Increased expression of profibrotic neutral endopeptidase and bradykinin type 1 receptors in stenotic aortic valves

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Received 26 October 2006; revised 21 March 2007; accepted 29 March 2007; online publish-ahead-of-print 15 May 2007

See page 1795 for the editorial comment on this article (doi:10.1093/eurheartj/ehm259)

Aims In aortic stenosis (AS), adverse remodelling of the valves may depend on altered local regulation of pro- and antifibrotic systems. We have recently shown that angiotensin-converting enzyme (ACE), which generates profibrotic angiotensin II and inactivates antifibrotic bradykinin (BK), is upregulated in stenotic aortic valves. Here, we analyse the expression of neutral endopeptidase (NEP), another profibrotic and BK-degrading enzyme, and of BK receptors in aortic valves in AS.

Methods and results Stenotic aortic valves (n = 86) were obtained at valve replacement surgery and control valves (n = 13) at cardiac transplantation. Expression levels of NEP and BK type 1 and 2 receptors (BK-1R and BK-2R) in aortic valves and in isolated valvular myofibroblasts were analysed by real-time PCR and immunohistochemistry, and NEP activity was quantified by autoradiography. NEP, BK-1R, and BK-2R mRNA levels were higher in stenotic than in non-stenotic valves (P < 0.05 for each) and the respective proteins localized to valvular endothelial cells and myofibroblasts. In stenotic valves, the proteolytic activity of NEP was significantly increased (4.5-fold, P < 0.001), and tumour necrosis factor-α induced the expression of NEP in cultured myofibroblasts. Finally, treatment of cultured myofibroblasts with an NEP inhibitor (phosphoramidon) downregulated the expression of profibrotic transforming growth factor-β1, whereas addition of BK decreased the expression of collagens I and III which was reversed by a BK-2R antagonist.

Conclusion NEP activity is increased in stenotic aortic valves in parallel with increased expression of BK-receptors. The upregulation of NEP and BK-1R have the potential to promote valvular fibrosis and remodelling while the increase in BK-2R may represent a compensatory antifibrotic response. These findings add novel pathogenic insight and raise potential new therapeutic targets in AS.

KEYWORDS Aortic stenosis; Bradykinin; NEP; Valve

Introduction Aortic valve stenosis (AS) is characterized by extensive calcification and extracellular remodelling of the valves including fibrosis, collagen deposition, and elastin degradation.¹-⁶ Importantly, inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), and proteases, such as chymase, cathepsin G, and MMP-1, secreted by the infiltrating inflammatory cells in the diseased valves promote adverse structural changes leading to AS progression.⁴-⁷-⁹ We have previously shown an upregulation of angiotensin-converting enzyme (ACE) activity in stenotic aortic valves.⁸ Besides forming the profibrotic factor angiotensin II (Ang II), ACE degrades the antifibrotic peptide bradykinin (BK),¹⁰ thus promoting fibrosis by two different mechanisms. Whether the other BK-degrading enzyme, neutral endopeptidase (NEP),¹¹,¹² is involved in the pathogenesis of AS has not been studied hitherto. Of note, NEP activity has been reported to be elevated in failing human hearts showing myocardial fibrosis and hypertrophy.¹³ The cardioprotective effects of the kinins, BK, and kallidin (Lys-BK), are mediated through the BK type-2 receptor (BK-2R), which triggers the production of nitric oxide (NO)¹⁴ and exerts anti-proliferative¹⁵ and anti-hypertrophic¹⁶ effects on myocytes and fibroblasts. Inhibition or genetic knockout of BK-2Rs leads to LV fibrosis, adverse remodelling, and heart failure, implying that kinin-mediated receptor signalling is essential for the functional and structural integrity of the heart.¹⁷ In animals with cardiac pressure overload, LV expression of BK-2Rs is increased in compensated hypertrophy but declines significantly during the transition to heart
failure. The expression of BK-2Rs is reduced also in human end-stage heart failure.

In contrast to BK-2R, which is constitutively expressed, BK-1R is expressed only at low levels in normal tissues but becomes induced in conditions such as inflammation and ischaemia. Overexpression of BK-1Rs makes mice more susceptible to inflammation, suggesting a role for BK-1R in modulating inflammatory responses. The proliferative and profibrotic potentials of BK-1R are activated by its ligand, des-Arg(9)BK [BK-(1–8)], which is produced from BK by either carboxypeptidase N in plasma or carboxypeptidase M in tissues. In addition, the expression of BK-1Rs is highly induced during the onset of myocardial fibrosis and transition to heart failure in spontaneously hypertensive rats.

The present study was set out to assess the expression and activity of NEP, and the expression and balance of BK-1R and BK-2R, in the diseased valves of human AS.

Methods

Samples and study population

Non-rheumatic stenotic aortic valves were obtained from 86 consecutive patients undergoing valve replacement surgery between August 2000 and January 2003. A detailed description of the patient recruitment was recently published in this journal. All patients had isolated AS, as those with more than mild aortic regurgitation or mitral valve disease were excluded. Other exclusion criteria included a history of myocardial infarction or proximal coronary artery disease excessing 50% of the luminal diameter at angiography, complicated diabetes, and renal insufficiency (serum creatinine >170 μmol/L). Non-stenotic control valves (n = 13) were obtained from patients undergoing cardiac transplantation due to dilated cardiomyopathy (n = 10), or from organ donors without cardiac disease whose hearts could not be used as grafts (n = 3) because of advanced age, suspected ischaemia, or resuscitation. The organ donors had died either of cerebrovascular accident or trauma. In both subgroups of controls, only valves without any visible morphological changes were accepted as controls and valves with early sclerotic changes were excluded from the control population. The main characteristics of the AS patients and of the individuals from whom the non-stenotic valves were removed, are shown in Tables 1 and 2, respectively. The investigation conforms to the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of Helsinki University Central Hospital. All participants signed an informed consent document.

Myofibroblast culture

In order to isolate aortic valve myofibroblasts, additional two stenotic aortic valves were obtained at valve replacement surgery and used immediately for cell culture. Control non-stenotic valves (n = 2) were obtained from patients undergoing aortic valve replacement due to aortic regurgitation. Freshly cut aortic valves were placed in Dulbecco’s modified Eagle’s medium (DMEM, Biowhitaker, Verviers, Belgium) supplemented with 10% heat-inactivated foetal bovine serum, 1% non-essential amino acids, 1% penicillin, 1% streptomycin, and 1% fungizone. The valves were rinsed in serum-free medium and placed in 0.2% type I collagenase diluted in a 1:1 mixture of phosphate-buffered saline (PBS) and medium for 30 min at 37°C. Endothelial cells were then removed from the valve surface by lightly scraping both surfaces of the leaflets. Then the valves were placed in collagenase solution (10 mL), and 20 mg of trypsin (Sigma, St Louis, MO, USA), 60 μL CaCl₂–stock, and 400 mg FFA-free albumin bovine (Sigma) were added, and the incubation continued for 30 min at 37°C. The leaflets were then rinsed in the medium and cut gently with small scissors into 1–8 mm² pieces, which were allowed to grow in 6-well plates covered by the above medium. After out-growing of the valvular interstitial cells, they were transferred with trypsin-EDTA into new 6-well plates and cultured until passages five to six.

### Table 1 Characteristics of patients with aortic valve stenosis (n = 86)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD or number of patients</th>
<th>Range</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>67 ± 10</td>
<td>39–82</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>40/46</td>
<td></td>
</tr>
<tr>
<td>NYHA class, 1/2/3/4</td>
<td>2/50/28/2</td>
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<tr>
<td>Aortic valve area index (cm²/m²)</td>
<td>0.36 ± 0.10</td>
<td>0.16–0.57</td>
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<tr>
<td>Mean pressure gradient (mmHg)</td>
<td>49 ± 16</td>
<td>15–95</td>
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<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>60 ± 12</td>
<td>21–80</td>
</tr>
<tr>
<td>Left ventricular mass index (g/m²)</td>
<td>155 ± 37</td>
<td>83–235</td>
</tr>
<tr>
<td>Pulmonary wedge pressure (mmHg)</td>
<td>13 ± 7</td>
<td>4–36</td>
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*Echocardiographic left ventricular mass index exceeding 110 g/m² in women or 134 g/m² in men.

### Table 2 Characteristics of the control group (n = 13)

<table>
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<tr>
<th>Characteristic</th>
<th>Mean ± SD or number of patients</th>
<th>Range</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>53 ± 10</td>
<td>27–62</td>
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<td>Sex (male/female)</td>
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<tr>
<td>NYHA class 1/2/3/4</td>
<td>3/1/8/1</td>
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</tr>
<tr>
<td>Transplantation/organ donor</td>
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<td></td>
</tr>
<tr>
<td>Bicuspid/tricuspid valve</td>
<td>0/13</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Smoking (no/yes/unknown)</td>
<td>7/2/4</td>
<td></td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE-inhibitor or</td>
<td>7 (54%)</td>
<td></td>
</tr>
<tr>
<td>AT1 blocker</td>
<td>3 (23%)</td>
<td></td>
</tr>
<tr>
<td>β-Blockers</td>
<td>7 (54%)</td>
<td></td>
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<tr>
<td>Diuretics</td>
<td>10 (77%)</td>
<td></td>
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<tr>
<td>Statins</td>
<td>1 (8%)</td>
<td></td>
</tr>
<tr>
<td>Digitalis</td>
<td>8 (62%)</td>
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Subconfluent cells were serum-starved and used in cell culture experiments as described below. The myofibroblast phenotype of the experiments was confirmed by immunohistochemistry using antibodies against alpha smooth muscle actin and vimentin.

**Experiments on cultured aortic valve myofibroblasts**

In order to study the expression levels of NEP, BK-1R, and BK-2R in isolated myofibroblasts, the cells were incubated with TNF-α (20 ng/mL, R&D Systems, Minneapolis, MN, USA), mast cell releasate (prepared as described by Kokkonen and Kovanen20), or medium alone for 24 h at 37°C, and the expression levels of NEP and BK receptors were quantified by real-time PCR. Furthermore, to examine the effect of exogenously added kinins and their receptor antagonists on myofibroblasts, subconfluent cells were incubated with BK (1 μM, Bachem, Bubendorf, Switzerland), BK-1/8 (1 μM, Bachem), the BK-2R antagonist icatibant (10 μM, Biomol International, Exeter, UK), the BK-1R antagonist [des-Arg10-HOE140 (10 μM, Sigma), the NEP inhibitor Phosphoramidon (1 μM, Sigma), or medium alone for 24 h at 37°C. The cellular mRNA expression levels of collagen I, collagen III, and transforming growth factor-β1 (TGF-β1) were determined by RT-PCR as described previously.4 Furthermore, the concentration of interleukin-6 (IL-6) in myofibroblast culture media was quantified using a commercially available enzyme immunoassay (Pierce, Rockford, IL, USA) according to manufacturers’ instructions.

**Real-time polymerase chain reaction**

Total RNA was isolated from a randomly selected subpopulation of 38 stenotic aortic valves and from all non-stenotic control valves (n = 13) using an ultra-pure TRIzol reagent (Gibco BRL, Gaithersburg, MD), and an RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Denmark) and an RNeasy Mini Kit (Qiagen) including DNase digestion. Purified total RNA from the aortic valve myofibroblasts was isolated using a universal TRIzol reagent (Gibco BRL). The mRNA expression levels of NEP, BK1R, and BK2R were determined by real-time PCR using a relative quantification method with GAPDH as an endogenous control.30 GAPDH, BK-2R, and BK-1R primers and probes were purchased from the Applied Biosystems Custom TaqMan® Gene Expression Array service, and were as follows: BK-1R: 5'-ACTTTTGTGCTTCTGGTTTCTTCT (F), 5'-AGTGTAAGAAGTGCTTCCAAAAG (R) and the probe 5'-FAM-CACGCTGGGACTGGTCTGG-MGB-3'; BK-2R: 5'-CCACACGGCTCTTTCTTCTT (F), 5'-GGGCAGAGGGTCCCTAAGA (R) and the probe 5'-FAM-TCACGGGCACATGC-MGB-3'; and GAPDH: 5'-GTAACCCGATTTGGTCTGATG-3' and the probe 5'-FAM-AAGAGCCTGTTGAAC-3'. NEP expression was analysed using pre-made standard gene expression assay provided by Applied Biosystems. Analyses were performed in 25 μL reaction volume in 96-well plates (Applied Biosystems) using TaqMan® Universal Master Mix (Applied Biosystems) with uracil-N-glycosylase (UNG) treatment. The samples were run on ABI Prism 7500 Sequence Detection System (Applied Biosystems) using a two-step programme consisting of 15 s at 95°C and 55 s at 60°C for 45 cycles. The threshold was set to the geometric phase of the amplification curve, and the amount of target was calculated using the formula 2^ΔΔCt.30

**Neutral endopeptidase autoradiography**

NEP autoradiography of frozen sections of aortic valves (20 μm thick) was performed in all stenotic (n = 86) and control (n = 13) aortic valves as described earlier.31 Briefly, slide-mounted aortic valve sections were pre-incubated in 50 mM Tris–HCl buffer for 50 min at room temperature, followed by a 2 h incubation at room temperature in Tris–HCl buffer containing 0.04 μCi of 125I-RB104. Slides were washed four times with Tris–HCl buffer for 1 min and once with distilled water for 5 s and dried under cool air. Non-specific binding was determined in parallel incubations in Tris–HCl buffer containing 100 mM Na2EDTA and 2.5 mM phenantrone. For quantification of NEP binding, the sections were placed on a Fuji Imaging Plate (BAS-TP2025, Tamro, Finland) and visualized by FUJIFILM BAS-5000 phosphorimager (Tamro).

**Immunohistochemistry and double immunofluorescence**

Immunohistochemistry of cryostat sections of aortic valves was performed using standard ABC-technique as described previously.2 NEP was detected using monoclonal mouse anti-CD10 antibodies (concentration 4 μg/mL, Novocastra, Newcastle, UK) and BK receptors were detected with rabbit-anti-BK-1R prepared as described previously by Raidoo et al.32 (1 μg/mL), and mouse-anti-BK-2R (5 μg/mL, BD Transduction Laboratories, Oxford, UK) antibodies, respectively. Myofibroblasts were identified using monoclonal antibodies against α-smooth muscle actin (2 μg/mL, DAKO, Glostrup, Denmark) and vimentin (1 μg/mL, Novocastra) and chondroblast-like cells in the ossified areas of the valves were identified with polyclonal rabbit-a-S100 antibody (9 μg/mL, DAKO). Endothelial cells were detected with a mixture of monoclonal mouse-anti-human CD31 (10 μg/mL, DAKO) and CD34 antibodies (0.5 μg/mL, Novocastra).33

Immunofluorescence stainings were performed using Alexa goat-anti-rabbit 546 IgG (red) and Alexa goat-anti-mouse 488 IgG (green) (Molecular Probes, Europe BV, Leiden, the Netherlands) as secondary antibodies at a concentration of 10 μg/mL each. The slides were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, St Louis, MO, USA) before mounting. Subcellular distributions of NEP, BK-1R, and BK-2R were visualized using confocal microscopy (Ultra View, Perkin-Elmer) with a section thickness of approximately 0.5 μm, as reported earlier.34 In all immunohistochemical and double immunofluorescence stainings, non-immune mouse, rabbit, and goat immunoglobulins served as negative controls.

**Statistics**

Group differences were analysed using Mann-Whitney U test for skewed data distribution and Student’s t-test for normally distributed data. The group data are summarized as medians and ranges or mean values ± SD. For correlations, either Pearson’s (r) or Spearman’s coefficients (rs) were calculated, depending on data distribution. The analyses were performed using SPSS (version 11.0) software program. Two-sided nominal unadjusted P-values <0.05 were considered statistically significant.

**Results**

Neutral endopeptidase, bradykinin receptors type-1, and -2 mRNA expression in aortic valves

Real-time PCR analysis revealed that NEP was locally produced in both stenotic and non-stenotic control aortic valves, with the stenotic valves showing significantly higher expression levels [median (range) 1.9 (0.2–123) vs. 1.0 (0.01–3.8) arbitrary units (AU), P = 0.02] (Figure 1A). Both BK-1R and BK-2R mRNA were also found in all studied valves. Compared with non-stenotic valves, BK-1R mRNA expression was increased in the stenotic valves [42 (1.5–417) vs. 8.2 (0.4–55) AU, P = 0.04] (Figure 1B), as was BK-2R expression [14 (4.2–58) vs. 9.6 (3.8–31) AU, P = 0.047] (Figure 1C). The mRNA levels of NEP, BK-1R, and BK-2R did not differ between tricuspid (n = 31) and bicuspid (n = 7) stenotic valves and were independent of the use of ACE-inhibitors (n = 11 AS patients). In patients with AS, NEP mRNA correlated with BK-1R (r = 0.55, P < 0.001) but...
not with BK-2R. There was also a direct correlation between the mRNA levels of BK-1R and BK-2R ($r = 0.48$, $P = 0.002$).

**Autoradiography of neural endopeptidase in aortic valves**

Autoradiography of aortic valves identified enzymatically active NEP in both non-stenotic and stenotic valves, with the stenotic ones showing, on average, 4.5 times higher activity ($P < 0.001$) (Figure 2A–C). In patients with AS, an inverse correlation ($r = -0.24$, $P = 0.026$) was found between logNEP activity and aortic valve area index, revealing that valvular NEP activity increased with increasing severity of AS.

**Immunohistochemical detection of neutral endopeptidase, bradykinin receptors type-1, and -2 in aortic valves and in isolated valvular myofibroblasts**

NEP was not immunohistochemically detectable in the control aortic valves (Figure 3A). In the stenotic valves, however, immunohistochemistry localized NEP to endothelial cells lining the valves as well as to cells deeper inside the valve leaflets (Figure 3B). Positive staining of NEP in cultured aortic valve myofibroblasts isolated from the stenotic valves was confirmed by confocal microscopy (Figure 3C).

The staining of BK-1R was more abundant in the stenotic than in the non-stenotic control valves (Figure 4A–C). In stenotic valves, the major cell type containing BK-1R was the valvular myofibroblast, as confirmed by double immunofluorescence against α-smooth muscle actin (data not shown) and vimentin (Figure 4D–F). In both groups, staining for BK-1R was found also in the endothelial lining of the valve cusps (Figure 4G–I). Importantly, in the stenotic valves, also the endothelial cells forming neovessels stained positively for BK-1R (Figure 4J–L). In addition, BK-1R was detected in the ossified areas of the valves, where it was localized to S-100 positive chondroblast-like cells (Figure 5A–C). Isolated aortic valve myofibroblasts stained positively for BK-1R, and confocal microscopy revealed the localization of BK-1R both intracellularly and on the cell surface (Figure 5D).

There was only weak staining for BK-2R in the control valves (Figure 6A), whereas the stenotic valves showed strong BK-2R positivity in the myofibroblasts of the valve leaflets (Figure 6B and D). Cultured myofibroblasts showed a strong signal of BK-2R in their nuclei and staining was...
also observed in the cytoplasm and on the cell surface (Figure 6C with inset).

**Neutral endopeptidase, bradykinin receptors type-1, and -2 mRNA expression in cultured aortic valve myofibroblasts**

Real-time PCR analysis of isolated aortic valve myofibroblasts confirmed that NEP, BK-1R, and BK-2R were locally produced by myofibroblasts originating from either stenotic or non-stenotic aortic valves (Figure 7A–C). Exposure to TNF-α induced a significant increase of mRNA expression of NEP ($P = 0.0003$) (Figure 7A). Likewise, NEP mRNA expression could be induced by adding mast cell releasate (i.e. constituents secreted by activated mast cells) to the myofibroblast culture ($P = 0.04$). With regard to BK-1R mRNA expression by the cultured myofibroblasts (Figure 7B), even the basal expression was higher in cells isolated from the stenotic valves ($P = 0.02$). Moreover, exposure to TNF-α increased BK-1R mRNA expression in cells derived from both non-stenotic ($P = 0.005$) and stenotic ($P = 0.004$) valves, but the induced level of BK-1R was markedly higher in cells derived from the stenotic valves ($P = 0.007$). Finally, as shown in Figure 7C, basal BK-2R expression was lower in the myofibroblasts derived from the stenotic than from the control valves ($P = 0.02$), but was upregulated by TNF-α in both cell populations ($P = 0.002$ for cells from control valves and $P = 0.0004$ for cells from stenotic valves). Cells from passages 4–6 showed comparable results.

**Effects of bradykinin, bradykinin-(1–8), bradykinin receptor antagonist, and neutral endopeptidase inhibition on cultured aortic valve myofibroblasts**

Finally, in cultured myofibroblasts, we tested the effects of exogenously added kinins [BK and BK-(1–8)], BK receptor antagonist, and NEP inhibition.
antagonists, and NEP inhibition on the expression of selected profibrotic components involved in valve remodelling (Figure 8A–D). First, stimulation of the myofibroblasts with BK for 24 h decreased the mRNA expression levels of collagen I ($P = 0.009$; Figure 8A) and collagen III ($P = 0.04$; Figure 8B), an effect that could be inhibited by simultaneously adding, icatibant, a BK-2R antagonist. Moreover, incubation of myofibroblasts with icatibant alone increased the expression of collagen I mRNA when compared with medium alone ($P = 0.01$; Figure 8A). Secondly, we studied the effects of exogenously added kinins on the ability of myofibroblasts to secrete the proinflammatory and profibrotic cytokine IL-6. As shown in Figure 8C, BK, the ligand of BK-2R, and in particular BK-(1–8), the ligand of BK-1R, stimulated the secretion of IL-6 by myofibroblasts ($P = 0.02$ and $0.01$, respectively). The observed BK-induced increase in IL-6 secretion was not inhibited by simultaneous addition of the BK-2R antagonist icatibant. In contrast, addition of the BK-1R antagonist [des-Arg(10)]–HOE140 inhibited the BK-(1–8)-dependent increase in IL-6 secretion ($P = 0.03$). Thirdly, treatment of the myofibroblasts with the NEP inhibitor phosphoramidon attenuated the mRNA expression of the profibrotic molecule TGF-β1 ($P = 0.04$; Figure 8D).

Discussion

Our novel data reveal the presence of two profibrotic components, NEP and BK-1Rs, as well as of antifibrotic BK-2Rs, in aortic valves, and further demonstrates their significant upregulation in stenotic aortic valves. Thus, the present study extends the role of NEP-kinin system into a new field, i.e. the pathophysiology of AS.

Stenosis of the aortic valves is characterized by extensive fibrosis and remodelling of the extracellular matrix, an adverse process in which both degradation of elastin and accumulation of collagen fibres contribute to the thickening and stiffening of the valve leaflets.$^{1,4}$ TGF-β1 is upregulated in stenotic valves,$^{4}$ and besides increasing collagen synthesis, this profibrotic molecule may also stimulate calcification of the valves.$^{35}$ Moreover, activation of ACE in the stenotic valves should lead to accumulation of the profibrotic and proinflammatory peptide Ang II$^{8,16}$ and to degradation of the antifibrotic peptide BK. In the stenotic valves, the increased expression of chymase$^{8}$ and cathepsin G$^{8}$ may also contribute to the generation of Ang II and inactivation of BK.$^{37}$ Here, we further show that another BK-degrading enzyme, NEP, is highly upregulated in the stenotic valves. Thus, our previous and present results suggest that the
Figure 5 Bradykinin type 1 receptor (BK-1R) in a stenotic aortic valve containing bony structures. In ossified areas of the valves, BK-1R (red) was found in chondroblast-like cells [lower magnification in (A) and higher magnification in (B)]. One BK-1R-positive chondroblast-like cell is shown with an arrow in (B). The chondroblast-like nature of the cells was confirmed by S-100 staining (C), (one S-100 positive cell is marked with an arrow). (D) Confocal microscopic image of BK-1R immunofluorescence staining (green) in a cultured aortic valve myofibroblast.

Figure 6 Bradykinin type 2 receptor (BK-2R, red) in a control (A) and a stenotic (B) aortic valve. Confocal microscopic image of BK-2R (green) in a cultured aortic valve myofibroblast (C). (D) represents an enlarged image of the square in (B). Examples of BK-2R-positive cells are marked with arrows.

The local balance between profibrotic and antifibrotic mechanisms is disturbed in the stenotic valves. With regard to the local kinin metabolism, activation of NEP may be even more important than activation of ACE since previous studies have shown that NEP is the major enzyme responsible for the local BK degradation at least in the heart muscle. In a hypertensive rat model, pharmacological inhibition of NEP, with or without concomitant ACE-inhibition, resulted in regression of cardiac hypertrophy and fibrosis as well as in attenuation of tissue inflammation. Our data therefore raise the logical question of whether NEP inhibitors could retard the adverse valvular remodelling in AS. This hypothesis is supported by the present observation that treatment with an NEP inhibitor (phosphoramidon) attenuated the expression of TGF-β1 in aortic valve myofibroblasts (Figure 8D). A recent retrospective study observed an association between the use of ACE-inhibitors and a lower rate of aortic valve calcification. In the presence of ACE-inhibitors, locally produced NEP in the valves may still continue to degrade BK, thus counteracting the BK-mediated beneficial effects of ACE-inhibitors. Thus, combined inhibition of NEP and ACE could offer an additional advantage in the pharmacological prevention of AS.

Since inflammation is a central component in the pathogenesis of AS, we tested the ability of TNF-α, a proinflammatory cytokine abundantly expressed in the stenotic valves, to regulate the expression of NEP and BK receptors in aortic valve myofibroblasts. Interestingly, myofibroblasts originating from both control and stenotic valves increased their expression of NEP, BK-1R, and BK-2R as a response to TNF-α. Moreover, NEP mRNA production was induced by factors released from activated mast cells. We have previously shown that mast cells accumulate and become
activated in the stenotic aortic valves, and we therefore believe that mast cells may be at least partly responsible for the upregulation of valvular NEP activity in AS. These findings suggest that the upregulation of valvular NEP, BK-1R, and BK-2R is unlikely a primary phenomenon in AS, but rather secondary to an ongoing pathological process, notably inflammation.

Since both types of BK receptors (1 and 2) were expressed on the surface of the valvular myofibroblasts, the locally formed kinins may exert both pro- and antifibrotic effects on the valves. Thus, the final outcome of the kinin-mediated effects on the valves depends on the valvular levels of the respective BK receptors and on the local levels of their ligands, i.e. the kinins. Kallidin (Lys-BK) binds to both BK-1R and BK-2R, whereas BK, the substrate of both ACE and NEP, binds to BK-2R. BK-1Rs are activated also by Lys-des-Arg(9)BK and BK-(1–8). Importantly, since four BK-degrading enzymes, i.e. ACE, chymase, cathepsin G, and NEP, are significantly upregulated in the stenotic valves, the overall net balance is shifted towards BK-degrading direction. Therefore, the augmented expression of the cardioprotective BK-2Rs in the diseased valves, which may occur in response to either decreased BK levels or increased TNF-α levels, may represent a compensatory attempt to protect the valves from further inflammatory and fibrotic damage. Indeed, the expression of BK-2Rs in myofibroblasts originated from the stenotic valves was markedly stimulated by TNF-α, although the basal level in these cells was lower than in myofibroblasts derived from the non-diseased valves. The antifibrotic role of BK in aortic valves is further supported by the finding that addition of BK to valvular myofibroblasts resulted in decreased collagen expression, which was reversed by a BK-2R antagonist, icatibant. Interestingly, icatibant alone upregulated collagen I expression in myofibroblasts, suggesting the presence of autocrine effects by endogenously produced BK. Increasing amounts of such endogenously produced unbound BK may also be subjected to cleavage by endogenous carboxypeptidase M, which can lead to increased levels of BK-1R ligands. Thus, icatibant may disturb the balance of BK-2R and BK-1R signalling in myofibroblasts by indirectly promoting BK-1R-mediated effects.

The upregulation of BK-1R in the stenotic relative to control valves was markedly higher than the upregulation of BK-2R (5.1-fold vs. 1.5-fold). BK-1R is induced in pathological conditions such as ischaemia and inflammation, and interestingly, we observed that myofibroblasts isolated from stenotic valves had higher basal expression level of BK-1R than the myofibroblasts derived from control valves. Moreover, myofibroblasts derived from the stenotic valves were more susceptible to TNF-α-induced upregulation of BK-1Rs (Figure 7B). In the present study, addition of both BK and BK-(1–8) to cultured myofibroblasts stimulated the secretion of IL-6, a cytokine with both proinflammatory and profibrotic properties. Importantly, this effect was inhibited by a BK-1R antagonist, but not by a BK-2R antagonist.
antagonist (Figure 8C). Thus, this experiment suggested that the BK-induced IL-6 secretion was not mediated via the BK-2R. One possibility is that BK was converted to BK-(1–8) under these culture conditions (e.g. by carboxypeptidase M) and, accordingly, the increased secretion of IL-6 actually was a response to locally generated BK-(1–8). The regulation and function of the BK-1Rs are less well-defined than those of BK-2Rs, and the role of BK-1Rs in cardiovascular diseases has been largely obscure. Interestingly, besides myofibroblasts, we observed BK-1R protein in chondroblast-like cells in bony areas of the valves and also in endothelial cells lining the neovessels of the stenotic valves. Therefore, in addition to promoting fibrosis and inflammation, BK-1Rs may also regulate calcification and neovascularization of the stenotic aortic valves.

In conclusion, here we report increased expression of BK-degrading NEP and the two BK receptors in stenotic aortic valves. Enhanced expression of BK-1Rs can lead to increased binding of its ligand BK-(1–8) and, as a response, to a local profibrotic and proinflammatory state. Accentuated degradation of BK by NEP may result in compensatory upregulation of BK-2Rs. Since valvular NEP may participate in the profibrotic progression of AS, NEP inhibitors could represent a novel therapeutic possibility to retard AS progression.

Acknowledgements

Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. This work was supported by the Finnish Foundation for Cardiovascular Research, Helsinki, Finland (SH); by the Sigrid Juselius Foundation, Helsinki, Finland; and by the EVO research funds of Helsinki University Central Hospital. We thank sincerely Mrs Liisa Blubaum, Mrs Elina Kaperi, Ms Suvi Mäkinen, and Mrs Jaana Tuomikangas for excellent technical assistance.

Conflict of interest: none declared.

References


Increased expression of profibrotic NEP and BK type 1 receptors in stenotic aortic valves


