

# B-Type Natriuretic Peptide Prevents Acute Hypertrophic Responses in the Diabetic Rat Heart

## Importance of Cyclic GMP

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**Stimulation of cardiomyocyte guanosine 3',5'-cyclic monophosphate (cyclic GMP) via endothelial-derived nitric oxide (NO) is an important mechanism by which bradykinin and ACE inhibitors prevent hypertrophy. Endothelial NO dysfunction and cardiac hypertrophy are morbid features of diabetes not entirely prevented by ACE inhibitors. In cardiomyocyte/endothelial cell cocultures, bradykinin efficacy is abolished by high-glucose-induced endothelial NO dysfunction. We now demonstrate that antihypertrophic actions of natriuretic peptides, which stimulate cyclic GMP independently of NO, are preserved in cardiomyocytes despite high-glucose-induced endothelial dysfunction. Further, streptozotocin-induced diabetes significantly impairs the effectiveness of acute antihypertrophic strategies in isolated rat hearts. In hearts from citrate-treated control rats, angiotensin II-stimulated [<sup>3</sup>H]phenylalanine incorporation and atrial natriuretic peptide and  $\beta$ -myosin heavy chain mRNA expression were prevented by B-type natriuretic peptide (BNP), bradykinin, the ACE inhibitor ramiprilat, and the neutral endopeptidase inhibitor candoxatrilat. These antihypertrophic effects were accompanied by increased left ventricular cyclic GMP. In age-matched diabetic hearts, the antihypertrophic and cyclic GMP stimulatory actions of bradykinin, ramiprilat, and candoxatrilat were absent. However, the blunting of hypertrophic markers and accompanying increases in cyclic GMP stimulated by BNP were preserved in diabetes. Thus BNP, which increases cyclic GMP independently of NO, is an important approach to prevent growth in the diabetic myocardium, where endothelium-dependent mechanisms are compromised. *Diabetes* 52:2389–2395, 2003**

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ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; cyclic GMP, cardiomyocyte guanosine 3',5'-cyclic monophosphate; ET-1, endothelin-1; LV, left ventricular;  $\beta$ -MHC,  $\beta$ -myosin heavy chain.

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**C**ardiac hypertrophy develops to maintain contractile function when cardiac workload is chronically elevated (1), but in the longer term increases cardiovascular risk (2). We previously demonstrated that bradykinin prevents acute myocardial hypertrophy in vitro by stimulating the release of endothelial nitric oxide (NO) (3) to elevate cardiomyocyte guanosine 3',5'-cyclic monophosphate (cyclic GMP) (4). This pathway also contributes to the antihypertrophic actions of ACE inhibitors (4,5). Endothelial NO dysfunction induced by high glucose abolishes the antihypertrophic and cyclic GMP-stimulatory effects of bradykinin in isolated cardiomyocytes (6), and there is evidence that antihypertrophic ACE inhibitor efficacy is compromised in experimental diabetes (7–9) and diabetic patients (10). It is therefore important to identify novel strategies to prevent growth specifically in the diabetic myocardium via mechanisms independent of endothelium.

Natriuretic peptides stimulate cyclic GMP independently of endothelial NO, by activating sarcolemmal receptors coupled to particulate guanylyl cyclase (11). We recently reported that atrial, B-type, and C-type natriuretic peptides (ANP, BNP, and CNP, respectively) prevent isolated cardiomyocyte hypertrophy via activation of particulate guanylyl cyclase (12). It is unknown if similar antihypertrophic natriuretic peptide effects are evident in whole hearts or if these are affected by chronically elevated glucose levels. In vivo, natriuretic peptide bioactivity is limited by neutral endopeptidase-mediated hydrolysis (13), and inhibition of neutral endopeptidase attenuates cardiac hypertrophy and remodelling (14–16). Neutral endopeptidase inhibition confers additional antihypertensive (and thus antihypertrophic) benefits in diabetes, compared with ACE inhibition alone (7). However, acute antihypertrophic actions of neutral endopeptidase inhibitors have not been evaluated in isolated diabetic myocardium. Neutral endopeptidase is markedly upregulated in heart failure (17), and given the existence of a specific *diabetic* cardiomyopathy (18), neutral endopeptidase inhibitor efficacy may also be blunted in diabetes. Our objective was to 1) determine whether high-glucose-induced endothelial dysfunction affects antihypertrophic efficacy of the natriuretic peptides in isolated rat cardiomyocytes, 2) examine the impact of chronic diabetes on the acute antihypertrophic actions of BNP and a neutral

TABLE 1

18S			
Forward primer	5'-TGTTCCACCATGAGGCTGAGATC		120 nmol/l
Reverse primer	5'-TGGTTGCCTGGGAAAATCC		120 nmol/l
Probe	VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA		125 nmol/l
ANP			
Forward primer	5'-GCCCTTGC GG TGTGTCA		100 nmol/l
Reverse primer	5'-TGCAGCTCCAGGAGGGTATT		150 nmol/l
probe	6FAM-CAGCTTGTTCGCATTGCCACT-TAMRA		100 nmol/l
$\beta$ -MHC			
Forward primer	5'-GTCAAGCTCCTAAGTAATCTGTT		100 nmol/l
Reverse primer	5'-GAAAGGATGAGCCTTTCTTTGC		150 nmol/l
Probe	6FAM-CTACAGGTGCATCAGCT-MGBNF2		200 nmol/l

Optimal sequences (5'→3') and concentrations of Taqman primers and probes.

endopeptidase inhibitor (candoxatrilat) in the whole rat heart, and 3) compare this with the protection elicited by bradykinin and an ACE inhibitor (ramiprilat).

## RESEARCH DESIGN AND METHODS

**Materials.** Rat ANP-28, rat BNP-32, and human/porcine/rat CNP-22 were from Bachem Feinchemikalien (Bubendorf, Switzerland). Bradykinin was from Auspep (Parkville, Australia), and ramiprilat and candoxatrilat were gifts from Hoechst (Frankfurt, Germany) and Pfizer (Sandwich, U.K.), respectively. Real-time PCR reagents were purchased from Applied Biosystems (Scoresby, Australia). All other materials were purchased from Sigma Biochemicals (St. Louis, MO), except where indicated, and were of analytical grade or higher. All protocols were approved by the Animal Experimentation Ethics Committee of the Howard Florey Institute.

**Cardiomyocyte/endothelial cell cocultures.** Male Sprague-Dawley rats (180–280 g) were anesthetized intraperitoneally with ketamine hydrochloride (100 mg/kg)/xylazine (12 mg/kg). Cardiomyocytes were isolated and plated into laminin (Collaborative Biomedical Products, Bedford, MA)-coated six-well plates (19) and used within 24 h. Sufficient cardiomyocytes were obtained from one heart to study six different treatments concurrently (i.e., paired), with all treatment groups studied at least in triplicate. A total of six cardiomyocyte preparations, from six normal rat hearts, were used. Bovine aortic endothelial cell cultures (passage 4–6) were maintained in 10% FCS and grown to confluence on 30-mm tissue culture inserts (0.4- $\mu$ m membrane, Millipore, North Ryde, Australia) as described (6). Endothelial cell-coated inserts were then pretreated with 25 mmol/l D-glucose for 24 h. We have previously demonstrated that this abolishes the ability of the calcium ionophore A23187 to stimulate endothelial release of NO; hence these endothelial cells have impaired NO function (6). At the end of the pretreatment period, endothelial cell-coated inserts were washed in serum-free medium and placed over cardiomyocytes. Cocultures were incubated in serum-free medium containing L-[2,3,4,5- $^3$ H]-phenylalanine (2  $\mu$ Ci/ml; NEN Lifesciences, Boston, MA)  $\pm$  angiotensin II (1  $\mu$ mol/l)  $\pm$  ANP, BNP, CNP (1  $\mu$ mol/l) or 8-bromo-cyclic GMP (1 mmol/l) for 2 h at 37°C.

**Induction of diabetes.** Male Sprague-Dawley rats (170–200 g) were treated with streptozotocin (55 mg/kg i.v. bolus; Sigma) after an overnight fast to induce type 1 diabetes. Age-matched controls received citrate buffer vehicle (0.42% in sterile saline, pH 4.5). Diabetes was confirmed after 4–5 days by urinalysis (Clinistix Reagent Strips; Bayer, Pymble, Australia) and progressed untreated for 8 weeks. In total, 64 and 69 rats were studied after citrate and streptozotocin injection, respectively.

**Perfusion of isolated rat hearts.** On the day of study, tail vein blood (300  $\mu$ l) was collected for determination of plasma glucose (using a glucose oxidase method, Beckman Coulter Synchron Clinical Analyser). Hearts were removed from anesthetized rats and arrested in ice-cold Krebs-Henseleit buffer (4). The aorta was cannulated and attached to a modified Langendorff rodent heart perfusion system (Petersen Scientific Glass Blowing, Preston, Australia) under constant pressure (60 mmHg). Heart rate and coronary flow were monitored throughout the perfusion. Hearts perfused by this method maintained regular contractions for 2.5–3 h.

Following equilibration (15 min), hearts were perfused for 90 min with buffer alone (vehicle) or buffer containing study drugs: 1) angiotensin II (10 nmol/l), 2) bradykinin (100 nmol/l) + angiotensin II, 3) ramiprilat (100 nmol/l) + angiotensin II, 4) BNP (10 nmol/l) + angiotensin II, and 5) candoxatrilat (50  $\mu$ mol/l) + angiotensin II. Angiotensin II was added after the first 30 min (4,20). Low-dose ramiprilat (10 nmol/l) was present in bradykinin-containing solutions to prevent degradation (21) without itself affecting [ $^3$ H]phenylalanine

incorporation. The lowest concentrations of BNP and candoxatrilat that reproducibly prevented angiotensin II-induced [ $^3$ H]phenylalanine incorporation were determined in preliminary studies; concentrations of bradykinin and ramiprilat were previously described (4). Hearts then incorporated [ $^3$ H]phenylalanine (0.25  $\mu$ Ci/ml) for a further 60 min with perfusion buffer containing 0.1% BSA and L-amino acids, as described previously (4). At the conclusion of the perfusion protocol, left ventricular (LV) free walls were dissected into pieces, snap-frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  for later biochemical assays.

**[ $^3$ H]phenylalanine incorporation.** [ $^3$ H]phenylalanine incorporation and ANP and  $\beta$ -myosin heavy chain ( $\beta$ -MHC) mRNA expression are conventional in vitro markers of hypertrophy. [ $^3$ H]phenylalanine incorporation was measured in cardiomyocytes or LV homogenates as previously described (4). Cardiomyocyte DNA content was determined using PicoGreen fluorimetric reagent (Molecular Probes, Eugene, OR). Total LV protein was quantified by Lowry assay. [ $^3$ H]phenylalanine incorporation was normalized to nanograms DNA per sample in cardiomyocytes (to correct for cell number) (6) or to milligrams protein content in LV samples.

**ANP and  $\beta$ -MHC mRNA expression.** Total RNA was extracted from LV tissue using RNeasy (Qiagen, Crawley, Australia) and reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems). ANP and  $\beta$ -MHC mRNA expression levels were quantified by real-time PCR using the  $\Delta\Delta C_t$  method (12,22), relative to the internal standard 18S ribosomal RNA. Primers and fluorogenic probes were designed from rat-specific sequences published on GenBank at previously determined optimal concentrations (Table 1). Probes were 5'-labeled with the reporter dyes FAM (ANP,  $\beta$ -MHC) or VIC (18S) and 3'-labeled with the quencher molecules TAMRA or MGBNF2. Reactions were performed in the ABI Prism 7700 sequence detection system (Applied Biosystems) (12).

**LV cyclic GMP content.** Cyclic GMP was diethyl ether-extracted from LV homogenate (4) and resuspended in sodium acetate buffer (0.05 mol/l, pH 6.2) before  $^{125}\text{I}$ -cyclic GMP radioimmunoassay (NEN DuPont).

**Statistical analysis.** Cardiomyocyte treatment groups were studied at least in triplicate, and the averaged result was taken as  $n = 1$  for each experiment from a total of six myocyte preparations. [ $^3$ H]phenylalanine incorporation per nanogram DNA was expressed as percent paired control, mean  $\pm$  SE. Statistical comparisons utilized two-way ANOVA or paired  $t$  test as indicated; Bonferroni correction for multiple comparisons was applied where appropriate. Isolated hearts were perfused in series of 10–12 hearts, each including at least one negative (vehicle) and one positive hypertrophic control (angiotensin II). Results were normalized to the mean vehicle result of that series for both control and diabetic groups. Statistical comparisons between treatment groups used one-way ANOVA on ranks with Dunn's method for multiple comparison. Effects of diabetes or angiotensin II alone were compared with controls using unpaired Student's  $t$  test. Data are presented as mean  $\pm$  SE from one-way ANOVA.  $P < 0.05$  was accepted as significant.

## RESULTS

**Natriuretic peptide effects in cultured cells.** We have previously demonstrated that cardiomyocyte protein synthesis is increased (by  $\sim 25\%$ ) on coculture with high-glucose-treated endothelial cells. Furthermore, angiotensin II elicits a 30–35% increase in cardiomyocyte protein synthesis, regardless of the presence or the absence of endothelial cells or whether the endothelial cells have

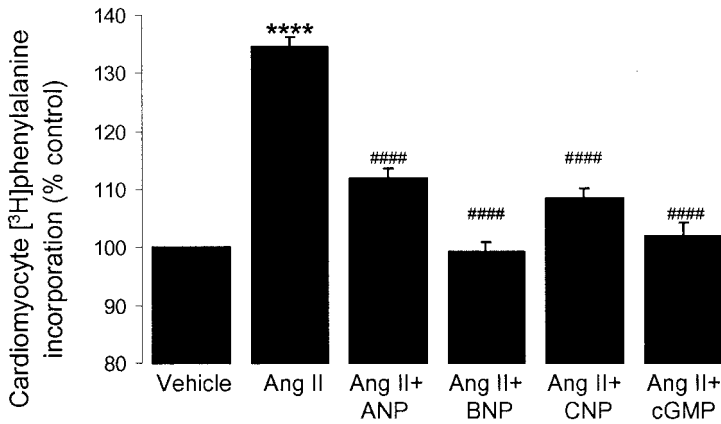


FIG. 1. Antihypertrophic actions of ANP, BNP, and CNP ( $n = 6$ ) or 8-bromo-guanosine 3',5'-cyclic GMP ( $n = 6$ ) against angiotensin II (Ang II;  $n = 6$ )–induced [ $^3\text{H}$ ]phenylalanine incorporation by cardiomyocytes cocultured with high-glucose–treated endothelial cells. \*\*\*\* $P < 0.001$  vs. control; #### $P < 0.001$  vs. angiotensin II.

been incubated in high-glucose conditions (6). Similar results are observed with phenylephrine (19). In the present study, angiotensin II increased [ $^3\text{H}$ ]phenylalanine incorporation to  $134 \pm 2\%$  of control ( $n = 6$ ,  $P < 0.001$ ) in cardiomyocytes cocultured with high-glucose–treated endothelial cells (Fig. 1). This was prevented by ANP, BNP, CNP, and the cyclic GMP analog 8-bromo-cyclic GMP (all  $n = 6$ ,  $P < 0.001$  vs. angiotensin II). Subsequent studies in isolated hearts utilized BNP.

**Baseline parameters in diabetes.** Plasma glucose was significantly increased to  $36.0 \pm 0.8$  mmol/l ( $P < 0.001$ ,  $t$  test) in 8-week diabetic rats compared with  $12.5 \pm 0.6$  mmol/l in control animals ( $n = 41$ ). Absolute heart weights were not different ( $1.5 \pm 0.1$  vs.  $1.6 \pm 0.1$  g in diabetic versus control rats, respectively, NS). Body weight gain was slower in diabetic rats (final body weight  $357 \pm 9$  g,  $P < 0.001$ ) compared with controls ( $502 \pm 7$  g); thus, diabetic heart-to-body weight ratios were greater,  $4.2 \pm 0.1$  g/kg, ( $P < 0.001$ ) compared with  $3.3 \pm 0.1$  g/kg. In the isolated hearts (perfused with buffer alone), heart rate tended to be higher in the diabetic group,  $188 \pm 6$  bpm ( $n = 20$ ,  $P = 0.06$ ) compared with  $176 \pm 6$  bpm in control hearts ( $n = 23$ ). Heart rate was not significantly altered by any of the drug treatment protocols (range 160–210 bpm; data not shown). Coronary flow during perfusion with vehicle was not different between control ( $10.6 \pm 0.5$  ml/min,  $n = 23$ ) and diabetic hearts ( $10.3 \pm 0.4$  ml/min,  $n = 18$ ). Coronary flow was increased transiently (before the addition of angiotensin II) by bradykinin in control hearts (to  $12.6 \pm 1.2$  ml/min,  $n = 5$ ,  $P < 0.05$  vs. vehicle), and by BNP in diabetic hearts (to  $12.0 \pm 0.8$  ml/min,  $n = 4$ ,  $P < 0.05$  vs. vehicle). Addition of angiotensin II restored flow to resting levels. Angiotensin II alone or in combination with other drug treatments did not alter coronary flow (data not shown). Observed influences on hypertrophic markers were thus attributed to a pharmacological action of drug treatments rather than being secondary to a vasoactive effect.

**LV [ $^3\text{H}$ ]phenylalanine incorporation.** In control hearts, angiotensin II stimulated LV [ $^3\text{H}$ ]phenylalanine incorporation to  $170 \pm 7\%$  ( $n = 14$ ,  $P < 0.001$ ) of vehicle alone ( $n = 15$ ) (Fig. 2A). This was prevented by bradykinin ( $n = 9$ ,  $P < 0.001$  vs. angiotensin II), ramiprilat ( $n = 8$ ,  $P < 0.005$  vs. angiotensin II), BNP ( $n = 9$ ,  $P < 0.001$  vs. angiotensin II), and candoxatrilat ( $n = 9$ ,  $P < 0.01$  vs. angiotensin II). Diabetes increased basal (perfusion with vehicle) LV [ $^3\text{H}$ ]phenylalanine incorporation to  $143 \pm 10\%$  of age-

matched controls ( $n = 15$ ,  $P < 0.001$ ) (Fig. 2 inset). Addition of angiotensin II further increased [ $^3\text{H}$ ]phenylalanine incorporation to  $137 \pm 8\%$  of the diabetic vehicle ( $n = 15$ ,  $P < 0.001$ ) (Fig. 2B). This hypertrophic response was abolished by BNP ( $n = 10$ ,  $P < 0.005$  vs. angiotensin II) but

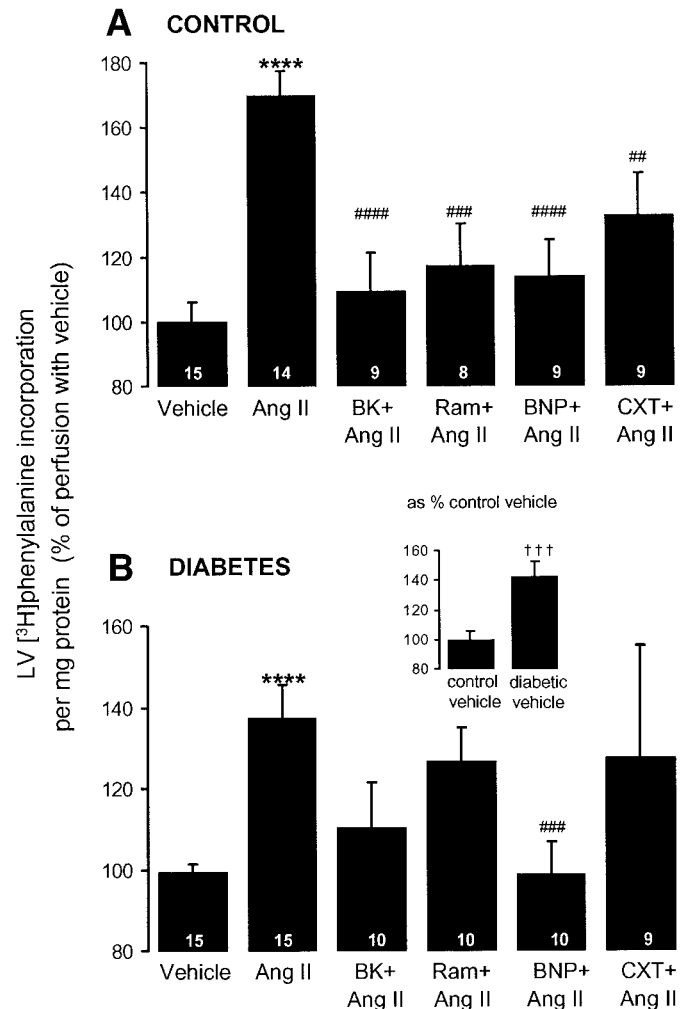
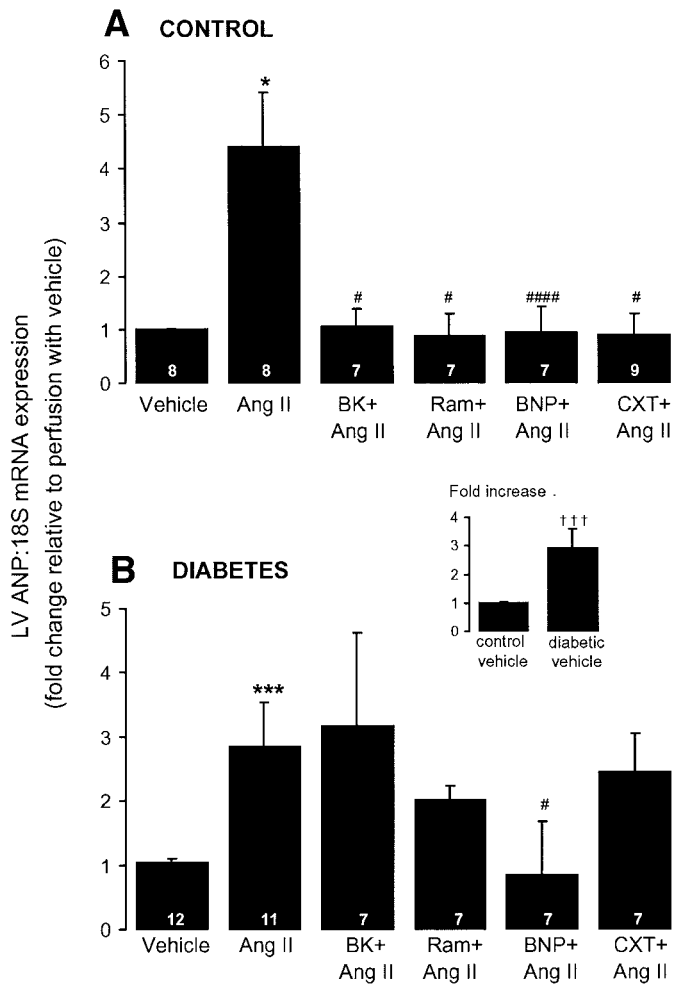


FIG. 2. Left ventricular (LV) [ $^3\text{H}$ ]phenylalanine incorporation in isolated control (A) and diabetic (B) rat hearts perfused with angiotensin II alone or in combination with bradykinin (BK), ramiprilat (Ram), BNP, or candoxatrilat (CXT). Numbers on bars indicate number of hearts per treatment group. \*\*\*\* $P < 0.001$  vs. vehicle; #### $P < 0.001$ , ### $P < 0.005$ , and ## $P < 0.01$  vs. matched angiotensin II alone; ††† $P < 0.001$  vs. control vehicle.



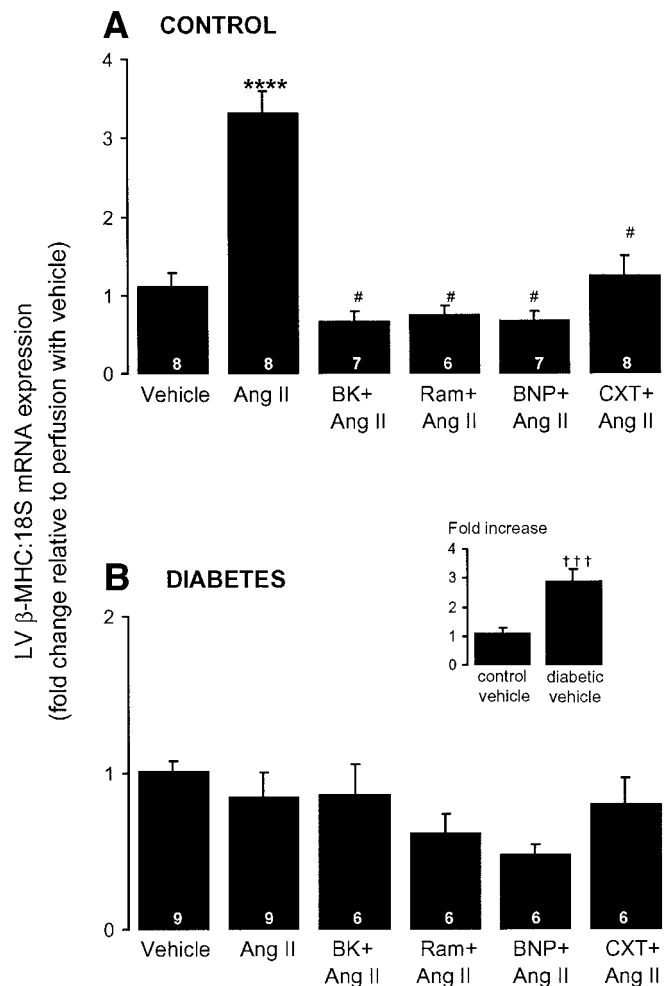


**FIG. 3.** LV ANP mRNA expression in isolated control (A) and diabetic (B) rat hearts perfused with angiotensin II alone or in combination with bradykinin, ramiprilat, BNP, or candoxatrilat. Numbers on bars indicate number of hearts per treatment group. \* $P < 0.05$  and \*\*\* $P < 0.005$  vs. vehicle; #### $P < 0.001$  and # $P < 0.05$  vs. matched angiotensin II alone; ††† $P < 0.001$  vs. control vehicle.

not by bradykinin ( $n = 10$ ), ramiprilat ( $n = 10$ ), or candoxatrilat ( $n = 9$ ).

**ANP and  $\beta$ -MHC mRNA expression.** In control hearts, angiotensin II increased ANP mRNA expression by 4.4  $\pm$  1.2-fold compared with vehicle ( $n = 8$ ,  $P < 0.05$ ) (Fig. 3A). This was prevented by bradykinin or ramiprilat (both  $n = 7$ ,  $P < 0.05$  vs. angiotensin II), by BNP ( $n = 7$ ,  $P < 0.001$  vs. angiotensin II), and by candoxatrilat ( $n = 9$ ,  $P < 0.05$  vs. angiotensin II). In diabetic hearts, basal expression levels of ANP ( $n = 12$ ) were approximately threefold greater compared with control hearts ( $P < 0.05$ ) (Fig. 3 inset). Addition of angiotensin II increased ANP mRNA further (to  $2.9 \pm 0.7$ -fold of vehicle,  $n = 11$ ,  $P < 0.05$ ); this was not significantly modulated by any of the treatments (Fig. 3B). Although BNP tended to reduce ANP mRNA to baseline, this trend was not significant when corrected for multiple comparisons ( $P = 0.16$ ).

Angiotensin II-stimulated  $\beta$ -MHC mRNA expression (to  $3.3 \pm 0.3$ -fold,  $n = 8$ ,  $P < 0.001$ ) (Fig. 4A) was also abolished by bradykinin ( $n = 7$ ), ramiprilat ( $n = 6$ ), BNP ( $n = 7$ ), and candoxatrilat ( $n = 8$ , all  $P < 0.05$  vs. angiotensin II) in control hearts. Basal expression levels of  $\beta$ -MHC mRNA ( $n = 9$ ) were approximately threefold



**FIG. 4.** LV  $\beta$ -myosin heavy chain ( $\beta$ -MHC) mRNA expression in isolated control (A) and diabetic (B) rat hearts perfused with angiotensin II alone or in combination with bradykinin, ramiprilat, BNP, or candoxatrilat. Numbers on bars indicate number of hearts per treatment group. \*\*\*\* $P < 0.001$  vs. vehicle; # $P < 0.05$  vs. angiotensin II; ††† $P < 0.001$  vs. control vehicle.

greater in control hearts ( $P < 0.05$ ) (Fig. 4 inset) than diabetic hearts. However, angiotensin II induced no further acute increase in  $\beta$ -MHC mRNA expression above the diabetic baseline ( $n = 9$ ) (Fig. 4B). BNP tended to reduce  $\beta$ -MHC mRNA expression in diabetic hearts, although this trend was again not significant ( $P = 0.3$ ,  $n = 6$ ) (Fig. 4B).  $\beta$ -MHC mRNA expression in diabetic hearts was not modulated by any of the drug treatments (all  $n = 6$ ).

**LV cyclic GMP content.** Angiotensin II alone did not influence cyclic GMP in hearts from control or diabetic rats ( $n = 9$ , NS versus vehicle) (Fig. 4). In control hearts, addition of bradykinin, BNP, or candoxatrilat increased cyclic GMP to  $155 \pm 14\%$  ( $n = 9$ ,  $P < 0.001$  vs. angiotensin II alone),  $150 \pm 10\%$  ( $n = 9$ ,  $P < 0.005$  vs. angiotensin II alone), and  $164 \pm 9\%$  of vehicle, respectively ( $n = 10$ ,  $P < 0.001$  vs. angiotensin II alone). Ramiprilat-induced increases in LV cyclic GMP ( $125 \pm 9\%$ ) were not significant ( $P = 0.09$ ,  $n = 9$ ) (Fig. 4A). Basal cyclic GMP was not altered in diabetic hearts ( $n = 10$ ) (Fig. 4 inset), but cyclic GMP responses to bradykinin, candoxatrilat, or ramiprilat were absent ( $n = 9$ ) (Fig. 4B). BNP, by contrast, retained cyclic GMP-stimulatory effects in diabetic hearts (to  $125 \pm$

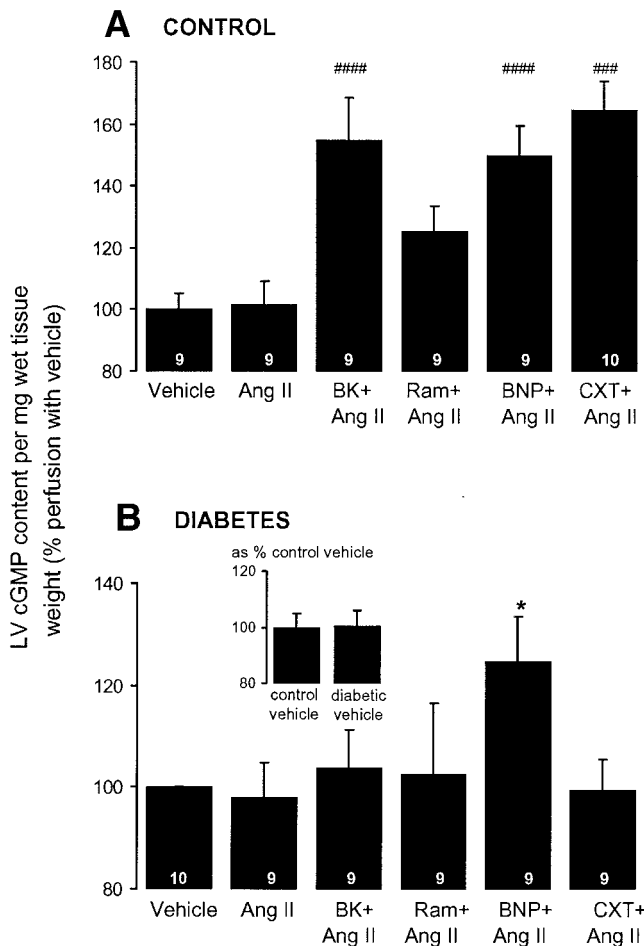


FIG. 5. LV guanosine 3',5'-cyclic monophosphate (cGMP) content in isolated control (A) and diabetic (B) rat hearts perfused with angiotensin II alone or in combination with bradykinin, ramiprilat, BNP, or candoxatrilat. Numbers on bars indicate number of hearts per treatment group. \* $P < 0.05$  vs. vehicle; ### $P < 0.001$  and ### $P < 0.005$  vs. angiotensin II alone (two-way ANOVA).

9%,  $n = 9$ ,  $P < 0.05$  vs. diabetic vehicle,  $P = 0.06$  vs. angiotensin II alone).

## DISCUSSION

The major finding in this study is that BNP exerts acute antihypertrophic actions in isolated hearts and that these effects were preserved following long-term diabetes. Protective effects of BNP, as well as those of ANP, CNP, and a cyclic GMP analog, were also retained in isolated cardiomyocytes cocultured with high-glucose-treated endothelial cells, where we previously reported bradykinin to fail (6). Our findings are particularly important because the antihypertrophic and cyclic GMP stimulatory actions of bradykinin, as well as those of ACE or neutral endopeptidase inhibitors, failed in diabetic rat hearts.

BNP prevents cardiac remodeling in vivo (23) and may contribute to the regulation of cardiac growth, since disruption of natriuretic peptide NP<sub>A</sub> receptors (activated by both BNP and ANP) promotes cardiac hypertrophy (24). In the present acute model of hypertrophy, BNP abolished angiotensin II-induced increases in LV protein synthesis and expression of ANP and  $\beta$ -MHC mRNA. This action was associated with significant stimulation of LV cyclic GMP, confirming findings in isolated cardiomyo-

cytes (12). In streptozotocin-induced diabetes, these acute effects of BNP were preserved, unlike those of bradykinin or ACE or neutral endopeptidase inhibition. The cyclic GMP-response to BNP was blunted in diabetic hearts, suggesting a potential downregulation of cardiac NP<sub>A</sub> receptors, as has been reported in the diabetic kidney (25). However, the BNP-induced rise in cyclic GMP was clearly sufficient to counter the response to angiotensin II. Moreover, the antihypertrophic actions of natriuretic peptides in cardiomyocytes/endothelial cell cocultures were mimicked by a cyclic GMP analog. Thus, the natriuretic peptide BNP has a selective advantage in preventing angiotensin II-induced cardiac growth in diabetes, due to its ability to act directly on the myocyte, avoiding the dependence on the endothelium.

Endothelial NO function is often compromised in diabetes (26). In this study, chronic diabetes attenuated the acute antihypertrophic and cyclic GMP-stimulatory actions of bradykinin and ramiprilat in isolated hearts. In control hearts, both bradykinin and the ACE inhibitor completely abolished angiotensin II-induced [<sup>3</sup>H]phenylalanine incorporation and expression of ANP and  $\beta$ -MHC mRNA. These effects were accompanied by increased LV cyclic GMP, in accordance with our previous finding that stimulation of cardiomyocyte cyclic GMP via endothelial NO is essential for the antihypertrophic effects of bradykinin in vitro (3,4). Diabetes impairs this antihypertrophic mechanism and may also compromise the long-term benefits of ACE inhibition on heart size clinically (10) and in experimental animals (7–9). Other investigators report no loss of antihypertrophic ACE inhibitor efficacy in diabetes (27–29), but in those studies, the reduction in heart size was secondary to a lowering of blood pressure rather than to a direct antihypertrophic effect as described here. Moreover, in the present study, 8 weeks of streptozotocin-induced diabetes per se increased a number of markers of cardiac hypertrophy, including LV protein synthesis and LV ANP and  $\beta$ -MHC mRNA expression. We speculate that the inability of angiotensin II to further stimulate  $\beta$ -MHC mRNA expression in these hearts suggests the induction of this isoform is maximally driven by the chronic diabetic state.

Our findings have identified BNP as a suitable antihypertrophic strategy, particularly in diabetic myocardium where bradykinin-dependent mechanisms fail. However, the therapeutic usefulness of the natriuretic peptides may be limited in vivo by low bioavailability and rapid clearance from the circulation (30). Neutral endopeptidase contributes significantly to the hydrolysis of natriuretic peptides, and selective neutral endopeptidase inhibitors reduce cardiac remodeling and hypertrophy in vivo (15,16). We therefore investigated whether the neutral endopeptidase inhibitor candoxatrilat was an effective alternative to ACE inhibition in preventing acute hypertrophic responses in diabetic hearts. Candoxatrilat attenuated angiotensin II-stimulated [<sup>3</sup>H]phenylalanine incorporation in control hearts and abolished the accompanying increases of ANP and  $\beta$ -MHC mRNA. These actions were accompanied by significant elevation of LV cyclic GMP, consistent with the association between the cardiac and vascular antihypertrophic effects of neutral endopeptidase inhibitors and

increased urinary cyclic GMP in vivo (14). Surprisingly, candoxatrilat exerted no net antihypertrophic or cyclic GMP stimulatory effect in hearts from streptozotocin-diabetic rats. Neutral endopeptidase inhibition may also fail to reduce cardiac size in diabetic rats in vivo (7), unless secondary to a lowering of blood pressure (31). However, the question remains as to why acute neutral endopeptidase inhibition, which potentiates the actions of endogenous natriuretic peptides, did not act like BNP in diabetic heart. Limitation of natriuretic peptide substrate is unlikely to be a factor, given that diabetes is associated with increased myocardial expression of BNP (32). However, neutral endopeptidase also cleaves endothelin-1 (ET-1) (13), which is upregulated in the diabetic heart (33). Concurrent inhibition of neutral endopeptidase-mediated ET-1 hydrolysis could oppose the benefits of augmented natriuretic peptide levels. Thus, infused ANP lowers systemic blood pressure in humans, while the neutral endopeptidase inhibitor candoxatrilat exerts a hypertensive effect (34) attributable to increased ET-1 activity (35). Moreover, combined inhibition of neutral endopeptidase/ACE or neutral endopeptidase/endothelin-converting enzyme lowers blood pressure more effectively than either inhibitor alone in diabetes with concomitant hypertension (31), suggesting that dual inhibitors are a better therapeutic strategy particularly in diabetes. Alternatively, neutral endopeptidase (in addition to ACE) also metabolizes kinins in the heart (36). Thus, the antihypertrophic effects of candoxatrilat could depend in part upon activation of the endothelium-dependent bradykinin/NO pathway, which would be consistent with the failure of candoxatrilat to stimulate cyclic GMP in diabetic hearts, where bradykinin pathways are impaired.

In conclusion, antihypertrophic and cyclic GMP stimulatory actions of BNP are preserved in diabetic rat hearts, unlike those of bradykinin, ramiprilat, or candoxatrilat. This supports the proposal that exogenous natriuretic peptide administration is an effective strategy to prevent cardiac growth in diabetes, where endothelium-dependent pathways are compromised. Recent reports indicate that exogenous ANP or BNP administration exerts acute hemodynamic benefits in patients with heart failure, despite elevated circulating natriuretic peptide levels (37,38). Whether the benefits of targeting the natriuretic peptide system are also evident in the longer term in vivo, particularly in patients resistant to conventional antihypertensive therapy including ACE inhibitors (39) has yet to be investigated. Diabetes attenuated the acute antihypertrophic effects of *selective* ACE and neutral endopeptidase inhibitors in isolated hearts, but the long-term antihypertensive and antihypertrophic effects of *dual* ACE/neutral endopeptidase inhibition are preserved in vivo (31). Given that neutral endopeptidase may also modulate local levels of angiotensin II and ET-1, future therapeutic strategies are likely to include the recently described compounds that inhibit all three key metallopeptidases, neutral endopeptidase, ACE, and endothelin-converting enzyme (40). These may be of particular benefit in diabetic patients, who often require multiple therapies to achieve adequate blood pressure control and thereby reduce cardiovascular risk.

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## REFERENCES

- Morgan H, Gordon E, Kira Y, Chua H, Russo L, Peterson C, McDermott P, Watson P: Biochemical mechanisms of cardiac hypertrophy. *Ann Rev Physiol* 49:533–543, 1987
- Messerli FH, Ketelhut R: Left ventricular hypertrophy: a pressure-independent cardiovascular risk factor. *J Cardiovasc Pharmacol* 22 (Suppl. 1):S7–S13, 1993
- Ritchie RH, Schiebinger RJ, LaPointe MC, Marsh JD: Angiotensin II-induced hypertrophy of adult rat cardiomyocytes is blocked by nitric oxide. *Am J Physiol* 275:H1370–H1374, 1998
- Rosenkranz AC, Hood SG, Woods RL, Dusting GJ, Ritchie RH: Acute antihypertrophic actions of bradykinin in the rat heart: importance of cyclic GMP. *Hypertension* 40:498–503, 2002
- Linz W, Wiemer G, Scholkens BA: Contribution of bradykinin to the cardiovascular effects of ramipril. *J Cardiovasc Pharmacol* 22 (Suppl. 9):S1–S8, 1993
- Rosenkranz AC, Dusting GJ, Ritchie RH: Endothelial dysfunction limits the antihypertrophic action of bradykinin in rat cardiomyocytes. *J Mol Cell Cardiol* 32:1119–1126, 2000
- Tikkanen I, Tikkanen T, Cao Z, Allen TJ, Davis BJ, Lassila M, Casley D, Johnston CI, Burrell LM, Cooper ME: Combined inhibition of neutral endopeptidase with angiotensin converting enzyme or endothelin converting enzyme in experimental diabetes. *J Hypertens* 20:707–714, 2002
- Black MJ, Briscoe T, Bertram JF, Jackson B, Johnston CI: Cardiac hypertrophy in diabetic spontaneously hypertensive rats: role of angiotensin II? *Clin Exp Pharmacol Physiol* 24:445–448, 1997
- Gerritsen TA, Bak AA, Stolk RP, Jonker JJ, Grobbee DE: Effects of nitrendipine and enalapril on left ventricular mass in patients with non-insulin-dependent diabetes mellitus and hypertension. *J Hypertens* 16:689–696, 1998
- Tamow L, Sato A, Ali S, Rossing P, Nielsen FS, Parving HH: Effects of nisoldipine and lisinopril on left ventricular mass and function in diabetic nephropathy. *Diabetes Care* 22:491–494, 1999
- de Bold AJ, Ma KK, Zhang Y, de Bold ML, Bensimon M, Khoshbaten A: The physiological and pathophysiological modulation of the endocrine function of the heart. *Can J Physiol Pharmacol* 79:705–714, 2001
- Rosenkranz AC, Woods RL, Dusting GJ, Ritchie RH: Antihypertrophic actions of the natriuretic peptides in adult rat cardiomyocytes: importance of cyclic GMP. *Cardiovasc Res* 57:515–522, 2002
- Kenny AJ, Bourne A, Ingram J: Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. *Biochem J* 291:83–88, 1993
- Kimura M, Umemura K, Ohashi K, Nakashima M: Effect of ecadotril, a neutral endopeptidase inhibitor, on myocardial hypertrophy in the rat aortic insufficiency model. *Can J Cardiol* 14:63–68, 1998
- Monopoli A, Ongini E, Cigola E, Olivetti G: The neutral endopeptidase inhibitor, SCH 34826, reduces left ventricular hypertrophy in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 20:496–504, 1992
- Richards AM, Crozier IG, Kosoglou T, Rallings M, Espiner EA, Nicholls MG, Yandle TG, Ikram H, Frampton C: Endopeptidase 24.11 inhibition by SCH 42495 in essential hypertension. *Hypertension* 22:119–126, 1993
- Fielitz J, Dendorfer A, Pregla R, Ehler E, Zurbrugg HR, Bartunek J, Hetzer R, Regitz-Zagrosek V: Neutral endopeptidase is activated in cardiomyocytes in human aortic valve stenosis and heart failure. *Circulation* 105:286–289, 2002
- Spector KS: Diabetic cardiomyopathy (Review Article). *Clin Cardiol* 21:885–887, 1998
- Ritchie RH, Marsh JD, Lancaster WD, Diglio CA, Schiebinger RJ: Bradykinin blocks angiotensin II-induced hypertrophy in the presence of endothelial cells. *Hypertension* 31:39–44, 1998
- Schunkert H, Sadoshima J, Cornelius T, Kagaya Y, Weinberg EO, Izumo S, Riegger G, Lorell BH: Angiotensin II-induced growth responses in isolated adult rat hearts: evidence for load-independent induction of cardiac protein synthesis by angiotensin II. *Circ Res* 76:489–497, 1995
- Grafe M, Bossaller C, Graf K, Auch-Schwelk W, Baumgarten CR, Hilde-



- brandt A, Fleck E: Effect of angiotensin-converting-enzyme inhibition on bradykinin metabolism by vascular endothelial cells. *Am J Physiol* 264: H1493–H1497, 1993
22. Winer J, Jung CK, Shackel I, Williams PM: Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* 270:41–49, 1999
  23. Ogawa Y, Tamura N, Chusho H, Nakao K: Brain natriuretic peptide appears to act locally as an antifibrotic factor in the heart. *Can J Physiol Pharmacol* 79:723–729, 2001
  24. Oliver PM, Fox JE, Kim R, Rockman HA, Kim HS, Reddick RL, Pandey KN, Milgram SL, Smithies O, Maeda N: Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc Natl Acad Sci* 94:14730–14735, 1997
  25. Sechi LA, Valentin JP, Griffin CA, Lee E, Bartoli E, Humphreys MH, Schambelan M: Receptors for atrial natriuretic peptide are decreased in the kidney of rats with streptozotocin-induced diabetes mellitus. *J Clin Invest* 95:2451–2457, 1995
  26. De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, Vanhoutte PM: Endothelial dysfunction in diabetes. *Br J Pharmacol* 130:963–974, 2000
  27. Gerds E, Svarstad E, Aanderud S, Myking OL, Lund-Johansen P, Omvik P: Factors influencing reduction in blood pressure and left ventricular mass in hypertensive type-1 diabetic patients using captopril or doxazosin for 6 months. *Am J Hypertens* 11:1178–1187, 1998
  28. Striffler JS, Bhathena SJ, Michaelis OE 4th, Campbell JD, Hansen CT, Scalbert E, Thibault N, Velasquez MT: Long-term effects of perindopril on metabolic parameters and the heart in the spontaneously hypertensive/NIH-corpulent rat with non-insulin-dependent diabetes mellitus and hypertension. *Metabolism* 47:1199–1204, 1998
  29. Al-Shafei AI, Wise RG, Gresham GA, Bronns G, Carpenter TA, Hall LD, Huang CL: Noninvasive magnetic resonance imaging assessment of myocardial changes and the effects of angiotensin-converting enzyme inhibition in diabetic rats. *J Physiol* 538:541–553, 2002
  30. Chen HH, Burnett JC Jr: C-type natriuretic peptide: the endothelial component of the natriuretic peptide system. *J Cardiovasc Pharmacol* 32 (Suppl. 3):S22–S28, 1998
  31. Tikkanen T, Tikkanen I, Rockell MD, Allen TJ, Johnston CI, Cooper ME, Burrell LM: Dual inhibition of neutral endopeptidase and angiotensin-converting enzyme in rats with hypertension and diabetes mellitus. *Hypertension* 32:778–785, 1998
  32. Walther T, Heringer-Walther S, Tschöpe R, Reinecke A, Schultheiss HP, Tschöpe C: Opposite regulation of brain and C-type natriuretic peptides in the streptozotocin-diabetic cardiopathy. *J Mol Endocrinol* 24:391–395, 2000
  33. Chen S, Evans T, Mukherjee K, Karmazyn M, Chakrabarti S: Diabetes-induced myocardial structural changes: role of endothelin-1 and its receptors. *J Mol Cell Cardiol* 32:1621–1629, 2000
  34. Ando S, Rahman MA, Butler GC, Senn BL, Floras JS: Comparison of candoxatril and atrial natriuretic factor in healthy men: effects on hemodynamics, sympathetic activity, heart rate variability, and endothelin. *Hypertension* 26:1160–1166, 1995
  35. Ferro CJ, Spratt JC, Haynes WG, Webb DJ: Inhibition of neutral endopeptidase causes vasoconstriction of human resistance vessels in vivo. *Circulation* 97:2323–2330, 1998
  36. Dumoulin MJ, Adam A, Rouleau JL, Lamontagne D: Comparison of a vasopeptidase inhibitor with neutral endopeptidase and angiotensin-converting enzyme inhibitors on bradykinin metabolism in the rat coronary bed. *J Cardiovasc Pharmacol* 37:359–366, 2001
  37. Kitashiro S, Sugiura T, Takayama Y, Tsuka Y, Izuoka T, Tokunaga S, Iwasaka T: Long-term administration of atrial natriuretic peptide in patients with acute heart failure. *J Cardiovasc Pharmacol* 33:948–952, 1999
  38. Colucci WS, Elkayam U, Horton DP, Abraham WT, Bourge RC, Johnson AD, Wagoner LE, Givertz MM, Liang CS, Neibaur M, Haught WH, LeJemtel TH: Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure: Nesiritide Study Group. *N Engl J Med* 343:246–253, 2000
  39. Mahmud A, Feely J: Favourable effects on arterial wave reflection and pulse pressure amplification of adding angiotensin II receptor blockade in resistant hypertension. *J Human Hypertens* 14:541–546, 2000
  40. Inguibert N, Coric P, Poras H, Meudal H, Teffot F, Fournie-Zaluski MC, Roques BP: Toward an optimal joint recognition of the S1' subsites of endothelin converting enzyme-1 (ECE-1), angiotensin converting enzyme (ACE), and neutral endopeptidase (NEP). *J Med Chem* 45:1477–1486, 2002