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Inhibition of calcifying nodule formation in cultured porcine aortic valve cells by nitric oxide donors

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ABSTRACT

Calcific aortic stenosis displays some similarities to atherosclerosis including evidence of endothelial dysfunction. Whether nitric oxide (NO), which is produced by valvular endothelium, has direct protective effects extending to calcification processes in aortic valve cells has not previously been examined. *In vitro* calcifying nodules in porcine aortic valve interstitial cell cultures, formed in response to transforming growth factor-beta1 (TGF-β1) 5 ng/ml, were inhibited by NO donors DETA-NONOate 5–100 μM, and sodium nitroprusside (SNP) 3 μM. Raising intracellular cGMP concentrations, via 8-bromo cGMP 1 mM or via brain natiuretic peptide and C-type natiuretic peptide 0.1 μM, inhibited TGF-β1-induced nodule formation, potentially implicating the cGMP pathway in the NO effect. Stimulation of interstitial cells with substance P or calcium ionophone (A23187) caused NO release and increased intracellular cGMP respectively. However in the presence of TGF-β1 basal levels of NO production via nitric oxide synthase (NOS) were insufficient to affect nodule formation. Increased dihydroethidium (DHE) fluorescence in response to TGF-β1, which was inhibited by DETA-NONOate and TEMPOL, suggested a role for intracellular superoxide in TGF-β1 signalling. Moreover, nodule formation was suppressed by superoxide scavengers TEMPOL, hydralazine and polyethylene glycol-superoxide dismutase (PEG-SOD), but not SOD. In conclusion, NO donors, or agents raising intracellular cGMP levels, may protect aortic valve interstitial cells from early events leading to calcification.

1. Introduction

Aortic valve stenosis poses an increasing burden as populations age. While progression to symptomatic aortic stenosis has a very poor prognosis, the treatment of choice, surgical valve replacement, may be limited as an option for elderly patients with co-morbidities (Bouma et al., 1999; Gilbert et al., 1999). Unfortunately there are currently no proven pharmacotherapies to delay aortic valve fibrosis and calcification, and the identification of potential therapeutic approaches has been limited by incomplete understanding of the aetiology of aortic valve stenosis.

Calcification of the aortic valve is increasingly appreciated to reflect a dynamic, regulated process with some similarities to atherosclerosis (reviewed by Chan, 2003; Freeman and Otto, 2005; O'Brien, 2006; Rajamannan et al., 2003). The stenotic valve itself displays some evidence of both lipid (O'Brien et al., 1996; Olsson et al., 1999) and immune cell infiltration (Otto et al., 1994; Srivatsa et al., 1997; Mohler et al., 2001). As regards the possible pathogenetic role of valvular endothelial dysfunction, aortic stenosis has been associated with evidence of systemic endothelial dysfunction (Poggianti et al., 2003) and reduced responsiveness to nitric oxide (NO) at the level of platelets (Chirkov et al., 2002).

Aortic valve endothelial cells have been shown in a number of species to produce endothelial mediators such as NO and prostacyclin (Amano et al., 1999; Pompilio et al., 1998). These mediators are likely to play a role in protecting the valve itself from thrombosis and immune cell infiltration (reviewed by Chan, 2003). Intriguingly, bicuspid aortic valves, which are associated with premature valve calcification, have been linked to NO deficiency in the endothelial nitric oxide synthase (eNOS) knock-out mouse model (Lee et al., 2000), and in a recent clinical study (Aicher et al., 2007).

In the hypercholesterolaemic rabbit, statins increase valvular eNOS expression and retard aortic valve calcification (Rajamannan et al., 2005). However, whether NO is able to exert a direct inhibitory effect on some of the processes within the valve leading to fibrosis and calcification has not been tested directly.

TGF-β1 is a cytokine which has been implicated in clinical aortic valve stenosis (Jian et al., 2003). Furthermore, it has been demonstrated to induce the formation of cell aggregates which then form calcific nodules in aortic valve cell cultures *in vitro* (Jian et al., 2003).

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The present study was designed to test the direct effects of NO on cultured porcine aortic valve interstitial cells, and in particular on TGF-β1 induced nodule formation as an early manifestation of TGF-β1 induced calcification in these cells. Furthermore, in light of the evidence for inflammation within the diseased valve (Otto et al., 1994; Srivatsa et al., 1997; Mohler et al., 2001), potential interactions of both TGF-β1 and NO with superoxide were also explored.

2. Materials and methods

2.1. Cell culture model

Hearts were obtained from female white domestic pigs, body weight 30 kg, killed after induction of anaesthesia with ketamine/xylazine and maintenance with 2% halothane in accordance with the NIH principles of laboratory animal care and procedures approved by the institutional animal ethics committees of the University of Adelaide and the Queen Elizabeth Hospital. The aortic valve leaflets were dissected under sterile conditions. Valvular endothelial cells were digested from the leaflets with collagenase type II (4 mg/ml), lima bean trypsin inhibitor (0.5 mg/ml) and fatty acid free bovine serum albumin (1 mg/ml) in calcium- and magnesium-free Hanks balanced salt solution. Digestion was carried out at 37 °C in a shaking water bath for 50 min. The leaflets were then rinsed in Hanks balanced salt solution and transferred to Dulbecco's modified Eagles medium and any remaining endothelial cells removed by gently rolling a sterile scalpel blade over the surface. After rinsing the leaflets were chopped into small pieces and the explants cultured in Dulbecco's modified Eagles medium with 10% fetal calf serum and penicillin/streptomycin at 37 °C with 5% CO_2 in air, at 95% relative humidity. When cells had emerged from the explants and formed a monolayer they were transferred with trypsin/EDTA and subcultured. The cell phenotype was assessed as predominantly myofibroblast as previously described using immunohistochemical staining for smooth muscle α-actin and vimentin (Kennedy et al., 2006). Cells were used for experiments between passages 2 and 4.

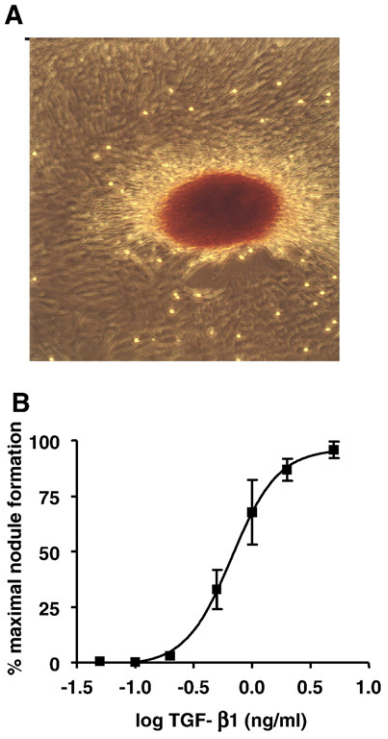

Fig. 1. A. Porcine AV interstitial cells treated with TGF-β1 5 ng/ml produce nodules which stain positive for calcium with alizarin red S. Original magnification 100×. B. Concentration dependent response to porcineTGF-β1 as % of maximum nodule formation (n=3–5 cultures). Peak nodule formation per well in the absence and presence of TGF-β1 5 ng/ml was 22±15 and 92±33 respectively, P<0.005 (n=14 cultures, paired t test).

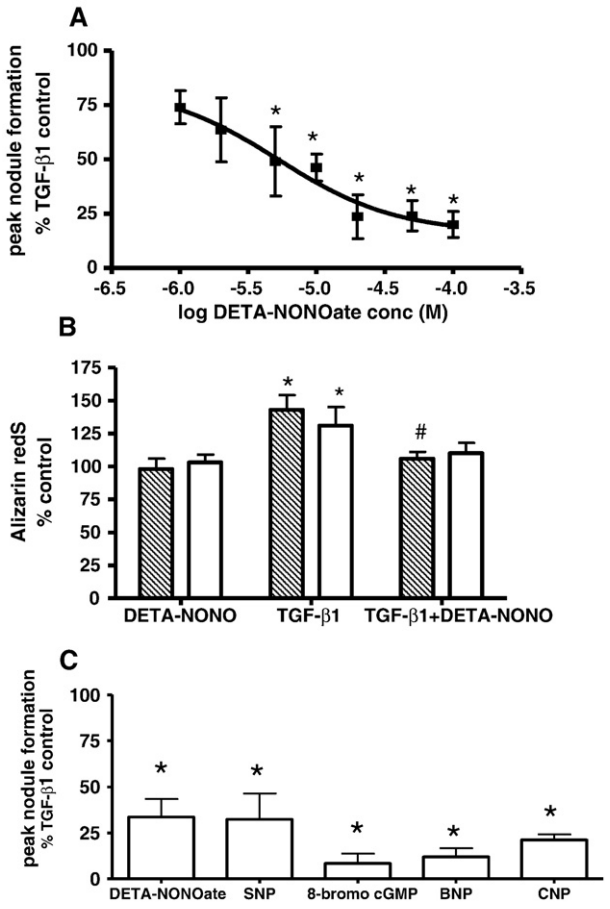

Fig. 2. A. Concentration response curve for inhibition of TGF-β1 induced nodule formation by DETA-NONOate (*P<0.05, compared with control, repeat measures ANOVA with Dunnett's post hoc test, n=3 cultures). B Effect of DETA-NONOate on total (open bars) and nodular (shaded bars) calcium deposition in AV interstitial cell cultures: alizarin red S stain was released from stained cells by incubation at room temperature with cetylpyridinium chloride 100 mM for 10 min for non-nodular staining and 48 h to release stain from nodules. Released stain was measured by absorbance at 595 nM and expressed relative to control cells. *P<0.05, relative to control, #P<0.05 relative to TGF-β1 alone (one-way ANOVA, with Tukey's post hoc test, n=4 cultures) C. Effect of NO donors, DETA-NONOate 20 μM and SNP 3 μM, and agents raising intracellular cGMP concentrations [8-bromo cGMP 1 mM, C-type natiuretic peptide (CNP) and brain natiuretic peptide (BNP) 0.1 μM] on TGF-β1 induced nodule formation. *P<0.05 compared with control (one-way ANOVA with Dunnett's post-hoc test, n=3–6 cultures).

2.2. Calcifying nodule formation

Valvular interstitial cells were plated into 6 or 12 well culture plates at a density of 0.5×10^6 and 0.2×10^6 cells/well respectively. When cells were 90% confluent the media was changed to Dulbecco's modified Eagles medium with 0.67% fetal calf serum. Nodule formation was assessed in the presence and absence of TGF-β1 (5 ng/ml) essentially as described by Mohler et al. (1999). Drugs under investigation were added to the wells at the same time as the TGF-β1 or its vehicle. The medium and study drugs were replenished every three days. Because of instability in solution, the NO donor, DETA-NONOate, was replaced daily. Treatments were carried out in triplicate in at least three cultures from different pigs. Nodule counts were carried out every two days by an observer who was blinded to the drug treatments. Nodules were verified as calcifying by staining with alizarin red S. Because of variability in absolute nodule counts

between different primary cultures, the effect of drugs on nodule formation was expressed as a percentage of the peak nodule formation in the corresponding TGF-β1 treatment control wells in each experiment. Peak nodule number occurred between days 4 and 8. For assessment of calcium deposition interstitial cells were incubated with TGF-β1 in medium containing β-glycerophosphate 5 mM and ascorbic acid 50 μg/ml to enhance calcification. Calcium staining was carried out between days 6 to 8. Alizarin red S staining was quantified as described by Huitema et al. (2006) for calcifying vascular cells, with the exception that a longer incubation (48 h) at room temperature with 100 mM cetylpyridinium chloride was required to release calcium from nodules. The absorbance of released alizarin red S was measured at 595 nm using a Bio-rad microplate reader.

2.3. Staining for alkaline phosphatase

Cells were stained on day 4 for alkaline phosphatase using Vector Blue substrate kit according to the manufacturer's instructions.

2.4. Assessment of endogenous NO formation

The ability of valvular interstitial cells to generate NO was assessed both directly via an NO electrode, and indirectly via intracellular cGMP accumulation.

2.4.1. NO release

Acute NO transients elicited in response to application of substance P or vehicle control were measured essentially as described previously (Rosenkranz et al., 2006). Valvular interstitial cells were seeded onto glass coverslips, cultured as above, and after 4 days in low serum Dulbecco's modified Eagles medium, they were rinsed in Krebs–Hepes buffer and placed into an organ bath chamber with Krebs–Hepes at 25 °C. A NO selective microsensor (ISO-NOP200, World Precision Instruments) was positioned 240–280 μm above the cell monolayer, and NO release was recorded online using DUO18 Data Acquisition software (WPI, Sarasota, USA). Following baseline stabilisation (15–30 min), 50 μl of substance P (3 nM) or buffer were applied to elicit a transient (1–2 min) increase in the NO signal. Between 5 and 10 measurements were recorded from across the entire cell monolayer, and the mean peak (delta pA) response was taken for each experiment.

2.4.2. cGMP formation

The valvular interstitial cells (90% confluence in 6 well culture plates) were washed with Krebs/Hepes and pre-incubated at 37 °C in Krebs/Hepes containing isobutyl methyl xanthine (IBMX) 500 μM for 30 min. NO release was induced by exposure to calcium ionophore (A23187 0.5 μM), or vehicle (0.01% ethanol) for 2 min at 37 °C. To confirm that cGMP accumulation was dependent on NO generation, parallel wells were stimulated with A23187 in the presence of the NOS

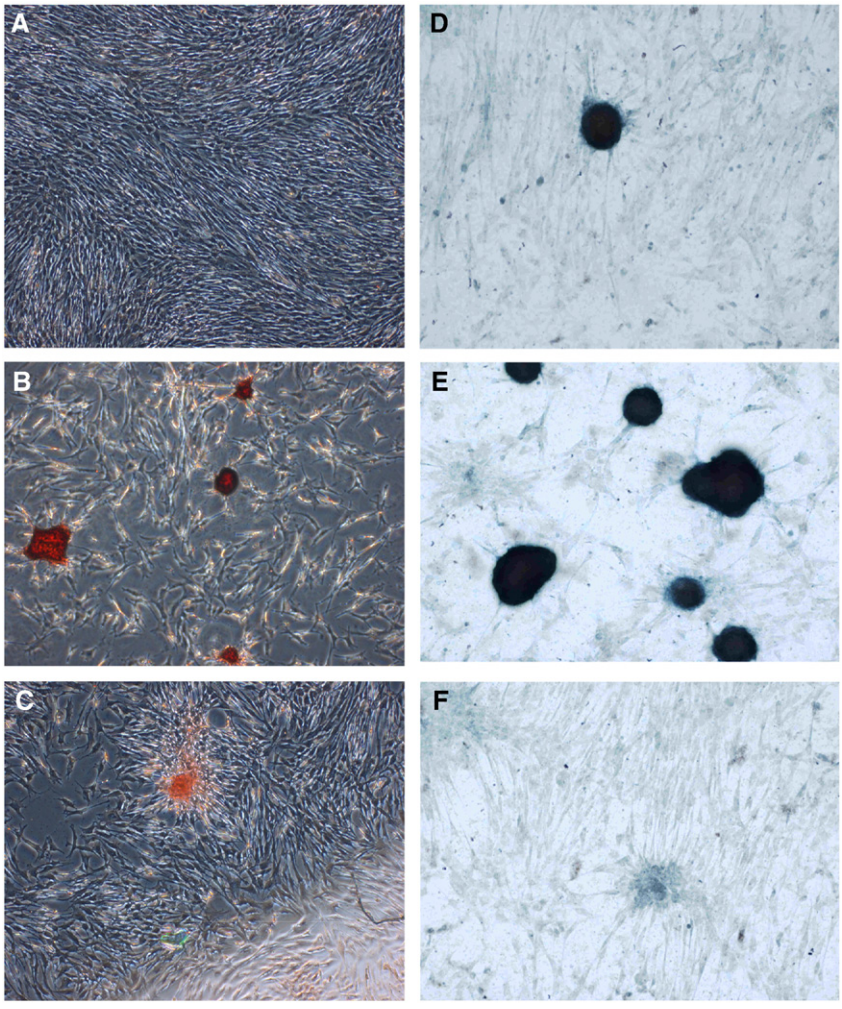

Fig. 3. Left hand panel shows alizarin red S staining in A control, B TGF-β1 and C, TGF-β1 + DETA-NONOate treated aortic valve interstitial cells. Alizarin red S staining is associated with nodules, and these are increased with TGF-β1 treatment and reduced by DETA-NONOate 20 μM. Original magnification 40×. Right hand panel shows alkaline phosphatase staining in D control, E TGF-β1treated cells, and F TGF-β1 + DETA-NONOate treated cells. Alkaline phosphatase staining is dense over nodules with paler staining associated with background cells. Positively staining nodules were increased by TGF-β1, and reduced by DETA-NONOate 20 μM, while background staining was similar for control and DETA-NONOate treated cells. Original magnification 100×.

inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) 300 μM, which was added to the cells at the same time as the IBMX. Treatments were performed in duplicate in each experiment. At the end of incubation the Krebs/Hepes was removed and the cell monolayer was treated with 65% ice cold ethanol and scraped from the wells. Intracellular cGMP was measured using a Biotrac radioimmunoassay (Amersham).

2.5. Dihydroethidium (DHE) staining for intracellular superoxide

Aortic valve interstitial cells grown to 90% confluence in 4- or 8-well chamber slides (Sybron, USA), were treated in 0.67% fetal calf serum containing media as for calcifying nodule estimation. After 4 days the Dulbecco's modified Eagles medium was replaced with Krebs/Hepes containing DHE 5 μM and the SOD inhibitor, diethyldithiocarbamic acid (DETCA) 3 mM, and the cells were incubated for 45 min at 37 °C in a humidified atmosphere, protected from the light, essentially as described previously (Miller et al., 1998). The fluorescent product formed from DHE and superoxide was detected via a 590 nm long pass filter using a fluorescent microscope (Nikon Eclipse TE300, Japan). At least five areas of confluent cells per slide were digitally photographed (Panasonic CCD) and the intensity of fluorescence per unit area was analysed using Video Pro 32 colour image analysis software (version 4, Leading Edge, Australia). No auto-fluorescence was detectable in the absence of DHE. Analysis was performed by an observer blinded to the treatment regimes. Fluorescence intensity was expressed relative to control treatments.

2.6. NADPH oxidase activity

AV interstitial cells were cultured in T-25 flasks for 4 days as per superoxide estimation via DHE staining. NADPH oxidase activity was estimated as previously described using 5 μM lucigenin mediated chemiluminescence (Kennedy et al., 2006) to quantify superoxide formation over 15 min in response to addition of NADPH 100 μM.

2.7. Analysis of data

Data are expressed as mean and S.E.M. Concentration response data were analysed using Graphpad Prism version 4 software. Multiple comparisons were analysed by one-way ANOVA with Tukey or Dunnett's post hoc tests. Single comparisons were by paired Student *t*-tests. A critical value of $P=0.05$ was adopted.

2.8. Materials

Porcine TGF-β1 was obtained from R&D systems Inc., USA, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amio] diazen-1-ium-1, 2-diolate (DETA-NONOate) from Cayman Chemical Co (Ann Arbour, MI, USA), alizarin red S, fatty acid-free bovine serum albumin, cetylpyridinium chloride, dihydroethidium (DHE), diethyldithiocarbamic acid (DETCA), ebselen, 4-hydroxy-TEMPO (TEMPOL), hydralazine HCl, l-arginine, isobutyl methyl xanthine (IBMX), L-NAME, sodium nitroprusside, superoxide dimutase (SOD), superoxide dismutase-polyethylene glycol (PEG-SOD), uric acid, from Sigma Chemical Co., St. Louis MO USA, 8-bromo-guanosine 3,5-cyclic monophosphate (8-Br-cGMP) from Alexis Corp, Switzerland, brain natiuretic peptide-32 and C-type natiuretic peptide 32-35 from Bachem AG, Bubendorf, Germany, collagenase type II and lima bean trypsin inhibitor from Worthington (Lakewood, NJ), benzyloxycarbonyl-Val-Ala-Asp(oMe)-fluoromethylketone (ZVAD) from Tocris Cookson Ltd. (Avonmouth, UK). All other chemicals were analytical grade purity.

3. Results

Porcine aortic valve interstitial cells formed nodules which stained positively for calcium with alizarin red S (Fig. 1A). Although 5 out of 14 cultures formed small numbers of nodules spontaneously, addition of exogenous TGF-β1 markedly increased nodule formation in a concentration dependent manner (Fig. 1B) over 4–8 days. Addition of calcifying media enhanced both nodule number per well and alizarin red S staining in TGF-β1 treated cells (185±5 compared with 268±17 nodules/well and 0.056±0.006 compared with 0.261±0.033 alizarin red S, OD595, both $P<0.05$, paired *t*-test $n=3$, in control versus calcifying media respectively).

The NO donor DETA-NONOate produced a significant and concentration-dependent inhibition of TGF-β1-stimulated nodule formation (Fig. 2A) with an IC50 of 5.3 μM and a maximum reduction to 19% of control. In those cultures which formed nodules in the absence of exogenous TGF-β1, DETA-NONOate was also inhibitory (mean peak nodule count:— control 7.1±0.8, 20 μM DETA-NONOate 0.6±0.4, $P<0.005$, $n=4$). TGF-β1 treatment increased total calcium staining by alizarin red S (total calcium by 31±14% and nodular calcium by 43±11%, $n=4$) and this was also inhibited by DETA-NONOate 20 μM (Fig. 2B). Alizarin red S staining was confined predominantly to nodules which were increased by TGF-β1 and decreased by DETA-NONOate 20 μM (Fig. 3A–C). A similar pattern of staining was evident for alkaline phosphatase, with staining being most dense over nodules, which was increased by TGF-β1, and decreased by DETA-NONOate 20 μM (Fig. 3D–F).

Nodule formation was also inhibited by a second NO donor, SNP 3 μM. Agents which raised intracellular cGMP levels, either directly (8-bromo cGMP 1 mM), or via stimulation of particulate guanylyl cyclase (brain natiuretic peptide and C-type natiuretic peptide, both 0.1 μM) also markedly inhibited nodule formation (Fig. 2C).

The possibility that treatment with DETA-NONOate might be able to reverse TGF-β1 induced nodule formation once initiated was investigated by addition of DETA-NONOate 20 μM 2 days after addition of TGF-β1. Although significantly reducing nodule number per well, later addition of DETA-NONOate had a significantly smaller effect on nodule number (Fig. 4A), and did not significantly reduce total alizarin red stain (Fig. 4B).

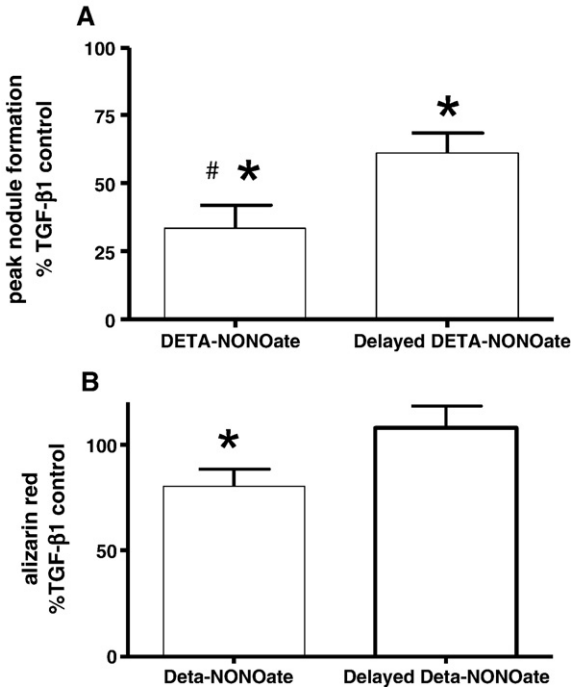

Fig. 4. Effect of delayed addition of DETA-NONOate 20 μM on TGF-β1 induced nodule formation(A) and total alizarin red S staining/well (B). DETA-NONOate was added 2 days after TGF-β1. *$P<0.05$, compared with TGF-β1 control, #$P<0.05$ compared with delayed addition of DETA-NONOate (paired *t*-test, $n=4$ cultures).

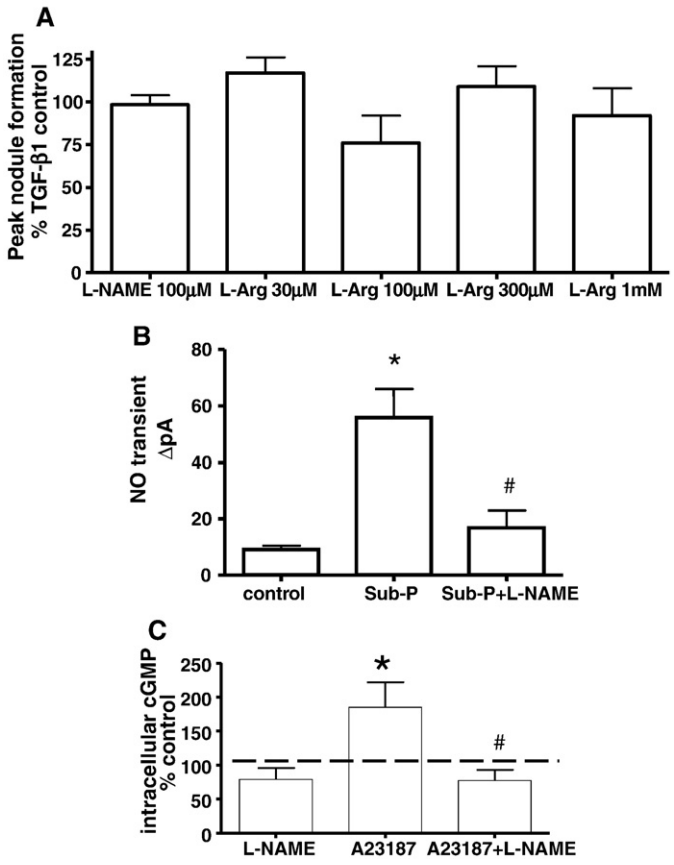

Fig. 5. A. Effect of inhibition of NOS with L-NAME 100 μM, and supplementation of media with L-arginine (L-Arg) 30 μM–1 mM on AV interstitial cell peak nodule formation in response to TGF-β1. $P=0.18$ (one-way ANOVA, $n=5$–11 cultures). B. Effect of substance P (Sub-*P*) 3 nM on real time NO release (ΔpA) from AV interstitial cells in the presence and absence of L-NAME 100 μM. *$P<0.05$ versus control, #$P<0.01$ versus substance P alone (one-way ANOVA with Tukey's post-hoc test, $n=3$ cultures). C. Effect of L-NAME on basal and stimulated cellular cGMP content. *$P<0.05$ versus control, #$P<0.05$ versus A23187 treated cells (one-way ANOVA with Tukey's post-hoc test, $n=6$ cultures).

In order to determine whether endogenous NO formation from these cells plays a role in modulating nodule formation, we examined the effects of the NOS inhibitor L-NAME 100 μM (Fig. 5A). NOS inhibition did not influence TGF-β1-stimulated nodule formation, suggesting that this process is unlikely to be limited by NO production by the interstitial cells themselves. Furthermore there was no significant dose response effect of exogenous NOS substrate (L-arginine 30 μM–1 mM) on TGF-β1-stimulated nodule formation ($P=0.18$, $n=5$–11, one-way ANOVA).

The ability of valvular interstitial cells to generate NO was confirmed by direct measurement of NO transients in response to substance *P*, which were inhibited by L-NAME (Fig. 5B). The delta pA in response to substance P was equivalent to 8 nM of NO as determined from electrode calibration curves to KNO_2. Furthermore, stimulation by the calcium ionophore A23187 0.5 μM produced a significant increase in intracellular cGMP which was inhibited by L-NAME, consistent with the presence of a constitutive Ca^{2+} dependent NOS in aortic valve interstitial cells (Fig. 5C). Conversely, low basal NO generation in these cells was reflected by lack of effect of L-NAME on basal intracellular cGMP.

The potential involvement of superoxide generation in TGF-β1-induced nodule formation was assessed by DHE fluorescence, a measure of intracellular superoxide. TGF-β1 produced a marked increase in DHE fluorescence by 250% (Fig. 6A and Fig. 7B). Nodules also displayed significant DHE fluorescence (Fig. 7C). DHE fluorescence was significantly inhibited by both TEMPOL 100 μM and DETA-NONOate 20 μM (Fig. 6A and Fig. 7E). A possible role of increased superoxide levels in nodule formation was examined using other scavengers of superoxide besides TEMPOL. In addition to TEMPOL, scavenging of intracellular superoxide with PEG-SOD 100 U/ml (Fig. 7D), but not extracellular scavenging with SOD 500 U/ml, inhibited TGF-β1 induced nodule formation by 70% (Fig. 6B). Hydralazine, at a superoxide scavenging concentration, viz. 100 μM (Daiber et al., 2005), also significantly attenuated nodule formation. In addition a possible role for inhibition of apoptosis was investigated using the pan-caspase inhibitor ZVAD. Unlike, DETA-NONOate, ZVAD 50 μM had no significant effect on TGF-β induced nodule formation (Fig. 6B).

A potential inhibitory effect of DETA-NONOate on superoxide formation via inhibition of NADPH oxidase was assessed in these cells, but this did not appear to be the mechanism of reduced superoxide levels since DETA-NONOate 20 μM had no significant effect on NADPH

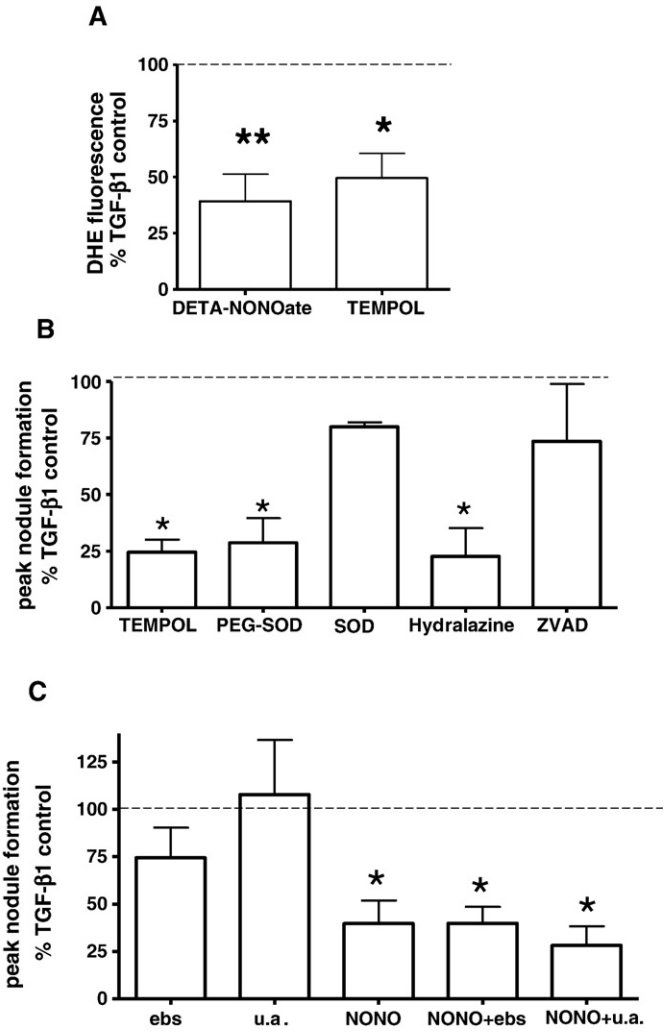

Fig. 6. A. Effects of DETA-NONOate 20 μM, and TEMPOL 100 μM on DHE fluorescence. B. Effects of superoxide scavengers (TEMPOL 100 μM, PEG-SOD 100 U/ml, SOD 500 U/ml and Hydralazine 100 μM) and caspase inhibitor (ZVAD 50 μM) on nodule count in TGF-β1 treated AV interstitial cells. Data are expressed relative to TGF-β1 controls. TGF-β1 alone increased DHE fluorescence (control 1.15±0.13, TGF-β1 3.83±1.49, *$P<0.05$, Mann–Whitney *U* test, $n=4$). *$P<0.05$, **$P<0.01$ (one-way ANOVA, with Dunnett's post-hoc test, $n=3$–6 cultures). C. Effect of peroxynitrite scavengers, ebselen (ebs)15 μM and uric acid (u.a.) 200 μM, alone and in the presence of DETA-NONOate, on TGF-β1 treated AV interstital cells. *$P<0.05$, DETA-NONOate 20 μM versus control (one-way ANOVA, with Tukey's post-hoc test, $n=4$–5 cultures). Uric acid and ebselen had no significant effect on TGF-β1-stimulated nodule formation, or on the inhibitory effect of DETA-NONOate.

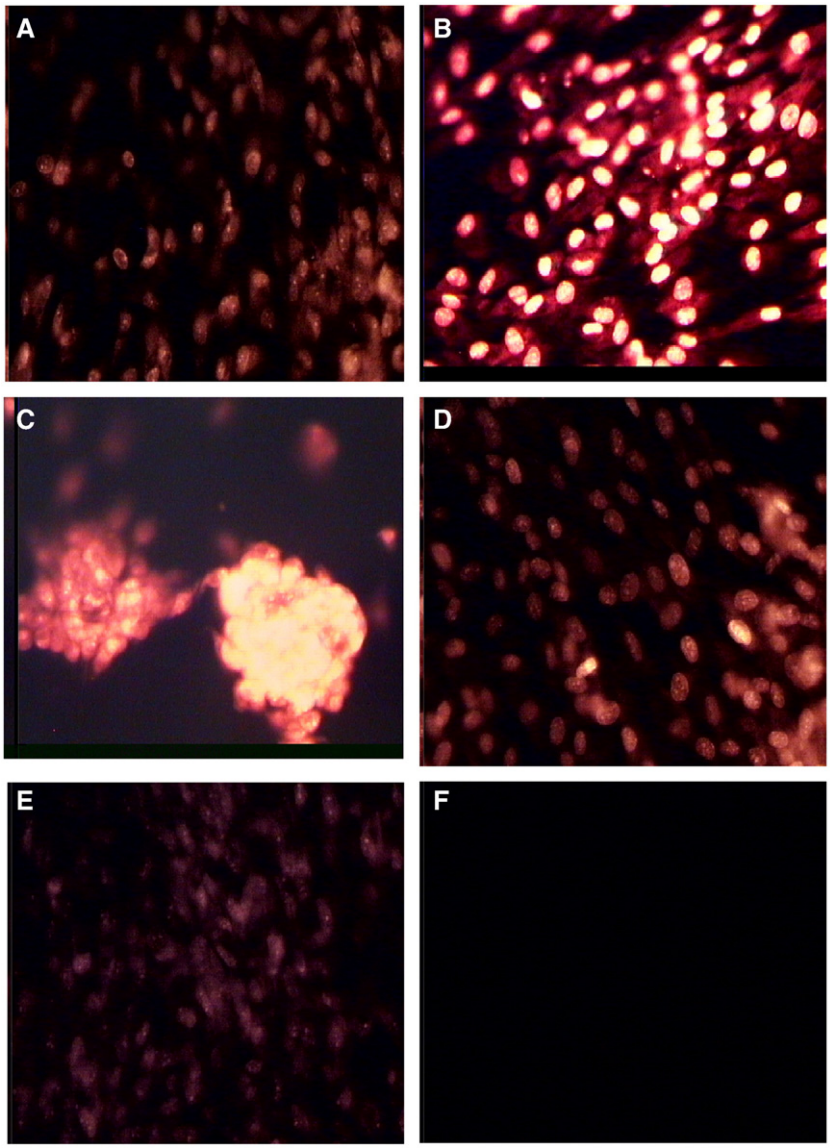

Fig. 7. DHE fluorescence in AV interstitial cells after A) control and B) TGF-β1 treatment. Fluorescence also localised to nodules C), and was inhibited by treatment with D)PEG-SOD 100 U/ml, and E) DETA-NONOate 20 μM. No fluorescence was evident in TGF-β1 treated cells in the absence of DHE (F).

oxidase activity (CPM × 10^3/μg protein; TGF-β1 41.8 ± 13.4, TGF-β1 + DETA-NONOate 57.3 ± 13.3, n = 4 cultures).

The observations on superoxide scavengers raised the possibility that the inhibitory effects of DETA-NONOate, in addition to effects on cGMP, might be modulated by peroxynitrite, generated via interaction of NO with superoxide. However, neither TGF-β1-induced nodule formation nor the inhibitory effect of DETA-NONOate was affected by peroxynitrite scavengers uric acid 200 μM or ebselen 15 μM (Fig. 6C).

4. Discussion

The cytokine TGF-β1 is found in calcified aortic valve and stimulates formation of calcific nodules in cultured human and sheep valve interstitial cells (Jian et al., 2003). We have now shown formation of nodules in monocultures of pig aortic valve interstitial cells in low serum Dulbecco's modified Eagles medium particularly when exposed to exogenous TGF-β1. These nodules stain positively for calcium with alizarin red S, and for alkaline phosphatase, consistent with the data for sheep aortic valve interstitial cells (Clark-Greuel et al., 2007). The major finding of the present study was that nitric oxide donors such as DETA-NONOate and SNP inhibited the TGF-β1-stimulated formation of nodules by aortic valve interstitial cells by more than 70%. In the case of DETA-NONOate this effect was shown to be concentration responsive with an IC_{50} of approximately 5 μM. This effect of DETA-NONOate was reflected in reduced alizarin red S staining and reduced alkaline phosphatase staining predominantly associated with nodules. Inhibition was evaluated predominantly in those cultures exposed to exogenous TGF-β1. However in those cultures in which nodules formed without the addition of exogenous TGF-β1, DETA-NONOate inhibited their formation also. However, once nodule formation had been initiated by TGF-β1 treatment, DETA-NONOate was much less effective in reducing the number of nodules. The inhibitory effect of NO donors on nodule formation was mimicked by increasing intracellular cGMP levels with the analogue, 8-bromo cGMP, suggesting that this action of NO is probably mediated via soluble guanylyl cyclase (sGC) activation. Unfortunately this could not be confirmed with the sGC inhibitor ODQ because this compound was toxic to cells when present in effective concentrations over the time course of these experiments. However elevation of cGMP via particulate GC receptor activators, brain natiuretic peptide and C-type natiuretic peptide, confirmed a protective role for cGMP signalling.

NO is an important functional antagonist of superoxide, and redox stress contributes significantly to development and progression of atherosclerosis. We have found that TEMPOL, a cell-permeable nitroxide SOD mimetic, suppressed TGF-β1-stimulated intracellular superoxide generation to the same extent as DETA-NONOate, and also decreased TGF-β1-induced nodule formation to a comparable degree (over 70% inhibition). A similar inhibitory effect was also seen with another cell-permeant superoxide scavenger, PEG-SOD, but not with the cell-impermeable form, SOD. In addition, hydralazine at a concentration previously shown to scavenge superoxide (Daiber et al., 2005) inhibited nodule formation. These data raise the possibility that the effects of NO donors may partially reflect superoxide scavenging. However, this would not explain the efficacy of 8-bromo-cGMP.

Furthermore, increases in intracellular formation of superoxide in aortic valve interstitial cells appear to contribute to the induction of nodule formation by TGF-β1. TGF-β1 possesses multiple intracellular signalling pathways including activation of the SMAD pathway (Derynck and Zhang, 2003), the Rho kinase pathway (Clements et al., 2005; Edlund et al., 2002) and the MAP kinase pathways (Kamaraju and Roberts, 2005). In addition there is some evidence in both smooth muscle cells (Mata-Greenwood et al., 2005) and cardiac fibroblasts (Cucoranu et al., 2005) that increases in intracellular superoxide induced via NADPH oxidase (Nox) activation, mediate some of the cellular effects of TGF-β1. Of particular relevance is the recent finding that Nox 4 regulates TGF-β1 mediated differentiation of cardiac myofibroblasts by regulating SMAD 2/3 activation (Cucoranu et al., 2005). The degree of cross talk between these different signalling pathways in different cell types is currently the subject of intense investigation (Javelaud and Mauviel, 2005), and multiple pathways are likely to be involved in calcific nodule formation under the influence of TGF-β1, of which superoxide formation may be but one. Unpublished data from our laboratory suggests that raising intracellular superoxide, for example by addition of high glucose, is insufficient in itself to induce nodule formation. Nevertheless the current data suggest that intracellular superoxide generation contributes to TGF-β induced nodule formation, an early manifestation of calcification in AV interstitial cells, and that both antioxidant as well as cGMP-stimulatory actions are likely to contribute to the observed inhibitory response to DETA-NONOate. Although DETA-NONOate has recently been shown to inhibit NADPH oxidase activity in endothelial cells via S-nitrosylation (Selemidis et al., 2007) we could detect no effect on superoxide generation via NADPH oxidase in valvular interstitial cells. This may reflect a difference in cell type or the lower concentration of DETA-NONOate employed in the present study. However the concentration which we employed, namely 20 μM, was near maximal indicating that reduction in NADPH oxidase activity is not the mechanism of its inhibitory effect on nodule formation.

Although nodule formation by aortic valve interstitial cells is accompanied by apoptosis (Jian et al., 2003), apoptosis does not appear to be necessary for nodule formation, since the caspase inhibitor, ZVAD, was unable to reduce nodule formation in both the previous study of Jian et al. (2003) and in the present study. Accordingly, although not directly tested, inhibitory effects of DETA-NONOate on nodule formation are unlikely to reflect an effect on apoptosis.

cGMP appears to be a key mediator of inhibition of TGF-β1-induced nodule formation, although the downstream mechanisms require elucidation. There is recent evidence in aortic endothelial cells that NO, acting via a cGMP-dependent mechanism, can inhibit TGF-β/SMAD regulated gene transactivation (Saura et al., 2005), and the possibility that a similar mechanism exists in valvular interstitial cells requires further study.

The present study also demonstrated that pig aortic valve interstitial cells can generate NO via a calcium-sensitive constitutive NOS, since A23187 acutely increased intracellular cGMP 2–3 fold and this increase could be prevented by the NOS inhibitor L-NAME. Accordingly it is possible that, in addition to paracrine control via NO from the valve endothelium, an autocrine control of calcification could be exerted via NO formation in valvular interstitial cells themselves. Although in the current experiments NOS blockade with L-NAME did not modulate nodule formation induced by TGF-β1, suggesting minimal autocrine counter-regulation by basally produced NO, the concentration of TGF-β1 employed in these experiments was near maximal, and the possibility remains that L-NAME treatment may have been effective against lower concentrations of TGF-β1. An alternative explanation is that basal formation of NO in the interstitial cells is too low to affect nodule formation in an autocrine manner. These data raise the possibility that agents which promote NO formation by the endothelium, a potential incremental source of NO *in vivo*, and to a lesser degree by the interstitial cells themselves, may be protective against early events leading to valvular calcification. In this regard, simvastatin and pravastatin have been shown to inhibit calcification in porcine aortic valve myofibroblasts (Wu et al., 2005), while atorvastatin was shown recently to inhibit the expression of osteogenic markers, alkaline phosphatase and osteocalcin, in human valve interstitial cells (Osman et al., 2006). Moreover, the data of Rajamannan et al. (2005) suggest that elevation of NO formation is relevant in the *in vivo* setting in that increased NO production by atorvastatin treatment was associated with a reduction of calcification in the hypercholesterolaemic rabbit aortic valve. In these cases, the protective effects of NO were applicable to the initiation stage of calcification. As shown in the present study, enhancement of NO at a later stage of nodule formation may be less effective in inhibiting calcification. In this regard, clinical investigations of progression of aortic stenosis to date have considered patients with relatively advanced disease. For example, high dose atorvastatin which stimulates eNOS expression (Rajamannan et al., 2005), proved ineffective in the SALTIRE study (Cowell et al., 2005). However, the recent development of reproducible technology for quantitation of aortic sclerosis (Ngo et al., 2004) in patients will facilitate investigations of earlier stages of disease progression.

Limitations of the study: The effect of NO donors was investigated in cultures in which calcifying nodules were produced only with TGF-β1. The relevance of this inhibitory effect to nodule formation induced by other agents was not evaluated. The experiments undertaken also do not permit delineation of a possible cGMP-independent component of the inhibitory effects of NO donors. Although we did not assess nitrotyrosine in these cells, concentrations of peroxynitrite scavengers, uric acid and ebselen, utilised were in excess of those used conventionally in the presence of much higher levels of NO donor (Walford et al., 2004; Heigold et al., 2002), and should have provided optimal peroxynitrite scavenging. With this reservation, the lack of definite effects of uric acid and ebselen, together with the extensive effects of 8-bromo-cGMP, suggest that the effects of NO are largely cGMP mediated.

In conclusion, NO donors inhibit calcifying nodule formation induced by TGF-β1 in pig aortic valve interstitial cells. This effect is potentially mediated both via increases in intracellular cGMP and by reduction in intracellular superoxide concentrations. This finding suggests that agents which donate NO, or augment the endogenous NO/cGMP pathway in aortic valve, and/or reduce intracellular superoxide concentrations, may afford protection against early processes leading to aortic valvular calcification.

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