# 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-β1). 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-β1 (5ng/ml) or vehicle, +/- 20μM Deta-NONOate (nitric oxide donor) or 10μM SB431542 (TGF-β1 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 ± 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-β1 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 ($x10^4/cm^2$), $P<0.001$, 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-β1-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 ($x10^4/cm^2$), $P<0.01$, n=4), and decreased TXNIP IF intensity/cell (7918±310 vs. 17059±204 (arbitrary units), $P<0.001$, n=3), compared with TGF-β1 treatment alone. SB431542 significantly decreased TGF-β1-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 ($x10^4/cm^2$), $P<0.001$, n=4 ), and decreased TXNIP IF intensity/cell (7670±798 vs. 17059±204 (arbitrary units), $P<0.001$, n=3), compared with TGF-β1 treatment alone.

**CONCLUSION:** TGF-β1-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- β1 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 57$^{th}$ 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
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