

**The Role of Oxidant Stress in a
Cellular Model of Aortic Valve Cell
Calcification**

by

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ABSTRACT

INTRODUCTION: Calcific aortic valve stenosis (AS) is associated with a significant increase in morbidity and mortality in affected individuals, especially with advancing age. However, the pathogenesis of AS has not been fully understood, in particular, the role of oxidative stress and its contribution towards the development of AS.

STUDY OBJECTIVE: The aim of the current study was to further delineate the role of redox stress, in particular as modulated by the endogenous anti-oxidant, thioredoxin (TRX) and the pro-oxidant, thioredoxin-interacting protein (TXNIP) following stimulation of cellular calcification / nodule formation induced by transforming growth factor-beta 1 (TGF- β 1). In addition, the hypothesis that nitric oxide (NO) suppresses TXNIP expression in this system was also tested.

METHODS: Cultured porcine aortic valve interstitial cells (AVICs) at 90% confluence were treated with TGF- β 1 (5ng/ml) or vehicle, +/- 20 μ M Deta-NONOate (nitric oxide donor) or 10 μ M SB431542 (TGF- β 1 inhibitor). TRX activity was quantified using the insulin disulphide reduction method with absorbance measured at 415 nm. Experiments were conducted in triplicate, and repeated in at least 3 cultures, between cell passages 2 and 4. Nodules were counted by an observer blinded to treatments. Experiments were also conducted in parallel, whereby TXNIP was measured by immunofluorescence and subsequently underwent image analysis. Cell survival quantification was performed in all experiments in response to various treatments as described above. Results were expressed as mean \pm SEM. Multiple comparisons between the effects of treatments

relative to respective controls were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. A critical $P < 0.05$ was considered statistically significant.

RESULTS: TGF- β 1 significantly increased calcific nodule formation compared to controls (37.19 ± 2.67 vs. 0.33 ± 0.12 (nodule count/well), $P < 0.001$, $n=4$), and correspondingly decreased TRX activity (39.94 ± 0.66 vs. 58.96 ± 2.22 (mU/mg protein), $P < 0.001$, $n=4$, figure 3.2) and cell survival/area (11.98 ± 0.74 vs. 20.10 ± 0.56 ($\times 10^4/\text{cm}^2$), $P < 0.001$, $n=4$), and increased TXNIP immunofluorescence (IF) intensity/cell (17059 ± 204 vs. 7984 ± 423 (arbitrary units), $P < 0.001$, $n=3$). Deta-NONOate significantly suppressed TGF- β 1-induced nodule formation (9.40 ± 1.28 vs. 37.19 ± 2.67 (nodule count/well), $P < 0.001$, $n=4$), and correspondingly increased TRX activity (59.21 ± 2.49 vs. 39.94 ± 0.66 (mU/mg protein), $P < 0.001$, $n=4$) and cell survival/area (16.93 ± 0.95 vs. 11.98 ± 0.74 ($\times 10^4/\text{cm}^2$), $P < 0.01$, $n=4$), and decreased TXNIP IF intensity/cell (7918 ± 310 vs. 17059 ± 204 (arbitrary units), $P < 0.001$, $n=3$), compared with TGF- β 1 treatment alone. SB431542 significantly decreased TGF- β 1-induced nodule formation (0.42 ± 0.25 vs. 37.19 ± 2.67 (nodule count/well), $P < 0.001$, $n=4$), and correspondingly increased TRX activity (59.94 ± 1.25 vs. 39.94 ± 0.66 (mU/mg protein), $P < 0.001$, $n=3$) and cell survival/area (20.50 ± 0.78 vs. 11.98 ± 0.74 ($\times 10^4/\text{cm}^2$), $P < 0.001$, $n=4$), and decreased TXNIP IF intensity/cell (7670 ± 798 vs. 17059 ± 204 (arbitrary units), $P < 0.001$, $n=3$), compared with TGF- β 1 treatment alone.

CONCLUSION: TGF- β 1-induced aortic valve interstitial cell calcific nodule formation is related to an increase in redox stress, involving a decrease in the endogenous anti-oxidant activity of thioredoxin (TRX), with a corresponding increase in the pro-oxidant, thioredoxin-interacting protein (TXNIP). In addition, TGF-B1-induced aortic valve interstitial cell calcific nodule formation results in a decrease in cell survival. These effects are ameliorated by nitric oxide (NO).

GLOSSARY OF COMMON ABBREVIATIONS

Abbreviations	Definition
ACE	Angiotensin-converting enzyme
ACEIs	Angiotensin-converting enzyme inhibitors
ADMA	Asymmetric dimethylarginine
AF	Atrial fibrillation
ALP	Alkaline phosphatase
Ang	Angiotensin
ANOVA	Analysis of variance
A2RBs	Angiotensin-2 receptor blockers
ARIC study	Atherosclerosis Risk in Communities study
AS	Aortic valve stenosis
ASc	Aortic valve sclerosis
ASK-1	Apoptosis signalling kinase-1
ASTRONOMER substudy	Aortic Stenosis Progression Observation Measuring Effects of Rosuvastatin substudy
AV	Aortic valve
AVA	Aortic valve area
AVC	Aortic valve calcium/calcification
AVECs	Aortic valve endothelial cells
AVICs	Aortic valve interstitial cells
AVR	Aortic valve replacement
AV-Vel	Peak aortic jet velocity
BMI	Body mass index
BMP(s)	Bone morphogenic protein(s)
BSA	Bovine serum albumin
CAC	Coronary artery calcification
CAD	Coronary artery disease

Cbfa1	Core-binding factor α1
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CHS	Cardiovascular health study
CRF	Chronic renal failure
CT	Computed tomography
DETA-NONOate	(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's media
DNA	Deoxyribonucleic acid
EBCT	Electron-beam-computed-tomography
ECs	Endothelial cells
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediamine tetra-acetic acid
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ESRD	End-stage renal disease
ET-1	Endothelin-1
FCS	Fetal calf serum
FZD	Frizzled
HCL	Hydrochloric acid
HDL	High-density lipoprotein
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMG-CoA	Hydroxymethylglutaryl coenzyme-A
HR	Hazard ratio
hs CRP	High sensitivity C-reactive protein
5-HT	5-hydroxytryptamine
IF	Immunofluorescence

IL	Interleukin
KLF-2	Kruppel-like factor-2
LDL	Low-density lipoprotein
L-NAME	N-Nitro-L-arginine-methyl ester
Lrp	LDL receptor-related protein
LV	Left ventricular
LVEF	Left ventricular ejection fraction
MAP	Mitogen-activated protein
MESA	Multi-Ethnic Study of Atherosclerosis
MI	Myocardial infarction
MMP	Matrix metalloproteinases
mRNA	Messenger Ribonucleic acid
MS	Metabolic syndrome
NADPH	Nicotinamide adenine dinucleotide phosphate
NEP	Neutral endopeptidase
NFATc1	Nuclear factor of activated T cells c1
NF-κB	Nuclear factor-κB
NK	Natural killer
NO	Nitric oxide
OPG	Osteoprotegerin
OSP	Osteopontin
Osx	Osterix
oxLDLs	Oxidized LDLs
PAECs	Porcine aortic endothelial cells
PAVECs	Porcine aortic valve endothelial cells
PBS	Phosphate-buffered solution
PPAR-γ	Peroxisome proliferator-activated receptor-γ
RAA	Renin-angiotensin-aldosterone
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand

ROS	Reactive oxygen species
SB431542	4-(5-Benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide hydrate, 4-[4-(1,3-Benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]-benzamide hydrate, 4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide hydrate
SEAS substudy	Simvastatin Ezetimibe in Aortic Stenosis substudy
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
siRNA	Single interrupting ribonucleic acid
SMCs	Smooth muscle cells
SOD	Superoxide dismutase
SPARC	Secreted protein, acidic and rich in cysteine/osteonectin
TAVR	Transcatheter aortic-valve replacement
Tempol	4-hydroxy-TEMPO
TF	Tissue factor
TGF-β1	Transforming growth factor-β1
TIMPS	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TRX	Thioredoxin
TXNIP	Thioredoxin-interacting protein
VCAM-1	Vascular cell adhesion molecule-1
VDUP1	Vitamin D3 up-regulated protein 1
VECs	Vascular endothelial cells
VEGF	Vascular endothelial growth factor
VICs	Valve interstitial cells

DECLARATION

The work within this thesis contains no material which has been accepted in its entirety for the award of any other diploma or degree in any university or 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 also give my consent to this copy of the thesis being made available for viewing, loan and photocopying at the University Library, subject to the provisions of the Copyright Act 1968. In addition, I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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PUBLICATIONS, PRESENTATIONS & SCHOLARSHIPS

Published abstracts related to this thesis

- **S Kanagasingam N**, Horowitz JD & Kennedy JA 2009, 'Transforming growth factor- β 1 increases thioredoxin-interacting protein (TXNIP) in calcifying aortic valve cells: attenuation by nitric oxide', *Heart Lung and Circulation*, **18**, supp. 3, abstract 436, pp. S193.
- **S Kanagasingam N**, Horowitz JD & Kennedy JA 2009, 'Development of aortic valve cellular calcification is associated with intracellular redox stress: amelioration by nitric oxide', *European Journal of Heart Failure*, **8**, supp. 2, abstract 1596, pp.ii825.

Local, national and international scientific meetings/conferences whereby presentations relating to this thesis were accepted

- *Heart Failure Congress 2009* (Nice, FRANCE)
- *Cardiac Society of Australia and New Zealand (CSANZ) 2009 57th Annual Scientific Meeting* (Sydney, New South Wales, AUS)
- *The Queen Elizabeth Hospital Research Day 2009* (Adelaide, South Australia, AUS)

Scholarships related to this thesis

- The University of Adelaide Divisional Scholarship
- The Queen Elizabeth Research Foundation Scholarship
- The Queen Elizabeth Hospital Medical Staff Society Educational Scholarship