the other hand, it has been suggested that the blood pressure-lowering effects of fat may be mediated by fat digestion products, non-esterified fatty acids. It is known that fat digestion (lipolysis of triglycerides to fatty acids) is required for its appetite suppressant effect (Matzinger et al. 2000; Feinle et al. 2003), for the stimulation of CCK, GLP-1, and PYY (Hildebrand et al. 1998; Feinle et al. 2003), suppression of ghrelin (Feinle-Bisset et al. 2005), slowing of gastric emptying (Carney et al. 1995; Pilichiewicz et al. 2003; O'Donovan et al. 2004), and pancreatic enzyme secretion (Hildebrand et al. 1998). In healthy older subjects, oral fat ingestion has been shown to decrease blood pressure more slowly than a high-carbohydrate drink, with the fall in systolic blood pressure corresponding with the rise in plasma triacylglycerols at approximately 30 minutes after ingestion of the drink (Visvanathan et al. 2006). If fat digestion is required for its hypotensive effect, inhibition of fat digestion should attenuate the fall in blood pressure after fat consumption, and this may have therapeutic implications for the management of postprandial hypotension in older persons.

3.7 Conclusions

Postprandial reductions in blood pressure have been consistently demonstrated in healthy older, but not younger, adults. The magnitude of the fall depends on macronutrient composition of the meal, with carbohydrates having a more rapid, but comparable, effect than fat or protein, in reducing postprandial blood pressure in healthy older adults. Thus, modification of the type of food eaten, in favour of macronutrients other than carbohydrates, may minimise the rapid fall in blood pressure after a meal. Gastric distension with a larger volume of water, along with the nutrient (eg. carbohydrate) load, may also attenuate the fall in postprandial blood pressure.

Furthermore, the addition of acarbose or guar gum to meals, which delays the rate of delivery of glucose to the small intestine by slowing the rate of gastric emptying and also intestinal glucose absorption, can reduce the postprandial fall in blood pressure, and may represent a therapeutic option in treating this clinically significant, potentially preventable, problem. Although ingestion of fat also decreases blood pressure, the onset of its effect is delayed compared with carbohydrate, which may possibly reflect the time taken for the absorption and digestion of fat into fatty acids. It is unknown whether the products of fat digestion are necessary for the hypotensive response to fat ingestion. The study described in Chapter 8 compares the effects of fat ingestion on blood pressure and heart rate in healthy young and older adults, and the effects of lipase inhibition.

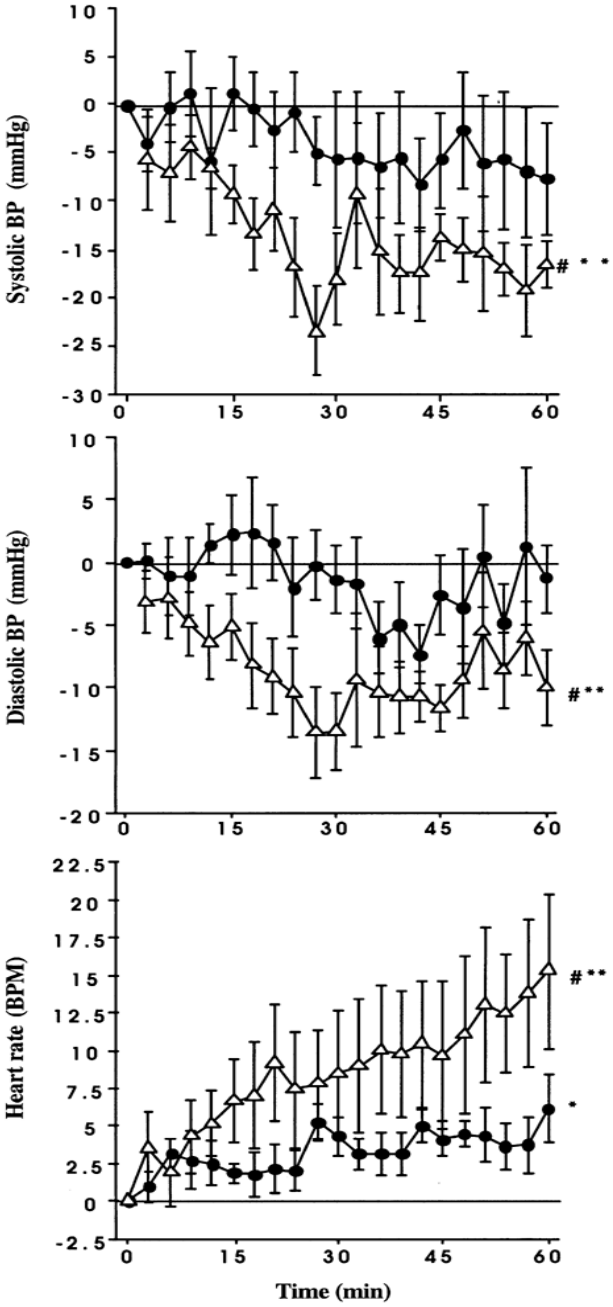


Figure 3.1: Effect of intraduodenal glucose infusion at a rate of either 1 kcal/min (●) or 3 kcal/min (△) on systolic blood pressure, diastolic blood pressure, and heart rate, in 8 healthy older subjects. Data are presented as changes from baseline and are mean values ± SEM. *change from baseline for 1 kcal/min ($P = 0.0001$); **change from baseline for 3 kcal/min ($P < 0.001$); #3 kcal/min vs 1 kcal/min ($P < 0.001$). Adapted from O'Donovan et al. (2002).

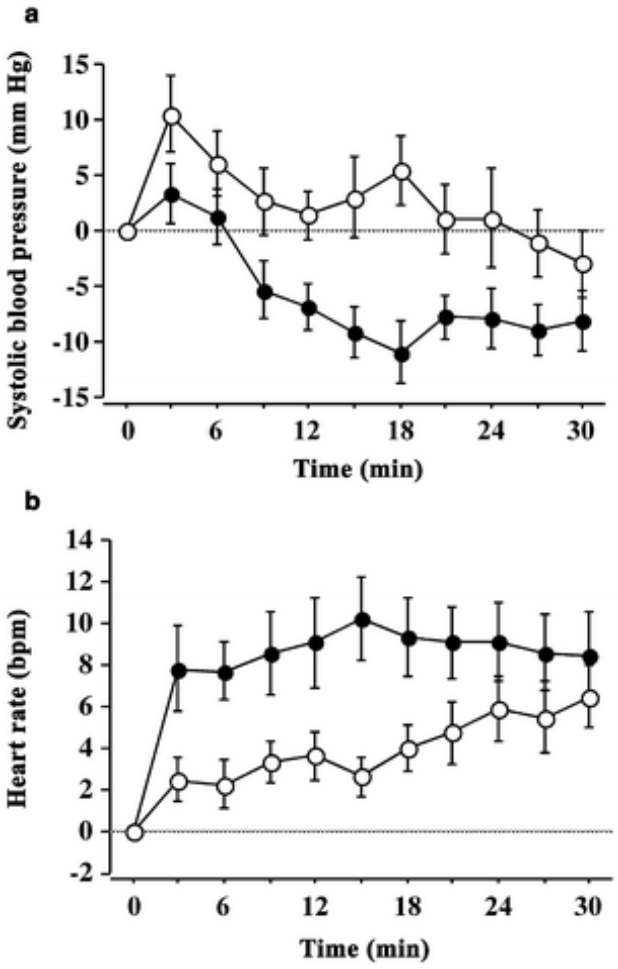


Figure 3.2: Effect of ingestion of a high-fat mashed potato meal containing orlistat (●) vs control (○) on (a) systolic blood pressure and (b) heart rate in 8 patients with type 2 diabetes. Data are presented as changes from baseline and are mean values ± SEM. $P <$ 0.03 for change in systolic blood pressure from baseline over the first 30 minutes following the orlistat-contaning meal; $P = 0.02$ for magnitude of the fall in systolic blood pressure with orlistat compared with control. Adapted from O'Donovan et al. (2004).

Chapter 4

VITAMIN D DEFICIENCY AND THE ROLE OF VITAMIN D IN GLUCOSE AND INSULIN METABOLISM AND INSULIN SENSITIVITY

4.1 Introduction

In recent years, actions of vitamin D, other than on calcium, bone and mineral metabolism have been identified. Vitamin D deficiency is common (Lips 2001; Souberbielle et al. 2001), as are abnormal glucose tolerance and diabetes mellitus (Wild et al. 2004), and these conditions may be connected. Both vitamin D deficiency and type 2 diabetes are conditions which carry a significant burden of illness and morbidity, and are becoming increasingly common as populations age.

This chapter summarises current knowledge of the relationships of vitamin D with glucose and insulin metabolism, and the possible pathophysiological role of vitamin D in disorders of glucose metabolism.

4.2 Vitamin D metabolism

Vitamin D is made in the skin in response to sunlight exposure and also obtained from the diet (Figure 4.1) (Lips 2001; Mathieu et al. 2005; Holick 2005). The major dietary sources of vitamin D are oily fish, eggs and meat. Even in countries where certain foods

are fortified with vitamin D, dietary intake of vitamin D alone is usually insufficient to maintain adequate serum levels of 25-hydroxyvitamin D (McKenna 1992; Holick 1994; Lips 2001; Nowson and Margerison 2002; Holick 2003; Mathieu et al. 2005; Holick 2005). Vitamin D3 (cholecalciferol) is converted by two hydroxylation steps via 25-hydroxyvitamin D to the biologically active metabolite 1,25-dihydroxyvitamin D, which acts by binding to nuclear vitamin D receptors within cells (Holick 2005). Vitamin D plays a major role in calcium metabolism and maintenance of bone mineralisation (Norman et al. 1982; Reichel and Norman 1989).

Circulating levels of 25-hydroxyvitamin D are generally used as a marker of vitamin D status. They are usually lowest in winter and highest in summer (Bouillon et al. 1987; Dawson-Hughes et al. 1997; Holick 2001; Working Group of the Australian and New Zealand Bone and Mineral Society 2005), due to differences in day length and, hence, sun exposure. Serum 25-hydroxyvitamin D concentrations decrease with increasing age due to reduced sun exposure and skin production in response to sunlight (MacLaughlin and Holick 1985; Reid et al. 1986; Bouillon et al. 1987; McKenna 1992; Need et al. 1993; Holick 2003). There are also regional differences in vitamin D levels, due to variations in sunlight exposure, latitude, ultraviolet radiation levels, skin pigmentation, and dietary intake of vitamin D (McKenna 1992; Lucas et al. 2005).

4.3 Vitamin D insuficiency/deficiency

4.3.1 Definition

Vitamin D deficiency, often with resultant osteomalacia and myopathy, is present at serum levels of 25-hydroxyvitamin D below 12.5 nmol/L (Need 2006). At higher levels

there is debate as to what constitutes vitamin D insufficiency and the plasma 25-hydroxyvitamin D concentration at which treatment should be initiated (Lips 2001; Vieth 2004). As serum 25-hydroxyvitamin D concentrations fall below an optimum level, calcium homeostasis is affected and there is a compensatory increase in serum parathyroid hormone (PTH) level (Need 2006). This seems to occur most predictably below 50 nmol/L (Need et al. 2004) and this cut-off point is widely considered to represent vitamin D insufficiency in need of treatment (Malabanan et al. 1998; Grey et al. 2005; Vanlint 2005).

4.3.2 Epidemiology

Older people are at particular risk of vitamin D deficiency due to their reduced skin vitamin D production (MacLaughlin and Holick 1985; Need et al. 1993; Holick 2003). Other groups at risk include dark-skinned individuals and women veiled for religious reasons. Certain conditions predispose to malabsorption of vitamin D, including gastric bypass surgery for obesity (Rogers et al. 1980) and untreated, or refractory coeliac disease (Keaveny et al. 1996).

Borderline, or inadequate body vitamin D stores are common, and the rates of vitamin D insufficiency/deficiency in various populations are outlined in Table 4.1. For example, in the Third National Health and Nutrition Examination Survey (NHANES III 1988-1994) in the United States, the prevalence of vitamin D insufficiency (defined as 25-hydroxyvitamin D concentrations below 62.5 nmol/L) was as high as 57% during winter in lower latitude regions (Looker et al. 2002). In one study 69 - 81% of South Asian children living in England had levels of 25-hydroxyvitamin D less than 50

nmol/L (Lawson and Thomas 1999), with up to one third having 25-hydroxyvitamin D levels less than 25 nmol/L (Lawson and Thomas 1999).

4.3.3 Established consequences of vitamin D insufficiency/deficiency

Vitamin D deficiency causes rickets in children (Lips 2001) and osteomalacia in adults (Lips 2001; Holick 2003). It is associated with increased bone turnover, reduced bone density, increased fracture rates (Villareal et al. 1991; Chapuy et al. 1992; Mowe et al. 1999; Pfeifer et al. 2000; Bischoff et al. 2003; Flicker et al. 2003), reduced muscle strength and even myopathy (Bischoff et al. 1999; Visser et al. 2003; Working Group of the Australian and New Zealand Bone and Mineral Society 2005) in adults. There is an inverse relationship between circulating 25-hydroxyvitamin D levels and the rate of falls in older people, which is likely to be related to effects of low vitamin D levels on neuromuscular function, including reduced muscle strength and walking speed, and increased body sway (Flicker et al. 2003). Correction of vitamin D insufficiency, or deficiency with vitamin D replacement (often combined with calcium), has been shown to increase bone density (Dawson-Hughes et al. 1991; Bischoff-Ferrari et al. 2004) and reduce fracture rates (Chapuy et al. 1992; Jackson et al. 2006), improve muscle strength, and reduce the risk of falls (Flicker et al. 2005). These effects appear to be dependent on a dose of at least 400-600IU vitamin D daily (Chapuy et al. 1992; Jackson et al. 2006), which is probably the dose required to increase serum 25-hydroxyvitamin D to within the normal range.

4.3.4 Newly-identified associations with vitamin D deficiency

A number of possible "novel" pathological consequences of vitamin D deficiency have been identified in recent years, mostly on the basis of associations that have yet to be examined for causation by appropriate intervention studies in humans. These include possible increases in the risk of cancer, particularly of the colon, breast, ovary and prostate (Garland et al. 2006; Wactawski-Wende et al. 2006), ischaemic heart disease (Enquselassie et al. 1993; Grimes et al. 1996), and hypertension (Kristal-Boneh et al. 1997; Pfeifer et al. 2001). Vitamin D is also immune-modulating (Walters 1992). Recent in vitro studies have revealed vitamin D-dependent pathways in the microbicidal activity of monocytes and macrophages (Liu et al. 2006).

4.4 Associations between Vitamin D, glucose and insulin

A number of observations have linked vitamin D deficiency to alterations in circulating glucose and insulin concentrations and, possibly, insulin sensitivity.

4.4.1 Type 1 diabetes mellitus

Administration of 1,25-dihydroxyvitamin D and its analogues inhibits the development of insulitis and the onset of type 1 diabetes in non-obese, diabetic-prone, mice (Mathieu et al. 1992; Mathieu et al. 1994; Mathieu et al. 1995; Gregori et al. 2002; Zella et al. 2003), and reduces the propensity of rats to develop streptozocin-induced diabetes (Del Pino-Montes et al. 2004). Administration of an analogue of 1,25-dihydroxyvitamin D to mice with immune diabetes stops progression of the inflammation in pancreatic islet cells, by altering the T lymphocyte response (Gregori et al. 2002).

Consistent with these results in animals, studies in humans suggest that increased vitamin D intake early in life may reduce the subsequent risk of type 1 diabetes. In one study, infants who received dietary supplementation with cod liver oil, a rich source of vitamin D, during their first year of life were found to have a reduced risk of type 1 diabetes (Stene and Joner 2003). Similarly, the EURODIAB study found a 33% reduction in the risk of developing childhood-onset type 1 diabetes before the age of 15 years in those who received vitamin D supplementation during the first year of life compared to non-supplemented children (combined odds ratio 0.67 [95% CI 0.53 - 0.86]) (EURODIAB Substudy 2 Study Group 1999), and a study in Finland found an association between dietary vitamin D supplementation in the first year of life and a reduced risk of type 1 diabetes, even after adjustment for social confounders (Hypponen et al. 2001). In a study of 233 females, maternal dietary vitamin D intake during the third trimester was associated with a decreased risk of anti-islet cell autoantibodies in their offspring (Fronczak et al. 2003), suggesting that maternal intake of vitamin D during pregnancy may protect against the subsequent development of type 1 diabetes in their offspring. This remains uncertain, however, as, in another study, maternal use of cod liver oil or vitamin D supplements during pregnancy was not associated with a reduced risk of the offspring subsequently developing type 1 diabetes (Stene and Joner 2003).

If vitamin D supplementation in early childhood does protect against the subsequent development of type 1 diabetes this may reflect the immunosuppressant properties of vitamin D (EURODIAB Substudy 2 Study Group 1999).

4.4.2 Insulin resistance and type 2 diabetes mellitus

Several lines of evidence from animal and human studies suggest a possible link between vitamin D deficiency and insulin resistance / type 2 diabetes. Non-obese young diabetes-prone mice fed on a vitamin D-depleted diet have an increased risk of developing glucose intolerance, diabetes at an earlier age, and more severe diabetes mellitus than control, non-vitamin D-depleted mice (Giulietti et al. 2004). Vitamin D has beneficial effects in animal models of diabetes. In obese Wistar rats, with type 2 diabetes induced by streptozocin, vitamin D3 (cholecalciferol) treatment for two weeks significantly reduced plasma glucose concentrations by approximately 40% compared to glucose levels shortly after diabetes onset (de Souza Santos and Vianna 2005).

Studies in humans also suggest a possible connection between vitamin D and glucose metabolism. For example, in the NHANES III cross-sectional survey of American adults aged 40 to 74 years, serum 25-hydroxyvitamin D levels were inversely related to the presence of type 2 diabetes and to increased insulin resistance, with odds ratios for diabetes of 0.25 (95% CI 0.11 - 0.6) in non-Hispanic whites and 0.17 (95% CI 0.05 - 0.37) in Mexican Americans with 25-hydroxyvitamin D levels > 81 nmol/L compared to those with levels < 43.9 nmol/L (Scragg et al. 2004). An inverse relationship has also been identified between serum 25-hydroxyvitamin D concentrations and the prevalence of the metabolic syndrome in American adults (Boucher 1998; Ford et al. 2005), with approximately twice the rate (27.5% vs 13.5%) in those with 25-hydroxyvitamin D levels ≤ 48.4 nmol/L compared to those with levels ≥ 96.4 nmol/L (Ford et al. 2005). Regular consumption of fish, a rich source of vitamin D, has been associated with a 60% reduction in the risk of developing glucose intolerance (Feskens et al. 1991).

In general, observations derived from population studies tend to be supported by laboratory studies. In one study of healthy adults there was a negative correlation between plasma 25-hydroxyvitamin D and glucose concentrations following an oral glucose tolerance test (Chiu et al. 2004), and a positive correlation between 25-hydroxyvitamin D concentrations and insulin sensitivity as measured by a hyperglycaemic clamp, even after correction for confounding factors, such as body composition as measured by body mass index ($R = 0.25$, $P = 0.007$) (Chiu et al. 2004). Similarly, in a study of 142 elderly Dutch men, during an oral glucose tolerance test serum 25-hydroxyvitamin D levels were inversely related to insulin ($R = -0.18$ to -0.25, $P < 0.05$) and glucose ($R = -0.26$, $P < 0.01$) concentrations (Baynes et al. 1997). In a recent study of 753 postmenopausal women with low bone density, fasting serum glucose and 25-hydroxyvitamin D concentrations were inversely correlated ($R = -0.15$, $P < 0.001$) (Need et al. 2005).

The observed associations in humans between vitamin D and insulin and glucose metabolism have not yet been confirmed by intervention studies and, hence, causal association has not been established. There are alternative possible explanations. For example, increased participation in outdoor exercise could increase both serum 25-hydroxyvitamin D concentrations due to greater sun exposure and insulin sensitivity due to effects of exercise, unrelated to vitamin D. The lack of confounding effects of reported leisure physical activity levels on the inverse association between 25-hydroxyvitamin D levels and the risk of type 2 diabetes in the NHANES III 1988-1994 study (Scragg et al. 2004), might suggest, however, that the association between

vitamin D insufficiency and increased diabetes risk is not mediated by a reduction in outdoor exercise (Scragg et al. 2004).

4.5 Possible mechanisms of action of vitamin D on glucose and insulin metabolism

If vitamin D does alter glucose and insulin metabolism, several mechanisms could be responsible (Figure 4.2).

4.5.1 Stimulation of insulin secretion

There is evidence that vitamin D may stimulate pancreatic insulin secretion directly. Vitamin D exerts its effects via nuclear vitamin D receptors (Zeitz et al. 2003), which are found in a wide variety of tissues, including T and B lymphocytes, skeletal muscle and the pancreatic islet beta cells (Walters 1992). Glucose- and sulphonylurea-stimulated insulin secretion is less from islets of vitamin D deficient rats, both in vitro and in vivo, than from islets of vitamin D sufficient rats, or vitamin D deficient rats treated with vitamin D (Norman et al. 1980; Chertow et al. 1986; Cade and Norman 1987). Some, but not all of this reduction, may be due to a concomitant decrease in reduction in food intake (Chertow et al. 1986).

Circulating insulin concentrations are also lower, and blood glucose concentrations higher, in mice with non-functioning vitamin D receptors than in wild-type mice (Zeitz et al. 2003). Interestingly, however, these vitamin D receptor mutant mice had preserved pancreatic islet mass and composition, with no change in the rate of formation of new beta cells (Zeitz et al. 2003). Acute administration of small, single

doses of 1,25-dihydroxyvitamin D3 to vitamin D-deficient rats has been shown to increase insulin secretion and reduce the blood glucose response to an intravenous glucose load (Cade and Norman 1987). An improvement in insulin secretion and the insulin response to an intravenous glucose tolerance test is also seen with 1,25-dihydroxyvitamin D3 replacement in vitamin D-deficient rabbits (Nyomba et al. 1984). The beneficial effects of vitamin D on glucose-induced insulin secretion are seen within three hours of its administration, while fasting glucose and insulin concentrations are apparently not affected by vitamin D treatment (Cade and Norman 1987). Intravenous administration of 1,25-dihydroxyvitamin D3 increases insulin concentrations in rats (Ishida et al. 1983) and prevents streptozocin-induced diabetes, an effect not seen in mice lacking the vitamin D receptor (Mathieu et al. 2001).

The stimulatory effects of vitamin D on insulin secretion may only be manifest when calcium levels are adequate. Glucose-stimulated insulin secretion is lower in vitamin D-deficient rats when concurrent hypocalcaemia is not corrected, than when it is (Beaulieu et al. 1993), while in vitro glucose-stimulated insulin release from pancreatic islet cells is stimulated by 1,25-dihydroxyvitamin D3 treatment in the presence, but not absence, of relatively high levels of calcium (Ishida et al. 1983). Dietary correction of hypocalcaemia reduces blood glucose concentrations in wild-type mice, but not in mice lacking a functioning vitamin D receptor (Zeitz et al. 2003).

Individual case reports suggest that in vitamin D deficient individuals, vitamin D treatment may increase insulin secretion (Kumar et al. 1994a). In a non-randomised study of ten women with type 2 diabetes, seven of whom were vitamin D-deficient at

baseline, there was a statistically significant 34% increase from baseline in first-phase insulin secretion during an intravenous glucose load, following one month of treatment with oral cholecalciferol (D3) 1332 IU daily (Borissova et al. 2003).

Vitamin D administration has thus been shown to increase insulin secretion in both animal (Cade and Norman 1987) and human (Kumar et al. 1994a; Borissova et al. 2003) studies, and to reduce the blood glucose response to intravenous glucose in rodents (Cade and Norman 1987).

4.5.2 Stimulation of parathyroid hormone (PTH)

There is some evidence that increased PTH activity is associated with, and possibly causes, reduced insulin sensitivity. The prevalence of both impaired glucose tolerance and type 2 diabetes is increased in patients with primary hyperparathyroidism (Taylor 1991; Taylor and Khaleeli 1997; Procopio et al. 2002). In one study of patients with primary hyperparathyroidism, insulin sensitivity during an intravenous glucose infusion was less than in control subjects, although fasting levels of glucose and insulin were comparable (Kumar et al. 1994b). Plasma PTH concentrations were found to be inversely related to insulin sensitivity in a study of healthy adults (Chiu et al. 2000), although administration of 'physiological' doses of parathyroid hormone by intravenous infusion to healthy men in a double-blinded randomised placebo-controlled study did not affect insulin sensitivity measured during an hyperinsulinaemic euglycaemic clamp (Fliser et al. 1997).

The secondary hyperparathyroidism that often accompanies vitamin D deficiency may thus mediate some of the effects of vitamin D on insulin sensitivity and glucose tolerance.

4.5.3 Effects on insulin sensitivity

There is little, and inconsistent, information about the effects on insulin sensitivity of alterations in vitamin D status. Administration of vitamin D has been shown to decrease, rather than increase, uptake of glucose by rat adipocytes in vitro (Huang et al. 2002). Administration of vitamin D to ethnically Asian British people with both vitamin D deficiency and diabetes was found to decrease insulin sensitivity (Taylor and Wise 1998). In contrast, in a study of men with impaired glucose tolerance there was a positive relationship between insulin sensitivity and serum 25-hydroxyvitamin D, although subsequent treatment of these men with the active vitamin D alphacalcidiol for up to 18 months did not affect insulin sensitivity as evaluated by an intravenous glucose tolerance test (Lind et al. 1989). In ten women with type 2 diabetes treated with vitamin D for one month, there was a non-significant increase in insulin sensitivity after vitamin D therapy, as well as an increase in first-phase insulin secretion (Borissova et al. 2003), while in another study of patients with type 2 diabetes, there was no change in insulin secretion or sensitivity during a meal-stimulation test after treatment with 1,25-dihydroxyvitamin D at a dose of 1.0 microgram per day for four days (Orwoll et al. 1994). In women with gestational diabetes mellitus, a single intravenous injection of 1,25-dihydroxyvitamin D lowered insulin levels, consistent with an increase in insulin sensitivity, whereas oral administration had no effect (Rudnicki and Molsted-Pedersen 1997). In a more recent double-blinded, placebo-controlled study in 445 patients with

osteoporosis followed up over three years, there were no differences in fasting plasma glucose between those treated with vitamin D3 (700IU daily) plus calcium compared to placebo (Pittas et al. 2007). A sub-analysis of this study showed that, those with impaired fasting glucose at baseline demonstrated a smaller rise in fasting glucose over the three-year study period with vitamin D therapy compared to placebo (0.02 ± 0.09 mmol/L vs 0.34 ± 0.11 mmol/L respectively, $P = 0.042$), although only after adjustment for various diabetes risk factors (Pittas et al. 2007). Insulin resistance, assessed by HOMA, increased in the placebo-treated group, but not in the vitamin D-treated impaired fasting glucose group (0.91 ± 0.31 vs 0.05 ± 0.19 respectively, $P = 0.031$). However, there was no difference between treatment groups in the number of subjects who developed diabetes (Pittas et al. 2007). It is not possible to determine whether these observed effects were related to vitamin D therapy, calcium therapy or both, as only combined therapy was given. Calcium treatment alone may have beneficial effects on glucose homeostasis (Choi et al. 2005; Pittas et al. 2006). Another recent double-blind placebo-controlled study of vitamin D3 plus calcium in 63 overweight pre-menopausal women undergoing a weight-loss program showed that, although fasting plasma glucose and insulin concentrations decreased over 15 weeks, weight loss alone could have accounted for these changes, and there were no significant treatment effects with combined vitamin D and calcium therapy (Major et al. 2007).

Patients with chronic renal failure on dialysis commonly have co-existent insulin resistance (DeFronzo et al. 1981). Short and longer term treatment with vitamin D has been shown to increase glucose uptake, insulin secretion (Mak 1992a), and insulin

sensitivity (Mak 1992b; Kautzky-Willer et al. 1995; Gunal et al. 1997; Mak 1998) in patients on haemodialysis.

Thus, in individuals with diabetes mellitus, 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D treatment may increase insulin secretion and improve glucose tolerance (Rudnicki and Molsted-Pedersen 1997; Borissova et al. 2003), but the effects in healthy individuals or in those with impaired glucose tolerance (Lind et al. 1989) remain unclear.

4.6 Vitamin D receptor polymorphisms

Four vitamin D receptor restriction site polymorphisms have been described, and linked to susceptibility to type 1 diabetes (Pani et al. 2000; Zeitz et al. 2003). A study in a Bangladeshi Asian population found that changes in glucose and insulin secretion after a standard glucose tolerance test were independently associated with vitamin D receptor gene polymorphisms, irrespective of vitamin D status (Hitman et al. 1998). In families of type 1 diabetic subjects, there is an association between vitamin D receptor polymorphisms and type 1 diabetes (McDermott et al. 1997; Pani et al. 2000). Vitamin D receptor genotype was found to be predictive of fasting glucose levels in a large group of younger healthy males, but only in those who had low levels of physical activity (Ortlepp et al. 2003). In this same, military, population the high physical activity group engaged in outdoor exercises such as long-distance running and cycling, and serum vitamin D levels were not performed to allow comparison to those with low physical activity levels (Ortlepp et al. 2003).

Interactions of 1,25-dihydroxyvitamin D with its receptor thus appear to be important in its subsequent effects on glucose metabolism.

4.7 Conclusions

Low plasma 25-hydroxyvitamin D levels in animals are associated with higher glucose concentrations and reduced insulin sensitivity, and may also be associated with an increased risk of developing type 1 diabetes (Giulietti et al. 2004). These associations are also evident in humans, with negative correlations between serum 25-hydroxyvitamin D concentrations and risk of developing glucose intolerance, type 2 diabetes and the 'metabolic syndrome' (Boucher 1998; Scragg et al. 2004; Ford et al. 2005). Animal studies suggest that correcting vitamin D insufficiency / deficiency increases insulin secretion and reduces glucose concentrations (Cade and Norman 1987; Huang et al. 2002). In type 2 diabetes, there is a suggestion that insulin secretion increases and insulin sensitivity improves following vitamin D treatment (Borissova et al. 2003), however, the available evidence in both vitamin D-deficient and diabetic individuals is conflicting (Lind et al. 1989; Orwoll et al. 1994).

Although there is mounting evidence linking vitamin D deficiency with abnormalities of glucose and insulin metabolism, the links clearly require further evaluation. Comparisons of 25-hydroxyvitamin D levels, glucose tolerance, and insulin sensitivity in healthy individuals with varying levels of indoor and outdoor physical activity would be useful to provide insight into the possible confounding role of exercise. The role of vitamin D treatment of vitamin D deficient-, and possibly even vitamin D replete-,

individuals in the prevention and treatment of diabetes, insulin resistance and the metabolic syndrome, requires further exploration.

The intervention study described in Chapter 10 assesses the effects of vitamin D therapy in individuals with low vitamin D concentrations in the setting of normal or impaired glucose tolerance, particularly evaluating the glucose and insulin response to an oral glucose load.

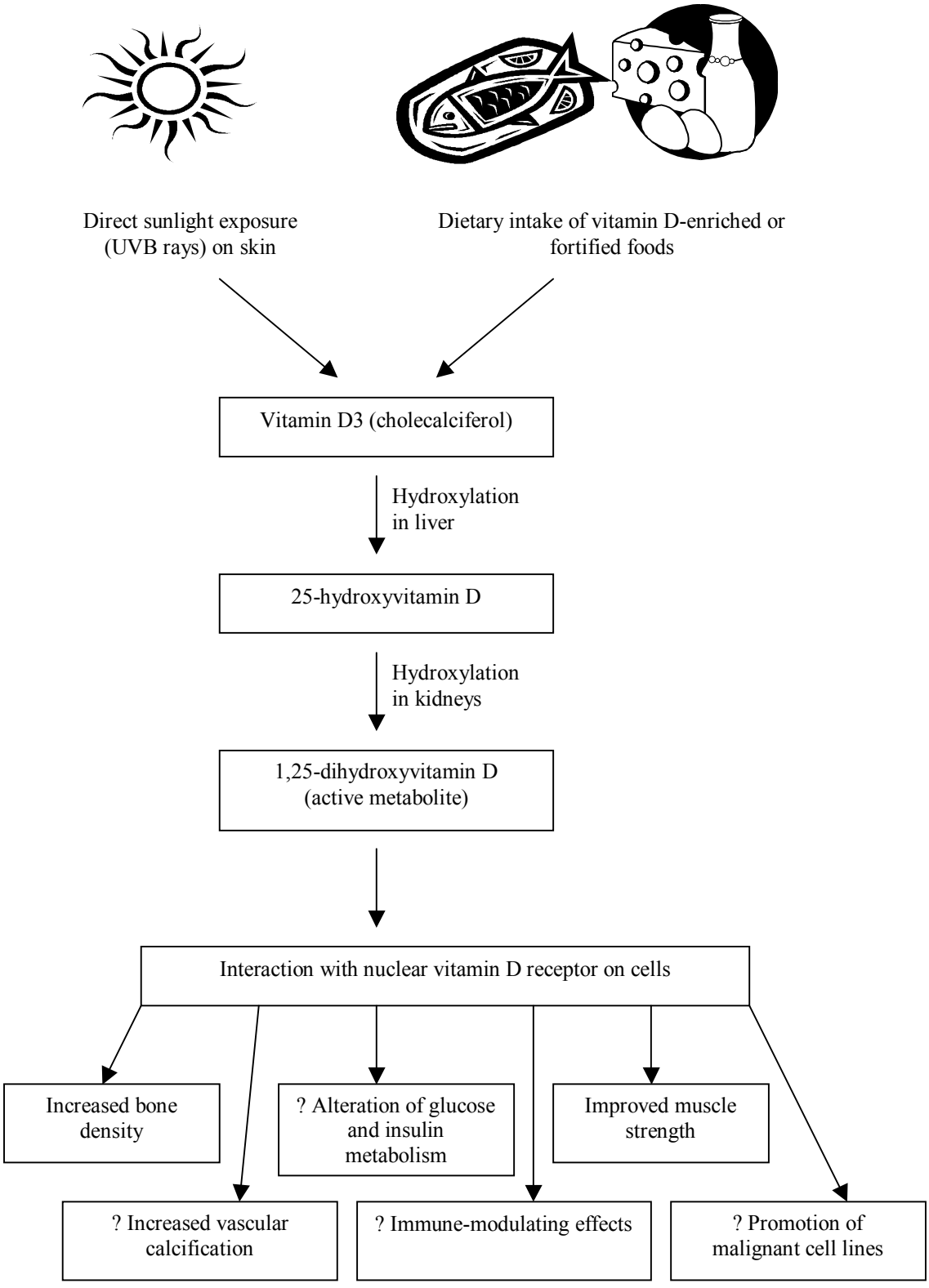


Figure 4.1: Sources of vitamin D and its metabolism.

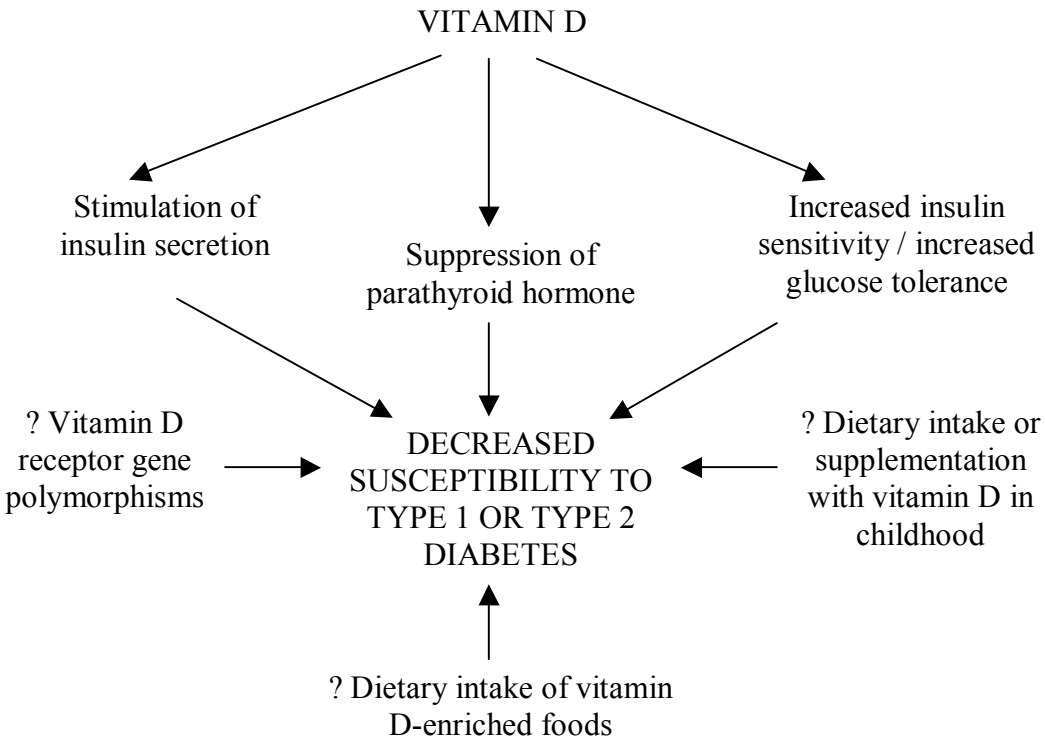


Figure 4.2: Potential mechanisms of action of vitamin D on glucose and insulin metabolism.

Table 4.1: Rates of vitamin D insufficiency/deficiency in various populations.

Study	**Population**	**Age (years); gender**	**Serum 25-hydroxyvitamin D concentration (nmol/L)**	**Prevalence of vitamin D insufficiency/ deficiency (%)**
Looker and Gunter, 1998	Community-dwelling U.S. adults	≥ 20 years; males and females	≤ 37.5	5 - 15%
Thomas et al., 1998	U.S. adults admitted to hospital	62 ± 19 years; males and females	≤ 37.5	57%
Lawson and Thomas, 1999	Children living in England; Pakistani, Indian, or Bangladeshi ethnicities	1.5 - 2.5 years; males and females	< 50	69 - 81%
Looker et al., 2002	Community-dwelling U.S. adults	12 – 29 years; males and females	< 37.5	4 - 6% in non-Hispanic whites; 32% in non-Hispanic blacks; 7 - 19% in Mexican Americans
Looker et al., 2002	Community-dwelling U.S. adults	30 – 59 years; males and females	< 37.5	7 - 10% in non-Hispanic whites; 31 - 48% in non-Hispanic blacks; 13 - 24% in Mexican Americans
Looker et al., 2002	Community-dwelling U.S. adults	≥ 60 years; males and females	< 37.5	5 - 12% in non-Hispanic whites; 27 - 43% in non-Hispanic blacks; 9 - 23% in Mexican Americans
Scragg et al., 2004	Community-dwelling U.S. adults	≥ 20 years	≤ 43.9	9.8% in non-Hispanic whites; 49.8% in non-Hispanic blacks; 24% in Mexican Americans
Need et al., 2005	Ambulant postmenopausal women attending Osteoporosis Clinics	35 - 94 years (mean = 63 years)	< 40 - 50	51% had vitamin D concentration < 60 nmol/L; 35% had vitamin D concentration < 50 nmol/L; 22% had vitamin D concentration < 40 nmo/L.

Chapter 5

COMMON METHODOLOGIES

5.1 Introduction

The methods presented in this chapter are techniques common to the studies presented in Chapters 6 - 10 of this thesis. All of these techniques have been validated previously and are accepted methods of assessment of appetite and eating behaviour. Where a new technique has been established, this is described in detail in the relevant chapter.

5.2 Subjects

Healthy young subjects (18 - 35 years) were recruited by advertisement, for the studies described in Chapters 7 and 8. Healthy older subjects (65 - 85 years) were recruited by advertisement, for the studies described in Chapters 8 and 9. Healthy subjects aged 18 - 64 years were recruited by advertisement for the studies described in Chapter 6.

No subject had a history of gastrointestinal illness, significant respiratory, renal, hepatic or cardiovascular disease, diabetes, epilepsy, and none was taking medication known to influence gastrointestinal function. All subjects were non-smokers, and none had a current alcohol intake of > 20 g per day.

For studies involving the administration of intravenous cholecystokinin, or oral cholecalciferol (Chapters 9 and 10), blood samples were taken to exclude those with

abnormalities in serum alanine aminotransferase or aspartate aminotransferase (greater than three times the upper limit of normal), serum creatinine (> 120 μmol/L), or in calculated creatinine clearance (< 50mls/min; calculated using the Cockcroft-Gault formula: creatinine clearance = [(140-age) × weight)]÷[0.814 × serum creatinine (μmol/L)]; multiplied by 0.85 for women).

Subjects were asked to refrain from alcohol for 24 hours prior to each study day and to maintain their usual physical activity during the study period. Written, informed consent was obtained from each subject prior to their participation in a study. All subjects were informed that they could withdraw at any time. Subjects were offered an honorarium for their participation.

5.3 Ethics approval

All protocols were approved by the Royal Adelaide Hospital Research Ethics Committee (Chapters 6 - 10) and, in addition, the Royal Adelaide Hospital Investigational Drug Sub-Committee for studies involving medication or drug administration (Chapters 7 - 10), prior to the recruitment of subjects.

5.4 Study environment

All studies presented in this thesis were conducted in the clinical research study rooms of the Department of Medicine at the Royal Adelaide Hospital. Subjects remained in these rooms for the duration of the study, except to use the toilet. Subjects were thus isolated from the external environment. The room temperature was kept constant at 24ºC by reverse-cycle air conditioning. Subjects were permitted to read, listen to the

radio or pre-recorded music, or to study. Subjects were kept resting in bed with the head of the bed elevated to 90º for the studies described in Chapters 7, 8, and 9 or seated at 90º for the studies described in Chapters 6 and 10. During buffet meals (Chapter 9), subjects were not allowed to read or converse with investigators, and were seated at a table to reproduce normal eating arrangements.

5.5 Assessment of feeding behaviour

5.5.1 Three factor eating restraint questionnaire

The studies presented in Chapters 7 - 9 evaluated the effects of specific interventions on appetite sensations and energy intake in healthy individuals. Subjects were thus excluded if there was evidence of 'abnormal' eating behaviour. The Three-Factor Eating Questionnaire (Stunkard and Messick 1985) is the most commonly used method for screening for 'abnormal' eating behaviour. This questionnaire consists of 51 questions and was devised to measure three factors related to eating habits: (1) cognitive restraint of eating, (2) disinhibition, and (3) hunger. Subjects were excluded if there was evidence of 'dietary restraint' on Factor 1, described as the tendency of individuals to restrict food intake in order to control their body weight (Herman and Mack 1975). In the study by Stunkard and Messick (1985), Factor 1 scores were 14.3 ± 3.6 (mean ± SEM) for 'restrained' eaters, compared to a lower score of 6.0 ± 5.5 among 'unrestrained' eaters (Stunkard and Messick 1985). Prior to participation in the studies described in Chapters 7 - 9, subjects were excluded if they recorded a score of > 11, indicating 'dietary restraint'. This cut-off score has been used in previous studies assessing appetite in young and older subjects, in which interventions such as

intravenous cholecystokinin (versus saline) and different nutrient preloads, were found to have effects on appetite and food intake (Rolls et al. 1990; MacIntosh et al. 2001).

5.5.2 Diet diaries

Validated methods of measuring food and energy intake in an individual include 24-hour food recalls (Beaton et al. 1979), 1-day records (Hartman et al. 1990), 2-day records (Hartman et al. 1990), and longer records over 3, 7, or 14 days (Basiotis et al. 1987; Bingham 1994; Bathalon et al. 2000).

Recording of food intake becomes less accurate towards the end of a 7-day food diary, due to declining subject compliance, and a 3-day or 5-day food diary can provide a valid estimate of dietary intake, especially if at least one weekend day is included (Gersovitz et al. 1978). In the study described in Chapter 6, subjects were instructed to complete a 3-day food diary (two weekdays and one weekend day), to evaluate baseline energy intake (Appendix 1). In the study described in Chapter 9, subjects were instructed to complete a 5-day food diary (three weekdays and two weekend days), to measure baseline energy intake, prior to commencement of the first 14-day diet period. Subjects were provided with detailed instructions as to how to complete each of the food diaries, including the need for accurate weighing and recording of all food and drink consumed.

5.5.3 Visual analogue scales

The most common form of assessment of appetite perceptions is the visual analogue scale (VAS) questionnaire. The reproducibility and validity of the VAS in measuring symptoms related to food intake, and in predicting subsequent food intake has been assessed previously in young and older subjects (Flint et al. 2000; Parker et al. 2004). In an analysis of four studies assessing appetite, with a total of 45 healthy young adults and 45 healthy older adults, energy intake at a test meal was positively correlated with pre-meal VAS ratings of hunger (R = 0.33, $P < 0.0001$) and desire to eat (R = 0.38, $P < 0.0001$) in older adults, and inversely related to fullness in both young (R = -0.21, $P = 0.02$) and older adults (R = -0.195, $P = 0.03$) (Parker et al. 2004).

The VAS consists of an 100 mm horizontal line with two extremes of a particular sensation, and subjects were instructed to place a vertical mark on each line to indicate how they were feeling at each point in time. In all VAS questionnaires used in this thesis (Chapters 7 and 9), eight variables were evaluated, which included appetite sensations (hunger, fullness, nausea, satiety, desire to eat, prospective consumption of food), and non-appetite sensations (anxiety, drowsiness), such that subjects would not be aware of the real purpose of the questionnaire (Appendix 2). For example, the two extremes of sensation 'hungry' versus 'not hungry' and 'empty' versus 'full'. Prospective consumption was assessed by asking 'How much food do you think you could eat?', and desire to eat by asking 'How strong is your desire to eat?'. Each of the sensations was then quantified as the distance (mm) along the horizontal line to the point of the vertical mark.

5.5.4 Food intake

In Chapter 9, subjects were offered a cold buffet style meal, in quantities in excess of what they would normally be expected to consume (Lavin et al. 1996). The total energy content of the food offered was approximately 11,800 kJ. The buffet meal consisted of 125 g white bread, 125 g wholemeal bread, 100 g sliced ham, 100 g sliced chicken, 85 g cheese slices, 100 g sliced tomato, 100 g sliced cucumber, 100 g lettuce, 200 g strawberry yoghurt, 140 g fruit salad, 150 g chocolate custard, 1 apple, 1 banana, 500 g unsweetened orange juice, 600 g iced coffee, 600 g spring water, 20 g margarine, and 20 g mayonnaise (Appendix 3).

All food items were weighed (to the nearest 0.1 gram) before and after subjects consumed the buffet meal. The total energy content (kJ) and macronutrient composition (% of energy from carbohydrate, fat, and protein) of the food consumed was determined using Foodworks Version 3.01 software (Xyris software, Highgate Hill, Queensland, Australia) (Cook et al. 1997).

5.6 Measurement of antral area by two-dimensional ultrasound imaging

Ultrasound measurement of the antrum of the stomach has been shown to be a precise and reproducible method of measuring gastric emptying of liquids (Holt et al. 1986; Ricci et al. 1993; Hveem et al. 1996). Gastric emptying times obtained from two-dimensional ultrasonographic measurement of the antral area correlate closely with scintigraphy, the established 'gold standard' for measurement of gastric emptying (R =

0.94, $P < 0.005$ for a 75 gram oral dextrose load; R = 0.97, $P < 0.001$ for a soup preload) (Figure 5.1) (Hveem et al. 1996).

Ultrasound has advantages over scintigraphy, being a non-invasive technique, without the need for exposure of the individual to radiation (Hveem et al. 1996). Furthermore, hunger sensations (R = -0.59, $P < 0.0001$) and energy intake (R = -0.9, $P < 0.0001$) have been shown to be significantly inversely related, and postprandial fullness related directly (R = 0.66, $P < 0.0001$) to antral area measurements (Sturm et al. 2004).

Antral area was measured in the studies described in Chapters 7 and 8. Measurements of antral area were performed using an Aloka SSD-650 CL Ultrasound Machine (Aloka Co., Ltd. Tokyo) using either a 3.5 or 5 MHz sector transducer. The transducer was positioned vertically to obtain a parasagittal image of the antrum with both the superior mesenteric vein and the abdominal aorta in longitudinal section and measurements were performed at the end of inspiration. This technique has been described and validated previously (Hveem et al. 1996; Jones et al. 1997). Two-dimensional antral area (cm^2) was measured using a caliper and calculation programme built into the machine, after outlining the circumference of the antrum. The area recorded during the fasted state was subtracted from the subsequent measurements made after a meal. Gastric emptying was expressed at any time point as: $A_{C(t)} = 100 - ((A_{(t)} / A_{max}) \times 100)$ where $A_{C(t)}$ = corrected antral area at a time point, $A_{(t)}$ = area measured at a given time point, and A_{max} = maximum antral area recorded after meal ingestion (Hveem et al. 1994).

5.7 Measurement of blood glucose and gastrointestinal hormone concentrations

Ten-millilitre venous blood samples were collected in ice-chilled ethylendiamine tetra-acetic acid (EDTA)-treated tubes containing 400 kIU aprotinin (Trasylol; Bayer Australia, Pymble, Australia) per millilitre of blood for measurement of plasma cholecystokinin (CCK), ghrelin, and insulin concentrations. Plasma was separated by centrifugation (3200 rpm for 15 min at 4°C) within 30 min of collection and stored at -70°C until assayed.

5.7.1 Blood glucose concentrations

Blood glucose was measured using a portable glucometre (MediSense Precision QID System, Abbott Laboratories, MediSense Products Inc., Bedford, MA, USA), using the glucose oxidase method. The accuracy of this method has been confirmed using the hexokinase technique (Horowitz et al. 1991).

5.7.2 Plasma ghrelin concentrations

Total plasma ghrelin (picograms per millilitre, pg/mL) was measured by radioimmunoassay (RIA) using a commercial antiserum (RAST-4745, Bachem, CA, USA) that does not cross-react with secretin, orexin, motilin, galanin or vasoactive intestinal peptide, with some modifications to a previously published method (Parker et al. 2005).

Human ghrelin was labelled with a ^{125}I-labeled tracer (Amersham IM347, GE Healthcare, Biosciences). Iodohistidyl-ghrelin was separated from free ^{125}I and

unlabelled ghrelin by reverse-phase high-performance liquid chromatography (HPLC) on a Phenomenex Jupiter C4 300 A 5 µm column cat no. 00B-4167-EO 250 × 4·6 mm. The column was eluted isocratically with 27% acetonitrile in triethylamine phosphoric acid buffer pH 3·0 (Prosearch International, Victoria, Australia). Standards were serially diluted from ghrelin peptide (Phoenix Pharmaceuticals, CA, USA) in a range from 4 to 256 pg/tube in buffer [50 mM phosphate pH 7·4 containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 2 g/l gelatin]. Incubation was for 3 days at 4 °C and second antibody precipitation was used to separate the antibody-bound peptide from free peptide. The second antibody was added after the incubation of antibody and label with standard (100 µl of sheep anti-rabbit antibody, Prosearch International, and 100 µl of 2% normal rabbit serum and 1 m); 8% PEG 6000 was added immediately before centrifugation. After centrifugation for 25 min at 4 °C, the tubes were decanted and counted on a Crystal LKB gamma counter.

The intra-assay coefficient of variation (CV) was 5% and the inter-assay CV was 18%, with a sensitivity of 40 pg/mL.

5.7.3 Plasma insulin concentrations

Plasma insulin concentrations (milliunits per litre, mU/L) were measured using the Abbott Imx Microparticle Enzyme Immunoassay (Abbott Laboratories, Diagnostic Division, Dainabot, Tokyo, Japan). The intra-assay coefficients of variation (CV) were 4.5% at 8.3 mU/L and 3.4% at 40.4 mU/L, with a detection limit in the assay of 1.0 mU/L.

5.7.4 Plasma CCK concentrations

Plasma CCK concentrations (pmol/L) were determined following ethanol extraction using an established radioimmunoassay (Santangelo et al. 1998; MacIntosh et al. 2001). Standards (synthetic sulfated CCK-8, Sigma, St. Louis, MO) were prepared in charcoal-stripped plasma and extracted in 66% ethanol along with the samples. Extracts were dried under N_2 and resuspended in assay buffer (50 mM phosphate, 10 mM EDTA, 2 g/litre gelatin, pH 7.4). A commercially available antibody (C2581, lot 105H4852, Sigma Chemical, St Louis, MO, USA) was added at a dilution of 1/17,500, and sulfated CCK-8 ^{125}I-labeled with Bolton and Hunter reagent (74 Tbq/mmol, Amersham International, Amersham Pharmacia Biotech, Bucks, UK) was used as tracer. Incubation was for 3 days at 4º C. The antibody-bound fraction was separated using dextran-coated charcoal containing gelatin (0.015 g gelatin, 0.09 g dextran, 0.15 g charcoal in 30 ml assay buffer). The antibody used binds to all CCK peptides containing the sulfated tyrosine residue in position 7, has a cross-reactivity of 26% with unsulfated CCK-8, less than 2% cross-reactivity with human gastrin, and does not bind to structurally unrelated peptides.

The intra-assay coefficient of variation (CV) was 9% and the inter-assay CV was 27%, with a sensitivity of 2.5 pmol/L.

5.8 Cardiovascular autonomic function

For the study described in Chapter 8, autonomic nerve function was evaluated using standardised cardiovascular reflex tests (Ewing and Clarke 1982; Piha 1991).

Parasympathetic function was evaluated by the variation (R-R interval) of the heart rate during deep breathing and upon standing (ratio of R-R interval at approximately beat 30 to R-R interval at approximately beat 15); both values are reduced in individuals with autonomic dysfunction. Sympathetic function was assessed by the fall in systolic blood pressure in response to standing; this increases in individuals with autonomic dysfunction. Each test result was scored according to age-adjusted criteria as 0 = normal, 1 = borderline or 2 = abnormal, for a total maximum score of 6. A score of 3 or more was considered to indicate definite autonomic dysfunction (Ewing and Clarke 1982; Piha 1991).

5.9 Statistical analysis

Data were analysed using statistical software packages Statview Version 5.0 (SAS Institute Inc., North Carolina, U.S.A.), SuperANOVA Version 1.11 (Abacus Concepts Inc., Berkeley, CA, U.S.A.), and SPSS for Windows Version 11.5 (Chicago, U.S.A.).

All data are expressed as mean values ± SEM. A P value of < 0.05 was considered statistically significant. The statistical analyses used for each study are provided in detail in each chapter.

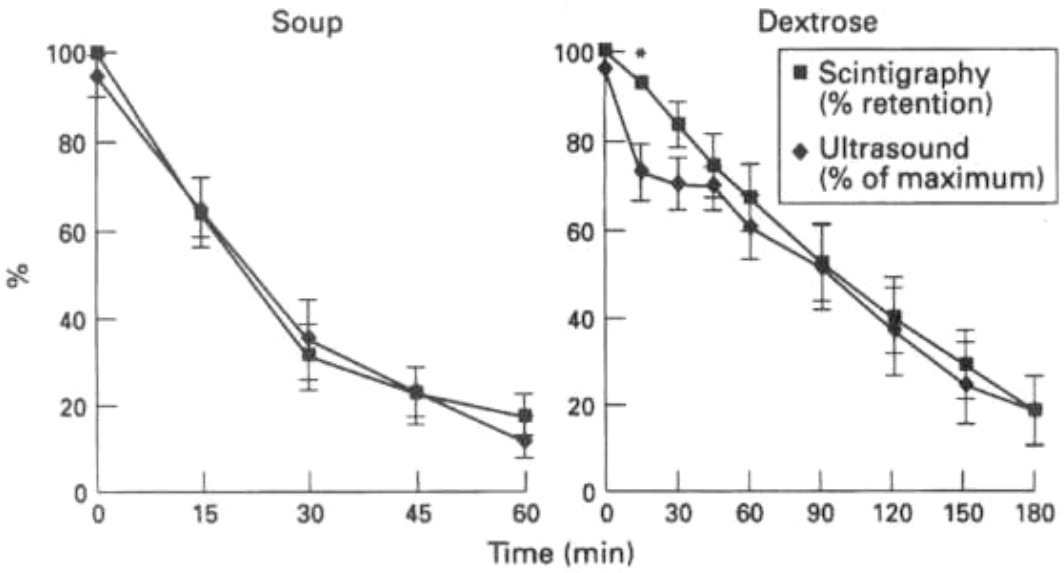


Figure 5.1: Gastric emptying of beef soup (350 mL, 20 kcal or 84 kJ) and dextrose (75 g dissolved in 350 mL water, 300 kcal or 1256 kJ) measured scintigraphically (intragastric retention of isotope) and by ultrasound (changes in antral area) in 7 healthy subjects. Data are mean values ± SEM. Adapted from Hveem et al. (1996).

Chapter 6

THE EFFECT OF AGE, GENDER, BODY COMPOSITION AND FOOD INTAKE ON CIRCULATING GHRELIN CONCENTRATIONS IN HEALTHY ADULTS

6.1 Summary

The determinants of plasma ghrelin concentrations, including the effects of ageing, gender and changes in body composition if any, are unclear. Appetite and energy intake decrease with advancing age, and there is a corresponding decline in total body lean tissue, and an increase in fat mass. Fasting plasma ghrelin and insulin concentrations were measured in 52 healthy subjects aged 22 - 82 years, and body composition assessed by dual energy X-ray absorptiometry. Energy intake was estimated from diet diaries. Fasting ghrelin concentrations were not significantly correlated with age or energy intake (R = 0.07, P = 0.62; and R = -0.14, P = 0.34 respectively) on univariate regression analysis, and ghrelin concentrations were higher in females than males (2887 ± 182 pg/mL vs 2083 ± 121 pg/mL; P = 0.001). Ghrelin was inversely related to body mass index (R = -0.328, P = 0.018), fat-free body mass (R = -0.428, P = 0.002), and total skeletal muscle mass (R = -0.439, P = 0.001), but not to body fat mass (R = 0.177, P = 0.208). On multiple regression analysis, total skeletal muscle mass (corrected for height) was the only significant (negative) predictor (P < 0.0001) of fasting ghrelin concentrations. In conclusion, in healthy adults plasma ghrelin concentrations are not

significantly influenced by age or energy intake *per se*, but are inversely related to skeletal muscle mass.

6.2 Introduction

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor. It has a number of actions, including stimulation of hunger and food intake (Kojima et al. 1999). Consumption of food suppresses ghrelin release, while plasma ghrelin concentrations increase in the fasted state (Druce et al. 2004; Natalucci et al. 2005), reaching a peak just before the next meal (Cummings et al. 2001; Natalucci et al. 2005). Ghrelin may thus play a role in meal initiation (Cummings et al. 2001). Possibly in response to chronic energy imbalance, circulating ghrelin concentrations are increased in undernourished states such as anorexia nervosa (Rigamonti et al. 2002; Krsek et al. 2003) and decreased in obesity (Tschop et al. 2001).

Healthy ageing is associated with a decline in appetite and food intake, which has been termed the anorexia of ageing. In at-risk older people this predisposes to pathological under-nutrition which is associated with increased morbidity and mortality (Chapman 2004). The causes of the physiological anorexia of ageing appear to be multiple, and are poorly understood. An understanding of these causes may help to develop ways of preventing and treating undernourished older people. One possible cause is age-related reductions in the orexigenic effects of ghrelin. Previous studies have produced conflicting data on the effect of healthy ageing on circulating ghrelin concentrations (Cummings et al. 2001; Rigamonti et al. 2002; Sturm et al. 2003; Purnell et al. 2003; Marchesini et al. 2004; Langenberg et al. 2005; Makovey et al. 2007; Bauer et al. 2007;

Schutte et al. 2007). In part, this uncertainty appears to arise from the confounding effects of age-related body composition changes on ghrelin concentrations. In several studies age-related differences in ghrelin concentrations evident on univariate analysis were no longer apparent when body composition measures were corrected for in multivariate analysis (Purnell et al. 2003; Marchesini et al. 2004). On average, body weight, as reflected in body mass index (BMI), increases until about age 60 years and declines somewhat thereafter (Rossner 2001), body fat stores increase throughout adult life whereas lean tissue declines (Baumgartner et al. 1995). Increased body mass index has been associated consistently with reduced ghrelin concentrations (Tschop et al. 2001; Langenberg et al. 2005; Makovey et al. 2007; Schutte et al. 2007), but it is not clear if this association is due to increased fat tissue, increased lean tissue or both (Tschop et al. 2001; Bunt et al. 2003; Fouladiun et al. 2005; Moran et al. 2005; Bertoli et al. 2006; Gravholt et al. 2006). Only limited analysis of the relationship between ghrelin concentrations and the components of lean tissue mass has been undertaken (Bertoli et al. 2006).

The aim of the present study was to examine the relationship of circulating ghrelin concentrations with age, body composition measures (including the components of lean tissue) and energy intake in healthy adults across a wide age range. It was hypothesised that circulating ghrelin concentrations would decline with age, independent of age-related changes in body composition and energy intake.

6.3 Materials and methods

6.3.1 Subjects

Fifty-two healthy subjects (26 men, 26 women), aged 22-82 years (49.2 ± 2.4 years), with a mean BMI of 23.7 ± 0.3 kg/m^2, were recruited through advertisement. The BMI of the subjects over 65 years was not significantly different to those under 65 years (24.6 ± 0.5 kg/m^2 vs 23.4 ± 0.4 kg/m^2, P = 0.12). Subjects were excluded if they smoked > 10 cigarettes/day, or consumed >20 g/day of alcohol. Pregnancy was excluded in women of reproductive age with a urine pregnancy test at the time of the screening visit. Pre-menopausal women were studied between days 1 to 14 of their menstrual cycle. All subjects were asked to maintain their usual diet and physical activity prior to, and throughout, their participation in the study.

The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital. Written, informed consent was obtained from each participant before inclusion in the study.

6.3.2 Study Protocol

On the initial study day, each subject arrived at 8:30 am, after an overnight fast, and blood was drawn for fasting ghrelin and insulin levels. On a separate study day, baseline body composition estimation was performed by dual energy X-ray absorptiometry (DEXA; Norland Densitometer XR36).

Each subject was asked to maintain a three-day food diary from which their total baseline energy intake was estimated using Foodworks version 3.1 software program.

A fasting morning venous blood sample (12-hour fast) was collected in ice-chilled EDTA tubes containing 1000 kallikrein inhibitory units aprotinin (Trasylol) per millilitre of blood. Plasma was separated by centrifugation (3200 rpm for 15 minutes at 4 °C) within one hour of collection and stored at -70 °C until assayed.

6.3.3 Measurements

6.3.3.1 Appetite

Sensations of hunger, fullness, and nausea were rated by each subject at regular intervals using 10-cm linear visual analogue scales (Sepple and Read 1989), as described in Chapter 5.5.3.

6.3.3.2 Food intake

The average energy intake was quantified from a three-day food diary, using Foodworks 3.10 (Xyris Software, Highgate Hill, Australia), as described in Chapter 5.5.2.

6.3.3.3 Body composition

Dual-energy X-ray absorptiometry (DEXA; Norland TM densitometer XR36 Norland Medical Systems, Fort Atkinson, Wisconsin, USA) was used to measure total body fat mass (BFM) and fat-free mass (FFM; which includes bone mineral content). Appendicular lean soft tissue (ALST) was calculated as the sum of the lean soft tissue mass in the upper and lower limbs, and included skeletal muscle, skin, and connective

tissue, minus the bone mineral content (Kim et al. 2002). These measurements by DEXA have been found to correlate well with those obtained by direct measures from computed tomography (CT) (Visser et al. 1999; Levine et al. 2000). In one study of 43 healthy young adults (mean age 39 ± 13 years), multislice CT-measured thigh muscle volume was highly correlated ($R^2 = 0.96$, $P < 0.0001$) with DEXA-measured thigh fat-free mass (Levine et al. 2000). Similarly, in another study of 60 healthy older adults aged 70 – 79 years, mid-thigh muscle mass measured by DEXA and multislice CT scan were well correlated ($R^2 = 0.94$, $P = 0.0001$).

Total body skeletal muscle mass (total SM) was calculated from DEXA using the following validated formula (Kim et al. 2002):
Total SM= (1.13 x ALST) – (0.02 x age) + (0.61 x sex) + 0.97, where sex = 0 for females, 1 for males).

The values for BFM, FFM, and total SM were corrected for height to give height-normalised indices (fat-free mass index FFMI, body fat mass index BFMI, and skeletal muscle mass index SMI), as suggested in a previous study (VanItallie et al. 1990).

6.3.3.4 Plasma ghrelin concentrations

Total plasma ghrelin (pg/mL) was measured by radioimmunoassay with some modifications to the previously published method (Parker et al. 2005), as described in Chapter 5.7.2.

6.3.4 Statistical analysis

Relationships between ghrelin and age and other variables were determined using simple regression analyses. All variables showing a correlation with a P value of < 0.2 were then entered into a stepwise multivariate regression model, with ghrelin concentration as the dependent variable. The residuals for ghrelin concentration as the dependent variable were distributed normally. Age and energy intake were included in the final regression model, regardless of P values, as these were pre-determined parameters of interest, and it was wished to determine the contribution of each component in predicting the variance in fasting ghrelin concentrations. All statistical analyses were performed using SPSS for Windows Version 11.5 statistical software. Results are expressed as mean ± SEM. A $P < 0.05$ was considered statistically significant.

6.4 Results

Subject details are shown in Table 6.1. The women were similar in age to the men, but weighed less, had lower BMIs, had more fat tissue, less lean tissue, and lower bone mineral content in the arms and legs.

As shown in Figure 6.1 there was no significant correlation between fasting ghrelin concentrations and age by univariate analysis ($R = 0.070$, $P = 0.621$). Fasting ghrelin concentrations were higher in females than males (2887 ± 182 pg/mL vs 2083 ± 121 pg/mL; $P = 0.001$).

The results of univariate correlation analyses between ghrelin and other variables are shown in Table 6.2. Body mass index and a number of measures of lean mass and skeletal muscle mass were significantly negatively correlated with ghrelin concentrations. There was a trend toward bone mass being a negative predictor, but this did not achieve statistical significance. There was a weak negative correlation between energy intake and ghrelin concentrations, which did not achieve statistical significance. In a separate analysis, energy intake was not significantly correlated with BMI (R = 0.13, $P = 0.37$) or age (R = 0.12, $P = 0.42$), but was positively correlated with lean body mass components ALST (R = 0.37, FFMI (R = 0.47), SMI (R = 0.41), BMC of arms (R = 0.37), LM of arms (R = 0.36) and LM of legs (R = 0.37) (all $P < 0.01$), and negatively correlated with the body fat mass variable BFMI (R = -0.43, $P = 0.001$).

As many of the measured variables are correlated with each other, multiple regression analysis with plasma ghrelin as the dependent variable was performed, with results shown in Table 6.3. The only significant independent predictor of ghrelin was total body skeletal muscle mass index, which was negatively correlated with fasting ghrelin concentrations ($P < 0.0001$).

6.5 Discussion

The major observations in this study are that: (1) healthy ageing is not associated with changes in circulating fasting ghrelin concentrations; (2) healthy women have higher fasting ghrelin concentrations than men, apparently related to differences in body composition; (3) body mass index and ghrelin concentrations are significantly

negatively correlated, even in non-obese individuals; and (4) skeletal muscle mass is an independent negative predictor of fasting ghrelin concentrations.

Previous studies have reported circulating ghrelin concentrations that are higher (Cummings et al. 2001; Purnell et al. 2003), lower (Rigamonti et al. 2002; Marchesini et al. 2004; Bauer et al. 2007), or no different (Sturm et al. 2003; Langenberg et al. 2005; Bertoli et al. 2006; Makovey et al. 2007) (as found in this current study), between healthy older and young adults on univariate analysis. There is thus no consistent evidence that circulating ghrelin levels increase or decrease with age, even before taking into account other factors. The results of previous studies may be inconsistent because of differences between the subject groups studied, particularly in age and body weight range, and the confounding effects of age-related body composition changes. In both the studies referred to above where an age-related difference was present on univariate analysis and a multivariate analysis was also performed, there was no remaining effect of age after body composition parameters were accounted for in the multivariate analysis (Purnell et al. 2003; Marchesini et al. 2004). In contrast, another study found an increase with age which was only present on multivariate analysis (Makovey et al. 2007). The finding in the current study of no relation between age and ghrelin concentrations on multivariate analysis including body composition and food intake parameters is, therefore, consistent with results of most previous studies. It appears that age *per se* has minimal, if any, influence on circulating ghrelin concentrations and that reduced circulating ghrelin concentrations are not a cause of the anorexia of ageing. The effect of ageing on the sensitivity to the orexigenic effects of ghrelin has, however, not been reported.

Fasting ghrelin concentrations were 38% higher in women than men in the current study, a difference that was highly significant. Some (Espelund et al. 2005; Moran et al. 2005; Makovey et al. 2007), but not all (Purnell et al. 2003; Langenberg et al. 2005; Bauer et al. 2007), previous studies have also found higher levels in women. The reason for these discrepant results is not clear. Multivariate analyses have been performed in three of the four studies, including ours (Moran et al. 2005; Makovey et al. 2007), where ghrelin concentrations were higher in women. In two of the three studies, (Moran et al. 2005) plus the current study, there was no gender difference, after correcting for body composition differences by multivariate analysis, whereas in the other ghrelin remained higher in women (Makovey et al. 2007). The interaction between body composition and ghrelin in our study appears to be due to the substantially lower skeletal muscle mass in women; skeletal muscle mass was a powerful and statistically significant negative predictor of ghrelin levels. The current study suggests that across a wide adult age range, ghrelin levels are higher in women than men, and that this difference is related to body composition, rather than other differences associated with gender.

The relative effects of changes in energy intake and body composition on circulating ghrelin concentrations have not been defined. Fasting acutely elevates ghrelin levels, which are also increased chronically in under-nourished young (Rigamonti et al. 2002) and older (Sturm et al. 2003) adults. Weight loss, whether induced by diet or surgery (which reduces food intake), is associated with an increase in ghrelin levels (Hansen et al. 2002; Cummings et al. 2002; Yukawa et al. 2006; Garcia et al. 2006). Conversely,

eating food acutely suppresses ghrelin levels (Cummings et al. 2001). The inverse relationship between energy intake and ghrelin appears to be mediated, at least in part, by the suppressive effects of insulin on ghrelin (Langenberg et al. 2005; Bauer et al. 2007). An increase in insulin after meal ingestion could result in decreased ghrelin levels, thereby reducing food intake (Saad et al. 2002). Fasting ghrelin concentrations have been shown to be negatively correlated with insulin levels (Rigamonti et al. 2002). Furthermore, in other studies, intravenous infusion of insulin resulted in a rapid decrease in plasma ghrelin concentrations within 30 minutes (Saad et al. 2002; Lucidi et al. 2002), with a maximal mean suppression of 32% in ghrelin levels (Saad et al. 2002).

In the stable state, energy intake, as assessed by diet diaries, is reported to be inversely related to circulating ghrelin concentrations (Marchesini et al. 2004; Bertoli et al. 2006). This relationship is not strong, however, perhaps due to limitations in measures of energy intake, and did not achieve statistical significance in the present study.

It is not clear how much of the increase in ghrelin levels with weight loss (and vice versa for weight gain) is due to the accompanying changes in body composition, rather than to reduced food intake *per se*. Most studies show an inverse relationship between body mass index and ghrelin concentrations (Tschop et al. 2001; Langenberg et al. 2005; Makovey et al. 2007; Schutte et al. 2007), and this was so in the present study even across a relatively narrow range of BMIs. Consistent with this, ghrelin concentrations are lower in obese than normal weight individuals (Shiiya et al. 2002). On average food intake is positively related to body weight on multivariate analysis ($F = 5.13$, $P = 0.001$ in men; $F = 13.78$, $P = 0.001$ in women) (Sherwood et al. 2000); extra

energy is needed to maintain the greater weight. It is possible, therefore, that the inverse relationship between body weight and ghrelin levels is accounted for by differences in food intake, at least in part. However, in the present study, daily energy intake did not significantly correlate with BMI. Furthermore, there was no significant relationship between food intake and ghrelin concentrations on either univariate or multivariate analysis. This suggests that there is an association between ghrelin levels and body composition independent of food intake.

The body composition component related to ghrelin in this study was skeletal muscle mass. Unlike some previous studies (Tschop et al. 2001; Bunt et al. 2003; Fouladiun et al. 2005; Gravholt et al. 2006) the amount of fat tissue was not related to ghrelin by either univariate or multivariate analysis. The finding of an inverse association with skeletal muscle mass agrees with that of studies where lean tissue was found to be inversely related to ghrelin levels (Foster-Schubert et al. 2005; Moran et al. 2005; Bertoli et al. 2006). Bertoli et al. (2006) also found no relation with fat mass and a significant inverse relationship between ghrelin levels and both fat-free mass and skeletal muscle mass measured by DEXA in young and older adults (Bertoli et al. 2006). They found a significant negative correlation between food intake and ghrelin concentrations, but did not undertake a multivariate analysis to test for independent associations. The current findings therefore confirm theirs of an inverse relation with skeletal muscle mass, and extend them to indicate no apparent association with nutritional intake.

It is important to understand the reasons why there might be an inverse association between skeletal muscle mass and circulating ghrelin concentrations. Both ghrelin and its receptor are found in skeletal muscle (Papotti et al. 2000; Gnanapavan et al. 2002) and short-term ghrelin administration increases lean body mass (Nagaya et al. 2004). Apart from the stomach and gastrointestinal tract, the growth hormone secretagogue receptors, through which ghrelin works, have also been found in skeletal muscle, and, to a lesser extent, in adipose tissue in humans (Papotti et al. 2000). Furthermore, *in vitro* studies have shown that ghrelin stimulates differentiation of rodent muscle cells (Zhang et al. 2007; Filigheddu et al. 2007). The inverse relationship identified between plasma ghrelin concentrations and skeletal muscle mass supports the existence of a connection between skeletal muscle and ghrelin secretory pathways and suggests that skeletal muscle may exert a negative feedback effect on ghrelin secretion. If so, this may have implications of practical importance. For example, it may enable dieting, overweight people to reduce the appetite-stimulating increase in ghrelin concentrations accompanying weight loss by increasing muscle mass, by engaging in resistance and strengthening exercises. Perhaps consistent with this possibility, subjects in the Rancho Bernardo Study who exercised at least three times per week, and therefore probably had greater muscle mass than those who did not, had lower levels of ghrelin, even after adjustment for BMI (Langenberg et al. 2005). It is therefore appropriate to postulate that ghrelin concentrations are determined by, and probably also a determinant of, total body skeletal muscle mass. Possible mechanisms for this connection may include an upregulation of growth hormone secretagogue receptors with an increase in skeletal muscle bulk, which subsequently has a negative feedback effect on ghrelin secretion.

The effects of acute exercise on plasma ghrelin concentrations have been previously studied. As plasma growth hormone concentrations increase with either high- or low-intensity exercise (Kallio et al. 2001; Dall et al. 2002; Kraemer et al. 2004a; Schmidt et al. 2004; Takano et al. 2005; Jurimae et al. 2007), and ghrelin is a growth hormone secretagogue which stimulates growth hormone secretion (Kojima et al. 1999; Takaya et al. 2000), it had been proposed that ghrelin could mediate the rise in growth hormone levels associated with exercise (Kraemer et al. 2004a; Schmidt et al. 2004; Christ et al. 2006; Jurimae et al. 2007). However, plasma ghrelin concentrations do not change significantly following a short session of moderate- or high-intensity exercise in healthy normal subjects (Kallio et al. 2001; Dall et al. 2002; Schmidt et al. 2004; Zoladz et al. 2005), or in male athletes (Kraemer et al. 2004a; Jurimae et al. 2007). As ghrelin concentrations increase during fasting and decrease following a meal (Cummings et al. 2001; Natalucci et al. 2005), previous studies have evaluated the effects of exercise in either the fasted (Kraemer et al. 2004a; Zoladz et al. 2005) or postprandial (Kallio et al. 2001; Dall et al. 2002; Zoladz et al. 2005; Christ et al. 2006; Jurimae et al. 2007) state, however no changes in ghrelin concentrations were found. On the other hand, one study in athletes assessing the effects of acute dietary modification showed that ghrelin concentrations increased following prolonged aerobic exercise, and were higher if the exercise was preceded by a low-fat compared to a high-fat diet (Christ et al. 2006). Whilst a short duration of resistance exercise (4 sets of 30 repetitions of bilateral leg extension, during which a pressure band was also applied to the thighs) had no effect on post-exercise ghrelin concentrations (Takano et al. 2005), the effects of such exercise in the longer term has not been defined. Interestingly, a comparison of different modalities of resistance exercise has shown that repetitive concentric, but not eccentric, muscle

contractions results in a suppression of ghrelin concentrations during the recovery period of the exercise session (Kraemer et al. 2004b). Furthermore, although intentional weight loss results in an increase in plasma ghrelin concentrations, weight maintenance over a longer duration results in a decrease in ghrelin concentrations at 12 months (Garcia et al. 2006). It may be that frequent repetitions of resistance-type exercise over a longer duration of time, rather than an acute session of either high- or low-intensity exercise, have a suppressive influence on ghrelin concentrations.

The present study has some limitations. Total plasma ghrelin concentrations were examined only in the fasted state. Fasting ghrelin concentrations are, however, known to correlate closely to 24-hour area under the curve or pooled ghrelin concentrations, in both young (Cummings et al. 2001; Purnell et al. 2003) ($R = 0.873$, $P = 0.0004$; $R = 0.89$, $P < 0.001$ respectively) and older ($R = 0.959$, $P < 0.001$) (Yukawa et al. 2006) individuals, so fasting levels are likely to reflect overall ghrelin levels. Furthermore, active ghrelin concentrations were not measured. Total (active and inactive) ghrelin has, however, been shown to correlate closely with active ghrelin concentrations ($R = 0.62$, $P < 0.001$) (Marzullo et al. 2004).

In conclusion, in this study of healthy adults across a sixty year age range, plasma concentrations of the orixegenic hormone ghrelin were not significantly influenced by age, were inversely correlated with BMI, and were higher in females than males, although not after correction of body composition differences. After accounting for all other covariates, total body skeletal muscle, but not fat, mass was a significant negative

predictor of ghrelin concentrations. Longer-term studies are indicated to address the effects of resistance and strengthening exercises on circulating ghrelin concentrations.

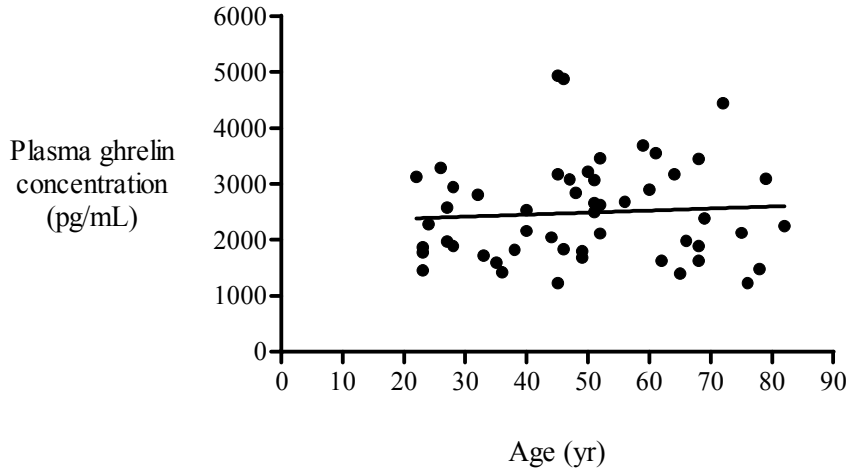


Figure 6.1: Relationship between fasting ghrelin concentrations and age for the entire group (R = 0.07, *P* = 0.62).

Table 6.1: Characteristics of the entire study group, with comparison between male and female and older and younger subgroups.

	All subjects (n = 52)	**Female (n = 26)**	**Male (n = 26)**	***P* value for male vs female subgroups**	***P* value for older (≥ 65 yrs; n = 12) vs younger (n = 40) subgroups**
Age, years	49.2 ± 2.4	48.2 ± 3.4	50.1 ± 3.3	0.69	< 0.0001
Body weight, kg	69.4 ± 1.3	64.2 ± 1.3	74.7 ± 9.2	< 0.0001	0.62
BMI, kg/m^2	23.7 ± 0.3	23.0 ± 0.4	24.3 ± 0.4	0.04	0.12
Bone mineral content (BMC) of arms, kg	0.41 ± 0.01	0.36 ± 0.01	0.46 ± 0.01	< 0.0001	0.25
Bone mineral content (BMC) of legs, kg	1.02 ± 0.02	0.94 ± 0.02	1.09 ± 0.03	< 0.0001	0.09
Fat-free mass (FFM), kg	45.1 ± 1.5	36.6 ± 0.8	53.7 ± 1.5	< 0.0001	0.47
Fat-free mass index (FFMI)*, kg/m^2	15.3 ± 0.4	13.1 ± 0.3	17.5 ± 0.4	< 0.0001	0.85
Body fat mass (BFM), kg	22.8 ± 0.9	26.3 ± 1.3	19.4 ± 4.3	< 0.0001	0.55
Body fat mass index (BFMI)*, kg/m^2	7.9 ± 0.3	9.4 ± 0.4	6.3 ± 0.3	< 0.0001	0.17
Appendicular lean soft tissue (ALST), kg	19.4 ± 0.7	15.4 ± 2.2	23.4 ± 0.7	< 0.0001	0.23
Total skeletal muscle mass (Total SM), kg	22.2 ± 0.8	17.4 ± 0.5	27.0 ± 0.8	< 0.0001	0.15
Total skeletal muscle mass index (SMI)*, kg/m^2	7.5 ± 0.2	6.2 ± 0.2	8.8 ± 0.2	< 0.0001	0.38
Energy intake / day, kJ	8593 ± 294	7820 ± 421	9365 ± 358	0.007	0.73
Fasting plasma ghrelin, pg/mL	2485 ± 122	2887 ± 182	2083 ± 121	0.001	0.35
Fasting plasma insulin, mU/L	5.6 ± 1.2	5.1 ± 1.5	6.1 ± 1.9	0.69	0.29

Data are mean values ± SEM.

* FFMI = FFM ÷ $(height)^2$; BFMI = BFM ÷ $(height)^2$; and SMI = total SM ÷ $(height)^2$

Table 6.2: Simple regression analyses of fasting plasma ghrelin concentrations with body composition and other variables (abbreviations as in Table 6.1).

Variable	R	*P* value
Age, years	0.070	0.621
Gender	0.461	0.001
BMI, kg/m^2	-0.328	0.018
BMC of arms, kg	-0.269	0.054
BMC of legs, kg	-0.234	0.096
Fasting plasma insulin, mU/L	0.097	0.492
Lean muscle mass (LM) of arms, kg	-0.396	0.004
LM of legs, kg	-0.429	0.001
FFM, kg	-0.428	0.002
FFMI, kg/m^2	-0.451	0.001
BFM, kg	0.177	0.208
BFMI, kg/m^2	0.223	0.113
ALST, kg	-0.432	0.001
Total SM, kg	-0.439	0.001
SMI, kg/m^2	-0.475	< 0.0001
Energy intake/day, kJ	-0.135	0.340

Data are mean values ± SEM.

Table 6.3: Stepwise multiple regression analysis of fasting plasma ghrelin with other covariates (abbreviations as in Table 6.1).

	B standardized coefficient	**t**	***P* value**
SMI	-0.475	-3.82	<0.0001
Age	0.043	0.340	0.735
BMI	-0.113	-0.779	0.440
BMC of arms	0.058	0.356	0.724
BMC of legs	0.072	0.462	0.646
ALST	0.111	0.309	0.759
FFMI	0.046	0.104	0.917
BFMI	-0.143	-0.875	0.386
LM of arms	0.048	0.195	0.846
LM of legs	0.129	0.358	0.722
Gender	0.220	1.021	0.312
Energy intake / day	0.070	0.513	0.610

Data are mean values ± SEM.

Chapter 7

THE EFFECTS OF CARBOHYDRATE AND FAT DIGESTION ON PLASMA GHRELIN CONCENTRATIONS IN HEALTHY YOUNG ADULTS

7.1 Summary

The details of how the ingestion of nutrients modulates ghrelin release have not been fully established. It is not certain whether the presence of nutrients in the small intestine is sufficient to alter ghrelin concentrations or whether digestion and absorption is required. Twenty-four healthy young adults with a mean age of 23 ± 0.6 years and BMI of 22.4 ± 0.5 kg/m^2 were examined on three separate days after an overnight fast. Twelve subjects participated in Part A of the study, and the other 12 subjects in Part B. In Part A, subjects received, in random order, one of three study drinks: 300 mL water (control); 300 mL high-fat drink (110 mL rich cream blended with 190 mL full-fat milk, and low-energy flavouring) given with and without 120 mg orlistat (lipase inhibitor). In Part B of the study, subjects received, in random order, one of three study drinks: 300 mL water; 300 mL sucrose (100 g sucrose with water and low-energy flavouring) given with and without 100 mg acarbose. Subjects were blinded to the addition of orlistat or acarbose in the drinks. In Part A, postprandial plasma ghrelin concentrations decreased following ingestion of the high-fat drink (P = 0.01), but did not alter with high-fat-orlistat drink or water (P > 0.1). Plasma insulin concentrations

increased after the high-fat drink, with and without, orlistat ($P = 0.0001$) and glucose concentrations did not change after either of the high-fat drinks. In Part B, there was a progressive suppression of plasma ghrelin concentrations following the sucrose drink ($P < 0.005$), which was significantly attenuated by acarbose ($P < 0.01$). Sucrose, with and without acarbose, resulted in an increase in plasma insulin ($P = 0.0001$) and glucose ($P = 0.0001$) concentrations, which were both higher after the sucrose drink than sucrose-acarbose drink ($P < 0.05$). In conclusion, fat and carbohydrate digestion is required for maximal suppression of ghrelin secretion.

7.2 Introduction

Ghrelin is an orexigenic hormone (Wren et al. 2001a; Wren et al. 2001b) secreted predominantly by the fundus of the stomach (Kojima et al. 1999; Ariyasu et al. 2001). Ghrelin expression and secretion is increased in negative energy balance states (Gualillo et al. 2003). Circulating ghrelin concentrations gradually increase in the fasted state, to reach a peak just before the next meal (Cummings et al. 2001). Consumption of food suppresses ghrelin release, and plasma ghrelin concentrations increase shortly before the start of a meal, reaching trough levels within one hour of meal ingestion (Cummings et al. 2001). The mix of macronutrients in a meal determines the timing and magnitude of suppression of ghrelin. Ghrelin suppression is noticeable from approximately 15 minutes after consumption of carbohydrates, reaching a nadir by 60 minutes, but occurs later and over a more prolonged course after a fat-enriched meal, from approximately 30 minutes after consumption (Erdmann et al. 2003).

An intravenous glucose injection decreases ghrelin concentrations, although significantly less and for a shorter duration than oral ingestion of the same amount of glucose (Shiiya et al. 2002). This difference highlights the role that small intestinal exposure plays in the suppression of ghrelin release by glucose. Furthermore, the suppresion of ghrelin in response to intraduodenal infusion of glucose requires the exposure of > 60 cm of the small intestine to glucose (Little et al. 2006a). Intraduodenal infusion of a triglyceride emulsion suppresses ghrelin concentrations in healthy young adults, and this effect is dependent on fat digestion (Feinle-Bisset et al. 2005). The details of how the ingestion of nutrients modulates ghrelin release have not, however, been fully established.

The aim of the present study was to investigate the role of carbohydrate and fat digestion in the suppression of plasma ghrelin concentrations by administering the disaccharide sucrose, with and without acarbose, an alpha-glucosidase inhibitor, and a high-fat drink, with and without the lipase inhibitor, orlistat. The hypothesis was that products of nutrient digestion are necessary for the small intestinal feedback inhibition of ghrelin, and that acarbose and orlistat would attenuate the suppression of ghrelin concentrations by sucrose and fat respectively.

7.3 Materials and methods

7.3.1 Subjects

Twenty four healthy, young adults (12 females and 12 males), with a mean age of 23 ± 0.6 years (range 18 - 35 years), were recruited through advertisements. Twelve subjects participated in Part A of the study and the other 12 subjects in Part B. Subjects had a

mean body mass index (BMI) of 22.4 ± 0.5 kg/m^2. Subjects were included if they were unrestrained eaters as determined by a score of < 11 on the eating restraint questionnaire component of the Three-Factor Eating Questionnaire (Stunkard and Messick 1985). Exclusion criteria included use of medications which may alter gastrointestinal motility or appetite, and a known history of diseases which may affect gastric motility (eg. diabetes mellitus). Females were studied between days 1 - 14 of their menstrual cycle, and pregnancy was excluded in women using a urine pregnancy test. The study was approved by the Research Ethics Committee of the Royal Adelaide Hospital. Written, informed consent was obtained from each participant before inclusion in the study.

7.3.2 Protocol

Each subject underwent three studies on separate days, at least 48 hours apart, in randomised order. On each of the three study days, the subject arrived at 8:30 am, after an overnight fast. They were positioned comfortably in bed at approximately 90 degrees. An intravenous cannula was placed in an antecubital vein for blood sampling. After a 20-minute recovery period, two baseline venous blood samples were collected at -5 and 0 mins. Subjects were then asked to ingest the study drink, which was removed from the 4 °C section of the refrigerator to room temperature five minutes before consumption. Part A involved ingestion of three study drinks (in random order): water (control drink; 300 mL); high-fat drink (300 mL total, made up of 110 mL rich cream blended with 190 mL full-fat milk, with low-energy flavouring for palatability; 87.6% energy as fat, 7.1% carbohydrate, 5.3% protein; total energy 2732 kJ); high-fat-orlistat drink (300 mL total, made up of 110 mL cream blended with 190 mL full-fat

milk, with low-energy flavouring, and 120 mg orlistat, Roche Products Pty., Dee Why, N.S.W., Australia). Part B included the three study drinks (in random order): water (control drink; 300 mL); sucrose drink (300 mL, made up of 100 g sucrose with water and low-energy lemon flavouring for palatability; 100% carbohydrate content; total energy 1674 kJ); sucrose-acarbose drink (300 mL, made up of 100 g sucrose with water and low-energy lemon flavouring, and 100 mg acarbose, Bayer Australia Ltd., Pymble, Australia). Subjects were blinded to the addition of orlistat and acarbose to the high-fat and sucrose drinks respectively.

Venous blood was subsequently collected at 15, 30, 45, 60, 90, 120, 150, and 180 minutes. Blood samples were collected in ice-chilled EDTA tubes containing 1000 kallikrein inhibitory units aprotinin (Trasylol) per millilitre of blood, for measurement of insulin, cholecystokinin (CCK), and ghrelin. Blood glucose concentrations were measured at the same time. Plasma was separated by centrifugation (3200 rpm for 15 min at 4 °C) within half an hour of collection and stored at -70 °C until assayed.

At -5, 0, 15, 30, 45, 60, 90, 120, 150, and 180 minutes, visual analogue scale (VAS) questionnaires were administered, to evaluate appetite-related sensations. The average of values at -5 and 0 min provided the baseline measurement.

Subjects were asked to record any gastrointestinal side-effects during, and over the 24 hours following, each study day.

7.3.3 Measurements

7.3.3.1 Appetite

Sensations of hunger, fullness, and nausea were rated by each subject at regular intervals using 10-cm linear visual analogue scales, as described in Chapter 5.5.3.

7.3.3.2 Gastric emptying

Measurements of the antral area were performed with an ultrasound machine (Aloka SSD-650 CL; ALOKA Co, Ltd, Tokyo), as described in Chapter 5.6. Antral area was measured immediately before the drink (t = 0 min), and at 5-minute intervals until 15 minutes, then every 15-minutes for a maximum of 180 minutes.

7.3.3.3 Blood glucose and plasma insulin concentrations

Blood glucose and plasma insulin concentrations were determined as described in Chapter 5.7.

7.3.3.4 Plasma ghrelin concentrations

Plasma ghrelin concentrations were determined as described in Chapter 5.7.2.

7.3.4 Statistical analysis

Gastric retention, appetite ratings (visual analogue scores), blood glucose, plasma insulin, and plasma ghrelin concentrations were evaluated using repeated-measures ANOVA. Post-hoc paired comparisons, adjusted for multiple comparisons by

Bonferroni correction, were performed when ANOVAs revealed significant effects. Peak plasma hormone concentrations were compared within subject groups using Student's paired *t* test. A *P* value of < 0.05 was considered statistically significant. Data are expressed as mean values ± SEM.

7.4 Results

7.4.1 Part A

The study drinks were well tolerated. The high-fat-orlistat drink induced mild transient diarrhoea or oily stools in five subjects, and mild flatulence in two subjects. The high-fat drink induced a mild bloating sensation in one individual. In all cases, the onset of the symptoms occurred after completion of the experiment, and resolved within 12 hours of drink ingestion.

7.4.1.1 Gastric emptying

Gastric emptying (Figure 7.1a) of water and high-fat-orlistat drinks were faster than that of the high-fat drink (treatment effect $P = 0.0007$), and there was a significant difference between high-fat and high-fat-orlistat drinks at t = 60 min ($P = 0.029$). The 50% gastric emptying time for the high-fat drink was longer compared to water (85.0 ± 12.9 mins vs 28.6 ± 3.8 min respectively, $P = 0.002$), and also longer than the 50% gastric emptying time for the high-fat-orlistat drink (68.0 ± 12.7 min), although this difference was not statistically significant ($P = 0.24$). The 50% gastric emptying time for the high-fat-orlistat drink was significantly longer than that of water ($P = 0.006$).

7.4.1.2 Plasma ghrelin concentrations

There were no differences in baseline plasma ghrelin concentrations between the three study days (Figure 7.2a). There was a significant treatment × time interaction (P = 0.006) for plasma ghrelin concentrations. Plasma ghrelin concentrations decreased following the high-fat drink from 1273 ± 255 pg/mL to a nadir of 1067 ± 251 pg/mL at 90 min (P = 0.01), i.e. a maximal suppression of ghrelin by 16%. Plasma ghrelin concentrations did not significantly change following either water or high-fat orlistat drink (P > 0.1). There was a significant difference in plasma ghrelin concentrations between high-fat and high-fat-orlistat drinks at t = 90 min (P = 0.03).

7.4.1.3 Blood glucose concentrations

There were no significant differences in baseline blood glucose concentrations between the three study days (Figure 7.3a). Blood glucose concentrations did not change following drink ingestion on any of the three treatment days (treatment × time interaction P = 0.23).

7.4.1.4 Plasma insulin concentrations

There were no significant differences in baseline plasma insulin concentrations between the three study days (Figure 7.4a). There was a rise in plasma insulin concentrations after the high-fat drink and high-fat-orlistat drinks, and no change after water (treatment × time interaction P = 0.0001). Plasma insulin concentrations were lower after the high-fat-orlistat, than high-fat drink, with significant differences at t = 15, 30, 90, and 120 min (P < 0.05), although the peak insulin concentrations, at t = 45 min, were

comparable between the two drinks (25.9 ± 3.4 mU/L for the high-fat drink versus 28.9 ± 4.7 mU/L for the high-fat-orlistat drink, P =0.09).

7.4.1.5 Appetite ratings

There were no differences in baseline scores for hunger (Figure 7.5a) or "prospective consumption" between study days (Figure 7.6a). Scores for hunger increased following the water drink (P = 0.0001), with no significant change from baseline following the high-fat and high-fat-orlistat drinks (P > 0.07), and no differences in hunger ratings between the high-fat and high-fat-orlistat drinks (P > 0.2). Hunger scores were lower for high-fat and high-fat-orlistat days when compared with control (treatment effect P = 0.02). Scores for prospective consumption progressively decreased over time after high-fat (P = 0.02) and high-fat-orlistat (P = 0.03) drinks, and increased after water (P = 0.0007). Prospective consumption was significantly less following the high-fat and high-fat-orlistat drinks compared with water (treatment effect P = 0.003), but there were no differences in prospective consumption between high-fat and high-fat-orlistat drinks (P > 0.1). Nausea scores did not change from baseline and remained low throughout all three study days (time effect P = 0.56) (Figures 7.7a), and there were no significant differences between high-fat, high-fat-orlistat, and water drinks (treatment effect P = 0.9).

7.4.2 Part B

The study drinks were well tolerated. The sucrose-acarbose drink induced mild transient nausea and mild flatulence in one subject. In this subject, the onset of the symptoms

occurred after completion of the experiment, and resolved within 7 hours of drink ingestion.

7.4.2.1 Gastric emptying

Gastric emptying (Figure 7.1b) of the sucrose-acarbose drink was slower than that of both the water and sucrose drinks (treatment effect $P = 0.0001$). Gastric emptying was slower following the sucrose-acarbose than sucrose drink, from t = 60 to 165 min ($P < 0.03$). Emptying of the sucrose-acarbose drink (50% gastric emptying time 86.2 ± 13.5 min) was significantly slower than that of sucrose (50.1 ± 5.7 min, $P = 0.03$), which was in turn longer than that of water (16.0 ± 2.3 min, $P = 0.0003$).

7.4.2.2 Plasma ghrelin concentrations

There were no differences in baseline plasma ghrelin concentrations between the three study days (Figure 7.2b). There was a significant treatment × time interaction ($P = 0.002$) for plasma ghrelin concentrations. Plasma ghrelin concentrations decreased following the sucrose drink from 819 ± 69 pg/mL to a nadir of 696 ± 49 pg/mL at 120 min, and were significantly suppressed from t = 30 min to 180 min compared to water ($P < 0.005$), i.e. a maximal 15% suppression of ghrelin. Plasma ghrelin concentrations were also significantly suppressed following the sucrose-acarbose drink compared to water, at t = 30, 60, 90, 150, and 180 min ($P \leq 0.01$). The degree of suppression of ghrelin concentrations following the sucrose-acarbose drink was attenuated when compared with sucrose, with approximately 30% higher ghrelin concentrations at t =

120 min (989 ± 152 pmol/L for the sucrose-acarbose drink versus 696 ± 49 pmol/L for the sucrose drink, $P = 0.0002$).

7.4.2.3 Blood glucose concentrations

There were no significant differences in baseline blood glucose concentrations between the three study days (Figure 7.3b). There was a rise in blood glucose concentrations after both sucrose and sucrose-acarbose drinks, to peaks of 8.1 ± 0.4 mmol/L and 6.6 ± 0.2 mmol/L respectively , with no change after control (treatment x time interaction $P = 0.0001$). Blood glucose concentrations were significantly lower after sucrose-acarbose than after sucrose, and this difference was significant from t = 15 to 120 min ($P < 0.04$).

7.4.2.4 Plasma insulin concentrations

There were no significant differences in baseline plasma insulin concentrations between the three study days (Figure 7.4b). There was a rise in plasma insulin concentrations after sucrose and sucrose-acarbose drinks, but no change after water (treatment × time interaction $P = 0.0001$). Plasma insulin concentrations were substantially lower after sucrose-acarbose than after the sucrose drink, with significant differences at t = 15 to 180 min ($P < 0.005$), and the peak insulin concentrations, at t = 30 min, were higher after the sucrose drink (68.4 ± 14.5 mU/L) than the sucrose-acarbose drink (25.2 ± 3.2 mU/L, $P = 0.0001$).

7.4.2.5 Appetite and nausea ratings

There were no differences in baseline scores for hunger or prospective consumption between study days (Figures 7.5b and 7.6b). Hunger scores progressively decreased after sucrose (P = 0.0001), but did not change from baseline after either water (P = 0.16) or sucrose-acarbose (P = 0.56) drinks. Scores for hunger were lower after both the sucrose and sucrose-acarbose drinks compared with water (treatment effect P = 0.049). Scores for prospective consumption were not significantly different between control, sucrose and sucrose-acarbose drinks (treatment effect P = 0.34). Nausea scores were very low throughout all three study days (time effect P = 0.08) (Figure 7.7b), and there were no significant differences between sucrose, sucrose-acarbose, and water drinks (treatment effect P = 0.36).

7.5 Discussion

In the present study, it was found that suppression of ghrelin by oral sucrose was attenuated by co-administration of the alpha-glucosidase inhibitor acarbose. Ingestion of a high-fat drink also suppressed plasma ghrelin concentrations, and this response was inhibited by the lipase inhibitor orlistat. As acarbose and orlistat inhibit the digestion of carbohydrates and fat respectively, these observations support the hypothesis that nutrient digestion is required for ghrelin suppression.

As discussed the postprandial suppression of ghrelin is dependent on macronutrient composition of a meal (Cummings et al. 2001; Erdmann et al. 2003; Erdmann et al. 2004), and is dependent on nutrient entry into the small intestine (Feinle-Bisset et al. 2005; Little et al. 2006a). In healthy adults, a 75 g oral glucose load in healthy adults

results in suppression of plasma ghrelin concentrations which reach their lowest level one hour after ingestion, with a subsequent increase (Shiiya et al. 2002). An intravenous glucose injection also reduces ghrelin concentrations, although for a shorter time than oral glucose (Shiiya et al. 2002). Parker et al. (2005) reported that, following intraduodenal infusion of glucose, plasma ghrelin concentrations were suppressed to at least 10% below baseline levels after 47.8 ± 10.5 minutes of commencing the infusion (Parker et al. 2005). The fall in ghrelin concentrations with an equivolaemic, equienergetic intragastric glucose infusion took slightly longer to occur, at 69.2 ± 7.7 minutes (P = 0.07), but was not different in magnitude to the fall following intraduodenal glucose infusion (19% for intragastric infusion versus 25% for intraduodenal infusion, P = 0.2) (Parker et al. 2005). These findings suggest that the presence of glucose in the stomach *per se* does not have a suppressive effect on plasma ghrelin concentrations. Studies in rats have produced similar findings (Williams et al. 2003). The above findings highlight the importance of nutrient exposure in the small intestine, and direct contact with the intestinal mucosa, in the suppression of ghrelin release by glucose.

Ingestion of a carbohydrate-rich meal in fasted subjects decreases plasma ghrelin levels within one hour (Cummings et al. 2001; Erdmann et al. 2003; Erdmann et al. 2004), and in some studies ghrelin levels remain suppressed for up to four hours (Erdmann et al. 2003). Fat administration, either orally (Erdmann et al. 2003; Erdmann et al. 2004) or by intraduodenal infusion (Feinle-Bisset et al. 2005) also suppresses plasma ghrelin concentrations, but the time course appears to be different to carbohydrate-induced suppression. There is an initial slight rise or little change in ghrelin levels up to one

hour after the meal (Erdmann et al. 2003; Erdmann et al. 2004), and subsequent fall towards baseline levels (Erdmann et al. 2004) or lower (Erdmann et al. 2003). Consistent with this, in the present study, the suppression of ghrelin concentrations after fat ingestion was not evident until one hour after drink ingestion. The role of protein in altering ghrelin secretion has not been clarified with studies reporting that ingestion of protein results in either a rise (Erdmann et al. 2003), or a fall in ghrelin concentrations (Al Awar et al. 2005; Bowen et al. 2006). In our study, both the sucrose and the high-fat drinks suppressed ghrelin concentrations, consistent with the findings of previous studies (Erdmann et al. 2003; Feinle-Bisset et al. 2005).

As indicated above, the presence of nutrients in the stomach *per se* appears not to influence plasma ghrelin concentrations in animals or humans (Williams et al. 2003; Parker et al. 2005); similarly gastric distension appears not to suppress ghrelin concentrations (Shiiya et al. 2002; Williams et al. 2003). Alterations in the rate of gastric emptying, by altering the duration, and timing, of nutrient contact with the small intestine, and thus affecting postgastric feedback mechanisms, may however modify ghrelin secretion. For example, Blom et al. (2006) found an inverse correlation between circulating ghrelin concentrations and the rate of gastric emptying following a standard breakfast, during both saline (R = -0.76, 95% CI -0.9 to -0.49) and GLP-1 infusions (R = -0.47, 95% CI -0.76 to -0.04) (Blom et al. 2006). This suggests that faster gastric emptying results in greater suppression of postprandial ghrelin concentrations, perhaps by enhancing the exposure of the small intestine to nutrients. In addition, the suppression of ghrelin may depend on the region of the gastrointestinal tract in contact with nutrients. Studies in rats have previously demonstrated that glucose delivered into

the stomach suppresses circulating ghrelin concentrations (Tschop et al. 2000; Williams et al. 2003; Overduin et al. 2005). However, the comparable suppression of ghrelin in rats in response to iso-energetic intragastric, intraduodenal, or intrajejunal infusions of glucose, suggests that the inhibitory effects on ghrelin secretion are not discretely dependent on the exposure of the stomach or duodenum to nutrients, and that exposure of the small intestine distal to the duodenum may play a role (Overduin et al. 2005). In humans, circulating ghrelin concentrations were decreased when the entire small intestine (> 60 cm in length) was exposed to an infusion of glucose, but not when glucose was infused into an isolated 60 cm segment of the proximal small intestine (Little et al. 2006a). Although it was not established whether this was a length-specific or region-specific response, it suggests that exposure of the more distal part of the small intestine is important for the suppression of ghrelin (Little et al. 2006a).

Acarbose delays digestion of disaccharides and absorption of glucose, decreases the breakdown of sucrose to fructose in the small intestine, and increases intestinal flow rates (Radziuk et al. 1984; Ruppin et al. 1988). The therapeutic effect of acarbose in patients with type 2 diabetes mellitus is related to the suppression of postprandial blood glucose concentrations (Chiasson et al. 1994), due to these actions and possibly also, in part, its inhibitory effect on gastric emptying (Ranganath et al. 1998; Gentilcore et al. 2005). The delay in gastric emptying in response to acarbose appears to require carbohydrate intake. In one study, the addition of a drink containing 100 mg of acarbose delayed gastric emptying of a high-carbohydrate mixed meal (61% carbohydrate, 23% fat, 16% protein) by approximately 25%, compared with that of a mixed meal without acarbose (Enc et al. 2001). However, in that same study, gastric emptying of a high-fat,

carbohydrate-free, meal (65% fat, 35% protein) was not affected when acarbose was included at the time of the meal, unless sucrose was ingested 120 minutes prior to the carbohydrate-free meal (Enc et al. 2001). This suggested that acarbose delays gastric emptying only in the presence of carbohydrates in the meal. Plasma concentrations of GLP-1, which may slow gastric emptying (Long et al. 1999; Naslund et al. 1999; Flint et al. 2001), increases to a greater extent in the presence of acarbose, following ingestion of sucrose (Ranganath et al. 1998; Gentilcore et al. 2005) or a high-carbohydrate mixed meal (Enc et al. 2001), and may be one of the mechanisms responsible for the delayed gastric emptying effects of acarbose when administered together with carbohydrates (Ranganath et al. 1998; Enc et al. 2001). In the present study, there was slowing of gastric emptying after combined ingestion of acarbose and sucrose, compared to ingestion of sucrose alone (Figure 7.1 b), as seen in previous studies. As slowing of gastric emptying is associated with an inhibition of the food-induced suppression of plasma ghrelin concentrations, the acarbose-induced attenuation of ghrelin suppression in the present study could have been due to inhibition of sucrose digestion, slowing of gastric emptying, or a combination of both. A study in which carbohydrate ingestion is inhibited, without effects on gastric emptying, i.e. by intraduodenal administration of acarbose and sucrose, would be necessary to separate out these effects. In the present study, the slowing of gastric emptying by the sucrose-acarbose drink was evident after 60 minutes, whereas ghrelin suppression was noted earlier, from 30 minutes. Blood glucose and plasma insulin concentrations were lower following the sucrose-acarbose, than sucrose drink, and this difference was significant from 15 minutes. It has previously been shown that ghrelin secretion is suppressed by an intravenous injection of glucose (Shiiya et al. 2002), and by hyperinsulinaemia,

independently of glucose (Flanagan et al. 2003). It can be speculated that the suppression of plasma ghrelin by the sucrose drink may have been mediated by either elevated blood glucose or plasma insulin, since the greater glycaemic and insulinaemic responses following the sucrose drink were associated with lower plasma ghrelin concentrations than the sucrose-acarbose drink.

Orlistat is a potent, specific, and reversible inhibitor of gastric and pancreatic lipases, and decreases fat absorption in the small intestine by approximately 30% (Hollander et al. 1998; Davidson et al. 1999), thus reducing feedback signals which slow gastric emptying. In patients with type 2 diabetes, orlistat accelerates gastric emptying of a mixture of pure fat (oil) and glucose (Pilichiewicz et al. 2003) and a high-fat, mashed potato, meal (O'Donovan et al. 2004). The standard dose of orlistat (120 mg) used in these studies would be expected to inhibit lipase activity by 75% (Borovicka et al. 2000), and reduce absorption of dietary fat by about 30% (Drent et al. 1995), and it has been shown that the slowing of gastric emptying by fat is dependent on lipolysis (Carney et al. 1995; Schwizer et al. 1997; Borovicka et al. 2000). Acceleration of gastric emptying by orlistat has also been demonstrated in healthy adults in previous studies (Schwizer et al. 1997; Borovicka et al. 2000; Chaikomin et al. 2006), and again in the present study. As indicated above, acceleration of gastric emptying with orlistat could be expected to be associated with an enhanced postprandial suppression of plasma ghrelin concentrations, whereas the opposite was observed in the present study. This finding is consistent with that of a previous study, in which the addition of 120 mg of orlistat completely blocked the approximately 60% suppression in plasma ghrelin concentrations produced by an intraduodenal infusion of a triglyceride emulsion

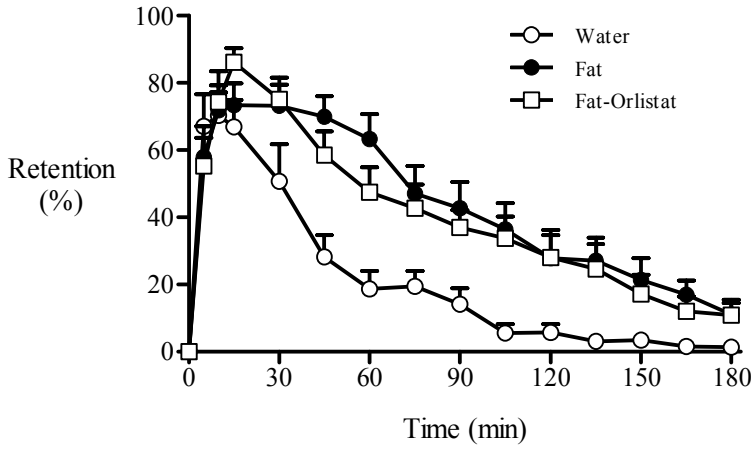
(Feinle-Bisset et al. 2005). Given that the use of orlistat resulted in about 75% inhibition of lipase activity, the lack of any ghrelin suppression suggests that there is a critical threshold in the amount of free fatty acids required to suppress plasma ghrelin (Feinle-Bisset et al. 2005). The present study demonstrates, for the first time, that the addition of orlistat also blocks the suppression of circulating ghrelin concentrations induced by oral ingestion of a high-fat drink. Together, the results of these two studies suggest that digestion of fat (as inhibited by orlistat) is necessary for its inhibitory action on plasma ghrelin concentrations. It seems highly likely that fatty acids, the products of fat digestion, are important in the suppression of ghrelin by fat. Furthermore, it has been demonstrated that suppression of plasma ghrelin occurs in response to an intraduodenal infusion of lauric acid, a fatty acid with an acyl chain length of 12 carbon atoms, but not with fatty acids with a chain length of 10 carbon atoms (decanoic acid), suggesting that ghrelin suppression is dependent on an acyl chain length of at least 12 carbon atoms (Feltrin et al. 2006).

In interpreting the findings of the present study, it should be noted that possible limitations may have been the amount of fat in the high-fat drink (88% of total energy as fat or 2393 kJ from fat) and the dose of orlistat (120 mg) used. In the present study, there was only a modest maximal suppression of ghrelin concentrations by 16% with the high-fat drink, compared to previous studies which have shown a 21% decrease in ghrelin concentrations after ingestion of a high-fat test meal (200 mL cream, 85% of energy as fat) (Erdmann et al. 2003), not unlike the high-fat drink in the present study, nevertheless this suppression was significant. The standard dose of orlistat used, 120 mg, abolished the suppression of ghrelin produced by the high-fat drink in the present

study, as it did in a previous study when administered with a fat emulsion intraduodenally (Feinle-Bisset et al. 2005). The results indicate that both the amount of fat in the drink, and the orlistat dose, were sufficient, and that it is unlikely that a higher dose of orlistat or a greater amount of fat than that used in the present study would have altered the results substantially. It should also be noted that the present study did not include a control condition of water with orlistat, or water with acarbose, however there is no evidence that orlistat or acarbose *per se* have any effects on the gastrointestinal tract, including gastrointestinal hormone secretion.

In summary, in this study of healthy young adults, plasma ghrelin concentrations were predictably suppressed by sucrose and fat. Inhibition of carbohydrate digestion by acarbose was associated with an attenuated suppression of ghrelin, which may also reflect slowing of gastric emptying. In order to examine the effects of carbohydrate digestion, further studies are warranted to assess if intraduodenal administration of sucrose with acarbose, which effectively bypasses the gastric emptying effects of acarbose, attenuates ghrelin suppression by sucrose. Reduction of fat digestion by orlistat both accelerates gastric emptying and attenuates the fat-induced ghrelin suppression, confirming that the digestion of fat is an important postgastric feedback mechanism potentiating the suppression of ghrelin secretion. The observations from this study have implications for the regulation of ghrelin secretion.

(a)



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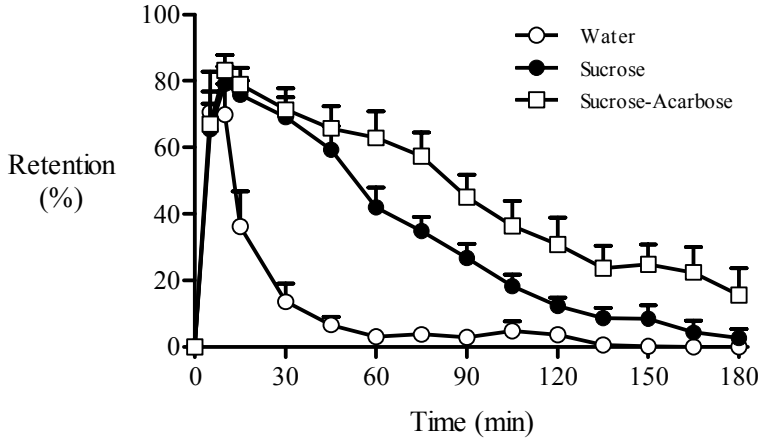
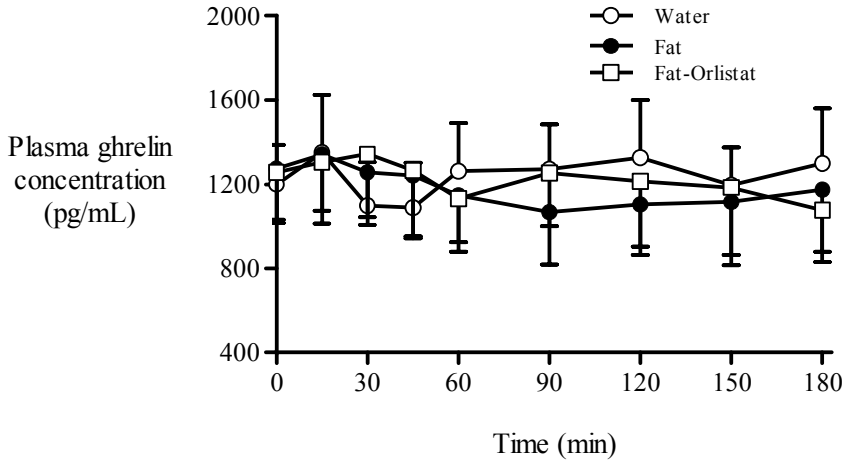


Figure 7.1: (a) Gastric emptying of the high-fat drink, with and without orlistat, and water; (b) Gastric emptying of the sucrose drink, with and without acarbose, and water. Data are mean values ± SEM. The presence of orlistat accelerated gastric emptying compared with the high-fat drink at t = 60 min (P = 0.029). Gastric emptying was slower following the sucrose-acarbose than sucrose drink, from t = 60 to 165 min ($P <$ 0.03).

(a)



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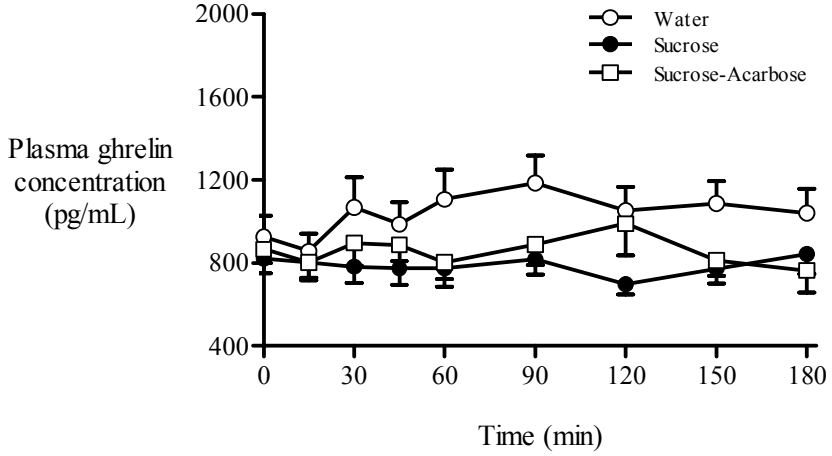
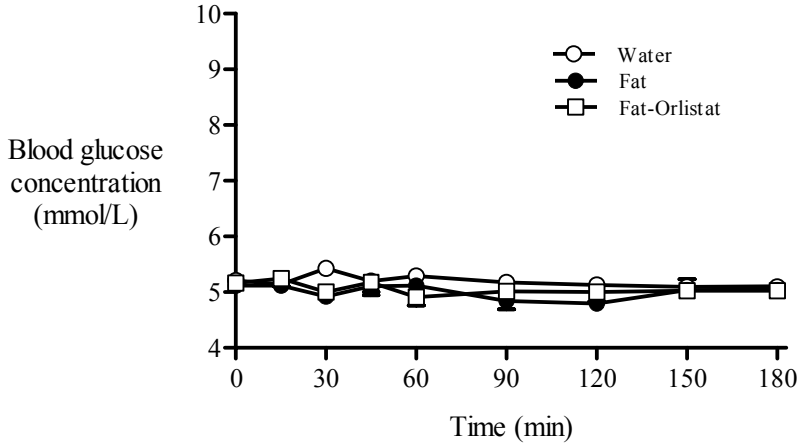


Figure 7.2: (a) Plasma ghrelin concentrations following ingestion of a high-fat drink, with and without orlistat, and water; (b) Plasma ghrelin concentrations following ingestion of a sucrose drink, with and without acarbose, and water. Plasma ghrelin was suppressed following the high-fat drink at t = 90 and 120 min (P = 0.01), and the presence of orlistat completely abolished this suppression (P = 0.03 compared to high-fat drink). The suppression of plasma ghrelin was greater following the sucrose than sucrose-acarbose drink (P = 0.0002).

(a)



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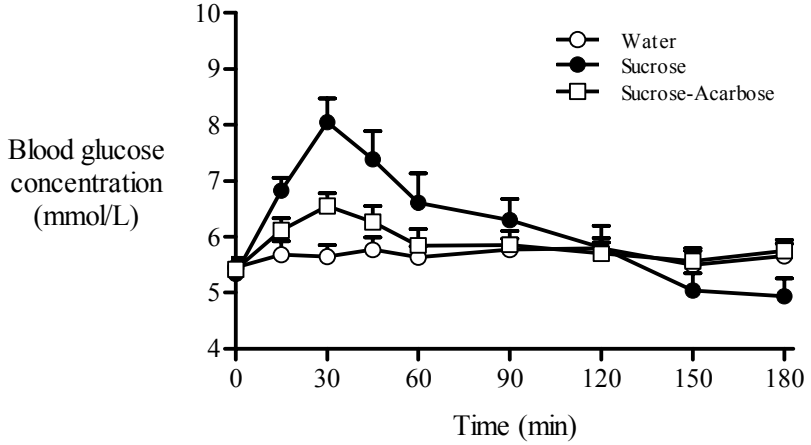
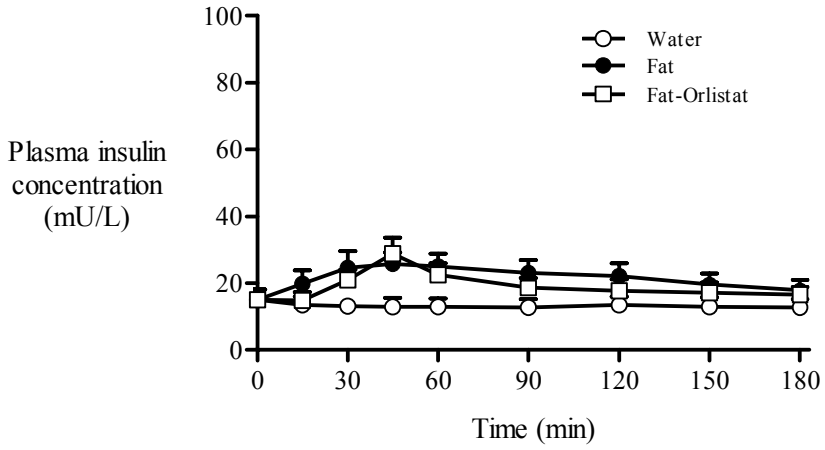


Figure 7.3: (a) Blood glucose concentrations following ingestion of a high-fat drink, with and without orlistat, and water; (b) Blood glucose concentrations following ingestion of a sucrose drink, with and without acarbose, and water. Blood glucose did not change following ingestion of high-fat and high-fat-orlistat drinks (treatment × time interaction $P = 0.23$). Blood glucose concentrations were significantly lower after the sucrose-acarbose, compared with sucrose drink from t = 15 to 120 min ($P < 0.04$).

(a)



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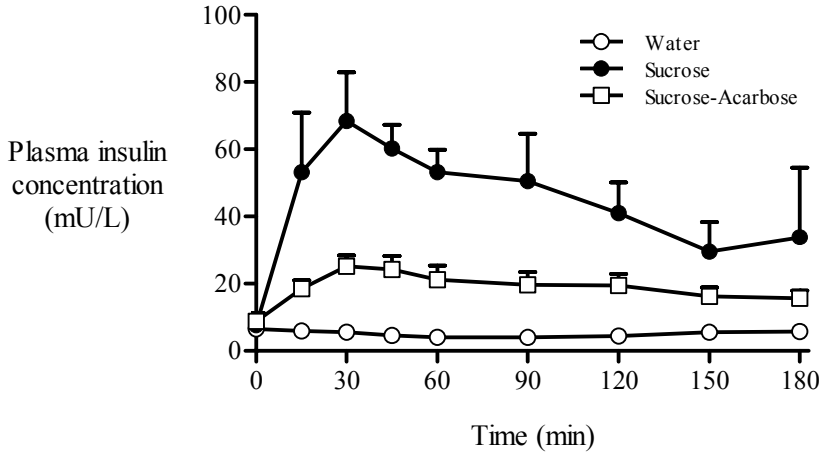
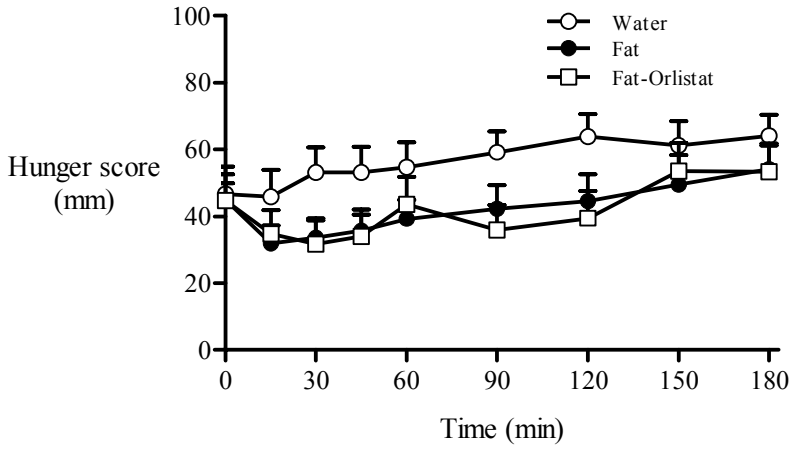


Figure 7.4: (a) Plasma insulin concentrations following ingestion of a high-fat drink, with and without orlistat, and water; (b) Plasma insulin concentrations following ingestion of the sucrose drink, with and without acarbose, and water. Plasma insulin was lower following the high-fat-orlistat, than high-fat drink, from t = 15 min ($P < 0.05$). Similarly, plasma insulin was lower after the sucrose-acarbose, than sucrose drink, from t = 15 to 180 min ($P < 0.005$).

(a)



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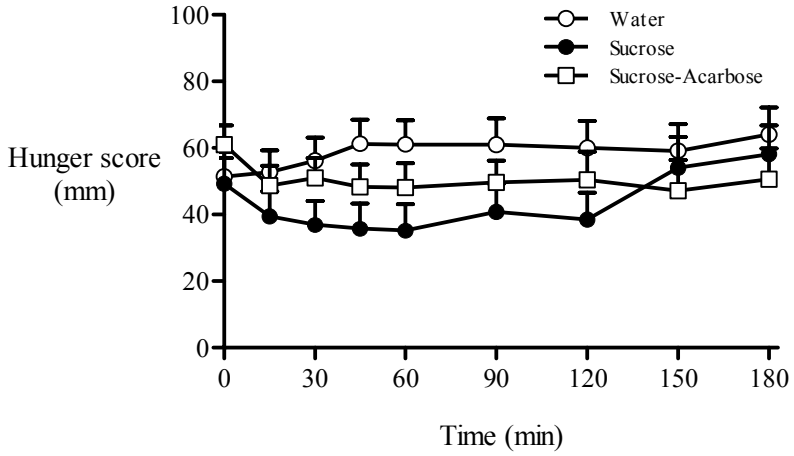
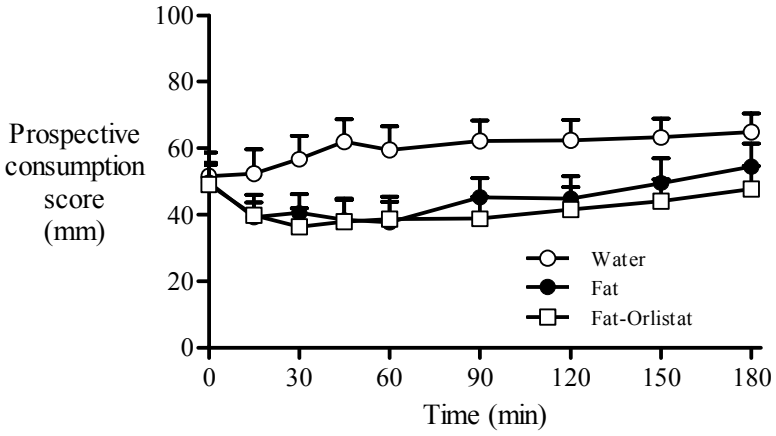


Figure 7.5: (a) Scores for hunger following ingestion of a high-fat drink, with and without orlistat, and water; (b) Scores for hunger following ingestion of a sucrose drink, with and without acarbose, and water.

(a)



(b)

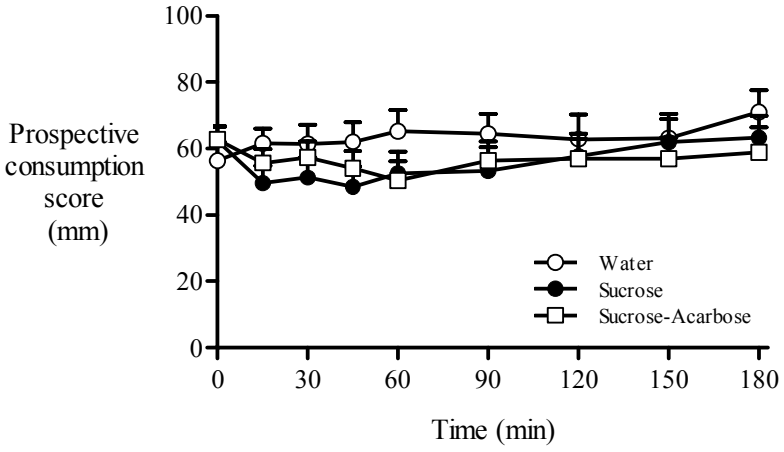
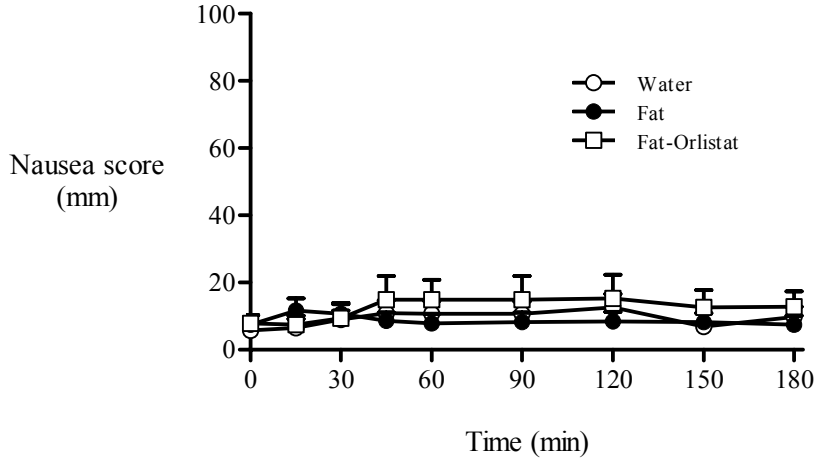


Figure 7.6: (a) Scores for prospective consumption following ingestion of a high-fat drink, with and without orlistat, and water; (b) Scores for prospective consumption following ingestion of a sucrose drink, with and without acarbose, and water.

(a)



(b)

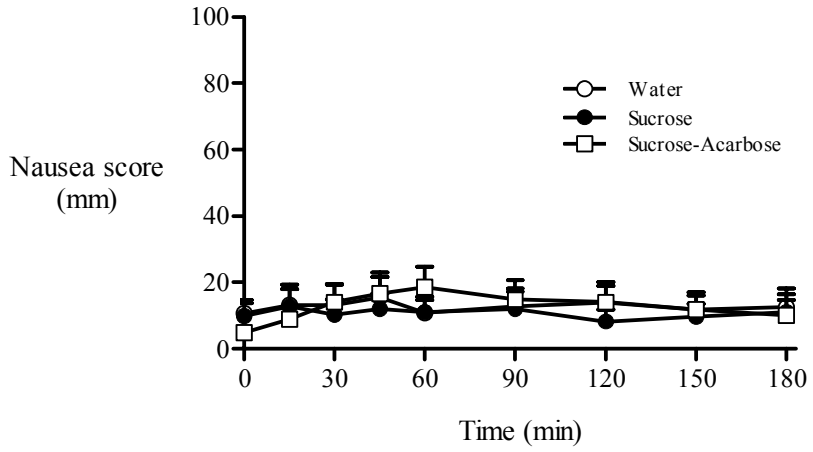


Figure 7.7: (a) Scores for nausea following ingestion of a high-fat drink, with and without orlistat, and water; (b) Scores for nausea following ingestion of a sucrose drink, with and without acarbose, and water.

Chapter 8

EFFECT OF DIETARY FAT AND ORLISTAT ON BLOOD PRESSURE AND HEART RATE IN HEALTHY YOUNG AND OLDER ADULTS

8.1 Summary

Postprandial blood pressure may be modified by the digestion of macronutrients such as fat. The effects of lipase inhibition with orlistat on the blood pressure and heart rate responses to a high-fat drink were examined in healthy young and older adults. Twelve young subjects aged 23.4 ± 1.2 years and nine older subjects aged 73.2 ± 2.0 years consumed a 300 mL high-fat drink (cream blended with milk, 88% fat) on two separate occasions, with, and without, 120 mg orlistat, and on a third occasion consumed 300 mL water (control). In both young and older subjects, gastric emptying of the high-fat drink was slower than the high-fat-orlistat drink, which was in turn slower than the water drink. In young subjects, neither systolic nor diastolic blood pressures significantly changed following any of the three drinks ($P \geq 0.1$), while heart rate increased after the high-fat ($P = 0.0003$) and high-fat-orlistat ($P = 0.0003$) drinks, and decreased following water ingestion ($P = 0.004$). In older subjects, however, systolic blood pressure progressively decreased following both the high-fat drink ($P = 0.0001$) and high-fat-orlistat drink ($P = 0.0001$), but not after water, with a greater reduction following the high-fat-orlistat than high-fat drink from 73 minutes ($P < 0.05$). Diastolic

blood pressure also decreased in the older subjects following the high-fat ($P = 0.0001$) and high-fat-orlistat ($P = 0.0001$) drinks, but not after water, with a greater reduction following the high-fat-orlistat drink than fat drink ($P < 0.05$). Heart rate was significantly higher in older subjects after the high-fat drink ($P = 0.0001$) and high-fat-orlistat drink ($P = 0.0001$), and not significantly different after water ingestion ($P = 0.09$). Thus, in older adults, ingestion of fat decreases systolic and diastolic blood pressures, and inhibition of fat digestion by orlistat exacerbates the reduction in postprandial blood pressure, possibly as a result of accelerating gastric emptying. Thus, fat digestion products do not appear critical for the hypotensive response to fat ingestion.

8.2 Introduction

Post-prandial hypotension (PPH) is defined as a decrease in systolic blood pressure of ≥ 20 mmHg within two hours of the start of a meal (Jansen and Lipsitz 1995). It is more common in older than young adults, and can result in significant morbidity, including an increased risk of falls and syncope (Aronow and Ahn 1994; Jansen and Lipsitz 1995; Puisieux et al. 2000). A 29-month follow-up study in 499 nursing home residents showed that, when compared to those without a new event, the maximal fall in postprandial systolic blood pressure is an independent risk factor for new falls, syncopal episodes, coronary events, stroke, and overall mortality (Aronow and Ahn 1997).

Apart from age, the magnitude of the fall in postprandial blood pressure is modified by baseline blood pressure (Sidery et al. 1993). Pre-existing hypertension exacerbates the decrease in postprandial blood pressure (Jansen et al. 1987b). Certain macronutrients in

a meal, in particular carbohydrate, may be important in reducing postprandial blood pressures (Potter et al. 1989; Jansen and Lipsitz 1995). This chapter focuses on the effects of different macronutrients on blood pressure.

One approach to the prevention and treatment of PPH, and hence a reduction in risk of the associated morbidity, may be to alter the type of food eaten in favour of macronutrients which minimise the fall in blood pressure. To do so systematically requires an understanding of the effects on blood pressure of ingesting different macronutrients and, given the much higher rate of PPH in older people, whether any differences in effects of the different macronutrients are modified by age. It has been established that ingestion of carbohydrate, either alone or as a predominant component of mixed foods, results in a 3 - 13 mmHg fall in systolic blood pressure in healthy older adults (Jansen et al. 1987b; Potter et al. 1989; Sidery et al. 1993; Visvanathan et al. 2005) and a decrease in systolic blood pressure of up to 15 mmHg in hypertensive older adults (Jansen et al. 1990). The magnitude of the fall in systolic blood pressure following an intraduodenal glucose infusion was significantly greater in older than young subjects (van Orshoven et al. 2008). It has been suggested that fat ingestion does not have a hypotensive effect in young adults (Jansen et al. 1990; Heseltine et al. 1990; Sidery et al. 1991). Observations relating to the effects of fat on blood pressure in older subjects are inconsistent, with some studies finding no fall (Jansen et al. 1990; Heseltine et al. 1990; Sidery et al. 1991; Sidery et al. 1993) or increase (Potter et al. 1989) in blood pressure after fat ingestion, while in others the extent of this fall was similar to that induced by carbohydrate, but the onset of the fall was delayed (Visvanathan et al. 2006). In a recent study of healthy older adults, intraduodenal

infusion of fat resulted in comparable maximal reductions in systolic blood pressure to those of intraduodenal glucose infused at the same rate (Gentilcore et al. 2008). If fat does have a less pronounced effect on blood pressure, one approach to PPH may be to increase fat at the expense of carbohydrate in the diet. No studies have compared the effects on blood pressure of fat in young and older adults. The study described in this chapter was performed to clarify the blood pressure response to ingestion of fat and determine whether it differs between older and young adults.

The products of carbohydrate and fat digestion may be particularly important to the blood pressure response to these macronutrients, as gastric distension alone, by ingestion of water, has been shown to increase blood pressure (Jordan et al. 1999; Shannon et al. 2002). For example, sucrose-induced postprandial blood pressure lowering is attenuated by acarbose, an alpha glucosidase inhibitor, which inhibits disaccharide digestion to glucose (Sasaki et al. 2001; Gentilcore et al. 2005). Orlistat, a lipase inhibitor which reduces the digestion, and hence absorption, of fat in the small intestine, has been used in the treatment of obesity, and lowers blood pressure compared to placebo, with a fall in systolic blood pressure of between 4 to 11 mmHg reported in studies with long-term use of orlistat, from three months to one year in duration, which may be secondary to weight loss with orlistat use (Bakris et al. 2002; Sharma and Golay 2002; Derosa et al. 2003; Derosa et al. 2005; Swinburn et al. 2005; Schneider et al. 2005; Zanella et al. 2006). When orlistat was administered acutely in patients with diet-controlled type 2 diabetes, systolic blood pressure fell by 11 mmHg in the first 30 minutes after ingestion of a high-fat/carbohydrate mixed meal with orlistat, compared with no fall in blood pressure without orlistat (O'Donovan et al. 2004). It is

known that, when administered with an intraduodenal triglyceride infusion, 120 mg of orlistat inhibits lipase activity by up to 75% (Borovicka et al. 2000; Feinle-Bisset et al. 2005). Inhibition of fat digestion by orlistat blocks the fat-induced increase in cholecystokinin (Hildebrand et al. 1998; Borovicka et al. 2000; Feinle et al. 2003; Feinle-Bisset et al. 2005), glucagon-like peptide-1 (Feinle et al. 2003), and peptide YY (Feinle-Bisset et al. 2005), and suppression of ghrelin (Feinle-Bisset et al. 2005). Orlistat also accelerates gastric emptying of high-fat meals in healthy adults (Schwizer et al. 1997; Borovicka et al. 2000; Chaikomin et al. 2006) and in patients with type 2 diabetes (Pilichiewicz et al. 2003; O'Donovan et al. 2004). In the current study, orlistat was added to a high-fat drink, to examine if co-administration of orlistat modifies the postprandial blood pressure response to fat ingestion acutely, and, in addition, to confirm that, analogous to the effect of carbohydrate ingestion on blood pressure, the effect of fat is more marked in older than young adults. We hypothesised that the products of fat digestion mediate the hypotensive response to fat in older adults, and that orlistat may, in fact, reduce the postprandial fall in blood pressure. Alternatively, the acceleration in gastric emptying of fat that occurs with orlistat may exacerbate the postprandial fall in blood pressure.

8.3 Materials and methods

8.3.1 Subjects

Twelve healthy, young adult subjects (6 males and 6 females), with a mean age of 23.4 ± 1.2 years (range 18-33 years), and nine healthy, older subjects (8 males and 1 female), with a mean age of 73.2 ± 2.0 years (range 66 - 85 years) were recruited by advertisement. Young subjects had a body mass index (BMI) of 22.5 ± 0.8 kg/m^2, and

older subjects had a BMI of 25.9 ± 0.6 kg/m^2. Subjects were included if they were unrestrained eaters as determined by a score of < 11 on the eating restraint questionnaire component of the Three-Factor Eating Questionnaire (Stunkard and Messick 1985). All subjects were non-smokers and none had a history of gastrointestinal disease or surgery, diabetes mellitus, significant respiratory, renal, hepatic or cardiac disease, autonomic dysfunction, chronic alcohol abuse or epilepsy. No subject was taking medication known to influence blood pressure or gastrointestinal function.

The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital and conducted in accordance with the guidelines in the Declaration of Helsinki. All subjects gave written, informed consent before inclusion into the study.

8.3.2 Study protocol

Each subject was studied on three separate occasions, separated by at least 48 hours. On each day, subjects attended the Discipline of Medicine, Royal Adelaide Hospital, at 8:30 a.m. following an overnight fast (12 hours for solids; 8.5 hours for liquids). An intravenous cannula was placed in the left antecubital vein for blood sampling and subjects were positioned comfortably in bed at approximately 90 degrees, to mimic normal physiological conditions during a meal. An automated blood pressure cuff was placed around the right arm for measurement of blood pressure and heart rate. Cardiovascular autonomic nerve function was evaluated at the end of one of the study days (Ewing and Clarke 1982; Piha 1991).

On each of the study days, at t = 0 min, subjects consumed the following equivolaemic drinks in randomised order: 1) water (control), 300 mL; 2) high-fat drink, 300 mL total, comprising 110 mL rich cream blended with 190 mL full-fat milk (88% fat, 7% carbohydrate, mostly lactose, 5% protein; total energy 2732kJ or 653 kcal), with low-energy flavouring; 3) high-fat-orlistat drink, 300 mL total, made up of 110 mL rich cream blended with 190 mL full-cream milk, with low-energy flavouring, with crushed and dissolved contents of one 120 mg capsule of orlistat (Roche Products Pty., Dee Why, N.S.W., Australia). All of the drinks were consumed within 3 minutes at room temperature. The dose of 120 mg of orlistat is a standard dose, and was chosen based on a previous study where orlistat was administered acutely with a meal, in patients with type 2 diabetes, and reduced postprandial systolic blood pressure (O'Donovan et al. 2004). Subjects were blinded to the addition of orlistat to the high-fat drink. Subjects were requested to report any adverse effects experienced during, or in the 24 hours following, each study day.

Venous blood samples were obtained at baseline immediately before ingestion of the drink (two baseline samples at t = -5 and -2 min), at 15-minute intervals for the first 60 minutes, then every 30 minutes until t = 181 min. Blood samples were collected in ice-chilled dipotassium EDTA tubes containing 400KIU aprotinin (Trasylol; Bayer Australia Ltd., Pymble, Australia) per millilitre of blood. Plasma was separated by centrifugation (3200 g, 15 min, 4ºC) within 30 minutes of collection and stored at -70ºC until assayed.

8.3.3 Measurements

8.3.3.1 Gastric emptying

Measurements of the gastric antral area were performed with an ultrasound machine (Aloka SSD-650 CL; ALOKA Co, Ltd, Tokyo), as described in Chapter 5 (Section 5.5.4). Antral area was measured immediately before the drink (t = 0 min), and at 5 minute intervals until 15 minutes, then every 15 minutes until t = 180 min, and gastric emptying time calculated as described in Chapter 5.6.

8.3.3.2 Blood pressure and heart rate

Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) were measured using an automated oscillometric blood pressure monitor (DINAMAP ProCare; GE Medical Systems, Sydney, NSW, Australia). Subjects were allowed to rest comfortably in the sitting position in bed for 30 minutes before two baseline measurements were taken at t = -5 and -2 min, before ingestion of the drink at t = 0 min. The study drink was consumed within 3 minutes. BP and HR were measured every 3 minutes from t = -5 min until t = 91 min, and then at 15-minute intervals until t = 181 min.

8.3.3.3 Blood glucose concentrations

Blood glucose concentrations (mmol/L) were determined as described in Chapter 5.7.1.

8.3.3.4 Cardiovascular autonomic nerve function

Autonomic nerve function was evaluated in the older subjects using standardised cardiovascular reflex tests (Ewing and Clarke 1982; Piha 1991), as described in Chapter 5.8.

8.3.4 Statistical analysis

Two-way repeated measures analysis of variance (ANOVA) was used to assess the overall effects of treatment and time and the treatment × time interactions on systolic and diastolic blood pressure, and heart rate changes from baseline. Mean contrasts were used to analyse individual point-by-point comparisons. Comparisons between the young and older groups in the effects of the drinks on blood pressure and heart rate were analysed using repeated measures three-way ANOVA, with time, age, and treatment as factors. Relations between 50% gastric emptying time (T50) and maximal decrease in systolic blood pressure, and between T50 and maximal increase in heart rate were assessed by Pearson's correlation analyses. The maximum fall in blood pressure and maximum rise in heart rate was defined as the greatest mean change from baseline in each subject at any given time point for each treatment. All analyses were performed using Statview version 5.0 (SAS Institute Inc., Cary, NC, USA) and SuperANOVA version 1.11 (Abacus Concepts Inc., Berkeley, CA, USA). Data are expressed as mean values ± SEM. A P value of < 0.05 was considered statistically significant.

8.4 Results

The study drinks were well tolerated in both younger and older subjects. The high-fat-orlistat drink induced mild transient diarrhoea or oily stools in six subjects, and mild

flatulence in two subjects. The high-fat drink induced a mild bloating sensation in one young individual, and mild abdominal discomfort and nausea in one older individual. In all cases, the onset of the symptoms occurred after completion of the experiment, and resolved within 19 hours of drink ingestion.

None of the older subjects had definite autonomic neuropathy; the median score for autonomic nerve dysfunction in the older subjects was 0 (range: 0 - 2); two subjects had a score of 1 and one subject had a score of 2.

8.4.1 Blood pressure and heart rate

8.4.1.1 Systolic blood pressure (SBP)

In young subjects (Figure 8.1a), there were no significant differences in baseline SBP between the three study days (high-fat drink 108.3 ± 2.3 mmHg vs high-fat-orlistat 108.9 ± 1.8 mmHg vs water 110.1 ± 2.1 mmHg). SBP did not change from baseline after the high-fat ($P = 0.18$), high-fat-orlistat ($P = 0.43$) or water ($P = 0.17$) drinks, and there was no treatment × time interaction ($P = 0.59$).

In older subjects (Figure 8.1b), there were no significant differences in baseline SBP between the three study days (high-fat drink 132.8 ± 4.8 vs high-fat-orlistat drink 131.6 ± 5.5 vs water 132.6 ± 4.8 mm Hg). One older subject had a fall in systolic blood pressure of > 20 mmHg (i.e. postprandial hypotension) following both the high-fat and high-fat-orlistat drinks. Mean baseline SBP was higher in older than young subjects (effect of age; $F = 25.21$, $P < 0.0001$). There was a significant treatment × age interaction ($F = 13.97$, $P < 0.0001$) and treatment × time × age interaction ($F = 10.25$, P

< 0.0001). There was a transient increase in SBP immediately (t = 0 - 4 min) following ingestion of the high-fat drink (P = 0.005), but not after water (P = 0.05) or high-fat-orlistat drink (P = 0.32).

For SBP (change from baseline) in older subjects, there was no significant treatment effect (P = 0.07), but a significant time (P = 0.0001) effect and treatment × time interaction (P = 0.017) after t = 55 minutes. SBP decreased progressively after the high-fat drink (P = 0.0001) and high-fat-orlistat drink (P = 0.0001), but not after water (P = 0.3). There was a greater reduction in SBP after the high-fat-orlistat than the high-fat drink, which was significant from t = 73 - 88 min (P < 0.05). The maximum fall in SBP during the high-fat (16.3 ± 2.1 mmHg) and high-fat-orlistat (20.1 ± 3.9 mmHg) drinks did not differ significantly (P = 0.27), and there was no significant difference in the time of maximum fall between high-fat and high-fat-orlistat drinks (66.3 ± 14.5 vs 68.3 ± 10.6 min; P = 0.9).

8.4.1.2 Diastolic blood pressure (DBP)

In young subjects (Figure 8.2a), there were no significant differences in baseline DBP between the three study days (high-fat drink 60.7 ± 1.7 mmHg vs high-fat-orlistat drink 62.2 ± 1.3 mmHg vs water 63.9 ± 1.8 mmHg). DBP did not change from baseline after the high-fat (P = 0.22), high-fat-orlistat drinks (P = 0.06), or water (P = 0.16), and there was no treatment × time interaction (P = 0.17).

In older subjects (Figure 8.2b), there were no significant differences in baseline DBP between the three study days (high-fat drink 74.2 ± 2.4 vs high-fat-orlistat drink 75.2 ±

2.6 vs water 75.6 ± 2.5 mmHg). Mean baseline DBP was higher in older than young subjects (effect of age; F = 27.12, $P < 0.0001$). There was a significant treatment × age interaction (F = 16.31, $P < 0.0001$), but no significant treatment × time × age interaction (F = 1.68, $P = 0.19$). There was a transient increase in DBP immediately (t = 0 - 4 min) following ingestion of the high-fat drink ($P = 0.01$), but not after water ($P = 0.27$) or high-fat-orlistat drink ($P = 1.0$).

For DBP (change from baseline) in older subjects, there were significant time ($P = 0.006$) effects and treatment × time interaction ($P = 0.0001$) over the duration of the study. DBP decreased after the high-fat (maximum decrease of 9.6 ± 1.2 mmHg, $P = 0.0001$) and high-fat-orlistat drinks (11.1 ± 2.9 mmHg, $P = 0.0001$), but not after water ($P = 0.31$). There was a slight, but significantly greater reduction in DBP following ingestion of the high-fat-orlistat drink than high-fat drink ($P < 0.05$), with a maximal fall in DBP of 11.1 mmHg vs 9.6 mmHg respectively.

8.4.1.3 Heart rate (HR)

In young subjects (Figure 8.3a), there were no significant differences in baseline HR between the three study days (high-fat drink 65.5 ± 1.3 vs high-fat-orlistat drink 64.9 ± 2.0 vs water 66.8 ± 2.0 bpm). HR increased after both the high-fat (maximum increase of 5.4 ± 1.9 bpm, $P = 0.0003$) and high-fat-orlistat (6.2 ± 1.7 bpm, $P = 0.0003$) drinks, with no significant difference in the magnitude of these increases, and decreased after water (6.8 ± 1.6 bpm, $P = 0.004$). For HR (change from baseline), there were significant treatment ($P = 0.009$) and time ($P = 0.0001$) effects and treatment × time interaction (*P*

= 0.03) over the duration of the study, and these effects continued for at least 180 minutes.

In older subjects (Figure 8.3b), there were no significant differences in baseline heart rate between the three study days (high-fat drink 59.7 ± 1.4 vs high-fat-orlistat drink 57.8 ± 1.8 vs water 59.2 ± 1.1 bpm), and mean baseline heart rates were lower in older than in young subjects (effect of age; F = 13.61, P = 0.002). There was a significant time × age interaction (F = 6.17; P = 0.01), but no significant treatment × age interaction (F = 0.06, P = 0.94) or treatment × time × age interaction (F = 0.51, P = 0.60). For HR (change from baseline), there were significant treatment (P = 0.0001) and time (P = 0.0001) effects, and treatment × time interaction (P = 0.0001). Heart rate increased after both the high-fat (P = 0.0001) and high-fat-orlistat (P = 0.0001) drinks, and decreased non-significantly after water (P = 0.09). The increase in heart rate was slightly greater following the high-fat drink than the high-fat-orlistat drink, but only at 55 and 58 minutes (P < 0.03). The maximum rise in heart rate during the high-fat (14.8 ± 2.6 bpm) and high-fat-orlistat (10.9 ± 1.6 bpm) drinks did not differ significantly (P = 0.13), and there was no significant difference in the time to maximum rise in heart rate between the high-fat and high-fat-orlistat drinks (79.3 ± 16.5 vs 71.7 ± 13.9 min; P = 0.63).

8.4.2 Blood glucose concentrations

In the younger subjects, there were no significant differences in baseline blood glucose concentrations between the high-fat drink, high-fat-orlistat drink, or water (Figure 8.4a). Blood glucose concentrations were not significantly altered after the high-fat drink (P =

0.28), high-fat-orlistat drink (P = 0.21), or water (P = 0.07). There was no significant time effect (P = 0.07) or treatment × time interaction (P = 0.23).

In the older subjects, there were no significant differences in baseline blood glucose concentrations between the high-fat drink, high-fat-orlistat drink, or water (Figure 8.4b). Blood glucose concentrations slightly increased after the high-fat drink (maximum increase of 0.6 ± 0.3 mmol/L at t = 15 min, P = 0.002) and the high-fat-orlistat drink (0.6 ± 0.2 mmol/L at t = 30 min, P = 0.004), and decreased after water (-0.6 ± 0.3 mmol/L, P = 0.0001). However, there was no significant treatment × time interaction (P = 0.21). Mean blood glucose concentrations were higher in older than young subjects throughout all three study days (effect of age; F = 31.26, P < 0.0001), with no significant effect of treatment (F = 0.80, P = 0.46), and no treatment × age interaction (F = 0.83, P = 0.44), in the combined group of young and older subjects.

8.4.3 Gastric emptying

In young subjects (Figure 8.5a), gastric emptying of water was substantially faster than that of the high-fat orlistat drink, which was in turn slightly faster than that of the high-fat drink (treatment effect P = 0.0007). The 50% gastric emptying time for the high-fat drink was significantly longer than that of water (77.0 ± 11.0 min vs 28.5 ± 3.7 min, P = 0.002), but not significantly different compared to high-fat-orlistat drink (76.3 ± 12.4 min, P = 0.24).

In older subjects (Figure 8.5b), gastric emptying of water was faster than that of the high-fat orlistat drink, which was in turn faster than that of the high-fat drink (treatment

effect $P = 0.0004$). Gastric emptying was significantly faster after high-fat-orlistat drink than after the high-fat drink from t = 45 to 120 min ($P < 0.04$). The 50% gastric emptying time (T50) for the high-fat drink was slower compared to water (60.3 ± 7.1 min vs 24.7 ± 5.8 min respectively, $P = 0.0003$), but not significantly different compared to the high-fat-orlistat drink (47.4 ± 5.0 min, $P = 0.24$). In the combined group of young and older subjects, there was an effect of treatment ($F = 17.55$, $P < 0.0001$) on the 50% gastric emptying time, but there was no effect of age ($F = 3.38$, $P = 0.08$), and no treatment × age interaction ($F = 1.34$, $P = 0.27$).

8.4.3.1 Relationships between gastric emptying, blood pressure and heart rate

In the older subjects, there were no significant relationships between the T50 and mean maximum fall in systolic blood pressure after the high-fat-orlistat drink ($R = -0.24$, $P = 0.54$) or the high-fat drink ($R = -0.08$, $P = 0.85$). There was a significant relationship between the T50 and mean maximum rise in heart rate after the high-fat-orlistat drink ($R = 0.83$, $P = 0.006$), but not after the high-fat drink ($R = 0.17$, $P = 0.65$).

8.5 Discussion

The present study in healthy young and older subjects confirms that ingestion of fat slows gastric emptying, and the addition of orlistat to a high-fat drink attenuates this slowing of gastric emptying. It also establishes that ingestion of a high-fat drink decreases systolic and diastolic blood pressure in older, but not in young, subjects, and demonstrates the novel finding that orlistat accentuates the hypotensive response to fat in older subjects. To our knowledge, this is the first study directly comparing the effects of fat ingestion, with and without orlistat, in young and older subjects.

In the present study, in healthy young subjects, fat ingestion was not followed by any significant change in systolic or diastolic blood pressures, and heart rate rose significantly after fat ingestion, regardless of whether orlistat was added. Consistent with these observations, previous studies have demonstrated that blood pressure does not change in healthy young subjects given a high-fat meal (66 - 71% energy as fat) (Heseltine et al. 1990; Sidery et al. 1991). Increases in heart rate have previously been noted after high-carbohydrate and high-fat meals in healthy younger subjects (Heseltine et al. 1990; Sidery et al. 1991), with no significant differences in the heart rate response between the two meals in one study (Sidery et al. 1991). This heart rate increase is probably due to activation of a baroreceptor reflex; as splanchnic blood flow increases after a meal, thus reducing systemic vascular resistance, there is a compensatory increase in heart rate. The observed increase in heart rate after orlistat in the present study is also consistent with previous studies – in eight patients with diet-controlled type 2 diabetes mellitus, aged 49 - 68 years, ranging from normal-weight to obese, administration of a mixed meal containing orlistat increased heart rate to a greater extent when compared with the same meal without orlistat ($P = 0.03$) (O'Donovan et al. 2004), possibly due to faster gastric emptying, with greater or earlier activation of the baroreceptor reflex. With long-term use of orlistat in weight-loss studies over 12 months, in obese patients with hypertension, concurrent with the reduction in blood pressure, heart rate either does not change (Bakris et al. 2002; Derosa et al. 2005) or decreases (Sharma and Golay 2002).

In a previous study of 12 healthy older adults, ingestion of a high-fat drink (total energy 2732 kJ, 88% energy as fat) resulted in a comparable lowering of systolic blood pressure compared with an iso-energetic high-carbohydrate drink (100% energy as carbohydrates) (maximum reduction of 13.4 mmHg for carbohydrate versus 15.6 mmHg for fat, non-significant difference $P = 0.47$), although the fall in blood pressure after fat occurred later than that after carbohydrate ingestion (onset 26.5 minutes versus 13 minutes) (Visvanathan et al. 2006). Another study in 10 healthy older individuals showed a maximum fall in diastolic blood pressure of 5 mmHg after a high-fat meal (total energy 2500 kJ, 71% energy as fat), but a non-significant fall in systolic blood pressure (Sidery et al. 1993). Consistent with the above findings, in the current study there was a fall in blood pressure after ingestion of fat in older, but not young, subjects. Heart rate increased in both young and older subjects. In contrast, a study in 7 healthy older adults demonstrated no significant changes in blood pressure following ingestion of a high-fat meal (total energy 2420 kJ, 66% energy as fat) (Potter et al. 1989). Similarly, in a previous study of 10 hypertensive older adults, ingestion of a high-fat drink (75 g of fat) did not induce a fall in blood pressure (Jansen et al. 1990). The fall in blood pressure observed after the high-fat drink in the present study is unlikely to be due to the small amount of carbohydrate (mainly lactose, 7% of total energy, or 192 kJ). Comparable amounts of carbohydrate were present in the aforementioned studies, where no changes in blood pressure were demonstrated after ingestion of a high-fat drink (7% energy as carbohydrate) (Jansen et al. 1990) or high-fat meal (6 g of carbohydrate, 25.2 kcal or 105 kJ) (Potter et al. 1989). Intraduodenal infusion of a greater amount of glucose at 1 kcal/min for 60 minutes (total amount of 60 kcal or 251 kJ) to that used in the present study, did not decrease blood pressure in healthy older

adults, while a faster infusion rate of 3 kcal/min over 60 minutes (752 kJ) decreased systolic and diastolic blood pressures (O'Donovan et al. 2002). Infusion of fat directly into the small intestine also appears to have similar blood pressure-lowering effects to carbohydrates. In a recent study of healthy older adults, intraduodenal infusion of fat resulted in comparable maximal reductions in systolic blood pressure (11.7 ± 4.8 mmHg) to those of intraduodenal glucose infused at the same rate (11.7 ± 2.8 mmHg) (Gentilcore et al. 2008). However, the maximal decrease in blood pressure occurred later after fat (46 ± 11 minutes) than glucose (18 ± 3 minutes, $P = 0.02$) infusion (Gentilcore et al. 2008). Thus, it appears that both oral ingestion and intraduodenal administration of fat lower blood pressure, with effects more marked in older than young adults. An effect of age on blood pressure responses to nutrient administration was also recently demonstrated in a study of young and older healthy adults, where the magnitude of the fall in systolic blood pressure following an intraduodenal glucose infusion was significantly greater in older than young subjects (mean maximal fall of 17.0 ± 4.1 mmHg vs 6.5 ± 1.6 mmHg, $P = 0.03$) (van Orshoven et al. 2008).

Cardiovascular responses to carbohydrate administration appear to be more consistent across studies compared to the effects of fat. The observed differences between young and older subjects in response to fat ingestion are analogous to the differences in response to carbohydrate ingestion. In healthy young subjects, systolic and diastolic blood pressures did not significantly change after eating a high-carbohydrate meal (Heseltine et al. 1990; Sidery et al. 1991). In contrast, in healthy older individuals, systolic and diastolic blood pressures decreased following consumption of carbohydrates (Jansen et al. 1987b; Potter et al. 1989; Jansen et al. 1990; Sidery et al.

1993; Jones et al. 1998; Visvanathan et al. 2005; Visvanathan et al. 2006), with smaller reductions in blood pressure after a high-protein meal (Potter et al. 1989). From the above observations, it appears that dietary modification, by altering the ratio of carbohydrate to fat content in a meal, or favouring one macronutrient over another, may not ameliorate the postprandial fall in blood pressure in older individuals. In addition to the "nutrient-driven" feedback signals from the small intestine, the magnitude of the postprandial fall in blood pressure is also influenced by 'intragastric' mechanisms, in particular gastric distension. Consumption of water, which increases gastric distension, has been shown to increase blood pressure, and was first studied in nineteen patients with severe orthostatic hypotension secondary to autonomic failure, where oral ingestion of 480 mL of water increased systolic blood pressure by 11 ± 2.4 mmHg 35 minutes after ingestion ($P < 0.001$) (Jordan et al. 1999). In another study of seven patients with autonomic failure, ingestion of 480 mL of water within 5 minutes, consumed just prior to a high-carbohydrate meal, increased systolic blood pressure by a maximum of 36 ± 23 mmHg after 20 minutes, compared with a fall in systolic blood pressure of up to 43 ± 36 mmHg when water was not ingested before the meal (Shannon et al. 2002). Thus, drinking water before a meal can attenuate the fall in postprandial blood pressure.

Several mechanisms have been proposed for the observed differences in postprandial blood pressures seen between young and older subjects and the variations in the magnitude of the blood pressure response among different macronutrients. Increased glucose and insulin concentrations secondary to carbohydrate ingestion in older compared to young adults have led some to hypothesise that the rise in circulating

insulin concentrations following meal ingestion, particularly carbohydrate ingestion, may mediate the blood pressure decrease, by blunting baroreflex sensitivity (Lipsitz et al. 1983). In support of a role of insulin, oral glucose ingestion was found to induce a larger increase in plasma glucose and insulin concentrations than an iso-energetic fructose load, and produce a significantly greater fall in blood pressure (Jansen et al. 1987b). Furthermore, fat and protein, both of which induced a minimal increase in blood glucose and plasma insulin concentrations, did not lead to a drop in blood pressure in either young or older subjects (Jansen et al. 1990). Intra-arterial infusion of insulin also induces vasodilation and increases forearm blood flow, and stimulates activation of the sympathetic nervous system (Creager et al. 1985). However, while insulin has vasodilatory properties, other studies do not support a role for insulin, by failing to show a correlation between the increase in plasma insulin concentrations and the fall in blood pressure after oral glucose loading (Jansen et al. 1987b; Jansen et al. 1990). In addition, it has been demonstrated that a 100 mL intravenous injection of 40% glucose is followed by a transient decrease in systolic blood pressure, which occurs before the glucose-induced increase in insulin concentrations (Jansen and Hoefnagels 1987). This has led to the suggestion that the action of insulin may not be an important mechanism of postprandial hypotension, and that ingestion of glucose results in direct vasodilatation of splanchnic veins and increased pooling of blood in the splanchnic circulation (Jansen et al. 1987b; Jansen and Hoefnagels 1987). However, some studies have demonstrated no apparent relationship between changes in blood glucose concentrations and blood pressure responses to a meal (Visvanathan et al. 2004; Visvanathan et al. 2006). In one study, the increase in plasma noradrenaline concentrations and heart rate, which reflect activation of the sympathetic nervous

system, were not significantly different between the glucose and fructose drinks, suggesting that differential activation of the sympathetic nervous system was not a major factor in the responses between young and older individuals (Jansen et al. 1987b). A limitation of the present study was that sympathetic nervous system activity and splanchnic blood flow were not assessed, so the precise mechanisms for postprandial hypotension could not be defined.

The extent of digestion of fat may modify the cardiovascular response. It has been previously suggested that the presence of non-esterified fatty acids (NEFA), the products of fat digestion, in the small intestine, may be a prerequisite for the hypotensive effect of fat (Visvanathan et al. 2006). In patients with diet-controlled type 2 diabetes mellitus, a single dose of orlistat has been shown to decrease systolic blood pressure (O'Donovan et al. 2004). Previous weight loss studies in overweight adults, which have included those with type 2 diabetes mellitus and hypertension, have generally shown a reduction in blood pressure and improvements in cardiovascular risk profile with longer use of orlistat upto one year (Bakris et al. 2002; Sharma and Golay 2002; Derosa et al. 2003; Swinburn et al. 2005; Schneider et al. 2005; Zanella et al. 2006). This may be partly related to the greater weight loss seen with orlistat compared to placebo (Bakris et al. 2002; Sharma and Golay 2002; Swinburn et al. 2005), however, equivalent degrees of weight loss with sibutramine failed to have an effect on blood pressure (Derosa et al. 2005). It is known that fat digestion (lipolysis of triglycerides to fatty acids) is required for its appetite suppressant effect (Matzinger et al. 2000; Feinle et al. 2003), for the stimulation of CCK, GLP-1, and PYY (Hildebrand et al. 1998; Feinle et al. 2003), suppression of ghrelin (Feinle-Bisset et al. 2005),

slowing of gastric emptying (Carney et al. 1995; Schwizer et al. 1997; Borovicka et al. 2000), and pancreatic enzyme secretion (Hildebrand et al. 1998). In the present study, in older subjects, orlistat increased the hypotensive effect seen with ingestion of fat. On the other hand, in young subjects, there were no changes in postprandial blood pressures after the high-fat or high-fat-orlistat drinks. These findings demonstrate that the hypotensive effect of fat is accentuated, rather than attenuated, when fat digestion is reduced by orlistat. While this suggests that the presence of fatty acids may not be mandatory for the hypotensive response, it does not exclude a role of fat digestion, as some of the hypotensive effects of orlistat may be explained by the faster rate of gastric emptying of fat in the presence of orlistat found in this study, which is concordant with the effects of orlistat in previous studies in healthy adults (Schwizer et al. 1997; Borovicka et al. 2000; Chaikomin et al. 2006) and in patients with diet-controlled type 2 diabetes (Pilichiewicz et al. 2003; O'Donovan et al. 2004). It is also possible that, following ingestion of the high-fat drink, some of the intragastric fat 'layered' on top of the aqueous phase due to its lower density (Horowitz et al. 1993), unlike a liquid fat emulsion (Schwizer et al. 1997). Due to the effects of gravity in the sitting position, the aqueous phase would empty faster than the oil phase (Horowitz et al. 1993; Carney et al. 1995) and, thereby, regulate gastric emptying of the remainder of the drink.

On the other hand, the delayed hypotensive effects of fat, compared to carbohydrate, ingestion seen in previous studies (Visvanathan et al. 2006) may reflect the known effect of fat in slowing gastric emptying (Cunningham and Read 1989). If gastric emptying is slowed, it can be hypothesised that the rate of delivery of nutrients to the

small intestine, and thus nutrient-driven effects on blood pressure, are delayed; in addition, the increase in gastric distension is prolonged.

In the current study, gastric emptying was slower after the high-fat drink compared to water, a finding consistent with previous studies when fat was infused into the small intestine (Cunningham and Read 1989). It has been previously demonstrated that the fall in postprandial blood pressure is directly related to the rate of gastric emptying in individuals with type 2 diabetes mellitus (Jones et al. 1998). Furthermore, previous studies have demonstrated that the reduction in postprandial blood pressure is attenuated with acarbose, an alpha-glucosidase inhibitor, which slows down glucose absorption (Sasaki et al. 2001; Gentilcore et al. 2005), and also delays gastric emptying (Ranganath et al. 1998; Enc et al. 2001; Gentilcore et al. 2005). However, whilst acarbose attenuates the fall in systolic and diastolic blood pressure occurring from within 39 minutes of ingestion of an acarbose-containing sucrose drink, the delayed gastric emptying effects of acarbose are seen later, at approximately 90 minutes (Gentilcore et al. 2005). Thus, the effects of acarbose on postprandial blood pressure are not accounted for by changes in the rate of gastric emptying (Gentilcore et al. 2005). In contrast, in the present study, in older subjects, orlistat increased the rate of gastric emptying from 45 minutes after ingestion of an orlistat-containing high-fat drink, and the hypotensive effects of orlistat were seen thereafter, from 73 minutes. Whilst the amount of digested fat in contact with the small intestine is lower in the presence of orlistat compared to fat alone, it can be speculated that the increased rate of gastric emptying with orlistat compensates by shortening the delay in small intestinal exposure

to fat digestion products. The blood pressure responses to intraduodenal infusion of fat with orlistat have not been evaluated.

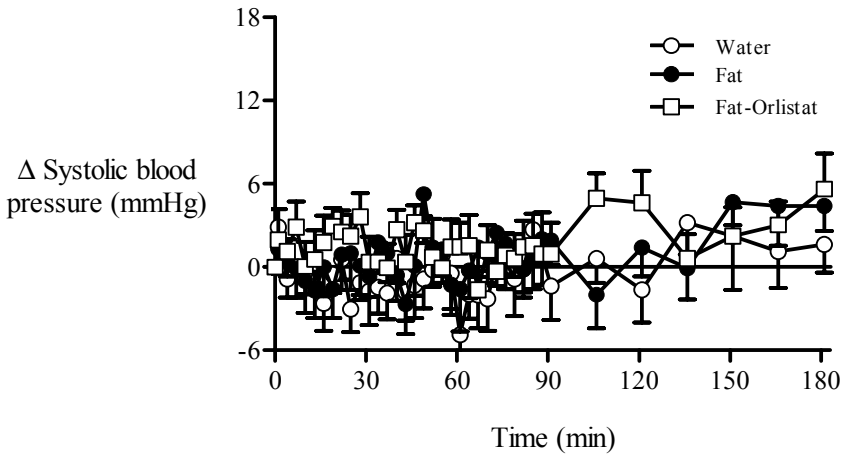
In the present study, blood pressures were measured at regular intervals up to three hours following ingestion of the high-fat drink, as the hypotensive effect of fat ingestion is quite prolonged. In a previous study using the same high-fat drink as in the present study, the suppressive effects on blood pressure in healthy older people were still fully present at 90 minutes, the end of the study period (Visvanathan et al. 2006). In the current study, the observation period was made twice the duration of that previous study (Visvanathan et al. 2006), to maximise the chances of characterising fully any blood pressure effects of the high-fat or high-fat-orlistat drinks during that time. Indeed, that proved the case, with suppressive effects of fat on systolic blood pressure in older people resolving by approximately 150 minutes, and those on diastolic blood pressure by 120 minutes (Figure 8.2). While the high-fat drink used in the present study had a relatively low carbohydrate content (88% fat, 7% carbohydrate, 5% protein), a slight increase in blood glucose concentrations up to a maximum of approximately 0.6 mmol/L was noted in the older, but not young, subjects.

Other factors may play a role in determining the degree of postprandial blood pressure reduction. Some studies in healthy young subjects have shown that the postprandial increases in heart rate and cardiac output were related to meal size, rather than meal constituents, with a larger increase in pulse rate seen after a bigger compared to a smaller meal, however there were inconsistent effects on postprandial mean arterial pressures (Waaler et al. 1991). Isovolaemic drinks were used in this study, similar to

those used in a previous study in healthy older adults (Visvanathan et al. 2006), to reduce the potential confounding effect of drink volume on gastric emptying rate or on cardiovascular responses.

In summary, a fall in blood pressure following a meal occurs more commonly in older compared to young adults. Fat ingestion was associated with a reduction in systolic and diastolic blood pressures in older, but not young, adults. Manipulation of the composition of a meal, in particular its carbohydrate and fat content, is thus unlikely to be of benefit in attenuating the cardiovascular response to a meal in older individuals. Co-administration of the lipase inhibitor orlistat accentuates the fat-induced reduction in blood pressure. This suggests that digestion of fat (which is inhibited by orlistat) is not an important factor in fat-induced hypotension, but rather explained by other actions of orlistat, most likely its action to accelerate gastric emptying. Whilst the blood pressure-lowering effects of orlistat may be beneficial in certain patient populations, such as in those who are overweight or have type 2 diabetes, it needs to be used with caution in older individuals who are at particular risk of postprandial blood pressure excursions. The exact role of fat digestion in determining fat-induced decreases in blood pressure remains to be determined.

(a)



(b)

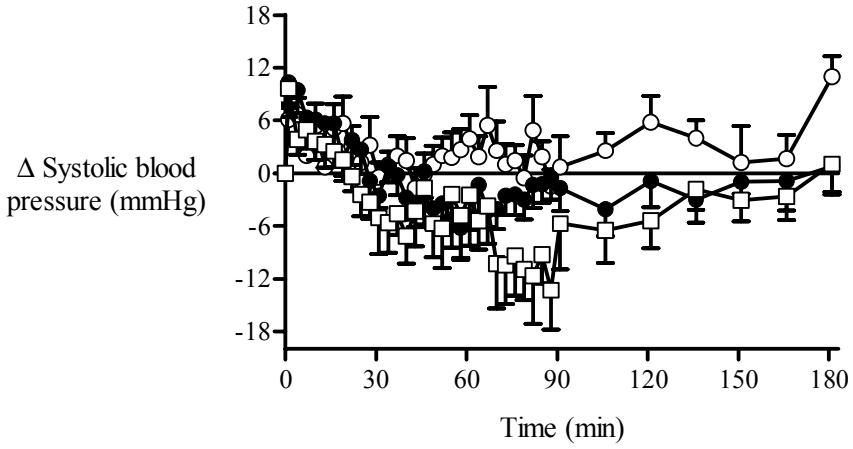
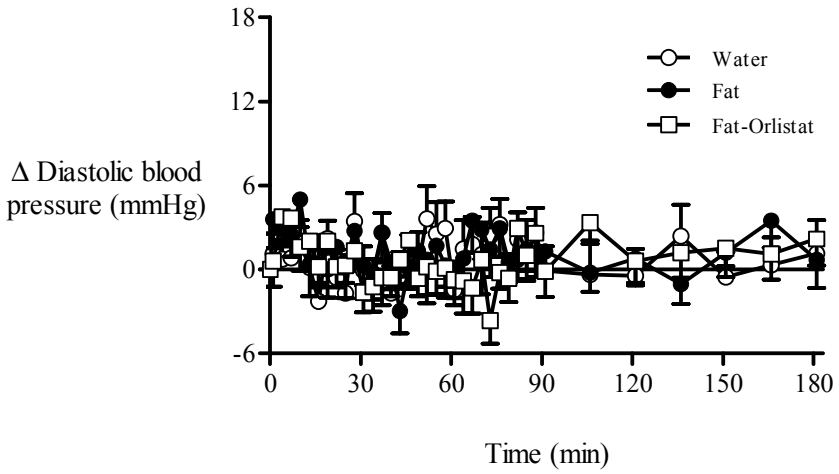


Fig. 8.1: Effects of water, fat, and orlistat drinks on systolic blood pressure in (a) young and (b) older subjects.

(a)



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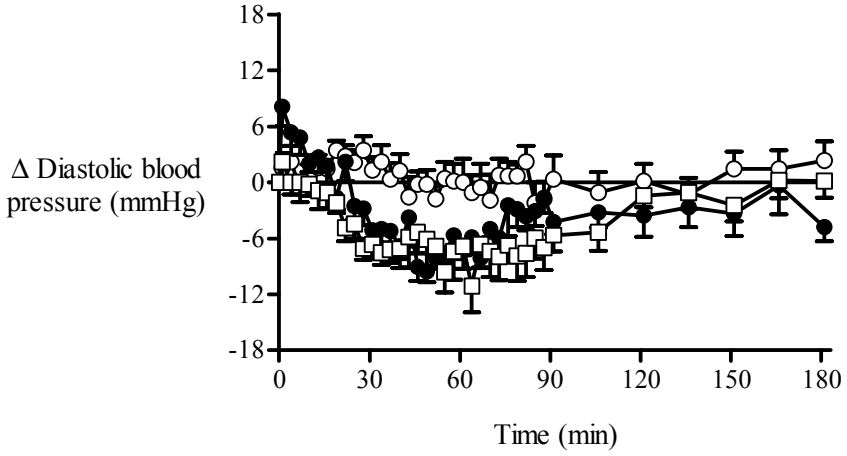
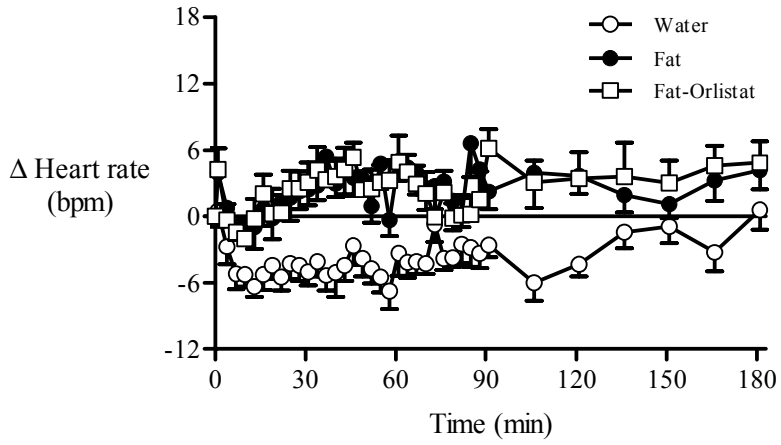


Fig. 8.2: Effects of water, fat, and orlistat drinks on diastolic blood pressure in (a) young and (b) older subjects.

(a)



(b)

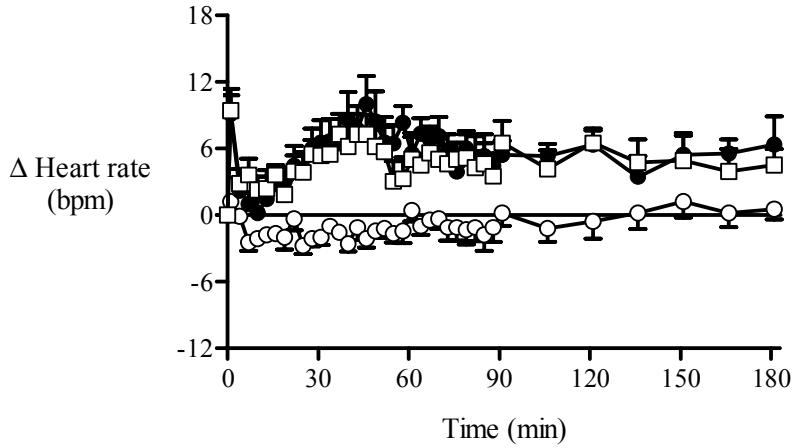
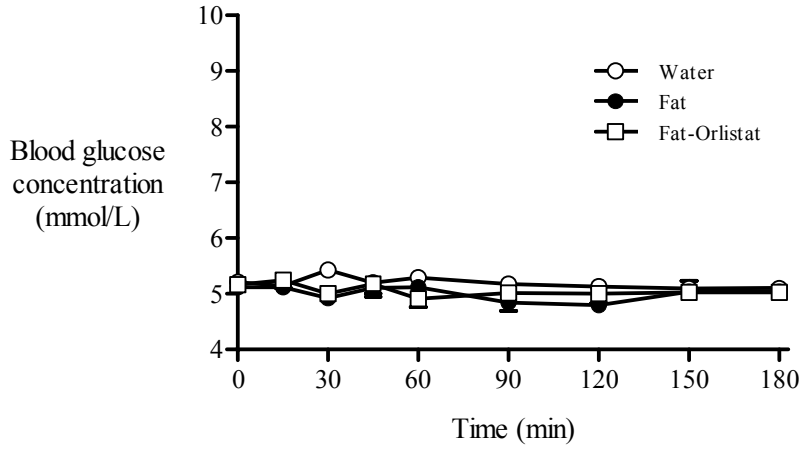


Fig. 8.3: Effects of water, fat, and orlistat drinks on heart rate in (a) young and (b) older subjects.

(a)



(b)

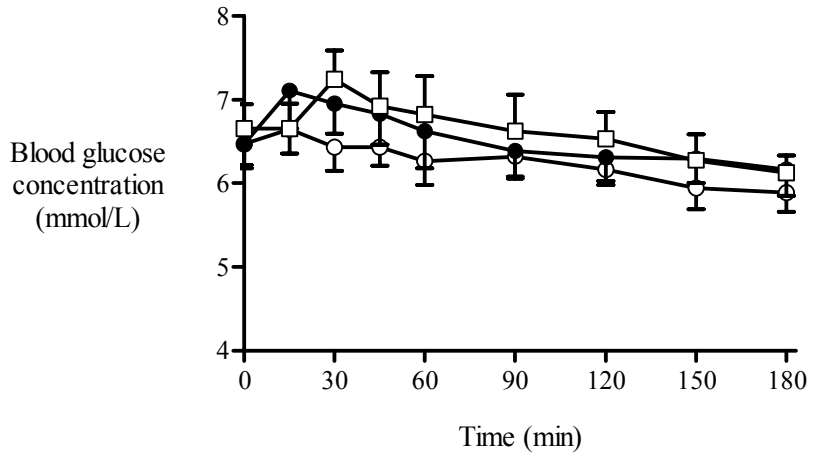
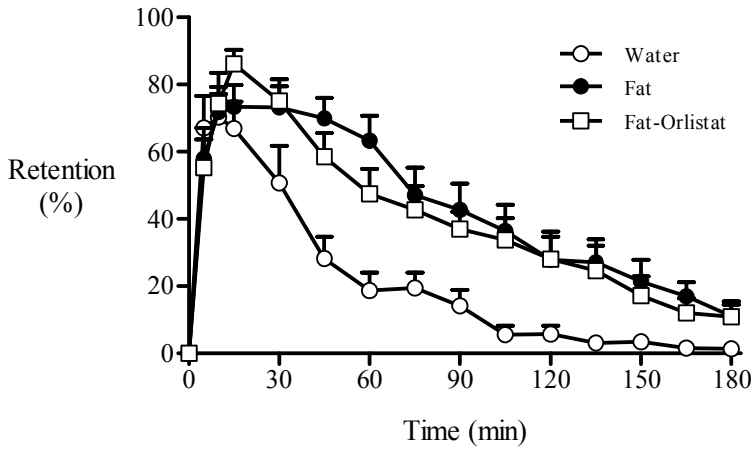


Figure 8.4: Blood glucose concentrations following water, fat, and orlistat drinks in (a) young and (b) older subjects.

(a)



(b)

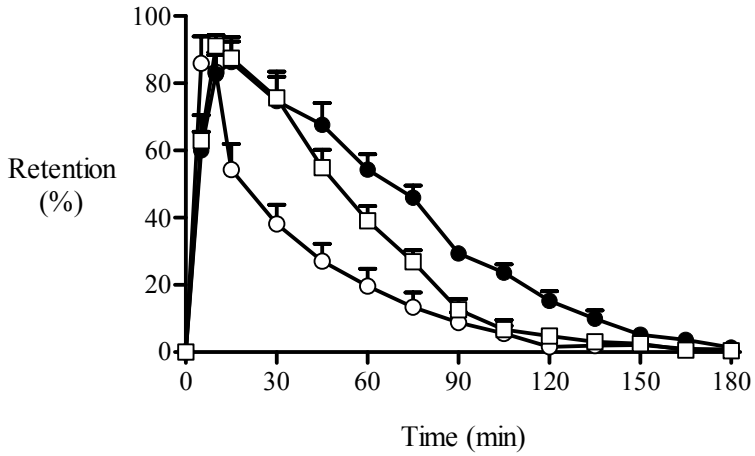


Figure 8.5: Gastric emptying of high-fat drink with and without orlistat, compared to water in (a) young and (b) older subjects.

Chapter 9

EFFECTS OF NUTRITIONAL SUPPLEMENTATION ON APPETITE AND ENERGY INTAKE IN RESPONSE TO INTRAVENOUS CHOLECYSTOKININ IN OLDER ADULTS

9.1 Summary

Increased dietary fat intake affects gastrointestinal function in animals and humans. The appetite-suppressive effects of exogenous CCK-8 are attenuated after exposure to a high-fat diet in rodents. The effects of exogenous CCK-8 on appetite and energy intake following a 14-day period on a high-fat diet compared with the subject's regular diet were examined in 14 healthy older adults (mean age 71.9 ± 1.4 years). Each subject completed three separate dietary periods- regular diet plus saline infusion (ND-SAL), regular diet plus CCK infusion (ND-CCK) in a dose of 1.5 ng/kg/min over 30 minutes, and a high-fat diet (supplementation with nutritional drinks) plus CCK infusion (HF-CCK) at the same dose. Following commencement of the infusion, appetite responses were assessed using visual analogue scales, and energy intake at a buffet-style meal was measured. Body weight was slightly higher after the high-fat diet than regular diet (ND-SAL 71.5 ± 3.5 kg vs ND-CCK 71.4 ± 3.3 kg vs HF-CCK 72.4 ± 3.3 kg, $P = 0.0001$). There were no significant differences in fasting or postprandial plasma CCK concentrations between the ND-CCK and HF-CCK days during the study ($P > 0.2$). There was no effect of diet on perceptions of hunger, fullness, desire to eat, or

prospective consumption ($P > 0.5$ for all). Energy intake at the buffet meal was higher on the ND-SAL study day (3349 ± 224 kJ) compared with either ND-CCK (3023 ± 317 kJ, $P = 0.005$) or HF-CCK (2905 ± 316 kJ, $P = 0.03$). There was no effect of diet on the suppression of energy intake by CCK-8 infusion ($P = 0.42$). Thus, under the conditions of this study, older adults exposed to a high-fat diet do not exhibit reduced sensitivity to the satiating effects of CCK.

9.2 Introduction

The gastrointestinal tract adapts to increased proportions of fat in the diet with changes to its morphology and function. In rats, a high-fat diet promotes hyperphagia (Savastano and Covasa 2005) and the suppression of energy intake and gastric emptying by an intraduodenal oleic acid infusion is attenuated (Covasa and Ritter 1999; Covasa and Ritter 2000). Furthermore, the inhibitory effects of an intraperitoneal injection of cholecystokinin (CCK-8) on both gastric emptying and energy intake are attenuated following a two to three week period on a high-fat, compared to an isoenergetic low-fat, diet (Covasa and Ritter 1998; Covasa and Ritter 2000; Savastano and Covasa 2005).

As in experimental animals, studies in humans have also shown that changes in nutrient intake, at least acutely, affect the gastrointestinal responses to food ingestion, which are known to modulate appetite and food intake. In a study of 12 healthy young males, gastric emptying and small intestinal transit of a high-fat test meal were faster after a 14-day period on a high-fat, hypercaloric diet (19300 kJ, 269.6 g fat) than a low-fat diet (9100 kJ, 11.6 g fat); gastrointestinal hormone concentrations were not measured and the energy content (as well as the fat content) of the two diets differed substantially

(Cunningham et al. 1991a). In another study of 12 healthy young males, ingestion of a high-fat, hypercaloric diet for 2 weeks (19170 kJ per day, 58% energy as fat) increased the mean 3-hour integrated plasma CCK-8 response to a standardised breakfast by approximately 43% ($P < 0.01$), when compared with the subject's regular diet (mean of 9590 kJ per day, energy from fat not specified) (French et al. 1995). Despite these increases in circulating concentrations of the satiety hormone, CCK, there was no decrease in energy intake at a meal consumed while on the high-fat diet, in fact, there was a non-significant increase in energy intake, compared to the regular diet (mean intake of 6919 kJ versus 6405 kJ, $P = 0.1$) (French et al. 1995), and the increased circulating CCK concentrations could, therefore, be a result of reduced sensitivity of CCK receptors responsible for feedback inhibition of CCK release (French et al. 1995). Alternatively, exposure to a high-fat diet may render the small intestine less 'sensitive' to fat, with a consequent increase in the rate of gastric emptying and intestinal transit, compared with a low-fat diet (Cunningham et al. 1991a), so that the reportedly high postprandial CCK concentrations (French et al. 1995) may be secondary to more rapid gastric emptying (Cunningham et al. 1991a).

There are differences in plasma CCK concentrations as well as the responses to exogenously administered CCK in young and older adults. Baseline fasting plasma CCK concentrations are higher in healthy older, compared to young, subjects (Khalil et al. 1985; MacIntosh et al. 1999; MacIntosh et al. 2000). In one study, after ingestion of a standardised liquid meal, mean maximal postprandial concentrations of CCK-8 were 137% higher in malnourished elderly than in older healthy adults, and 152% higher than in young healthy adults (Berthelemy et al. 1992). In another study, fasting plasma CCK

concentrations were 254% higher (P = 0.02) in under-nourished older than in healthy young adults, and remained elevated in the pre-meal period (Sturm et al. 2003). In that same study, however, CCK concentrations were not significantly different between under-nourished and healthy, older adults (Sturm et al. 2003). Infusion of CCK-8 suppressed energy intake by greater than two-fold in older than in young subjects (MacIntosh et al. 2001), consistent with the outcome of animal studies in which intraperitoneal injection of CCK-8 dose-dependently suppressed energy intake in older, to a greater extent than in young, mice (Silver et al. 1988). Thus, older adults have higher baseline CCK concentrations and apear to be more sensitive to the satiating effects of CCK than young adults, and these combined effects may contribute to the reduction in energy intake in older persons. Hence, a reduction in sensitivity to the satiating effects of cholecystokinin, may prove effective in improving appetite and energy intake in older persons with reduced food intake, particularly those who are under-nourished. i.e. a chronic reduction in energy intake in older adults increases plasma CCK, which in turn leads to further reduction in energy intake and increases sensitivity to the actions of CCK. Potentially, one way to break the cycle of perpetually decreased energy intake is by increasing the amount of energy consumed, particularly the fat content of the diet.

A recent study in healthy young males reported that fasting plasma CCK concentrations were about 1.4-fold higher after a three-week period on a high-fat diet (44% energy as fat) when compared with an isocaloric low-fat diet (9% energy as fat), however, energy intake at a buffet meal and appetite sensations were not influenced by the type of diet (Little et al. 2007). Thus, in healthy young adults, consumption of a high-fat diet does

not apparently decrease fullness, or increase hunger ratings (French et al. 1995) or decrease energy intake at a test meal (Cunningham et al. 1991a; French et al. 1995), when compared to the subject's regular diet (French et al. 1995), or a low-fat diet (Cunningham et al. 1991a). However, the appetite-suppressive effect of exogenous CCK following high-fat dietary exposure in older adults has hitherto, not been evaluated. Given the apparent greater sensitivity to the appetite-suppressant effects of CCK in older than young adults, it may be that dietary alterations have effects on CCK in older people, and not in young adults. The primary aim of the present study was to evaluate the hypothesis that exposure to a high-energy, high-fat diet for a two week period in older adults would attenuate the satiating effects of an intravenous CCK infusion.

9.3 Materials and methods

9.3.1 Subjects

Fourteen healthy older subjects (8 men, 6 women), with a mean age of 71.9 ± 1.4 years (range 66 - 85 years), and a BMI of 24.8 ± 0.7 kg/m^2, were recruited through advertisement. Subjects were included if they were unrestrained eaters as determined by a score of < 11 on the eating restraint section (Factor 1) of the Three Factor Eating Questionnaire (Stunkard and Messick 1985). Exclusion criteria included significant respiratory, renal, or cardiac disease, use of medications which may alter gastrointestinal motility or appetite, and a known history of disease which may affect gastric motility (eg. diabetes mellitus). All subjects were non-smokers and consumed <20 g of alcohol per day. Prior to inclusion in the study, subjects maintained a five-day food diary (including 3 week days and a weekend) to ensure that their usual fat intake

was within the range of 25 - 35% energy from fat, as recommended in the National Health and Medical Research Council Dietary guidelines for adult Australians. The study was approved by the Research Ethics Committee of the Royal Adelaide Hospital. All subjects provided written, informed, consent before their enrolment in the study.

9.3.2 Protocol

Each subject completed two, 14-day dietary periods, in randomised order, separated by a 7-day "washout" period, during which subjects consumed their regular diet: (1) regular 'normal' diet, followed by, in random order, 7 days apart, 0.9% saline infusion (ND-SAL) or CCK-8 infusion (ND-CCK), at the end of the diet period; (2) regular diet supplemented with high-fat liquid nutritional supplements, followed by CCK-8 infusion (HF-CCK) at the end of the 14-day diet period.

9.3.2.1 Diets

The high-fat diet comprised the subject's usual diet plus two high-fat supplement drinks per day. These supplement drinks were a mixture of two different nutritional supplements, Nepro (Abbott Australasia Pty. Ltd., Botany, New South Wales, Australia), and Calogen (Nutricia Clinical Care, Trowbridge, Wiltshire, England), chosen to provide a high amount of total fat and energy, whilst maintaining palatability. Subjects were asked to consume the supplement drink twice a day, between meals, in the mid-morning, mid-afternoon, or evening, and were provided with verbal, and written, instruction as to how to mix the two supplements. Each supplement drink was made by mixing 237 mL Nepro with 30 mL Calogen- 100 mL of Nepro provided 839 kJ (200 kcal) energy, 9.6 g fat, 7 g protein, and 22.3 g carbohydrate and 100 mL of

Calogen provided 1850 kJ (450 kcal) energy, 50 g fat (100% long-chain triglycerides in a water emulsion), nil carbohydrate, and nil protein. Thus 534 mL of the combined supplement was taken per day, providing an extra 5084 kJ (75.4 g of fat) daily.

Subjects were instructed to eat their usual diet as much as possible and not to reduce the intake of other foods. They were required to document, in a food diary, all food and liquids that they had consumed over each of the 14-day dietary periods.

9.3.2.2 Gastrointestinal and appetite responses during intravenous cholecystokinin (CCK-8) infusion

On the day immediately following the diet periods, subjects attended the research centre at 8:30 a.m. following an overnight fast from 10:00 p.m. the previous evening. Two intravenous cannulae were inserted, one into each forearm, for intravenous infusion and blood sampling. Following a 30 min rest (t = -15 min), a baseline blood sample was taken, and a visual analogue scale (VAS) questionnaire administered. At t = 0 min, an intravenous infusion of either normal (0.9%) saline, or cholecystokinin-8 (CCK-8 sulfated, Clinalfa, Merck Biosciences AG, Laufelfingen, Switzerland) at a rate of 1.5 ng/kg/min was commenced and maintained for 30 minutes. VAS were completed and a blood sample taken, at t = 0 min, prior to the commencement of the infusion, and thereafter at t = 15, 30, 45, and 75 min. At t = 15 min, subjects were offered a cold, buffet-style meal, as described in Chapter 5.5.4. This meal provided food in excess of what the subject would be expected to eat. Subjects were instructed to eat until comfortably full and allowed to eat from t = 15 min to 45 min. The CCK-8 infusion was ceased 15 minutes after the commencement of the meal at t = 30 min. The intravenous

cannulae were removed and the subject was allowed to leave the laboratory at t = 75 min.

9.3.3 Measurements

9.3.3.1 Appetite-related sensations and energy intake

Perceptions of hunger, fullness, desire to eat, and nausea were measured using validated VAS questionnaires (Flint et al. 2000; Parker et al. 2004), as described in Chapter 5 (Section 5.5.3).

All food items consumed at the buffet meal were weighed (to the nearest 0.1 g). The amount of food eaten (g), total energy intake (kJ), and macronutrient distribution (% energy) of food consumed during the 14-day diet periods and from the buffet-style meal was analysed using commercially available software Foodworks 3.01 (Xyris Software, Highgate Hill, Queensland, Australia) (Cook et al. 1997).

9.3.3.2 Blood glucose concentrations

Blood glucose was measured using a portable blood glucose metre (MediSense Precision QID System, Abbott Laboratories, MediSense Products Inc., Bedford, MA, USA), as described in Chapter 5.7.1.

9.3.3.3 Plasma CCK concentrations

Venous blood samples (10 mL) were collected in ice-chilled ethylendiamine tetra-acetic acid (EDTA)-treated tubes containing 400 kIU aprotinin (Trasylol; Bayer Australia,

Pymble, Australia) per mL of blood for measurement of plasma CCK-8 concentrations. Plasma was separated by centrifugation (3200 rpm for 15 min at 4°C) within 30 min of collection and stored at –70°C until assayed.

Plasma CCK concentrations (pmol/L) were determined following ethanol extraction using an established radioimmunoassay (Santangelo et al. 1998; MacIntosh et al. 2001), as described in Chapter 5.7.4.

9.3.4 Statistical analysis

The means of the values at t = -15 and 0 min for VAS scores and plasma CCK concentrations provided the baseline values. The primary endpoints of the study were plasma CCK concentrations, energy intake at the buffet meal, and appetite ratings (VAS scores). VAS scores, blood glucose, and plasma CCK concentrations were analysed by two-way repeated-measures analysis of variance (ANOVA) with treatment, and time, as factors. Energy intake was analysed using one-way ANOVA. Post-hoc paired comparisons, adjusted for multiple comparisons by Bonferroni correction, were performed when ANOVAs revealed significant effects. Statistical significance was accepted at $P < 0.05$, and data are presented as mean values ± SEM. All analyses were performed using statistical software packages Statview Version 5.0 (SAS Institute Inc., North Carolina, USA), SuperANOVA Version 1.1 (Abacus Concepts Inc., Berkeley, CA, USA). The number of subjects was based on power calculations derived from a previous study (MacIntosh et al. 2001).

9.4 Results

The study procedures were well tolerated by all subjects. There was a minimally higher nausea score at t = 15 min for the HF-CCK compared to the ND-PL (P = 0.001) and ND-CCK days (P = 0.02), however, there were no overall differences in nausea between the three study days (treatment effect: P = 0.08) (Figure 9.1). The high-fat diet was well tolerated by all subjects, and there was 100% compliance with the nutritional supplements, as calculated from the food diaries which subjects completed during the 14-day high-fat diet period. The subject's average energy intake on their habitual diet, as determined from the baseline 5-day diet diary, was 8275 ± 372 kJ/day (2547 ± 176 g of food) per day, with a macronutrient composition of 35.5 ± 0.9 % from fat, 42.9 ± 1.8 % from carbohydrate, and 18.0 ± 0.8 % from protein.

The total daily energy intakes during the 14-day dietary periods were 8272 ± 480 kJ/day on the regular diet, and 11642 ± 414 kJ/day on the high-fat diet (P = 0.0001) (Figure 9.2), consisting of 6558 kJ from their regular diet and 5084 kJ (75.4 g of fat) from the supplement. The macronutrient distribution during the 14-day diets (fat, carbohydrate, and protein) is outlined in Table 9. and Figure 9.3. During the high-fat diet, the contribution to total daily energy intake by fat was greater, and that of protein and carbohydrate less, compared with the subject's regular diet (all $P < 0.01$).

Body weight was slightly higher on the study day after the high-fat compared to regular diet (ND-SAL 71.5 ± 3.5 kg vs ND-CCK 71.4 ± 3.3 kg vs HF-CCK 72.4 ± 3.3 kg, P = 0.0001).

9.4.1 Plasma CCK concentrations

There were no significant differences between plasma CCK concentrations (Figure 9.4) at baseline on any of the three study days (ND-SAL 2.4 ± 0.2 pmol/L vs ND-CCK 2.6 ± 0.2 pmol/L vs HF-CCK 2.4 ± 0.2 pmol/L). There was a significant time (P = 0.0001) and treatment (P = 0.0001) effect and treatment × time interaction (P = 0.0001) for plasma CCK concentrations. As expected, plasma CCK concentrations were less during ND-SAL than both ND-CCK (P < 0.04) and HF-CCK (P < 0.03) CCK-8 infusion study days from t = 15 to 45 min. Plasma CCK concentrations increased on the saline infusion day (P = 0.0001) from the start of the buffet meal at 15 minutes, to peak at 5.9 ± 0.8 pmol/L at 45 minutes. On the two CCK infusion days, plasma CCK concentrations increased from the start of the CCK-8 infusion to peak at 15 minutes (ND-CCK 13.8 ± 1.3 pmol/L, P = 0.002, and HF-CCK 16.8 ± 1.7 pmol/L, P = 0.001), significantly higher than on the saline day, and then decreased to be similar to those on the saline infusion day at 75 minutes. There were no significant differences in plasma CCK concentrations between ND-CCK and HF-CCK for the duration of the study (P > 0.2).

9.4.2 Appetite-related sensations

There was no effect of diet on ratings of hunger, fullness, desire to eat, or prospective consumption throughout the study (all P > 0.5). After commencement of the CCK infusion, fullness did not change during either ND-CCK or HF-CCK study periods (Figure 9.5a), and only increased following commencement of the buffet meal for all dietary periods (time effect: P = 0.0001), with no differences between study days (treatment effect: P = 0.6). After commencement of the buffet meal, hunger decreased (time effect P = 0.0001; treatment effect P = 0.8) (Figure 9.5b), desire to eat decreased

(time effect P = 0.0001; treatment effect P = 0.7), and prospective consumption decreased (time effect P = 0.0001; treatment effect P = 0.7), on all study days, with no differences between the days.

9.4.3 Energy intake

Energy intake at the buffet meal was higher on the saline infusion study day than on either of the two CCK-8 infusion days (P = 0.03 vs ND-CCK; P = 0.005 vs HF-CCK) (Table 9.2). The amount of food consumed was also higher after ND-SAL study period compared to either ND-CCK (P = 0.04) or HF-CCK (P = 0.003). There was no effect of diet on the percentage contribution to energy intake by fat, carbohydrate, or protein at the buffet meal (all P > 0.1).

Compared to placebo, there was a 9.7% reduction in energy intake at the buffet meal with CCK-8 infusion during the regular diet and a 13.3% reduction in energy intake at the buffet meal during the high-fat diet, with no significant difference in the effects of diet on the suppression of energy intake by CCK-8 infusion (ND-CCK vs HF-CCK, P = 0.42).

9.5 Discussion

The present study in healthy older adults found that fasting plasma CCK concentrations were not significantly different after two weeks on a diet that delivered approximately 41% more energy than the subject's regular diet, and substantially more fat, both in absolute amounts (136 g vs 78 g of fat/day) and as a percentage of total energy intake (43% vs 35% energy as fat). Consistent with the results of previous studies (MacIntosh

et al. 2001), CCK-8 infusion suppressed energy intake at a buffet meal, by approximately 11% in the present study, compared to saline infusion. However, contrary to the underlying hypothesis, there was no significant effect of diet during the preceding two weeks on the appetite or energy intake response to CCK-8 infusion, suggesting that, in healthy older adults, the sensitivity to exogenous CCK-8, at a dose of 1.5 ng/kg/min for 30 minutes, is not affected by short-term exposure to an increased energy, high-fat diet.

There is evidence from animal studies that there is an increase in circulating CCK concentrations following intraduodenal fat infusion or exposure to a high-fat diet, compared with a low-fat diet. Early studies in rats showed that the increase in plasma CCK concentrations following an intraduodenal 10% triglyceride infusion was some 1.7-fold greater following a high-fat diet (20% energy as fat) for two weeks, than a low-fat diet (5% energy as fat) (Spannagel et al. 1996). In this same study, prior daily exposure to intraduodenal fat infusions over three days also resulted in higher plasma CCK, in response to an intraduodenal triglyceride infusion, on the third than the first day, indicating that exposure to high-fat loads in the small intestine or dietary fat supplementation has the capacity to increase circulating CCK concentrations in rodents (Spannagel et al. 1996). Furthermore, exposure to a high-fat diet increases lipolytic activity in the intraluminal contents of the small intestine, and increases pancreatic lipase secretion, when compared with a low-fat diet (Spannagel et al. 1996). Comparable increases in pancreatic lipase activity were seen in another study in rats fed a high-fat diet (67% energy as fat) compared with a low-fat diet (10% energy as fat) (Sabb et al. 1986). It has also been shown that overall fat absorption in the small

intestine is increased in rats maintained on a high-fat diet (20% energy as fat), than regular diet (4% energy as fat) for four weeks (Singh et al. 1972). Therefore, in rodents, exposure to a high-fat diet increases the capacity for digestion and absorption of fat, and potentiates fat-stimulated circulating CCK concentrations, although the effects on fasting CCK concentrations in animals have not been evaluated.

As in experimental animals (Spannagel et al. 1996), a high-fat diet has also been shown in humans to increase plasma CCK concentrations in response to an orally ingested test meal, consistent with down-regulation, or reduced sensitivity of CCK receptors responsible for feedback inhibition of CCK release (French et al. 1995). However, in another study, plasma CCK responses to an intraduodenal lipid infusion did not differ after a 14-day period on either a high-fat (44% energy as fat) or low-fat diet (10% energy as fat) (Boyd et al. 2003). Furthermore, in this same study, energy intake after a duodenal lipid infusion was not significantly different after the high-fat compared to the low-fat diet (Boyd et al. 2003). A recent study in healthy young men showed that fasting plasma CCK concentrations were higher after a three-week period on a high-fat (44% energy as fat) diet when compared with an iso-energetic low-fat (9% energy as fat) diet, however spontaneous energy intake and appetite sensations were not influenced by the type of diet (Little et al. 2007). In contrast, the present study found that plasma CCK concentrations were not significantly different, at baseline or in response to CCK infusion, following the high-fat diet, compared with the regular diet. It is clear that mean fasting plasma CCK concentrations are higher in healthy older compared to young subjects (Khalil et al. 1985; MacIntosh et al. 1999; MacIntosh et al. 2000; Sturm et al. 2003), up to four- or five-fold (MacIntosh et al. 2001; Sturm et al.

2003). From the preceding obervations, it can be assumed that fasting plasma CCK concentrations in the older adults in the present study would have been higher than those in healthy young adults during their regular diet, which may potentially account for the lack of any further changes in CCK concentrations after the high-fat diet. While fasting CCK concentrations in healthy young men were increased following a high-fat diet, compared to a low-fat diet (4.3 ± 0.4 pmol/L vs 3.1 ± 0.3 pmol/L; $P < 0.05$), it did not result in any differences between the two diets in appetite sensations or energy intake in response to CCK-8 infusion (Little et al. 2007). This difference in fasting plasma CCK was very small, albeit significant, and only reflects circulating concentrations, not local concentrations in the region of the small intestine (i.e. at the tissue level). Furthermore, the difference in the proportion of fat between the high-fat diet (44% energy from fat) and low-fat diet (9% energy from fat) in that study (Little et al. 2007) was greater than the differences in the proportion of fat between the high-fat and regular diets in the current study (43% vs 35% energy from fat). Nevertheless, in the current study, the absolute amount of fat consumed, as determined from the diet diaries, was substantially higher on the high-fat diet compared to regular diet (mean of 136 g vs 78 g), in fact an increase of approximately 74%. Consistent with the results of fasting plasma CCK concentrations in the present study, a previous study showed no apparent differences in fasting plasma CCK-8 concentrations, although the total 3-hour integrated CCK-8 concentrations (comprising predominantly postprandial levels) after a standardised meal were approximately 43% higher after a high-fat, hypercaloric diet (58% energy from fat), compared to the subject's usual diet (French et al. 1995). CCK occurs in a number of forms, and fragments with >12 amino acid residues (for example, CCK-33) are the most abundant circulating, biologically active form (Peikin 1989). It

has been demonstrated that exogenous CCK-8 suppresses endogenous CCK release (MacIntosh et al. 2001), however, the postprandial endogenous CCK response was not assessed in the current study.

In animals, a high-fat diet can also modulate the rate of gastric emptying, appetite and food intake in response to nutrient exposure. The suppression of food intake in rats by an intraduodenal oleic acid infusion is less pronounced on a high-fat diet compared with a low-fat diet (Covasa and Ritter 1999). In addition, in response to intraduodenal oleic acid infusion in rats, there is reduced inhibition of gastric emptying following a high-fat diet (54% energy as fat) compared with an iso-energetic low-fat diet (5% energy as fat) (Covasa and Ritter 2000). The mechanisms by which exposure to a high-fat diet may reduce the inhibitory effects of fat on gastric emptying in animals are not fully understood, but may be mediated by CCK, as the rate of gastric emptying following intraperitoneal CCK administration in rats is also less delayed following a two-week period on a high-fat diet, than a low-fat diet (Covasa and Ritter 2000). In addition, a greater length of intestine exposed to fat enhances the slowing of gastric emptying by an intragastric infusion of oleic acid in dogs (Lin et al. 1990). Exposure to a high-fat diet also increases the capacity for fat digestion and absorption (Singh et al. 1972; Sabb et al. 1986; Spannagel et al. 1996), thus, it is possible that there is reduced length of intestine exposed to nutrients following a high-fat diet (Covasa and Ritter 2000). In addition, the stimulation of CCK and, therefore, feedback inhibition of gastric emptying, by an intraduodenal lipid infusion in rats is dependent on lipolysis of triglycerides to free fatty acids (Raybould et al. 1998). Therefore, in animals, high-fat diet exposure may increase the efficiency of the small intestine to absorb and digest fat,

thus reducing direct contact of the small intestine with fat, and attenuating the inhibitory effects of fat on gastric emptying.

The present study was performed to test the hypothesis that two weeks of an increased energy, high-fat diet would reduce the sensitivity to the satiating effects of CCK in older persons. This hypothesis is supported by studies in animals. For example, in response to acute intraperitoneal injections of CCK, food intake in rats was reduced up to 39% less in animals on a high-fat diet (34% energy from fat) for three weeks, than in animals on an iso-energetic low-fat diet (5% energy from fat) (Covasa and Ritter 1998). Similarly, in another study, an intraperitoneal CCK-8 injection reduced food intake in rats by up to 22% less after a three-week period on a high-fat diet (30% energy from fat), when compared with an iso-energetic low-fat (6% energy from fat) diet ($F = 4.05$, $P = 0.024$) (Covasa et al. 2001). In that same study, chronic intraperitoneal CCK-8 infusion over 28 days in freely-fed rats maintained on a standard chow diet, attenuated the appetite-suppressive effects of an acute injection of CCK-8, when compared with saline-injected rats on the same diet. Although plasma CCK concentrations were not measured in this latter study, it was assumed by the authors that the high-fat diet also raised endogenous CCK concentrations (Covasa et al. 2001), and that chronically elevated CCK concentrations, either through maintenance on a diet high in fat, or through continuous intraperitoneal infusion of exogenous CCK, reduced the satiating effect of an additional dose of exogenous CCK, when compared with a low-fat diet (Covasa et al. 2001). It has been hypothesised that down-regulation of CCK receptors or changes in gastric emptying on a high-fat diet may explain the diminished satiating effect of exogenous CCK in this setting (Covasa and Ritter 1998; Covasa et al. 2001).

In support of the above findings, exposure to a high-fat diet over three weeks reduced the inhibitory effects of intraperitoneal CCK administration on energy intake in rats, and promoted hyperphagia, when compared with an iso-energetic low-fat diet (Savastano and Covasa 2005). Interestingly, rats maintained on a high-protein diet (71% protein) for three weeks also have reduced CCK-induced inhibition of food intake compared with an iso-energetic low-fat diet (Covasa et al. 2001). In addition, another study in rats found that gastric emptying was more rapid following a high-protein diet (55.9% energy as protein) for three weeks, compared with a low-protein diet (9.2% energy as protein) (Shi et al. 1997). Thus, maintenance on either a high-fat or high-protein diet attenuates the appetite-suppressive effects of exogenous CCK in rodents. However, unlike the elevated circulating CCK concentrations in rats fed a high-fat diet (Spannagel et al. 1996), postprandial CCK concentrations were decreased following a high-protein diet than a low-protein diet (Shi et al. 1997), suggesting decreased CCK secretion after a high-protein diet and, thus, reduced suppression of appetite.

Studies in humans have shown that chronic dietary changes affect the acute intestinal responses to food ingestion which modulate gut function, appetite, and food intake. In healthy young males, a slightly longer period of supplementation using a glucose polymer for 7 days modulated gastrointestinal function, such that the satiating effect of a subsequent intraduodenal lipid infusion was attenuated compared with no supplementation (Andrews et al. 1998). This study suggests that a nutrient can influence the gastrointestinal response of other macronutrients. In both young and older individuals, dietary glucose supplementation for 3 to 10 days modestly accelerated

gastric emptying of a challenge dose of glucose (Cunningham et al. 1991b; Horowitz et al. 1996; Beckoff et al. 2001), but, in older subjects, did not affect energy intake at a buffet meal (Beckoff et al. 2001). Thus, dietary supplementation with glucose results in more rapid gastric emptying, and attenuation of the satiating effects of intraduodenal fat, but does not appear to significantly influence energy intake. It is possibly easier to detect changes in gastric emptying, than energy intake, in response to dietary glucose supplementation.

The gastrointestinal effects of dietary fat supplementation have also been investigated. In young healthy individuals, a more rapid gastric emptying of a high-fat test meal was observed following a two-week period on a high-fat, hypercaloric, diet, which provided an extra 10200 kJ and 258g of fat daily, when compared with a low fat diet (Cunningham et al. 1991a). However, initial studies in healthy young adults showed no differences in *ad libitum* food intake after exposure to a high-fat, high-energy, diet compared with a low-fat diet (Cunningham et al. 1991a; French et al. 1995). Consistent with these findings, a comparison of medium- (40% of energy as fat) and high-fat (60% of energy as fat) diets with a low-fat (20% of energy as fat) diet, which were not isocaloric, over seven days, failed to show any significant differences in spontaneous energy intake (Stubbs et al. 1995). It has been suggested that high dietary fat exposure may render the small intestine less 'sensitive' to the usual inhibitory effects of fat on gastric emptying (Cunningham et al. 1991a). In a recent study in 52 normal-weight to obese adults, aged 18-64 years, the maximum tolerated volume of a liquid nutrient drink (volume ingested which resulted in maximum satiation) was higher following a 14-day period on a high-fat (44% energy as fat), compared to an isocaloric standard

(30% energy as fat) diet, in those who had a high baseline maximum tolerated volume, however, the gastric emptying half-time of a solid mixed meal and energy intake were not significantly different between diets (Park et al. 2007).

In contrast to animals, subjective appetite sensations can be assessed in humans. Interestingly, increased hunger and decreased fullness have been observed during the first or second week of a two-week period on a high-fat diet, when compared with an assessment at the end of the first day of the diet (French et al. 1995). However, in this same study, hunger and fullness sensations to a standard test breakfast at the end of the high-fat diet period did not significantly differ from pre-diet assessment, despite higher mean three-hour integrated total plasma CCK concentrations (1285.0 ± 153.0 versus 896.7 ± 78.2 pmol/L pre-diet, $P < 0.01$) (French et al. 1995). The observed increased circulating CCK concentrations, however, appear intuitively inconsistent with the decreased fullness and increased hunger sensations observed in this study (French et al. 1995), and requires further clarification. It may be that accelerated gastric emptying secondary to a high-fat diet and hence, increased exposure of the small intestine to nutrients, may be responsible for the increased postprandial CCK concentrations (French et al. 1995).

Sensitivity to the appetite-suppressive effects of CCK may alter with age. In animal studies, older animals have been found to be more sensitive to the satiating effects of intraperitoneal administration of CCK-8 than younger animals (Silver et al. 1988; Voigt et al. 1996). In healthy, young adults, intravenous CCK infusion resulted in a dose-dependent suppression of food intake (Kissileff et al. 1981; Stacher et al. 1982; Pi-

Sunyer et al. 1982; Muurahainen et al. 1991; Lieverse et al. 1995a; Greenough et al. 1998). In one study comparing the effects of low-dose and high-dose intravenous CCK in 12 young and 12 older subjects, CCK suppressed energy intake significantly more in older than in younger subjects, in a dose-dependent manner (mean suppression of 32% versus 15.5% respectively; effect of age $F = 5.70$, $P < 0.05$) (MacIntosh et al. 2001). It was postulated that this increased sensitivity may, in part or in full, account for the age-associated reduction in energy intake, and that increasing food intake, with both an increase in total energy intake and the amount of fat, would decrease the sensitivity to CCK. Contrary to this hypothesis, there was no difference in energy intake in the current study following the high-fat diet compared with the subject's regular diet.

There are some limitations with the present study. Firstly, nutritional supplement drinks were chosen to improve compliance in these older adults, as there were likely to have been difficulties with adherence to the high-fat diet if subjects were assigned to set food items over the two week period. Furthermore, subjects were not allocated to a low-fat diet, but assessed after a two-week period on their habitual diet. Secondly, although the total daily energy intake was significantly greater during the high-fat, than regular, diet, the difference between the high-fat diet and the individual's habitual diet (43% versus 35% energy from fat) was small. Furthermore, as the nutritional supplements provided an extra 75.8 g of fat per day (an increase of approximately 74% in the amount of fat from the subject's regular diet), some subjects may have compensated by reducing their fat intake at mealtimes. In a previous study, when healthy older individuals were given a high-fat liquid preload (containing 1255 kJ energy, and 25.5 g of fat), subsequent energy intake was higher when the preload was given $\geq$ 60 minutes before the test meal,

compared with immediately prior to the meal (Wilson et al. 2002). A recent Cochrane systematic review reported that, 29 of 35 studies in older people, where energy intake was reported following nutritional supplementation, showed an increase in total daily energy or protein intake or both, compared with usual diet (Milne et al. 2005), suggesting that older people do not fully compensate by eating less when offered nutritional supplementation, and show a modest weight gain (Milne et al. 2006). In the present study, in order to reduce the possible suppressive effects on total daily energy intake, subjects were instructed to consume the nutritional supplement drinks at least two hours away from mealtimes, in keeping with recommendations for under-nourished older individuals in nursing homes (Wilson et al. 2002). In response to an additional 5084 kJ energy, subjects reduced their energy at other times by 1714 kJ (i.e. a 20.7% compensation). In response to an extra 75.4 g of fat, subjects reduced their fat intake at other times by 17.4 g of fat (i.e. 22.3% compensation). This minor compensation of energy and fat intake is consistent with other reports of studies in older people (Milne et al. 2005), and, in the present study, resulted in a substantial increase in total energy intake of 40.7% and a mean increase in fat intake of 74.4% on a high-fat diet when compared with the subject's regular diet. Although this increase in fat intake from their usual diet was substantial, there was a relatively small increase of 8% in the contribution of fat to total daily energy intake following the high-fat diet. In comparison, two studies in healthy young adults, where standard set menus were used during a high-fat diet for two weeks, were designed to substantially increase both energy intake and fat intake, to a greater extent than those of our study (Cunningham et al. 1991a; French et al. 1995). Nevertheless, neither showed an increase in energy intake following the high-fat diet (Cunningham et al. 1991a; French et al. 1995). One of

those studies showed that daily energy intake was increased by 9580 kJ (an increase of 99.9%) on the high-fat diet compared to the subject's usual diet, and whilst the high-fat diet provided 58% of energy from fat, the difference between the contribution of fat to daily energy intake on the two diets was not specified (French et al. 1995). Another study in healthy young males showed an increase in daily energy intake by 10,250 kJ on the high-fat diet, and a 22-fold increase in fat intake, compared to a prescribed low-fat diet (Cunningham et al. 1991a).

Thirdly, two weeks may not have been sufficient to observe an effect on appetite. Nevertheless, previous studies assessing the effects of a high-fat diet on appetite and food intake were of the same duration (Cunningham et al. 1991a; French et al. 1995; Boyd et al. 2003), or of a slightly longer duration of 21 days (Little et al. 2007). In the present study, the dose of CCK-8 used significantly suppressed energy intake at a buffet meal by 9.7% compared with saline infusion. The dose and duration of the infusion of CCK-8 (1.5 ng/kg/min over 30 minutes) was based primarily on previous studies showing significant suppression of energy intake in young and older adults (MacIntosh et al. 2001; Brennan et al. 2005). While it could be argued that the resulting plasma CCK concentrations during the CCK infusions were moderately supraphysiological, being greater than two-fold higher compared with postprandial CCK concentrations during the saline infusion, they are comparable with CCK concentrations in response to ingestion of a 750 kcal mixed-nutrient meal (Sturm et al. 2004) or an intraduodenal infusion of a triglyceride emulsion (Pilichiewicz et al. 2006). Furthermore, in healthy young males, a significant increase in nausea ratings ($P = 0.028$) has been reported with infusion of CCK-8 at a dose of 2 ng/kg/min over 150 minutes, compared to isotonic

saline infusion (Brennan et al. 2005), which was a larger dose infused over a longer duration than that used in our study. In the study by MacIntosh et al. (2001), in both young and older subjects, there was a dose-dependent suppression of energy intake with both low-dose CCK-8 infusion (1 ng/kg/min) and high-dose (3 ng/kg/min) over 25 minutes, and nausea ratings were similar with the low- and high-dose CCK-8, and saline infusions (P = 0.53). Lastly, the possibility of a type 2 error should be considered, although, as stated, the number of subjects in the current study was based on power calculations from a previous study (MacIntosh et al. 2001). It should also be noted that there was no control day following the high-fat diet (i.e. intravenous infusion of saline), for logistical reasons.

In summary, while attenuation of the appetite-suppressive effects of CCK by manipulation of macronutrients in the diet would be beneficial in under-nourished older adults, as a means of improving their nutritional state, in the present study, there were no significant differences in the suppression of appetite and energy intake by CCK following a high-fat, high-energy diet for two weeks in older adults. While this suggests that manipulation of the diet in older persons favouring increased intake of fat and increased energy is unlikely to have beneficial effects on improving appetite and food intake, at least over this time course, it would be of interest to evaluate the satiating effects of endogenous CCK with the use of a CCK-1 receptor antagonist, such as dexloxiglumide (Degen et al. 2007), following exposure to a high-fat diet.

Table 9.1: Daily energy intake and macronutrient distribution during the 14-day regular diet and high-fat diet periods.

Diet	Energy intake (kJ/day)	Fat	Carbohydrate	Protein
Regular diet	8272 ± 480	78 ± 5 g	212 ± 18 g	90 ± 6 g
% energy		35 ± 1	43 ± 2	19 ± 1
High-fat diet	11642 ± 414 [(1)]	136 ± 4 g	275 ± 16 g	107 ± 5 g
% energy		43 ± 1 [(2)]	40 ± 1 [(3)]	16 ± 0.4 [(4)]

Data are mean values ± SEM.

[(1)] $P = 0.0001$ compared to regular diet.

[(2)] $P = 0.0001$ compared to regular diet.

[(3)] $P = 0.0075$ compared to regular diet.

[(4)] $P = 0.0001$ compared to regular diet.

Table 9.2: Energy and macronutrient intake at the buffet-style meal during an intravenous CCK-8 infusion.

Treatment	Energy intake (kJ)	Amount (grams)	Fat (% of energy intake)	Carbohydrate (% of energy intake)	Protein (% of energy intake)
ND-SAL	3349 ± 224	879 ± 51	29 ± 2	52 ± 3	19 ± 1
ND-CCK	3023 ± 317 [(1)]	801 ± 61 [(4)]	29 ± 2	51 ± 3	21 ± 1
HF-CCK	2905 ± 316 [(2,3)]	760 ± 68 [(5,6)]	28 ± 2	54 ± 3	18 ± 1

Data are mean values ± SEM.

[(1)] $P = 0.03$ compared to ND-SAL.

[(2)] $P = 0.005$ compared to ND-SAL.

[(3)] $P = 0.42$ compared to ND-CCK.

[(4)] $P = 0.04$ compared to ND-SAL.

[(5)] $P = 0.003$ compared to ND-SAL.

[(6)] $P = 0.27$ compared to ND-CCK.

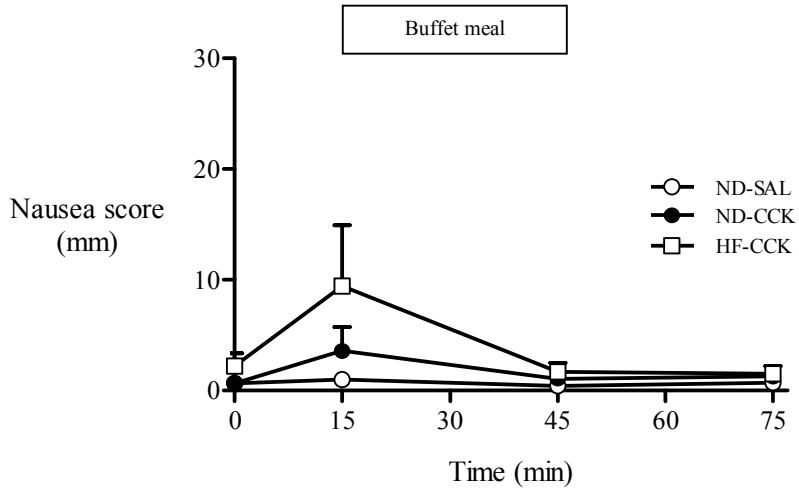


Figure 9.1: Nausea scores during each of the three study days ($P = 0.08$ for differences between study days). Data are mean values ± SEM.

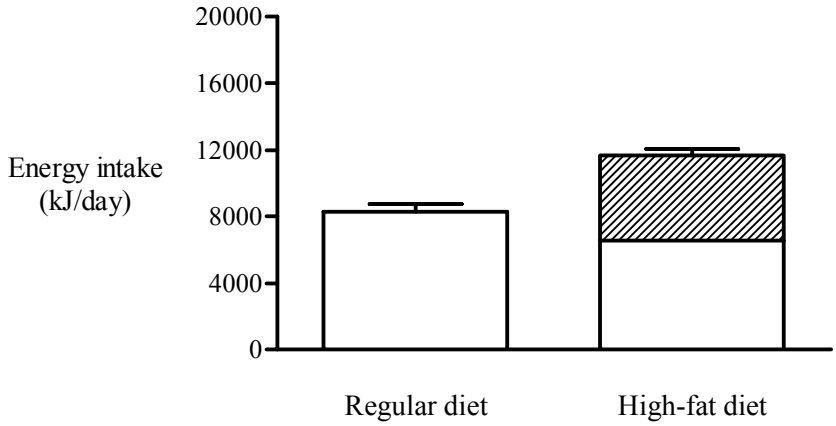


Figure 9.2: Daily energy intake on the regular and high-fat diets. The contribution of the high-fat nutritional supplement (5084 kJ/day) to total energy intake is shown (shaded). Data are mean values ± SEM.

* $P = 0.0001$ compared to regular diet.

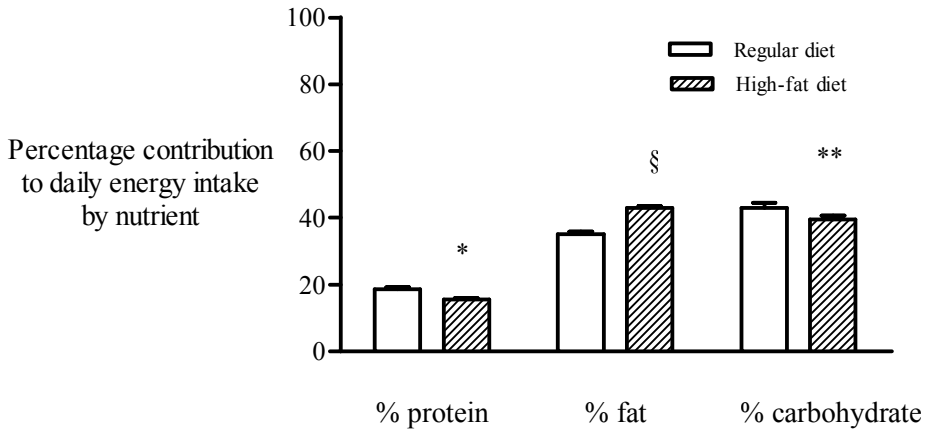


Figure 9.3: Macronutrient composition (protein, fat, carbohydrate) of average daily energy intake on the 14-day regular and high-fat diets. Data are mean values ± SEM.

* $P = 0.0001$ compared to regular diet.

§ $P = 0.0001$ compared to regular diet.

** $P = 0.0075$ compared to regular diet.

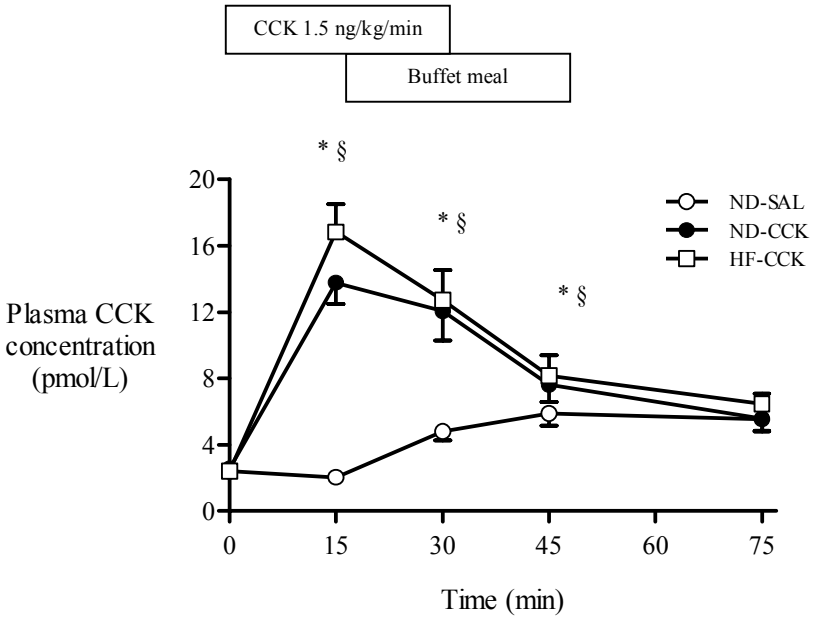


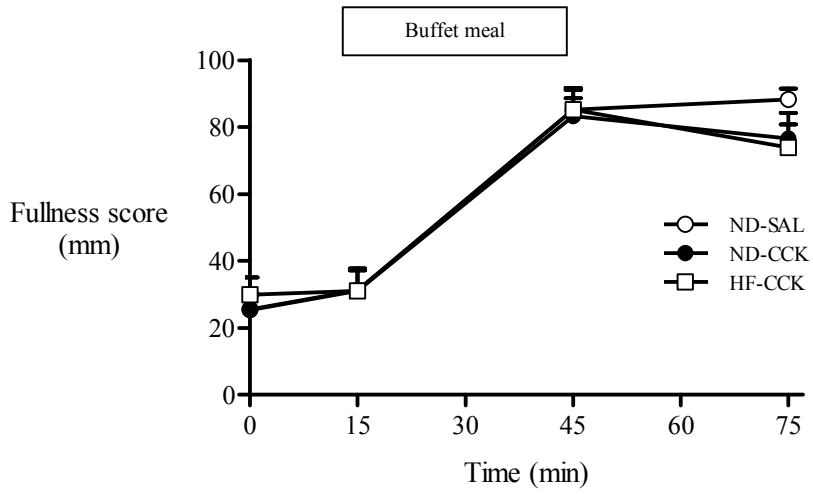
Figure 9.4: Plasma CCK-8 concentrations during each of the three study days. Data are mean values ± SEM.

§ HF-CCK versus ND-SAL ($P < 0.03$).

* ND-CCK versus ND-SAL ($P < 0.04$).

ND-CCK versus HF-CCK ($P > 0.2$).

(a)



(b)

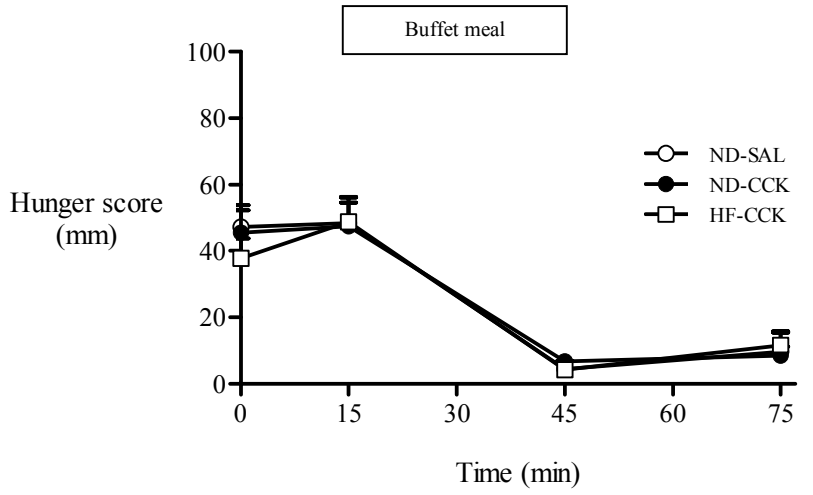


Figure 9.5: (a) Fullness and (b) hunger scores during each of the three study days. Data are mean values ± SEM.

Chapter 10

GLUCOSE TOLERANCE AND VITAMIN D: EFFECTS OF TREATING VITAMIN D DEFICIENCY

10.1 Summary

The aim of the study was to determine the effects of vitamin D treatment on plasma glucose, serum insulin and insulin sensitivity in vitamin D deficient individuals without diabetes mellitus. Thirty three adults with vitamin D insufficiency/deficiency (serum 25-hydroxyvitamin D concentration ≤ 50nmol/L) and without diabetes (12 with impaired glucose tolerance) were given two oral doses of 100,000 IU cholecalciferol, two weeks apart. Before the first dose, and two weeks after the second dose, a 75 g oral glucose tolerance test (OGTT) was performed. Plasma glucose, serum insulin, 25-hydroxyvitamin D and parathyroid hormone (PTH) concentrations were measured and insulin sensitivity calculated from the results of the OGTT.

Serum 25-hydroxyvitamin D increased from 39.9 ± 1.5 (SEM) to 90.3 ± 4.3 nmol/L ($P < 0.0001$) and serum PTH decreased from 6.7 ± 1.2 to 4.5 ± 0.6 pmol/L ($P = 0.055$). There was no change in blood glucose mean of 0-120 min (6.1 ± 0.3 before vs 6.2 ± 0.3 mmol/L, $P = 0.63$) or insulin mean of 0-120 min (47.8 ± 5.35 vs 48.9 ± 5.22 mU/L, $P = 0.67$) concentrations, and no change in insulin sensitivity (SiM $P = 0.97$, $ISI_{0,120}$ $P = 0.74$, QUICKI $P = 0.88$, HOMA $P = 0.99$) after vitamin D treatment. Results did not differ between subjects, with and without, impaired glucose tolerance.

In adults without diabetes, correction of vitamin D insufficiency/deficiency is not associated with any effect on blood glucose or insulin concentrations or insulin sensitivity as assessed during an OGTT. These observations do not support an association between glucose/insulin homeostasis and vitamin D, at least in the short term.

10.2 Introduction

Borderline, or inadequate, body vitamin D stores are common in both younger and older adults, but particularly the latter. In the Third National Health and Nutrition Examination Survey (NHANES III 1988-1994) in the United States, the prevalence of vitamin D insufficiency (defined as 25-hydroxyvitamin D concentrations below 62.5 nmol/L) was as high as 57% during winter in lower latitude regions (Looker et al. 2002). In the same community-dwelling adult population, 5-15% of people had moderate to severely reduced concentrations of 25-hydroxyvitamin D (serum 25-hydroxyvitamin D levels at or below 37.5 nmol/L) (Looker and Gunter 1998).

A number of observations have linked vitamin D deficiency to alterations in circulating glucose and insulin concentrations and, possibly, insulin sensitivity. There has been considerable interest in this possible link, with over 40 reviews related to this subject published in the last 5 years. Cross-sectional studies have shown inverse correlations between glucose concentrations, insulin resistance and the risk of diabetes mellitus with serum 25-hydroxyvitamin D (Baynes et al. 1997; Chiu et al. 2004). The observed associations between vitamin D deficiency and impaired glucose tolerance and diabetes

in humans have not, however, been evaluated fully by intervention studies. If vitamin D treatment has beneficial effects on glucose metabolism, these are intuitively most likely to be evident in people who are vitamin D deficient. This group has been little studied (Gedik and Akalin 1986; Kumar et al. 1994a; Borissova et al. 2003), with results that are inconclusive. A number of small studies of vitamin D treatment have been performed (Nyomba et al. 1986; Inomata et al. 1986; Gedik and Akalin 1986; Ljunghall et al. 1987; Lind et al. 1989; Zofkova and Stolba 1990; Orwoll et al. 1994; Boucher et al. 1995; Rudnicki and Molsted-Pedersen 1997; Fliser et al. 1997; Taylor and Wise 1998; Borissova et al. 2003) and recently a larger study of combined vitamin D and calcium therapy in older people (Pittas et al. 2007). Although in several studies there has been evidence of increased insulin sensitivity and/or decreased glucose levels (Kumar et al. 1994a; Rudnicki and Molsted-Pedersen 1997; Pittas et al. 2007), such changes have not been detected consistently.

The current study was conducted to specifically investigate individuals with known vitamin D deficiency without diabetes, and to evaluate the effects of normalisation of vitamin D on plasma glucose and serum insulin concentrations and insulin sensitivity. If treatment of vitamin D deficiency improves insulin sensitivity, it might reduce the rate of development of type 2 diabetes mellitus and the metabolic syndrome, an important component of which is insulin resistance.

10.3 Materials and methods

10.3.1 Subjects

A cohort of thirty seven adults (14 men and 23 women) age 19-75 years with vitamin D insufficiency/deficiency, with or without primary hyperparathyroidism (elevated plasma total and ionised calcium and PTH concentrations), and no history of diabetes mellitus, who represented successive referrals to Endocrinology outpatient clinics at the Royal Adelaide Hospital, were recruited, across a mix of seasons throughout the year. In all subjects, the baseline serum 25-hydroxyvitamin D concentrations was < 50 nmol/L (normal range 60-160 nmol/L). Subjects were allowed to take calcium supplements, and if doing so, continued on these unchanged throughout the study. Subjects were excluded from participation if they had taken any form of vitamin D supplementation in the preceding 3 months.

The protocol was approved by the Royal Adelaide Hospital Human Research Ethics Committee, and all subjects gave informed, written consent.

10.3.2 Protocol

At 8:00 a.m. – 9:30 a.m. after a 12-hour overnight fast from food and fluids, except water, subjects underwent a standard 75 g oral glucose tolerance test (OGTT) with collection of venous blood samples via an intravenous cannula at baseline (0 minutes), and then 60 and 120 minutes after the ingestion of the glucose drink.

On the same day, after completion of the OGTT, subjects received 100,000 units of cholecalciferol (vitamin D3) orally as a powder mixed in a drink, and were given a

further dose of 100,000 units of cholecalciferol to take 2 weeks later while at home. Two weeks after the second dose of cholecalciferol (i.e. 28 ± 2 days after the first OGTT), subjects underwent a second OGTT, using an identical protocol to the first. Subjects were asked not to alter their usual diet or exercise during the study and to continue calcium supplements unchanged if they were taking these. Diabetes mellitus was diagnosed at the OGTT when a fasting glucose concentration was ≥ 7 mmol/L or 2 hour glucose ≥ 11.1 mmol/L, fasting impaired glucose tolerance (IFG) if the fasting glucose was 5.6 - 6.9 mmol/L and impaired glucose tolerance (IGT) if the 2-hour glucose was 7.8 - 11 mmol/L (American Diabetes Association 2005). After completion of the study each subject was commenced on vitamin D replacement with 500 - 1000 international units (IU) per day of cholecalciferol (vitamin D3).

10.3.3 Measurements

Plasma glucose and serum insulin concentrations were measured on all blood samples; plasma total and ionised calcium, phosphate, albumin, sodium, potassium, creatinine, urea, and serum parathyroid hormone, 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D were measured on the baseline blood sample. Total and calculated ionised calcium, phosphate, albumin, sodium, potassium, creatinine, and urea were measured by routine automated laboratory methods on the Olympus AU 5400 analyser. Plasma glucose and serum insulin were measured as described in Chapter 5.7. Serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were assayed using a manual IDS radioimmunoassay method on Crystal 2 Packard Radioimmunoassay multidetector. Serum parathyroid hormone was measured using an automated immunoassay method on Immulite 2000.

10.3.3.1 Measurement of insulin sensitivity

Insulin sensitivity was evaluated using plasma glucose and serum insulin concentrations during the OGTT, according to the following formulae:

1. SiM (Avignon et al. 1999): [(w × Sib) + Si2h] / 2, where w = mean Si2h/mean Sib, Sib = 10^8/fasting insulin (microunits/mL) × fasting glucose (mg/dl) × VD), Si2h = 10^8/2-hr insulin (microunits/mL) × 2-hr glucose (mg/dL) × VD), and VD = 150mL per kg body weight.

2. $ISI_{0,120}$ (Gutt et al. 2000): (m/MPG) / log MSI, where m = [75,000mg + (fasting glucose – 2-hr glucose) × 0.19 × body weight] / 120 min; MPG = mean of fasting and 2-hr glucose concentrations (mg/dL).

3. QUICKI (Katz et al. 2000): $1 / [\log I_0 + \log G_0]$, where I_0 = fasting insulin, G_0 = fasting glucose.

4. HOMA (Matthews et al. 1985): $(I_0 \times G_0) / 22.5$

10.3.4 Statistical analysis

Statistical analysis was carried out using Statview Version 5.0 statistical software (SAS Institute Inc.) and SuperANOVA version 1.11 (Abacus Concepts, Inc., Berkeley, CA). The effects of vitamin D treatment on glucose and insulin concentrations were analysed by repeated-measurement analysis of variance (ANOVA), with time and treatment as factors. Insulin sensitivity indices (SiM, $ISI_{0,120}$, QUICKI, and HOMA) before and after vitamin D treatment were analysed by Student's *t* test for paired observations. Comparisons before and after treatment on BMI, serum PTH, 25-hydroxyvitamin D,

1,25-dihydroxyvitamin D, ionised calcium and creatinine were also performed using Student's paired *t* tests. Comparison of plasma glucose, serum insulin, and insulin sensitivity results between the impaired versus normal glucose tolerance groups were analysed using an interaction test in an ANOVA model. The results are presented as the mean ± SEM. A *P* value of < 0.05 was considered statistically significant. Pearson's correlation coefficients were used to determine the relations between variables. The primary endpoint was a change in insulin sensitivity in response to vitamin D treatment, with secondary endpoints of changes in plasma glucose and/or serum insulin.

From the results of a previous study examining the effects of vitamin D supplementation on insulin resistance (Borissova et al. 2003), it was calculated that a sample size of 30 subjects would have 80% power to detect a change in means for HOMA-IR index of 1.67 (i.e. approximately 21%), assuming a standard deviation of 3.10, using a paired t-test of 0.05 two-sided significance level.

10.4 Results

Demographic and biochemical variables at baseline and after treatment with vitamin D are summarised in Table 10.1.

Two subjects withdrew from the study after the first visit as they did not wish to have further blood tests. Two other subjects were excluded after the baseline visit and referred for appropriate treatment after their first oral glucose tolerance test (OGTT) revealed newly-diagnosed diabetes mellitus. Thirty three subjects completed the study.

Three of the 33 vitamin D deficient subjects had primary hyperparathyroidism (elevated plasma ionised calcium together with elevated parathyroid hormone concentrations) and ten were taking calcium supplements. Thirty one subjects were white and two non-white. Body weight did not change during the study.

With vitamin D treatment, mean serum 25-hydroxyvitamin D more than doubled, and 1,25-dihydroxyvitamin D increased by approximately a third. Only one subject had a very low 25-hydroxyvitamin D level of less than 25 nmol/L at baseline. Following treatment, thirty out of thirty-three subjects (91%) had 25-hydroxyvitamin D concentrations > 60 nmol/L, and in all subjects the vitamin D level was ≥ 50 nmol/L. There was a 33% reduction in serum parathyroid hormone (PTH) concentrations, which almost achieved statistical significance (P = 0.055). At baseline serum PTH was inversely related to 25-hydroxyvitamin D levels (R = -0.44, P = 0.01), but there was no significant correlation post-treatment (R = 0.075, P = 0.68).

There was no difference between baseline and post-treatment plasma glucose levels at 0, 60, or 120 minutes during the OGTT (treatment effect P = 0.94; treatment × time interaction P = 0.72) (Table 10.1). There was also no difference between baseline and post-treatment serum insulin concentrations (treatment effect P = 0.67; treatment × time interaction P = 0.28) (Figure 10.1).

Measures of insulin sensitivity remained unchanged following vitamin D treatment (SiM P = 0.97; $ISI_{0,120}$ P = 0.74; QUICKI P = 0.88; HOMA P = 0.99, Table 10.1).

At baseline 12 subjects had impaired glucose tolerance: 6 impaired fasting glucose (IFG) only, 2 impaired glucose tolerance at 2 hours only (IGT) and 4 subjects, both. When data in the 12 subjects with impaired glucose tolerance at baseline and 21 normal tolerance subjects were analysed separately, there were no significant effects of vitamin D treatment on blood glucose (treatment × time interaction $P = 0.26$ for IGT group), insulin (treatment × time interaction: $P = 0.93$ for IGT group), or insulin sensitivity (Table 10.2). When interaction effects were tested in a three-way ANOVA model, plasma glucose was higher at all time points in the impaired tolerance compared to the normal tolerance group (mean plasma glucose (0 - 120 min) 7.13 ± 0.32 versus 5.92 ± 0.27 mmol/l respectively; F = 3.79, $P = 0.03$), however, there was no effect of vitamin D treatment on glucose in either group (F = 1.23, $P = 0.30$). There was no difference in the pattern of rise in serum insulin from 0 - 120 min between the normal and impaired tolerance groups (F = 1.09, $P = 0.34$), and no effect of vitamin D treatment (F = 0.28, $P = 0.76$). At baseline, and following treatment with vitamin D, the normal tolerance group had higher mean values for SiM (F = 5.82, $P = 0.02$), $ISI_{0,120}$ (F = 4.01, $P = 0.05$), and QUICKI (F = 8.00, $P = 0.01$), and lower mean values for HOMA (F = 8.67, $P = 0.01$), compared to the impaired tolerance group (mean values listed in Table 10.2). However, there were no group × treatment interactions for SiM (F = 0.18, $P = 0.67$), $ISI_{0,120}$ (F = 0.60, $P = 0.44$), QUICKI (F = 0.04, $P = 0.85$), or HOMA (F = 0.01, $P = 0.90$).

When the 10 subjects taking calcium supplements were excluded from the analysis, there were no significant treatment effects of vitamin D on plasma glucose, serum insulin, or on markers of insulin sensitivity (data not shown). Similarly, when the 3

subjects with primary hyperparathyroidism, or the 2 non-white subjects were excluded separately from the analysis, there was no significant effect of vitamin D treatment on any measure of glucose or insulin concentration, or of insulin sensitivity (data not shown).

10.5 Discussion

This study investigated individuals without diabetes specifically selected for evidence of vitamin D insufficiency/deficiency, as the effects of repletion of vitamin D may be more apparent in this group than in those with vitamin D concentrations in the normal range. It was found that correction of low vitamin D concentrations in adults without diabetes mellitus had no effect on glucose or insulin concentrations, or measures of insulin sensitivity during an oral glucose tolerance test. Subject numbers were small in previous studies with vitamin D deficient individuals, and included either those with or without diabetes (Nyomba et al. 1986; Gedik and Akalin 1986; Kumar et al. 1994a; Boucher et al. 1995; Taylor and Wise 1998; Borissova et al. 2003). However, the number of subjects in the present study was sufficient to be able to detect a change in insulin sensitivity of approximately 20%, but all changes in insulin sensitivity and in glucose and insulin concentrations after vitamin D therapy were far smaller than this, with none approaching statistical significance.

Reduced circulating vitamin D concentrations have been associated with impaired insulin sensitivity and an increased risk of developing type 2 diabetes in cross-sectional studies (Scragg et al. 2004; Ford et al. 2005). Similarly, in observational studies, plasma 25-hydroxyvitamin D concentrations have been correlated negatively with glucose

(Chiu et al. 2004; Need et al. 2005) and insulin concentrations (Baynes et al. 1997), and positively with insulin sensitivity (Chiu et al. 2004). If vitamin D does affect glucose and insulin metabolism, several mechanisms could be responsible (Chertow et al. 1986; Cade and Norman 1987). There is evidence from animal studies that vitamin D may stimulate pancreatic insulin secretion directly. For example, glucose and sulphonylurea-stimulated insulin secretion is less from islets of vitamin D deficient rats, both in vitro and in vivo, than from islets of vitamin D sufficient rats or vitamin D deficient rats treated with vitamin D (Chertow et al. 1986; Cade and Norman 1987). Acute administration of small, single doses, of 1,25-dihydroxyvitamin D3 to vitamin D deficient rats has also been reported to increase insulin secretion and reduce the blood glucose response to intravenous glucose (Cade and Norman 1987).

We are aware of fourteen publications relating to studies in which vitamin D has been administered to humans with or without diabetes and the effects on glucose and/or insulin metabolism evaluated (Nyomba et al. 1986; Inomata et al. 1986; Gedik and Akalin 1986; Ljunghall et al. 1987; Lind et al. 1989; Zofkova and Stolba 1990; Kumar et al. 1994a; Orwoll et al. 1994; Boucher et al. 1995; Rudnicki and Molsted-Pedersen 1997; Fliser et al. 1997; Taylor and Wise 1998; Borissova et al. 2003; Pittas et al. 2007). The results of these studies have been inconsistent, and the majority have failed to detect any change in blood glucose concentrations. In a case report, glucose tolerance improved after vitamin D therapy in a vitamin D-deficient subject (Kumar et al. 1994a). Statistically significant reductions in fasting blood glucose after vitamin D treatment, alone (Rudnicki and Molsted-Pedersen 1997) or when combined with calcium (Pittas et al. 2007), were reported in two studies of 12 and 445 subjects respectively, but in the

latter study only after retrospective cohort analysis (Pittas et al. 2007) (see below). To our knowledge no significant changes in blood glucose concentrations have been reported in other studies. Secretion and blood levels of insulin and insulin sensitivity have been reported to increase (Gedik and Akalin 1986; Lind et al. 1989; Orwoll et al. 1994; Boucher et al. 1995; Taylor and Wise 1998; Borissova et al. 2003; Pittas et al. 2007), decrease (Rudnicki and Molsted-Pedersen 1997; Taylor and Wise 1998), or not change (Lind et al. 1989; Zofkova and Stolba 1990; Kumar et al. 1994a; Fliser et al. 1997; Borissova et al. 2003; Pittas et al. 2007) after vitamin D treatment.

The results of the longest, and largest, of the vitamin D treatment studies were recently reported (Pittas et al. 2007). A retrospective, post-hoc, analysis of a study in which 445 older people without diabetes were treated for three years with either placebo, or a combination of vitamin D and calcium, showed that the change in fasting plasma glucose, the primary endpoint of the study, was not significantly different between the placebo and vitamin D-calcium-treated groups (0.18 mmol/L versus 0.12 mmol/L respectively, $P = 0.29$) (Pittas et al. 2007). In the group who had impaired fasting glucose at baseline, the increase in fasting plasma glucose levels during the study was less with vitamin D-calcium therapy than placebo (0.02 ± 0.09 mmol/L vs 0.34 ± 0.11 mmol/L respectively, $P = 0.042$), after correcting for various diabetes risk factors. Insulin resistance, assessed by HOMA, increased in the placebo-treated group, but not in the vitamin D-treated impaired fasting plasma glucose group (0.91 ± 0.31 vs 0.05 ± 0.19 respectively, $P = 0.031$) (Pittas et al. 2007). However, there was no difference between treatment groups in the number of subjects who developed impaired fasting glucose or diabetes after 3 years (19% in the placebo group versus 20% in the vitamin

D-calcium-treated group, $P = 0.84$) (Pittas et al. 2007). It is not possible to determine whether the observed effect was related to vitamin D therapy, calcium therapy or both, as only combined therapy was given. Calcium treatment alone may have beneficial effects on glucose homeostasis (Liu et al. 2005; Choi et al. 2005; Pittas et al. 2006).

Viewed together the results of previous vitamin D treatment studies do not suggest that vitamin D therapy acts differently depending on the glucose tolerance of the person treated, or that it works better in people with diabetes or impaired glucose tolerance than with normal glucose tolerance. Of the eight intervention studies of vitamin D treatment performed specifically in subjects with abnormal glucose tolerance or diabetes that the author is aware of (Inomata et al. 1986; Ljunghall et al. 1987; Lind et al. 1989; Kumar et al. 1994a; Orwoll et al. 1994; Rudnicki and Molsted-Pedersen 1997; Taylor and Wise 1998; Borissova et al. 2003), two showed a reduction in blood glucose concentrations (Kumar et al. 1994a; Rudnicki and Molsted-Pedersen 1997), one an increase (Taylor and Wise 1998), and there were no effects on insulin sensitivity. Consistent with this, in the current study there was no difference between the responses of the subjects with normal compared to impaired glucose tolerance in our study.

It was hypothesised that if vitamin D therapy were to have beneficial effects on glucose tolerance and/or insulin sensitivity, these were likely to be most evident in individuals with vitamin D deficiency. If beneficial effects of vitamin D therapy were observed in vitamin-D deficient people without diabetes, a patient population that has not been well-assessed previously, such treatment might provide a means of preventing or delaying the development of diabetes. To date, only three small studies, containing a

total of 8 subjects (3 of them with type 2 diabetes), have exclusively studied vitamin D-deficient subjects (Gedik and Akalin 1986; Kumar et al. 1994a; Taylor and Wise 1998), with no consistent effects on glucose/insulin metabolism detected; moreover, those studies were unable to detect a consistent effect on blood glucose concentrations; glucose levels decreased (Kumar et al. 1994a) or increased (Taylor and Wise 1998), insulin concentrations and secretion were unchanged (Kumar et al. 1994a) or increased (Gedik and Akalin 1986; Taylor and Wise 1998) and insulin sensitivity was unchanged (Kumar et al. 1994a) or reduced (Taylor and Wise 1998). In another study, of predominantly vitamin D deficient subjects, 34% of them with type 2 diabetes, vitamin D treatment was reported to increase blood insulin concentrations during an OGTT but not to affect glucose tolerance (Boucher et al. 1995). With the adequate sample size in our study, the finding of a lack of effect of vitamin D therapy on glucose and insulin metabolism in vitamin D deficient individuals appears clear-cut and these results do not support an effect of vitamin D treatment to reduce blood glucose or improve insulin sensitivity.

The present study has a number of possible limitations. Firstly, there was no control group, with all subjects receiving vitamin D. It was considered ethically difficult not to treat individuals with known vitamin D insufficiency/deficiency. Subjects were instructed not to change their diet or exercise levels during the study, and there is no reason to believe they did; the lack of change in body weight also suggests they did not. Moreover, if they had, it would seem intuitively more likely that they would have made lifestyle changes that favoured reductions in blood glucose concentrations and improvements in insulin sensitivity, rather than the other way around. Nevertheless, the

unlikely possibility of a change in subject behaviour that acted to conceal a vitamin-D induced change in glucose/insulin metabolism cannot be totally excluded.

Secondly, one month may have been insufficient time to detect effects of vitamin D treatment on insulin and glucose metabolism. However, supposedly positive effects of vitamin D therapy have been reported for shorter studies (in one study insulin levels were reportedly lower, and insulin sensitivity higher, a day after a single intravenous injection of 1,25-dihydroxyvitamin D than at baseline (Rudnicki and Molsted-Pedersen 1997)) and a number of intervention studies of substantially longer duration than the current study have also produced negative, or conflicting results (Gedik and Akalin 1986; Lind et al. 1989). The individuals in our study had vitamin D insufficiency/deficiency and may not have had sufficiently low concentrations of 25-hydroxyvitamin D. However, in a previous study, glucose tolerance did not change with more severe vitamin D deficiency (Boucher et al. 1995).

The dose or formulation of vitamin D used in the current study may have been a limitation, however the dose of cholecalciferol was sufficient for the mean serum concentration of 25-hydroxyvitamin D to more than double to be well within the normal range at 93.3 nmol/L (and for the elevated mean serum PTH concentration to be suppressed into the normal range). Furthermore, there was a highly significant (approximately 30%) increase in serum 1,25-dihydroxyvitamin D concentrations, an increase that suggests both that the study duration was sufficient to detect any independent effect of 1,25-dihydroxyvitamin D (as opposed to 25-hydroxyvitamin D) on glucose and insulin metabolism, and that different findings would have been

unlikely with alternate vitamin D preparations. Indeed, conflicting and inconsistent, effects have been reported for administration of each of cholecalciferol, ergocalciferol (Nyomba et al. 1986; Gedik and Akalin 1986; Kumar et al. 1994a; Boucher et al. 1995; Borissova et al. 2003; Pittas et al. 2007), and active vitamin D (Inomata et al. 1986; Ljunghall et al. 1987; Lind et al. 1989; Zofkova and Stolba 1990; Orwoll et al. 1994; Rudnicki and Molsted-Pedersen 1997; Fliser et al. 1997) on glucose and insulin metabolism.

The oral glucose tolerance test, and the fasting glucose value within it, are the standard methods of diagnosing both diabetes and impaired glucose tolerance (American Diabetes Association 2005). As such they have considerable clinical importance, and the finding in the current study of an absence of effect of vitamin D therapy should be viewed in that light. The intravenous euglycaemic hyperinsulinaemic clamp is often considered the gold standard technique of measuring insulin sensitivity (Gutt et al. 2000), while results of the frequently sampled intravenous glucose tolerance test (FSIVGTT) correlate well with the euglycaemic clamp procedure ($R = 0.73$, $P = 0.004$) (Coates et al. 1995). We used the baseline, and baseline-plus-two hour glucose and insulin values to calculate insulin sensitivity using the HOMA method and the SiM and $ISI_{0,120}$ indices respectively. Results of the HOMA ($R = 0.65$, $P < 0.001$) (Ciampelli et al. 2005), $ISI_{0,120}$ ($R = 0.63$, $P < 0.001$) (Gutt et al. 2000) and SiM methods ($R = 0.78$, $P < 0.001$) (Ciampelli et al. 2005) correlate closely and significantly with those of the clamp technique and those of the SiM method with the FSIVGTT ($R = 0.92$, $P < 0.0001$) (Avignon et al. 1999). It is, therefore, most unlikely different results for insulin sensitivity would have been obtained with the use of a clamp technique.

There is evidence that elevated PTH levels may be associated with increased risk of metabolic syndrome (i.e. insulin resistance) (Reis et al. 2007) and diabetes (Procopio et al. 2002) independent of 25-hydroxyvitamin D levels. Coadministration of calcium with vitamin D produces a greater decrease in PTH level than the same dose of vitamin D given alone (Grant et al. 2005). It is possible that vitamin D may improve insulin sensitivity only when given with calcium and future studies in this area should take this into account.

In summary, although there is evidence from animal, in vitro and epidemiological studies of a link between abnormalities of glucose and insulin metabolism and vitamin D deficiency, the available evidence from vitamin D treatment studies in humans has been conflicting. In the current study, correction of vitamin D deficiency was associated with a clear-cut lack of effect on glucose and insulin metabolism as assessed by an oral glucose tolerance test after one month. This finding argues strongly against a beneficial effect of vitamin D therapy on glucose/insulin metabolism, at least in the short-term.

Table 10.1: Demographic and biochemical variables at baseline and one month after treatment with cholecalciferol (mean ± SEM).

Characteristic	**Baseline**	**Post-treatment**	***P* value (compared to baseline)**
Gender (male/female)	13 / 20		
Skin colour (white/non-white)	31 / 2		
Age (years)	55 ± 2.6		
Weight (kg)	67.5 ± 1.9	67.7 ± 1.9	0.46
BMI (kg/m^2)	24.1 ± 0.6	24.2 ± 0.6	0.21
Primary hyperparathyroidism (present/absent)	3 / 30		
Serum PTH (pmol/L)	6.7 ± 1.2	4.5 ± 0.6	0.055
Serum 25-hydroxyvitamin D (nmol/L)	39.9 ± 1.5	90.3 ± 4.3	< 0.00001
Serum 1,25-hydroxyvitamin D (pmol/L)	100.2 ± 6.4	129.1 ± 10.8	0.003
Serum ionised calcium (mmol/L)- calculated	1.16 ± 0.01	1.17 ± 0.01	0.16
Serum creatinine(μmol/L)	52.9 ± 6.2	61.8 ± 4.6	0.12
Plasma glucose (mmol/L)			
0 min	5.16 ± 0.09	5.18 ± 0.09	0.78
60 min	7.85 ± 0.41	7.76 ± 0.41	0.75
120 min	6.08 ± 0.27	6.19 ± 0.24	0.63
Mean (0-120 min)	6.36 ± 0.23	6.37 ± 0.24	0.63
Serum insulin (mU/L)			
0 min	6.9 ± 0.6	6.9 ± 0.6	0.94
60 min	81.6 ± 11.1	77.0 ± 7.6	0.55
120 min	54.8 ± 6.3	62.7 ± 8.8	0.12
Mean (0 - 120 min)	47.8 ± 5.3	48.9 ± 5.2	0.67
Insulin sensitivity index			
SiM	2.96 ± 0.36	2.98 ± 0.40	0.97
$ISI_{0,120}$	4.74 ± 0.27	4.66 ± 0.29	0.74
QUICKI	0.36 ± 0.01	0.36 ± 0.01	0.88
HOMA	29.21 ± 2.78	29.25 ± 2.87	0.99

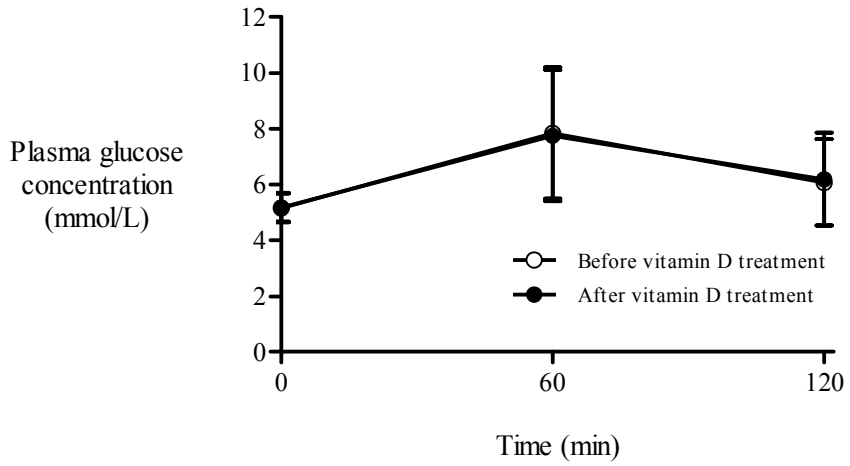
Table 10.2: Insulin sensitivity indices at baseline and one month after treatment with cholecalciferol (mean ± SEM) in subjects with normal (n = 21) or impaired glucose tolerance (IFG and/or IGT*, n = 12) at baseline.

Variables	**Normal tolerance (n = 21)**		**Impaired tolerance (n = 12)**	
	Baseline	Post-treatment (*P* value: compared to baseline)	Baseline	Post-treatment (*P* value: compared to baseline)
Insulin sensitivity index				
SiM	3.71 ± 0.49	3.65 ± 0.56 (*P* = 0.92)	1.65 ± 0.22	1.80 ± 0.37 (*P* = 0.52)
$ISI_{0,120}$	5.31 ± 0.34	5.09 ± 0.37 (*P* = 0.55)	3.74 ± 0.26	3.90 ± 0.37 (*P* = 0.54)
QUICKI	0.38 ± 0.01	0.38 ± 0.01 (*P* = 1.00)	0.34 ± 0.01	0.35 ± 0.01 (*P* = 0.72)
HOMA	23.76 ± 2.98	23.21 ± 1.93 (*P* = 0.84)	38.77 ± 4.53	39.82 ± 6.16 (*P* = 0.86)

** IFG = impaired fasting glucose tolerance (fasting glucose 5.6 - 6.9 mmol/L);*

IGT = impaired glucose tolerance (2-hour glucose 7.8 - 11.0 mmol/L).

(a)



(b)

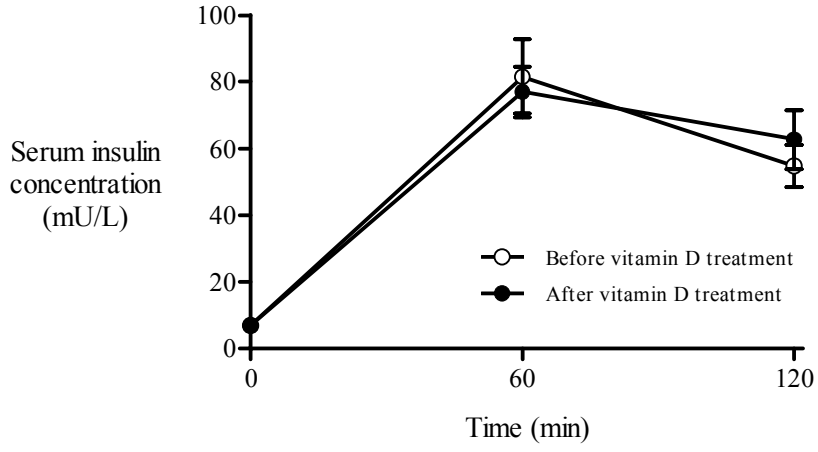


Figure 10.1: (a) Plasma glucose and (b) serum insulin concentrations during oral glucose tolerance test, prior to, and one month after, cholecalciferol treatment, in 33 subjects with vitamin D insufficiency. Data are mean values ± SEM.

Chapter 11

CONCLUSIONS

Undernutrition and nutritional frailty represent common, and significant, problems in persons aged 65 years and over, and require an understanding of the complex interplay of central and peripheral mechanisms which are involved in the control of appetite and energy intake, and the physiological decline of appetite with increasing age, termed the 'anorexia of ageing'. Ageing is associated with an increased prevalence of many disorders, including vitamin D deficiency and postprandial hypotension, which are both established risk factors for falls, which in turn are associated with increased morbidity and mortality. The research presented within this thesis has focused on the gastrointestinal control of appetite and food intake, with an emphasis on the role of ghrelin. It has also dealt with some of the sequelae of under-nutrition in older persons, in particular vitamin D deficiency and postprandial hypotension.

In Chapters 6 and 7, the effects of age and nutrient digestion on circulating ghrelin concentrations were evaluated. The role of fat digestion in altering the postprandial blood pressure response in young and older adults was evaluated in Chapter 8. In addition, the results of some intervention studies are described in Chapters 9 and 10, the former study relating to nutritional supplementation as a strategy for increasing energy intake, and the latter study to the effects of vitamin D replacement therapy on glucose and insulin metabolism.

Whilst ageing rodents have lower circulating ghrelin concentrations than young rodents, the consequences of healthy ageing on circulating plasma ghrelin concentrations in humans are unclear. The study described in Chapter 6 evaluated the variations in fasting ghrelin concentrations over a sixty year age range in healthy young and older subjects. Plasma ghrelin concentrations were higher in females than males, but did not correlate with age, and were inversely related to body mass index. Ghrelin was independently, and inversely, related to total body skeletal muscle mass, but not to any other body composition variable. These observations suggest that strategies for increasing muscle mass, through resistance exercises, may aid in abolishing the compensatory rise in ghrelin concentrations seen with under-nutrition and weight loss, and accordingly, enhance appetite and energy intake in under-nourished older adults.

Plasma ghrelin concentrations increase before a meal, and decrease to trough levels within one hour of ingestion of a meal. Macronutrients differ in their ability to suppress ghrelin, being earlier, and more pronounced, after carbohydrate ingestion, and relatively delayed after fat ingestion. The study described in Chapter 7 investigated the role of carbohydrate and fat digestion in the suppression of plasma ghrelin concentrations in healthy young adults. Plasma ghrelin concentrations were suppressed by sucrose and fat. Inhibition of carbohydrate digestion by acarbose was associated with an attenuated suppression of ghrelin concentrations, although it was not possible to exclude a contribution of delayed gastric emptying to this effect. Reduction of fat digestion by orlistat both accelerated gastric emptying and attenuated the fat-induced ghrelin suppression, confirming that the digestion of fat is an important postgastric feedback

mechanism potentiating the suppression of ghrelin secretion. In order to examine the effects of carbohydrate digestion, further studies are warranted to assess if intraduodenal administration of sucrose with acarbose, which effectively bypasses the gastric emptying effects of acarbose, attenuates ghrelin suppression by sucrose. Given that orlistat and acarbose are therapeutic agents in the management of overweight individuals and in those with type 2 diabetes, the attenuation of postprandial plasma ghrelin suppression may possibly have undesirable effects by enhancing appetite and energy intake.

Postprandial hypotension is defined as a 20mmHg or greater fall in systolic blood pressure within two hours of a meal. The magnitude of the fall in postprandial blood pressure is dependent, in part, on the macronutrient composition of the meal, and the effects are particularly discernable in older adults. Although carbohydrate ingestion is potent in reducing postprandial blood pressures in older adults, fat ingestion appears to have comparable, but relatively delayed effects. In the study described in Chapter 8, the role of fat digestion in modifying postprandial blood pressure responses was evaluated in healthy young and older adults. Fat ingestion was associated with a reduction in systolic and diastolic blood pressures in older, but not young, adults. Co-administration of the lipase inhibitor, orlistat, accentuated the fat-induced reduction in blood pressure in older adults. This suggests that digestion of fat (which is inhibited by orlistat) is not an important factor in fat-induced hypotension, but rather explained by other actions of orlistat, most likely its action to accelerate gastric emptying. Whilst the blood pressure-lowering effects of orlistat may be beneficial in certain patient populations, such as in those who are overweight or have type 2 diabetes, it needs to be used with caution in

older individuals who are at particular risk of postprandial blood pressure excursions. In order to discriminate between the gastric emptying effects of orlistat and those of fat digestion in determining fat-induced decreases in blood pressure, future studies would need to assess the effects of administration of orlistat and fat directly into the small intestine.

Gastrointestinal function and appetite can be modulated by dietary manipulation of the macronutrient composition of an individual's diet. The intervention study described in Chapter 9 evaluated the effects of two weeks of dietary fat supplementation on the sensitivity to the satiating effects of intravenous CCK-8 in healthy older subjects. There was no significant difference in the suppression of appetite and energy intake by CCK following a high-fat, high-energy, diet for two weeks in older adults. No differences were observed in fasting or postprandial plasma CCK concentrations after the dietary supplementation period compared to regular diet. Thus, manipulation of the diet in older persons favouring increased intake of fat and increased energy appears unlikely to have beneficial effects on improving appetite and food intake, at least over the time course of the present study. It would be of interest to evaluate sensitivity to the satiating effects of endogenous CCK with the use of a CCK-1 receptor antagonist, such as dexloxiglumide, following exposure to a high-fat diet.

Vitamin D deficiency is common, as is type 2 diabetes, and the two conditions may be linked. There is mounting evidence linking vitamin D deficiency with abnormalities of glucose and insulin metabolism. The study described in Chapter 10 evaluated the effects of vitamin D therapy in healthy young and older adults with low vitamin D

concentrations in the setting of normal, or impaired glucose tolerance. Correction of vitamin D insufficiency/deficiency was associated with a clear-cut lack of effect on glucose and insulin metabolism as assessed by an oral glucose tolerance test after one month. This observation argues strongly against a beneficial effect of vitamin D therapy on glucose/insulin metabolism, at least in the short-term. It remains to be determined whether correction of vitamin D deficiency in the longer term has any beneficial effects on glucose and insulin metabolism.

The observations of the studies presented in this thesis have provided insights into the gastrointestinal mechanisms regulating appetite and food intake, in particular the consequences of dietary manipulation, and determinants of ghrelin secretion, in healthy adults, and the contribution of the gastrointestinal system to the 'anorexia of ageing'. These observations have implications for the regulation of appetite in under-nourished older individuals. Further studies evaluating the effects of carbohydrate and fat digestion on circulating ghrelin concentrations, and dietary manipulation on gastrointestinal hormone release in older adults are warranted. The contribution of gastric and small intestinal mechanisms to the fat-induced reduction in postprandial blood pressure in older adults also remains to be determined.

Appendix 1

5 DAY FOOD DIARY

NAME:_____

Please return to:
Kamilia Tai
Department of Medicine
Royal Adelaide Hospital
Phone : (08) 8222 5039

GUIDELINES

- This is a diary for you to record all of the food and drink you consume over the next five days.
- To record in the food diary we would like you to ideally weigh as many of the foods as practical. Alternatively, use cup or spoon measures (metric) or common serves e.g. slice of bread etc. Do not guess weights unless you are eating out and there is no other alternative.
- Record everything you eat and drink from the time that you get up until the time you go to bed at night. Use a separate page for each day.
- Fill in the diary immediately after eating. Try to make your eating pattern as typical as possible.
- Don't forget to record all snacks and drinks such as tea/coffee (with or without milk or sugar), or alcoholic or soft drinks.
- Be as specific as possible e.g. specify the type of bread (white/wholemeal), the degree of fat trimming meat, type of margarine or oil, and the type of milk (whole milk, skim).
- If you follow a recipe, please record it at the back of the food diary. An example is listed.
- Indicate the method of cooking, e.g. boiling, frying. Also indicate the type of oil used for frying.
- List separate foods on a different line so that a ham sandwich should be recorded as bread (type), margarine (type), and ham all on separate lines.
- Please take the diary with you if you eat or drink anything outside of your home.
- Use the example given as a guide to record your foods.

EXAMPLE

Date:…….. Day of the Week:……

Time	**Description of food and drink consumed**	**Quantity**
7:00am	Weetbix	3
	Full-cream milk	1/2 cup
	Bread (white) toasted	1 slice
	Margarine (polyunsaturated)	2 tsp
	Orange juice (unsweetened)	1 glass
10:00am	Coffee (instant) black	1 cup
	sugar	2 tsp
	Milk arrowroot biscuits	2
12:30pm	Bread (white)	2 slices
	Margarine (polyunsaturated)	2 tsp
	Ham	1 slice
	Cheese	1 slice
5:30pm	Steak (beef) raw	200g
	Potato (with skin) baked	200g
	Beans (French) boiled	60g
	Bread (white)	1 slice
8:00pm	Milk (full cream)	250mL
	Milo	2 tsp
	Milk coffee biscuits	2

RECIPE EXAMPLE

Date: 12/5/2005 Day of the Week: Thursday

Time	**Description of Food and drink consumed**	**Quantity**
RECIPE	TUNA MORNAY	2 serves
	Tuna (in brine)	250g
	Flour (plain white)	1 tbsp
	Cheese (matured)	20g
	Breadcrumbs (white)	1 tbsp
	Margarine (polyunsaturated)	3 tsp
	Milk (skim)	1/2 cup

Date:…….. Day of the Week:……

Time	**Description of Food and drink consumed**	**Quantity**

Appendix 2 Visual analogue scale questionnaire

Subject: **Time:**

Visit: **Date:**

For each sensation please indicate how you are feeling at this moment by placing a **vertical mark** at the appropriate point on each scale below. Please **do not** make a cross or a sloping mark. Please mark **all** scales.

Not hungry _____ Hungry

Not nauseated _____ Nauseated

Empty _____ Full

Alert _____ Drowsy

Calm _____ Anxious

Not satiated _____ Satiated

How strong is your desire to eat?

Weak _____ Strong

How much food do you think you could eat?

None _____ A large amount

Appendix 3 Composition of the buffet meal used to quantify food intake.

Food items	Amount served, g	Energy content, kJ	Fat, g	Carbohydrate, g	Protein, g
Wholemeal bread, 4 slices[a]	125	1,304	3.6	50.0	12.6
White bread, 4 slices[a]	125	1,295	2.9	56.4	11.8
Ham, sliced[b]	100	453	3.6	0	18.8
Chicken, sliced[c]	100	677	7.0	0	24.6
Cheese, sliced[d]	85	1,436	28.3	0.9	21.9
Tomato, sliced	100	56	0.1	1.9	1.0
Lettuce	100	27	0	0.4	0.9
Cucumber, sliced	100	44	0.1	1.9	0.5
Strawberry yoghurt[e]	200	966	6.2	33.8	9.4
Fruit salad[f]	140	343	0.1	19.3	0.6
Chocolate custard[g]	150	662	5.3	22.7	4.8
Apple	170	359	0.2	21.3	0.5
Banana	190	680	0.2	37.8	3.2
Orange juice, unsweetened[h]	500	800	5.0	42.5	5.0
Iced coffee[i]	600	1,788	10.2	61.8	21.0
Water	600	0	0	0	0
Margarine[j]	20	609	16.4	0.1	0.1
Mayonnaise[k]	20	310	6.5	4.0	0.2
Total	3,425	11,808	95.7	354.6	136.9

[a] Sunblest, Tiptop, Australia;
[b] Deli leg ham, Woolworths, Australia;
[c] Virginian chicken, Woolworths, Australia;
[d] Coon Tasty Cheese slices, Australian Cooperative Foods Ltd., Australia;
[e] Yoplait, National Foods Ltd., Australia;
[f] Goulburn Valley, SPC, Ardmona Operations Ltd., Australia;
[g] Yogo, National Foods Ltd., Australia;
[h] Daily Juice Company, Australia;
[i] Farmers Union, Balemar Pty. Ltd., Australia;
[j] Flora, Unilever Australasia, Australia;
[k] Kraft, Kraft Foods Ltd., Australia.

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