RESEARCH PAPER

Ramipril retards development of aortic valve stenosis in a rabbit model: mechanistic considerations

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BACKGROUND AND PURPOSE

Aortic valve stenosis (AVS) is associated with significant cardiovascular morbidity and mortality. To date, no therapeutic modality has been shown to be effective in retarding AVS progression. We evaluated the effect of angiotensin-converting enzyme inhibition with ramipril on disease progression in a recently developed rabbit model of AVS.

EXPERIMENTAL APPROACH

The effects of 8 weeks of treatment with either vitamin D_2 at 25 000 IU for 4 days a week alone or in combination with ramipril (0.5 mg·kg^{-1}) on aortic valve structure and function were examined in New Zealand white rabbits. Echocardiographic aortic valve backscatter (AV_{BS}) and aortic valve : outflow tract flow velocity ratio were utilized to quantify changes in valve structure and function.

KEY RESULTS

Treatment with ramipril significantly reduced AV_{BS} and improved aortic valve : outflow tract flow velocity ratio. The intravalvular content of the pro-oxidant thioredoxin-interacting protein was decreased significantly with ramipril treatment. Endothelial function, as measured by asymmetric dimethylarginine concentrations and vascular responses to ACh, was improved significantly with ramipril treatment.

CONCLUSIONS AND IMPLICATIONS

Ramipril retards the development of AVS, reduces valvular thioredoxin-interacting protein accumulation and limits endothelial dysfunction in this animal model. These findings provide important insights into the mechanisms of AVS development and an impetus for future human studies of AVS retardation using an angiotensin-converting enzyme inhibitor.

Abbreviations

ACE, angiotensin converting enzyme; ACEi, angiotensin converting enzyme inhibitor; ADMA, asymmetric dimethylarginine; Ang II, angiotensin II; ARB, angiotensin-receptor blocker; AV_{BS}, aortic valve backscatter; AVS, aortic valve stenosis; AVv, aortic valve velocity; IVSd, interventricular septal dimension; LVOTv, left ventricular outflow tract flow velocity; NAD(P)H, nicotinamide adenine dinucleotide phosphate; PCR, polymerase chain reaction; ROS, reactive oxygen species; SeCr, serum creatinine; SNP, sodium nitroprusside; TGF-β1, transforming growth factor-β1; TXNIP, thioredoxin-interacting protein

Introduction

Aortic valve stenosis (AVS) is a major cause of cardiac morbidity and mortality (Aronow *et al.*, 1999; Otto *et al.*, 1999), and is associated with significant health-care costs in the Western community. Understanding the pathogenesis of AVS in humans is inherently restricted by the inability to examine aortic valves serially. However, models of the development of AVS have been developed, utilizing cell culture of valve fibroblasts (Jian *et al.*, 2003; Kennedy *et al.*, 2009) and a variety of animal models (Drolet *et al.*, 2003; Rajamannan *et al.*, 2005; Cuniberti *et al.*, 2006; Weiss *et al.*, 2006; Ngo *et al.*, 2008). In view of the previously prevailing hypothesis that AVS might result primarily from an 'atherogenesis-like' process (Rajamannan, 2009), many of these models have utilized (partially or entirely) hypercholesterolaemia as a basis for the induction of aortic valve calcification (Drolet *et al.*, 2003; Rajamannan *et al.*, 2005; Weiss *et al.*, 2006; Hekimian *et al.*, 2009). However, hypercholesterolaemia in such models has never been proven to be sufficient to induce AVS (Drolet *et al.*, 2003; Rajamannan *et al.*, 2005; Weiss *et al.*, 2006). Indeed, recent clinical studies have failed to show either any influence of cholesterol-lowering therapy on AVS progression (Cowell *et al.*, 2005; Rossebo *et al.*, 2008; Chan *et al.*, 2010), or any association between hypercholesterolaemia and aortic valve disease in an aging population (Ngo *et al.*, 2009).

In a rabbit model of AVS, we have recently shown that vitamin D_2 alone for 8 weeks induced the development of AVS with calcification and inflammatory infiltration of the aortic valve, which was coupled to changes in valve echogenicity as well as transvalvular haemodynamic changes (Ngo *et al.*, 2008), hallmark features of human AVS. Furthermore, this model exhibits marked elevation of intravalvular thioredoxin-interacting protein (TXNIP) (an endogenous inhibitor of the antioxidant thioredoxin, which plays a central role in regulating redox stress) (World *et al.*, 2006).

There is evidence to suggest that the activation of angiotensin II (Ang II)-producing systems may be involved in the pathogenesis of AVS. Angiotensin-converting enzyme (ACE) (O'Brien *et al.*, 2002) has been detected on stenotic valve lesions but not on normal valves. Furthermore, there is also evidence of local production of Ang II by chymase tryptase, and cathepsin G from activated mast cells identified on stenotic valve lesions (Helske *et al.*, 2004; 2006). Furthermore, interventions targeting the renin-angiotensin-aldosterone system may exert salutary effects on endothelial function, which is likely to be relevant to the pathophysiology of AVS, given that AVS is associated with valvular endothelial dysfunction (Chirkov *et al.*, 2006) and systemic endothelial dysfunction (as determined from biochemical evidence; Ngo *et al.*, 2007). Gkizas *et al.* (2010) have recently demonstrated that eplerenone limits the degenerative changes within the valve matrix of a rabbit model of aortic valve disease. Similarly, Osman *et al.* (2007) have demonstrated that β_2-adrenoceptor stimulation, which induces endothelial release of nitric oxide (NO) (Figueroa *et al.*, 2009), decreased calcification of human aortic valve interstitial cells.

However, to date, no randomized prospective study either in animal models or in a clinical setting has shown retardation of development of haemodynamically defined AVS by either ACE inhibitors, angiotensin-receptor blockers or aldosterone antagonists. The principal objective of the current study was therefore to determine whether the ACE inhibitor, ramipril, could modify the development of AVS in an animal model of AVS (Ngo *et al.*, 2008). We also planned to examine the effects of ramipril on vascular endothelial function, and on intravalvular accumulation of TXNIP.

Methods

Experimental protocol

Male New Zealand White rabbits (2–2.5 kg) were treated with vitamin D_2 alone ($n = 14$) or together with ramipril ($n = 16$) for 8 weeks. Vitamin D_2 25 000 IU was administered on 4 days per week ($n = 14$), as previously described (Ngo *et al.*, 2008). Ramipril was administered in drinking water at approximately 0.5 mg·kg^{-1} daily. Additionally, untreated rabbits were used as normal controls, for comparison ($n = 6$).

At baseline and after 8 weeks of treatment, echocardiography was performed under sedation with ketamine (3.5 mg·kg^{-1}) and xylazine (5 mg·kg^{-1}). Blood samples were taken at baseline, 4 and 8 weeks for the measurement of serum creatinine, calcium, phosphate and total cholesterol. Plasma asymmetric dimethylarginine (ADMA), a biochemical marker of endothelial dysfunction, was assayed at baseline and after 8 weeks' treatment.

After 8 weeks, the animals were killed under anaesthesia with ketamine/xylazine; hearts and thoracic aortae were rapidly removed and placed in ice-cold Krebs solution and aortic valve leaflets were excised within 30 min.

The experimental protocol was approved by the institutional animal ethics committee and conforms to the National Health and Medical Research Council of Australia guidelines for animal usage for experimentation.

Preparation of solutions

Vitamin D_2 (Ergocalciferol 40 000 IU·g^{-1}, Sigma Pharmaceuticals, South Croydon, Victoria, Australia) powder stored under nitrogen was weighed and dissolved in ethanol (100 µL), prior to dilution with tap water to a final volume of 55 mL for each rabbit. Vitamin D_2 solution was prepared fresh daily. For the untreated control group, an equal amount of ethanol was added to drinking water.

Ramipril (crushed Tritace® 5 mg, Aventis Pharmaceuticals, Bridgewater, NJ, USA) was suspended in ethanol (100 µL). Ramipril (±vitamin D_2) solution was diluted to a final volume of 55 mL. All rabbits received water and normal chow *ad libitum*.

Echocardiographic measurements

Ultrasound images were obtained with a 5 MHz sector phased-array probe, operated at 6.7 MHz, connected to a Vivid 5, GE Vingmed (Horten, Norway). Aortic valve backscatter (AV_{BS}) was utilized as the primary measure of increased valve echogenicity as previously described (Ngo *et al.*, 2004). Peak left ventricular outflow tract flow (LVOTv) and transaortic valve flow (AVv) were measured utilizing continuous-wave Doppler recordings from the highest velocity available; these were used as measures of AVS. The ratio of AVv/LVOTv was used to compare changes in haemodynamics between the two groups.

Reproducibility between different observers of AVv/LVOTv was 3.3% ($n = 6$). Left ventricular wall thicknesses as measured by interventricular septal dimension (indexed for body weight) were measured from 2D-guided M-mode echocardiographic tracings. Echocardiographic imaging and analyses were performed by observers blinded to the treatments.

Evaluation of aortic valve echogenicity

Valve echogenicity was quantified by the determination of AV_{BS} in rabbits, performed as previously described (Ngo et al., 2004; 2008). Two-dimensional ultrasonic backscatter images of the aortic valves were obtained from standard parasternal-like long-axis view over three cardiac cycles with a zoom of 2 cm. Three consecutive scans were performed per animal. A total of 10 square-shaped regions of interest (five on the anterior leaflet and five on the posterior leaflets) were obtained by placing 7×7 pixel sample volumes on the valves. Backscatter values from the blood pool adjacent to the left ventricular outflow tract and the aortic blood pool were used as reference values, as previously described (Ngo et al., 2008).

Aortic valve characterization

TXNIP protein was assessed immunohistochemically using goat anti-TXNIP (D-15) antibody (SC 33098, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as previously described (Ngo et al., 2008). Sections were incubated with the primary antibody diluted 1:200 with phosphate-buffered saline (PBS) overnight at 4°C; rabbit anti-goat biotinylated (DAKO, Carpinteria, CA, USA), diluted 1:200 with PBS was used as secondary antibody and then with an avidin-biotin peroxidase complex (Vector, Burlingame, CA, USA), diluted 1:200 with PBS. Localization of the peroxidase conjugates was achieved by using diaminobenizidine tetrahydrochloride as a chromagen for 1 min. Sections incubated with 1:10 normal goat serum, instead of the primary antiserum, served as negative controls. Immunostaining for TXNIP was quantified using computer-assisted image analysis, as previously described (Ngo et al., 2008).

Alazarin red S was utilized to quantify aortic valve calcification and RAM-11 to quantify macrophage infiltration as previously described (Ngo et al., 2008).

Real-time RT-PCR for TXNIP quantification

Rabbit aortic valves were snap-frozen and RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time PCR was used to assess transcript levels with negative controls included in each run. Specific primers TXNIP and 28S for the use of SYBR Green are TXNIP forward AGGATTCTGTGAAGGTGATG; TXNIP reverse GCCTCTGACTGATGACAACT; 28S forward TGGTAAGGA AACCAGCTCTA; 28S reverse ATGCAGTATCAGCCAGTCTT. Primers specificity in real-time PCR reactions was confirmed using RT-PCR. Briefly, total RNA (1 µg) was treated with DNase I (Invitrogen) and then RNA was used in 25 uL of real-time PCR reaction including Brilliant SYBR Green One-step QRT-PCR Master Mix according to manufacturer's instructions (Stratagene, La Jolla, CA, USA). Real-time quantitations were performed on 7000 Sequence Detection System thermal Cycler (Applied Biosystems, Mulgrave, Victoria, Australia). The calculation of relative change in mRNA was performed using the delta-delta method as previously described (Qi et al., 2006; 2007), with normalization for the housekeeping gene 28S.

Endothelial function

Plasma ADMA concentrations. Plasma concentrations of ADMA, an endogenous inhibitor of NO synthase, were measured by high-performance liquid chromatography with fluorescence detection using AccQ-Fluor as the derivatizing reagent as previously described (Heresztyn et al., 2004; Ngo et al., 2008).

Isolated aortic preparations. The thoracic aortae were cut into 2–3 mm rings and mounted under 2 g tension in organ baths as previously described (Ngo et al., 2008). Contractile responses were expressed as a percentage of the potassium physiological saline solution response. In aortic rings precontracted with phenylephrine to 70% of maximal, relaxation concentration-response curves were obtained for ACh and sodium nitroprusside, as measures of endothelium-dependent and direct NO-mediated relaxation respectively. Concentration-response curves were analysed by GraphPad Prism version 4 (GraphPad Software, Inc., La Jolla, CA, USA).

Study analysis/statistics

All statistical data involved comparisons between the vitamin D_2 and vitamin D_2/ramipril groups. The primary (null) hypothesis tested in this study was that ramipril would not affect AVS induced by vitamin D_2 alone, as measured by AVv/LVOTv ratio and AV_{BS} after 8 weeks; these analyses were performed using two-way analysis of variance (ANOVA) and Student's unpaired t-test respectively. For a study with 15 animals in each group, on the basis of our previously published results (Ngo et al., 2008), there was approximately 80% power to detect 1.50 SD difference between group means (approximately 50% reduction of AV_{BS}), corresponding to 6 dB of AV_{BS}. Two-way ANOVA was used to compare other echocardiographic parameters and biochemical parameters as well as comparing plasma ADMA concentrations over the 8 weeks of treatment. All other differences between groups were expressed as changes between baseline and after 8 weeks of treatment, and examined using unpaired t-test. Comparisons between non-parametric data were performed using Mann–Whitney U-test. The untreated rabbit (control) group was subjected to no formal statistical analysis as examinations of this group were subsidiary objectives of the study. Correlations between parameters of endothelial function and AV_{BS} were performed utilizing linear regression. Real-time RT-PCR results are expressed as a fold change compared with the control value. Results are expressed as mean ± SEM, unless otherwise stated. A level of probability of $P < 0.05$ was accepted as statistically significant. All statistical analyses were performed using Graphpad Prism version 4 (GraphPad Software, Inc.).

Results

Effects of ramipril on echocardiographic parameters

In untreated rabbits (control group), changes in AVv/LVOTv and AV_{BS} over 8 weeks were similar to those previously
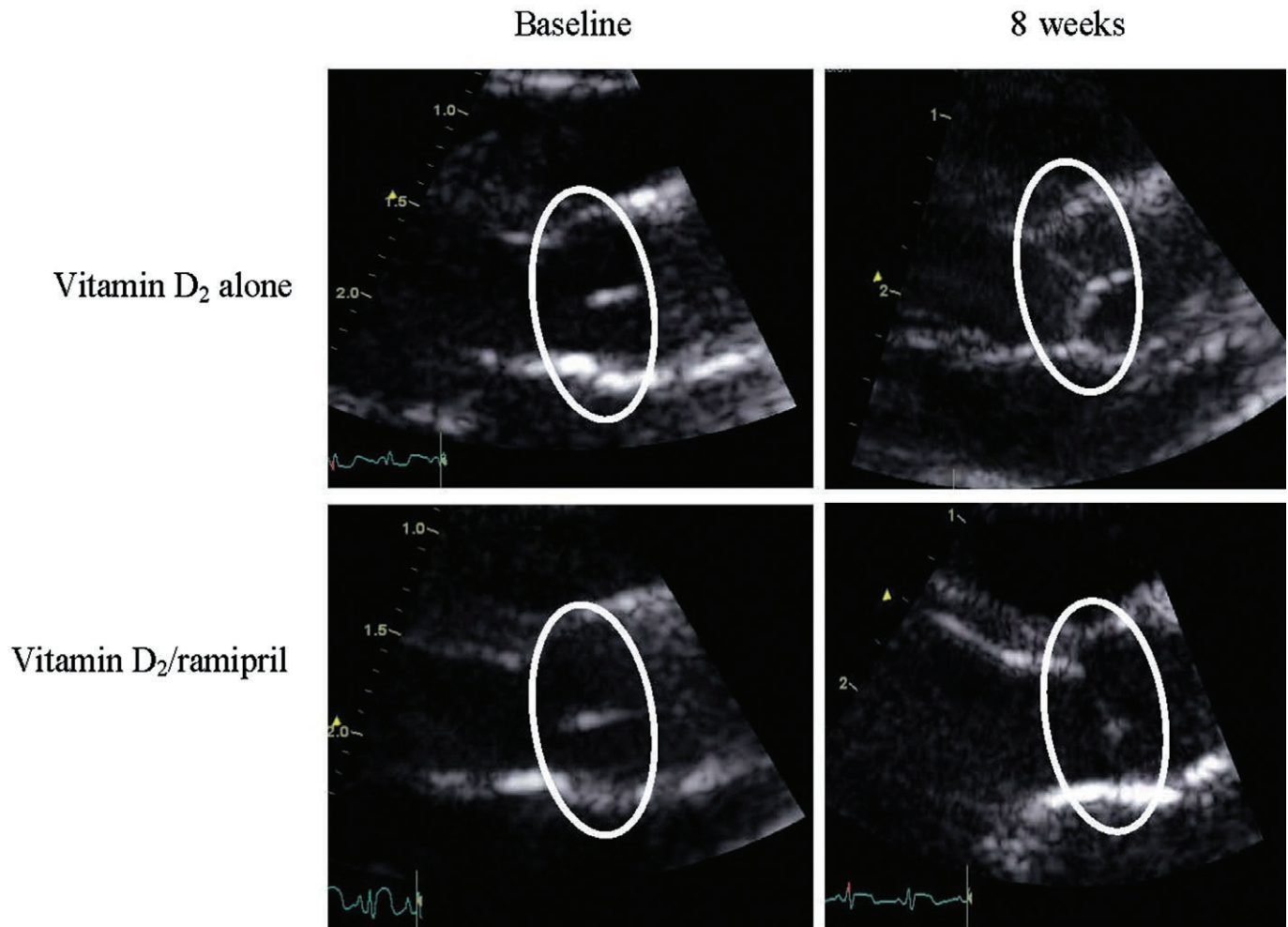
Figure 1

Echocardiographic views of rabbit aortic valves at baseline and after 8 weeks of treatment. Zoom pictures (2 cm) of parasternal-like long axis view of AV leaflets. Typical examples for vitamin-D_2 group and vitamin D_2/ramipril groups are shown at baseline and after 8 weeks. Regions of interest show position of valve. Note increased echogenicity in the vitamin D_2 group (in comparison with vitamin D_2/ramipril valves) at 8 weeks.

documented by us (Ngo *et al.*, 2008): AVv/LVOTv increased marginally, while mean AV_{BS} at 8 weeks was 4.7 ± 0.7 dB. Rabbits treated with vitamin D_2 developed similar degrees of AVS to those previously described over an 8 week period (Ngo *et al.*, 2008), as shown in Figure 1. Treatment with ramipril significantly retarded AVS development ($F = 5.6$, $P = 0.01$), as assessed by comparison of changes in AVv/LVOTv (Figure 2A). After 8 weeks, there was also significant diminution of AV_{BS} values ($P < 0.05$) in the vitamin D_2/ramipril-treated rabbits versus the vitamin D_2 alone-treated group (Figure 2B). Ramipril also limited the development of left ventricular hypertrophy compared with vitamin D_2 alone group ($F = 10.8$, $P < 0.01$) over 8 weeks (Figure 2C).

Valve characterization

Alazarin red S scores were significantly (Spearman's rank test: $P < 0.001$) correlated with AV_{BS}, while there was a borderline correlation (Spearman's rank test: $P = 0.07$) between AV_{BS} and RAM-11 scores (Figure 3A).

TXNIP immunostaining in vitamin D_2-treated rabbits was similar to that previously observed in this model (Ngo *et al.*, 2008). Concomitant treatment with ramipril markedly attenuated TXNIP accumulation, both as assessed via quantitative immunostaining and via real-time RT-PCR (Figures 3B–D).

Biochemistry

Treatment with vitamin D_2 in combination with ramipril was associated with a non-significant trend towards a fall in serum creatinine (0.15 ± 0.014 mmol·L^{-1} versus 0.11 ± 0.02 mmol·L^{-1}; $F = 1.9$, $P = 0.19$) and a significant decrease in calcium/phosphate product concentrations (9.9 ± 0.6 vs. 7.8 ± 0.6; $F = 10.6$, $P = 0.004$).

Vascular endothelial function

Plasma ADMA concentrations. As previously observed (Boger *et al.*, 1997; Ngo *et al.*, 2008), ADMA concentrations decreased with age in both groups of animals. Treatment with
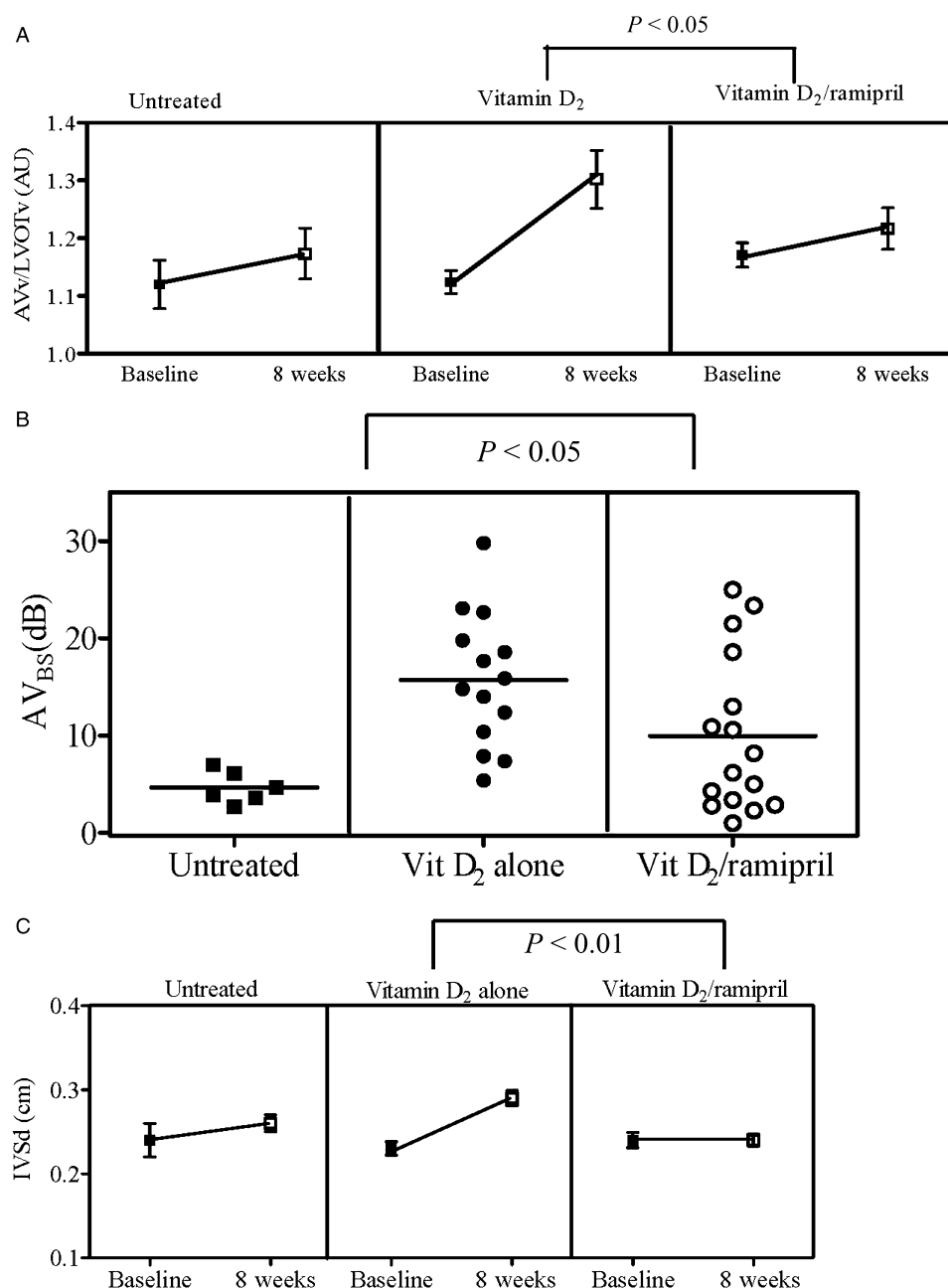
Figure 2

(A) Comparison of transvalvular flow between groups. In untreated rabbits, AVv/LVOTv was 1.12 ± 0.04 (baseline) versus 1.17 ± 0.04 (8 weeks); and AV_{BS} 4.7 ± 0.7 dB after 8 weeks. There was a statistically significant difference in the time-treatment interaction between the vitamin D_2 ($n = 14$) and vitamin D_2/ramipril ($n = 16$) groups [$F = 5.6$; $P = 0.02$; two-way analysis of variance (ANOVA)]. (B) AV_{BS} comparison between groups. Mean AV_{BS} in the vitamin D_2 alone group was higher compared with the vitamin D_2/ramipril group (15.7 ± 1.8 dB vs. 9.9 ± 2.0 dB, respectively, $P < 0.05$; unpaired t-test). Mean AV_{BS} for the untreated group was 4.7 ± 0.7 dB. (C) Interventricular septal dimension (IVSd) comparison between groups. Significant retardation of left ventricular hypertrophy as evident by a decrease in IVSd in the vitamin D_2/ramipril treated group versus vitamin D_2 treated alone over the 8 weeks of treatment was observed ($F = 10.8$, $P < 0.01$; two-way ANOVA). AV_{BS}, aortic valve backscatter score; AVv, aortic valve velocity; LVOTv, left ventricular outflow tract flow velocity.

ramipril reduced ADMA concentrations compared with vitamin D_2 treatment alone ($F = 7.9$, $P < 0.01$; Figure 4A); after 8 weeks, the ADMA concentrations were similar to those observed previously in untreated rabbits (Ngo et al., 2008).

NO-mediated vasodilatation. As shown in Figure 5A, treatment with vitamin D_2/ramipril was associated with significant increases in maximal vasodilator responses to ACh in isolated aortic ring preparations ($P < 0.05$). Responses to SNP
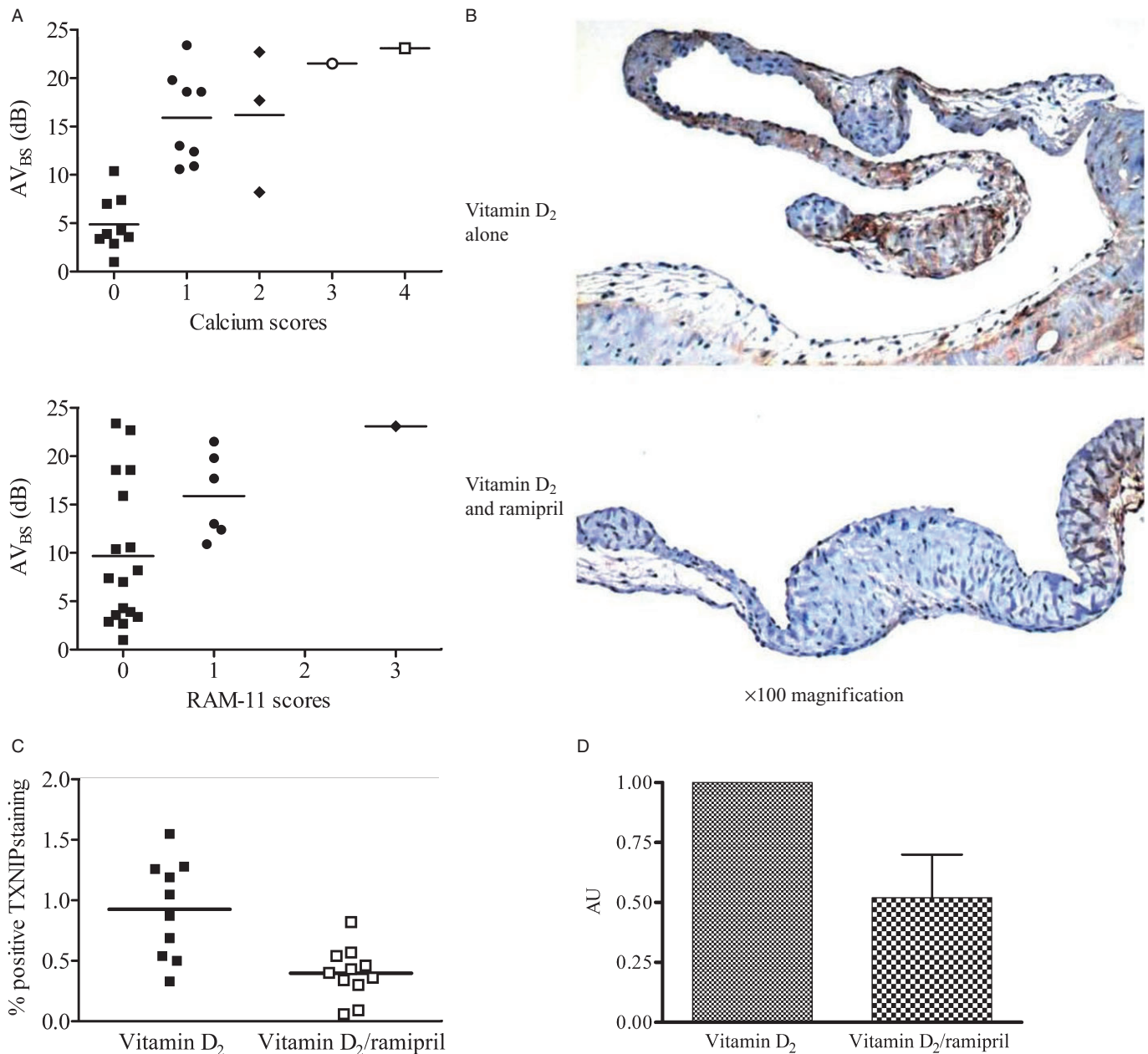
Figure 3

(A) Histological staining with Alizarin red S, and immunohistochemistry with RAM-11 for macrophages. Valves with lower AV_{BS} scores had a significantly reduced calcium content ($P < 0.001$) and tended to have a reduced inflammatory milieu ($P = 0.07$). (B) Ramipril co-treatment reduced the TXNIP immuno-positive staining in valve tissue. (C) Ramipril co-treatment ($n = 11$) significantly reduced valvular TXNIP compared with vitamin D_2 treatment alone ($n = 10$) (0.4 ± 0.06 vs. 0.9 ± 0.1, respectively, $P = 0.001$; unpaired t-test). (D) Real-time RT-PCR results showed that the vitamin D_2/ramipril ($n = 4$) group had a significantly reduced TXNIP mRNA compared with the vitamin D_2 alone group ($n = 3$) ($P = 0.02$). AV_{BS}, aortic valve backscatter score; TXNIP, thioredoxin-interacting protein.

were also significantly improved with ramipril treatment ($P < 0.05$).

Correlations between endothelial function parameters and AV_{BS}

Given that NO exerts an inhibitory effect both on TXNIP expression (Schulze et al., 2006) and on valve matrix calcifi-

cation (Kennedy et al., 2009), we sought correlations between the extent of endothelial dysfunction in the presence and absence of ramipril treatment and AV_{BS} scores. There were significant negative correlations between AV_{BS} and both change in ADMA concentrations (δ ADMA) for both treatment groups (vitamin D_2 alone, $r^2 = 0.6$ and vitamin D_2/ramipril, $r^2 = 0.8$, respectively, both $P < 0.001$) (Figure 4B); and maximum ACh-mediated vasorelaxation (vitamin D_2
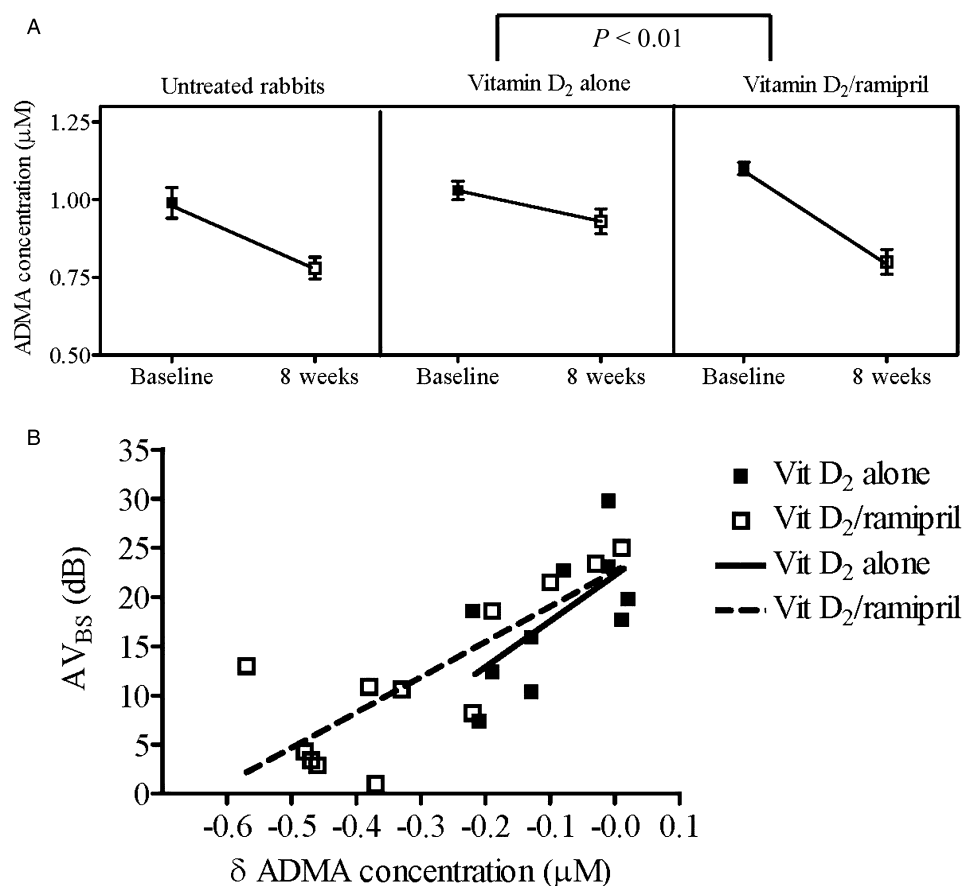
Figure 4

(A) Comparison of ADMA concentrations between the vitamin D_2 ($n = 10$) and vitamin D_2/ramipril ($n = 12$) groups over the 8 weeks of treatment. As previously demonstrated (Ngo et al., 2008), in untreated rabbits ADMA concentrations decrease substantially with aging (0.99 ± 0.05 to 0.78 ± 0.04 μM). Two-way analysis of variance showed there was statistical difference in ADMA concentrations between the two groups over time ($F = 9.5$; $P = 0.006$). (B) For both vitamin D_2 alone and vitamin D_2/ramipril, there were negative correlations between AV_{BS} and changes in ADMA concentration ($r^2 = 0.4$ and $r^2 = 0.6$, respectively, both $P < 0.05$). ADMA, asymmetric dimethylarginine; AV_{BS}, aortic valve backscatter score.

alone, $r^2 = 0.8$ and vitamin D_2/ramipril, $r^2 = 0.9$, respectively, both $P < 0.001$) (Figure 5B).

Discussion

The current study demonstrates that concomitant ramipril therapy retarded the development of vitamin D_2-induced AVS in the rabbit model, as measured by changes in transvalvular flow profile and AV_{BS}. This retardation of AVS development was associated with a reduction in TXNIP accumulation within the valve matrix, and the preservation of vascular endothelial function, as assessed both physiologically (ACh responses) and biochemically (ADMA concentrations). These findings therefore represent the first definitive demonstration that ACE inhibitors may retard the development of AVS.

A number of recent findings support the concept that TXNIP represents a fundamentally important modulator of intracellular oxidative stress, which is under the negative control of NO (Schulze et al., 2006; Kanagasingam et al., 2009; Shaked et al., 2009). Increased expression of TXNIP is

associated with the stimulation of profibrotic mechanisms (Kobayashi et al., 2003; Advani et al., 2009). Furthermore, it has recently become clear that TXNIP functions to link oxidative stress with inflammatory activation (Schroder et al., 2010; Zhou et al., 2010), which is a prominent feature of early AVS (Miller et al., 2008). Indeed, increased TXNIP tissue concentrations are both reactive oxygen species (ROS)-stimulated and promote further ROS release by inhibiting thioredoxin activity (Yamawaki et al., 2003; World et al., 2006; Berk, 2007; Schroder et al., 2010). In view of the role of ADMA as an inhibitor of NO synthesis (Leiper and Vallance, 1999), it might be expected that increased ADMA concentrations would also be associated with increased TXNIP expression; however, this has not been reported previously. Although this currently demonstrated endothelial dysfunction with elevations of ADMA/TXNIP associations and AVS development does not prove causation, it provides a potential physiological basis for the observed effects of ramipril. Furthermore, in cell culture models of aortic valve calcification, we have demonstrated that both NO (Kennedy et al., 2009) and TXNIP modulate calcification (Kanagasingam et al., 2009).
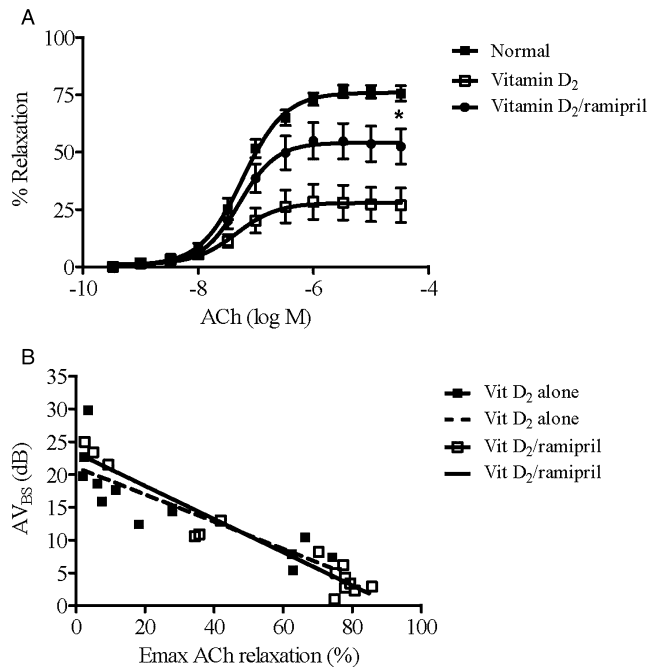
Figure 5

(A) Maximal ACh-induced relaxation responses of aortic rings from untreated rabbits ($n = 6$); vitamin D_2 group ($n = 13$); and vitamin D_2/ramipril group ($n = 15$). Maximal relaxation responses to ACh in untreated rabbits were 76.0 ± 1.5%. Relaxation was significantly greater in the vitamin D_2/ramipril group versus the vitamin D_2 alone group ($P = 0.02$). (B) Negative correlations between AV_{BS} and maximum ACh-induced vasorelaxation ($r^2 = 0.7$ and $r^2 = 0.9$, respectively, $P < 0.001$ for both). AV_{BS}, aortic valve backscatter score.

Evidence for a potential therapeutic role for ACE inhibitors in AVS comes from three categories of findings: increased generation of Ang II within diseased valve matrix, pathophysiological actions of Ang II which might contribute to the development of AVS and limited *in vivo* studies. Apart from the specific generation of Ang II in stenotic valve lesions (Helske *et al.*, 2004; 2006), Ang II exerts multiple effects, which may be conducive to the development of fibrosis and calcification. Specifically, Ang II exerts pro-inflammatory effects, mainly by increasing the expression and activation of NAD(P)H oxidase (Cai *et al.*, 2003). The associated production of ROS leads to an increased redox stress (Das *et al.*, 2004), 'scavenging' of NO (Cai and Harrison, 2000), activation of apoptosis (Dimmeler and Zeiher, 2000) and the associated monocyte/macrophage infiltration (Brasier *et al.*, 2002; Cathcart, 2004), which subsequently promotes accumulation of transforming growth factor-β1 (Saito *et al.*, 2005) and may contribute to fibrosis and calcification. These data are consistent with recent findings related to the association of redox stress with AVS. As regards *in vivo* evidence, Arishiro *et al.* (2007), using a hypercholesterolaemic rabbit model, showed that olmesartan significantly reduced atherosclerotic changes in aortic valves. Simolin *et al.* (2009) also recently reported that enalapril reduced AV thickening in mice with renal insufficiency. In retrospective, non-randomized human studies, inconsistent benefits of ACE inhibitors have been found: O'Brien *et al.* (2005) found that the use of ACE

inhibitors was associated with lower aortic valve calcium scores, while Rosenhek *et al.* (2004) demonstrated a non-significant trend for ACE inhibitors to be associated with the reduction of peak velocity and pressure gradient. Despite this considerable body of evidence, no other previous studies have been performed to test the hypothesis that ACE inhibitors retard AVS progression.

The current data also shed further light on the relationship between the development of AVS and potential impairment of NO effect, as measured in this study by ADMA concentrations and impaired NO-mediated vasodilatation. As AVS is characterized by the progressive loss of valve endothelium (Harasaki *et al.*, 1978; Riddle *et al.*, 1980; Otto *et al.*, 1994), valvular endothelial function cannot be assessed. The extent of change in ADMA concentrations with time and the impairment of ACh-induced relaxation were correlated to the extent of AV_{BS} in individual animals. The preservation of NO effect in early AVS might also be relevant to the limitation of the associated cardiovascular morbidity risk (Otto *et al.*, 1999). Furthermore, these data are consistent with our previous findings, obtained in a cell culture model (Kennedy *et al.*, 2009), in this model (Ngo *et al.*, 2008) and in studies of advanced human AVS (Chirkov and Horowitz, 2007; Ngo *et al.*, 2007). The effects of ramipril observed in the present study are also consistent with results from previous investigations with other ACE inhibitors with regard to ADMA kinetics (Ito *et al.*, 2001; Chen *et al.*, 2002) and endothelial function (Pitt *et al.*, 2001).

The observed changes in vascular endothelial function and valvular TXNIP content might arise either from decreased Ang II generation and/or decreased bradykinin degradation. For example, the inhibition of NAD(P)H oxidase expression would limit NO 'scavenging' while bradykinin would stimulate NOS activation (Skidgel *et al.*, 2006). In turn, NO has been shown to suppress TXNIP expression (Schulze *et al.*, 2006). Thus, the suppression of TXNIP effect, and therefore of intracellular redox stress, could potentially modulate valvular calcium deposition in the setting of early AVS. No previous studies have examined the effects of ACE inhibitors on TXNIP physiology. However, the current data do not demonstrate a primary role for TXNIP in the development of AVS.

The results of the current study should also be considered in light of the recent publication of three negative clinical investigations of statin therapy in AVS (Cowell *et al.*, 2005; Rossebo *et al.*, 2008; Chan *et al.*, 2010). As previously observed, hypercholesterolaemia, both in animal models (Drolet *et al.*, 2003; Rajamannan *et al.*, 2005; Weiss *et al.*, 2006) and in humans (Ngo *et al.*, 2009), is minimally associated with the development of AVS. The current experiments provide a basis for a 'cholesterol-independent' schema for the pathogenesis of the condition and for its limitation.

The results of the current study are subjected to several caveats. First, the development of AVS was induced by the exposure to supraphysiological doses of vitamin D_2 and therefore does not precisely recapitulate human AVS, despite the marked similarities, as previously described (Ngo *et al.*, 2008). Secondly, this model, despite the increase of transvalvular flow, is essentially one of 'mild' AVS; therefore, no conclusions can be drawn on the effects of ramipril in more advanced cases of the disease. On the other hand, the rabbit model of AVS reflects the major histological features of early

clinical AVS, including inflammatory infiltration, lipid deposition and calcification (as well as valve thickening and narrowing). The extent of retardation of AVS, and the salvage of endothelial function, varied markedly in ramipril-treated rabbits: we cannot exclude the possibility that intake of ramipril was variable. Finally, it is possible, although unlikely, on the basis of epidemiological data (Ngo *et al.*, 2009), that the hypotensive and afterload-reducing effects of ramipril might have contributed directly to the retardation of AVS development, and also to the retardation of the development of left ventricular hypertrophy. However, the effects of ramipril on intravalvular TXNIP accumulation, valve AV_{BS} and valve AVv/LVOTv all suggest direct, rather than indirect effects on the valve itself.

The current data provide a strong rationale for the inception of a randomized trial of ACE inhibition as a strategy for the limitation of AVS in humans. While the evidence showing the benefits of ACE inhibitors in the prevention of cardiovascular death and myocardial infarction is unquestionable, the results of the current study add AVS as a potential therapeutic area where ACE inhibition may be of pivotal importance.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

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