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Kinin B_2 receptor is not involved in enalapril-induced apoptosis and regression of hypertrophy in spontaneously hypertensive rat aorta: possible role of B_1 receptor

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1 Treatment with enalapril induces smooth muscle cell apoptosis and regression of aortic hypertrophy in spontaneously hypertensive rats (SHRs), whereas combined blockade of angiotensin II AT_1 and AT_2 receptors does not. We postulated that vascular apoptosis with enalapril involves enhanced half-life of bradykinin (BK) and kinin B_2 receptor stimulation.

2 SHR, 11-weeks old, were treated for 4 weeks with enalapril ($30\,mg\,kg^{-1}\,day^{-1}$), Hoe 140 ($500\,\mu g\,kg^{-1}\,day^{-1}$; B_2 receptor antagonist), alone or in combination. Controls received vehicle.

3 The half-life of hypotensive responses to intra-arterial bolus injections of BK were significantly increased in SHR anesthetized after 4 weeks of enalapril, an effect prevented by Hoe 140. The magnitude of BK-induced hypotension was significantly attenuated in all rats treated with Hoe 140.

4 As compared to placebo, enalapril treatment significantly reduced blood pressure ($-34\pm2\%$), aortic hypertrophy ($-20\pm3\%$), hyperplasia ($-37\pm5\%$) and DNA synthesis ($-61\pm8\%$), while it increased aortic DNA fragmentation by two-fold. Hoe 140 given alone or in combination with enalapril affected none of these parameters.

5 As a possible alternative mechanism, aortae isolated during the second week of enalapril treatment showed a transient upregulation of contractile responses to des-Arg[9]BK ($EC_{50}<1$ nM), which were significantly reduced by [Leu[8]]des-Arg[9]BK ($10\,\mu M$). Moreover, *in vitro* receptor autoradiography revealed an increase in expression of B_1 and B_2 receptor binding sites by 8–11 days of enalapril treatment.

6 Aortic apoptosis induction and hypertrophy regression with enalapril do not involve kinin B_2 receptors in SHR. Kinins acting *via* B_1 receptors remains a candidate mechanism.
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Keywords: Hypertension; vascular hypertrophy; apoptosis; enalapril; kinin B_2 receptor; kinin B_1 receptor

Abbreviations: ACE, angiotensin I-converting enzyme; ACEi, ACE inhibitor; AT_1, angiotensin II type 1 receptor; BK, bradykinin; dBK, des-Arg[9]-bradykinin; LVH, left-ventricular hypertrophy; SHRs, spontaneously hypertensive rats; SMCs, smooth muscle cells; WKY, Wistar-Kyoto

Introduction

The development of hypertension is accompanied by cardiovascular hypertrophy and by enhanced growth of cardiac fibroblasts and vascular smooth muscle cells (SMCs) (Folkow, 1982; Lee *et al*., 1995; Hamet *et al*., 2001). In a genetic model of primary hypertension with low renin but high tissue angiotensin I-converting enzyme (ACE) levels, the spontaneously hypertensive rat (SHR), dysregulation of cell proliferation and death contributes to increased cardiovascular mass and DNA content (Walter & Hamet, 1986; Hamet *et al*., 1996; Thorin-Trescases *et al*., 2001). We previously reported that a transient burst of SMC apoptosis participates in the early phase of aortic mass regression in SHR treated with a variety of antihypertensive drugs (deBlois *et al*., 1997; Tea *et al*., 2000; Marchand *et al*., 2003). In losartan-treated SHR, onset regression of vascular hypertrophy is blocked by the coadministration of the caspase inhibitor z-VAD-fmk, suggesting an obligatory role for SMC apoptosis in this model of rapid vascular mass regulation (Marchand *et al*., 2003). The transient increase in vascular SMC apoptosis follows a time window that occurs within 1 week of treatment with AT_1 receptor antagonists and around 2 weeks of treatment with an ACE inhibitor (ACEi) (deBlois *et al*., 1997; Tea *et al*., 2000). In contrast, blood pressure reduction follows a similar time course with both treatments. The different time course of apoptosis induction suggests that the underlying mechanisms differ for the two classes of drugs. We previously showed that regression of vascular mass and hyperplasia by valsartan is prevented completely by an antagonist of AT_2 receptors (PD123319), suggesting the implication of this receptor in SMC apoptosis induced by AT_1 receptor antagonists (Tea

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et al., 2000). Since ACEi reduces angiotensin II generation, this results in a reduced stimulation of AT_1 and AT_2 receptors. Since combined blockade of AT_1 and AT_2 receptors fails to induce vascular apoptosis in rats treated with valsartan + PD123319 for 2 weeks (Tea *et al*., 2000), these data further suggest a distinct pathway for ACEi and AT_1 antagonists in regulating SMC apoptosis in this model.

ACEi increases the half-life of kinins (Bhoola *et al*., 1992). Kinin B_2 receptors contribute to the prevention of cardiovascular growth by ACEi in several models (Linz & Schölkens, 1992; Farhy *et al*., 1993; Linz *et al*., 1995; Benetos *et al*., 1997), although the role of kinins in regression of cardiovascular mass is less well defined (Black *et al*., 1996). The augmentation of bradykinin (BK) in the circulating levels could lead to apoptosis *via* the increased stimulation of B_2 receptors and the subsequent increased release of nitric oxide, a known inducer of SMC apoptosis (Pollman *et al*., 1996; Ishigai *et al*., 1997). We therefore investigated the hypothesis that kinin B_2 receptors are involved in the regression of aortic and cardiac DNA accumulation *via* apoptosis induced in SHR treated with enalapril.

Methods

Animal procedure

Male SHR weighing 250–275 g were purchased from Charles-River (St-Constant, Canada) and housed for at least 1 week before initiation of drug treatment at 11 weeks of age. SHR ($n = 9$–10 per group) were randomly assigned to a 4-week treatment with the ACEi enalapril ($30\,mg\,kg^{-1}\,day^{-1}$) in drinking water, the selective kinin B_2 receptor antagonist Hoe 140 ($500\,\mu g\,kg^{-1}\,day^{-1}$), by continuous subcutaneous infusion with an 2ML4 osmotic minipump (Alzet, CA, U.S.A.) or a combination of both drugs. Hoe 140 was dissolved in saline. Control animals received vehicle. Age-matched Wistar-Kyoto (WKY) rats received vehicle and were used for the determination of vascular SMC number. Food and water were administered *ad libitum*. Systolic blood pressure and heart rate were determined by tail-cuff plethysmography in conscious, restrained rats at −1, 4 and 25 days of treatment, as previously described (deBlois *et al*., 1997). Rats were anesthetized with a single subcutaneous injection of ketamine ($80\,mg\,kg^{-1}$), xylazine ($4\,mg\,kg^{-1}$) and acepromazine ($2\,mg\,kg^{-1}$). To evaluate DNA synthesis *in vivo*, rats were injected intravenously with [^{3}H]-thymidine ($0.5\,mCi\,kg^{-1}$) 1.5 h before death (Tea *et al*., 1999). Rats were killed by an intravenous injection of 1.5 ml $CdCl_2$ ($100\,mmol\,l^{-1}$) *via* the vena cava to induce diastolic cardiac arrest. Ventricles were isolated, separated and weighed. The thoracic aorta was isolated as previously described (deBlois *et al*., 1997) and a 3-mm-long ring of aorta was cut between the third and fourth intercostal arteries, after weighing the vessel. The aortic rings were fixed in 4% paraformaldehyde overnight and processed according to routine histological procedures for morphometric measurements in cross sections of paraffin-embedded arteries. The cardiac tissue and aortic media (without endothelium) were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

To validate that chronic Hoe 140 treatment resulted in B_2 receptor blockade, we examined the magnitude and half-life (time of half-recovery from the maximal response) of the hypotensive response following an intra-arterial bolus injection of BK, as previously described (Drapeau *et al*., 1991). Before killing, catheters (PE-50) were placed in a carotid artery in a subset of anesthetized rats ($n = 4$–7 per group). Blood pressure was measured by a calibrated pressure transducer. When blood pressure stabilized, BK (0.01–1.00 μg in 0.1 ml saline) was administered intra-arterially and the hemodynamic response was recorded. All animal manipulations were conducted according to institutional guidelines.

Hypertrophy and hyperplasia measurements

The medial cross-sectional area was evaluated in 3-μm-thick, hematoxylin-stained sections of aorta. Photomicrographs were taken at ×40 magnification, digitized and analyzed using the NIH Image 1.61 program (developed at the National Institute of Health; http://rsb.info.nih.gov/nih-image/). Vascular cross sections were used to measure the SMC number per unit of length in the aortic media, using the three-dimensional disector method, as previously described (deBlois *et al*., 1997). The left and right ventricle weights to body weight ratio were evaluated separately.

DNA analysis

The whole aortic media was homogenized in liquid nitrogen using a mortar and pestle. An aliquot of the pulverized aortic media was weighed and total tissue DNA was extracted by the phenol and chloroform procedure, following tissue digestion steps with proteinase K and RNAse A in the presence of EDTA (Teiger *et al*., 1996). DNA concentration was determined by spectrophotometry and DNA content per unit of aortic length was calculated. DNA synthesis in the aortic media was quantified by evaluating [^{3}H]-thymidine incorporated using the extracted DNA. The degree of oligonucleosomal DNA fragmentation, a hallmark of apoptosis, in the aortic media was quantified as previously described (deBlois *et al*., 1997). Briefly, 1 μg of extracted DNA was radiolabeled on free 3′OH ends using terminal deoxynucleotidyl transferase and [^{32}P]-dCTP. Increasing quantities of radiolabeled DNA (0.05, 0.1, 0.2 and 0.4 μg) were loaded in adjacent lanes of 1.5% agarose gels. After electrophoresis, DNA was transferred onto nylon membrane (Hybond N+, Amersham) and exposed to a ^{32}P-sensitive screen. Radioactivity associated with 150–1500 bp DNA fragments (part of the 'DNA ladder') was quantified using a PhosphoImager (Molecular Dynamics). The mean optical density per pixel for each lane was divided by the corresponding value obtained with an internal standard in the gel (1 μg λDNA/*Hind*III fragments that underwent the same process). For each sample, the four values were plotted against the quantity of DNA loaded on the gel and the slope of the linear regression was defined as the 'DNA fragmentation index' (arbitrary units μg^{-1} DNA). The same procedures were performed on the homogenized left and right ventricles to determine their DNA content, DNA synthesis and DNA fragmentation. Rats that received the intra-arterial bolus injections of BK were not used for evaluation of DNA synthesis.

Vasoreactivity

To evaluate the possible involvement of kinin B_1 receptors in ACEi-induced SMC apoptosis, their function was evaluated in

11-weeks-old male SHR also purchased from Charles-River (St Constant, Canada). SHRs ($n=4$–7 per group) were treated or not with enalapril (30 mg kg^{-1} day^{-1}) in the drinking water for 8–14 days, a time period corresponding to SMC apoptosis induction with enalapril (deBlois *et al*., 1997). The animals were then killed by CO_2 inhalation and the aorta was immediately isolated, placed in ice-cold saline solution, and cleaned free of adventitial tissue. Vessels were cut into 3-mm-long rings and placed at 37°C in organ chambers filled with oxygenated Krebs solution containing (in mM): dextrose, 11; NaCl, 117.5; $MgSO_4$, 1.18; KH_2PO_4, 1.2; $NaHCO_3$, 25; KCl, 4.7; $CaCl_2$, 2.5. Isometric contractions and relaxations were measured with the use of isometric force transducers (Harvard Apparatus Canada), a digital data-acquisition system (Model MP100, Biopac System, Harvard). The arterial rings were placed under 2 *g* of tension and allowed to stabilize for 1 h before control stimulation with KCl (70 mM). After a 30 min recuperation period, acetylcholine (1 μM) was added to phenylephrine (100 nM)-precontracted vessels to verify the endothelial integrity. After a 1 h recuperation period, enalapril (0.1 mM) was added to prevent agonist degradation and the rings were stimulated with cumulative concentrations of des-Arg^9-bradykinin (dBK; 1–1000 nM) in the absence or presence of the B_1 antagonist [Leu^8]dBK (10 μM). In a subset of tissues, responses were recorded in the presence of indomethacine (100 μM – stock solution prepared in Na_2CO_3 0.1 mM). Vasoactive responses were expressed in mg.

Tissue preparation for autoradiography

Immediately after sacrifice, aortae were removed and a segment (with endothelium) was frozen in 2-methyl butane cooled at −45 to −55°C with liquid nitrogen, and then stored at −80°C until use. Matched aorta segments of 10 rats (four untreated SHR and six SHR treated for 8–11 days with enalapril) were mounted together in a gelatine block and serially cut into 20-μm-thick coronal sections with a cryostat fixed at a temperature varying between −10 and −13°C. A total of six sections per slide were then alternatively thaw-mounted on 0.2% gelatine/0.033% chromium potassium sulphate-coated slides. Two slides were taken for the total binding and one slide (adjacent sections) for the nonspecific binding. A total of nine slides were produced for each receptor per animal, giving 180 sections for all the study. Slides were then kept at −80°C until use.

In vitro *receptor autoradiography*

Mono-iodinated peptide radioligands were prepared as described previously (Cloutier *et al*., 2002; Ongali *et al*., 2003) and had a specific activity corresponding to 2000 c.p.m. fmol^{-1} or 1212 Ci mmol^{-1}. Aortic sections were thawed and incubated at room temperature for 90 min in 25 mM PIPES buffer (pH 7.4; 4°C) containing 1 mM 1,10-phenanthroline, 1 mM dithiothreitol, 0.014% bacitracin, 0.1 mM captopril, 0.2% bovine serum albumin (protease free) and 7.5 mM magnesium chloride in the presence of 150 pM of [^{125}I]HPP-Aca-Lys[β-D-Nal^7, Ile^8]des-Arg^{9}BK (for B_1 receptor) or 200 pM [^{125}I]HPP-Hoe 140 (for B_2 receptor). The concentration chosen for [^{125}I]HPP-Hoe 140 was based on previous studies (Cloutier *et al*., 2002), while that of [^{125}I]HPP-Aca-Lys[β-D-Nal^7,Ile^8]des-Arg^{9}BK was based on preliminary experiments that provided maximal specific binding (Bmax) at 150 pM, with a dissociation constant (K_d) of 54 pM (unpublished data). The nonspecific binding was determined in the presence of 1 μM of unlabelled ligands HPP-Aca-Lys[β-D-Nal^7, Ile^8]des Arg^{9}BK for B_1 receptor and HPP-Hoe 140 for the B_2 receptor. At the end of the incubation period, slides were transferred sequentially through four rinses of 4 min each in 25 mM PIPES (pH 7.4; 4°C) and dipped for 15 s in distilled water (4°C) to remove the excess of salts, and air-dried. Kodak Scientific Imaging Films BIOMAX TM MS® were juxtaposed onto the slides in the presence of [^{125}I]-microscales and exposed at room temperature for 2 days for both radioligands.

The films were developed in D-19 (Kodak developer) and fixed in Kodak Ektaflo. Autoradiograms were quantified by densitometry using an image analysis system (MCID™, Imaging Research Inc., Ontario, Canada). A standard curve from [^{125}I]-microscales was used to convert density levels into femtomoles per milligram of tissue (fmol mg^{-1} tissue). Specific binding was determined by subtracting values of nonspecific labelling from that of total binding.

Drugs and materials

Hoe 140 was graciously supplied by Aventis Pharma (Montreal, Canada). Ketamine and acepromazine were from Ayerst (Montreal, Canada). Xylazine was from Bayer (Etobicoke, Canada) and [^{3}H]-thymidine was from ICN (CA, U.S.A.). HPP-Aca-Lys[β-D-Nal^7,Ile^8]des-Arg^{9}BK (3–4 hydroxyphenyl-propionyl-Aca-Lys[β-D-Nal^7,Ile^8]des-Arg^{9}BK) and HPP-Hoe 140 (3–4 hydroxyphenyl-propionyl-D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK) were developed from the selective B_1 receptor antagonists R715 or Aca-Lys[β-D-Nal^7,Ile^8]des-Arg^{9}BK (Neugebauer *et al*., 2002) and the B_2 receptor antagonist Hoe 140 or Icatibant (Hock *et al*., 1991), respectively. They were kindly supplied by Dr W. Neugebauer (Department of Pharmacology, Université de Sherbrooke, Sherbrooke, Canada). Autoradiographic [^{125}I]-microscales (20 μm) and Kodak Scientific Imaging Films BIOMAX TM MS® (double-coated, 24×30 cm^2) were purchased from Amersham Pharmacia Biotech, Canada. All other chemicals were purchased from Sigma-Aldrich, Canada.

Statistical analysis

Data were analyzed by analysis of variance and unpaired Student's *t*-test with Bonferroni correction for multiple comparisons when appropriate. DNA synthesis in aorta was analyzed using the nonparametric tests of Kruskal–Wallis followed by Mann-Whitney, because of unequal variance between the groups. Values are presented as mean ± standard error of the mean (s.e.m.) and $P<0.05$ was considered statistically significant.

Results

Systemic and hemodynamic changes with therapy

The mean body weight of rats before treatment was 260 ± 2 g ($n=38$). All rats gained weight during the experimental period and the final values of body weight were similar between all groups (not shown). Daily water consumption significantly

increased by 35% in rats treated with enalapril or enalapril + Hoe 140. Rats used in this study were hypertensive before initiation of treatments, with an average systolic blood pressure of 183 ± 2 mmHg ($n = 38$). Figure 1 shows the evolution of systolic blood pressure in control and treated rats over the 4-week experimental period. A significant reduction ($P < 0.0001$) in systolic blood pressure was observed after 4 days of treatment in groups treated with enalapril with or without Hoe 140. At the end of the treatment with enalapril, systolic blood pressure was reduced by 34% vs control group (207 ± 4 mmHg, $P < 0.0001$). Coadministration of Hoe 140 had no effect on blood pressure lowering induced by enalapril. Heart rate was not different between control and experimental groups before and after treatment (control pretreatment values: 370 ± 8 bpm; $n = 9$). In order to validate the effective blockade of kinin B_2 receptors, a subset of rats were canulated *via* the carotid artery, as described in Methods. Figure 2a shows the dose–response relationship between BK and the magnitude of the hypotensive response. Administration of 0.01 and 0.10 μg BK intraarterially after the 4-week therapy induced a significantly smaller hypotension in both groups receiving Hoe 140 as compared to control and enalapril groups. As seen in Figure 2b, enalapril treatment induced a significantly longer half-life for BK-induced hypotensive responses vs control, and this effect was abolished by the cotreatment with Hoe 140 at doses of 0.01, 0.10 and 1.00 μg of BK.

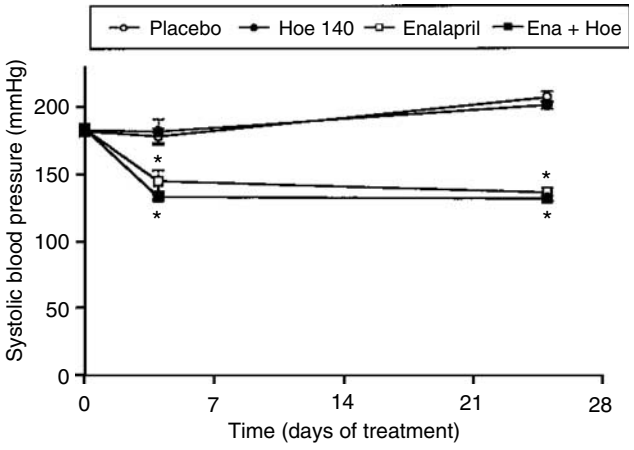

Figure 1 Systolic blood pressure regulation over the 4-week experimental period. The graph represents arterial systolic blood pressure of SHR treated with a placebo, enalapril, Hoe 140 or a combination of both treatments, and measured by plethysmography (mean ± s.e.m.; $n = 9$–10 per group). *Significantly different ($P < 0.05$) from placebo group.

Vascular remodeling

After 4 weeks of treatment, vascular mass was significantly reduced in enalapril-treated rats, as evaluated by the aortic cross-sectional area (20% reduction vs control: $521 \pm 12\ \mu m^2 \times 10^3$, $P < 0.0001$, Figure 3a). The vascular growth regression was further analyzed by evaluating aortic DNA content per length of vessel. Enalapril reduced aortic DNA content (37% reduction vs control: $1.65 \pm 0.15\ \mu g\ mm^{-1}$, $P = 0.003$). SMC number in the arterial wall was evaluated by the three-dimensional disector method (Figure 3b). The number of SMC per unit of vessel length in control SHR (114 ± 6 SMC μm^{-1}) was similar to previously published results (Owens, 1987; Tea *et al*., 2000). Control SHR showed SMC hyperplasia in comparison to the value obtained in age-matched WKY (85 ± 6 SMC μm^{-1}, $P < 0.0001$). In SHR treated with enalapril, this value was reduced to 72 ± 4 SMC μm^{-1} ($P < 0.0001$ vs untreated SHR). To test whether kinins play a role in enalapril-induced SMC apoptosis, we evaluated the DNA fragmentation index as described in Methods. A sustained elevation of fragmentation in DNA extracted from thoracic aorta was observed with enalapril when compared to untreated rats (Figures 3c and 4). Treatment with enalapril caused a significant reduction in DNA-specific activity in thoracic aorta, as shown in Figure 3d (61% reduction vs control: 406 ± 38 c.p.m. 100 $\mu g\ DNA^{-1}$, $P = 0.001$). The co-administration of Hoe 140 did not modulate any of these actions of enalapril, and Hoe 140 given alone affected none of these parameters (aortic cross-sectional area, SMC number, DNA content, DNA fragmentation and DNA synthesis).

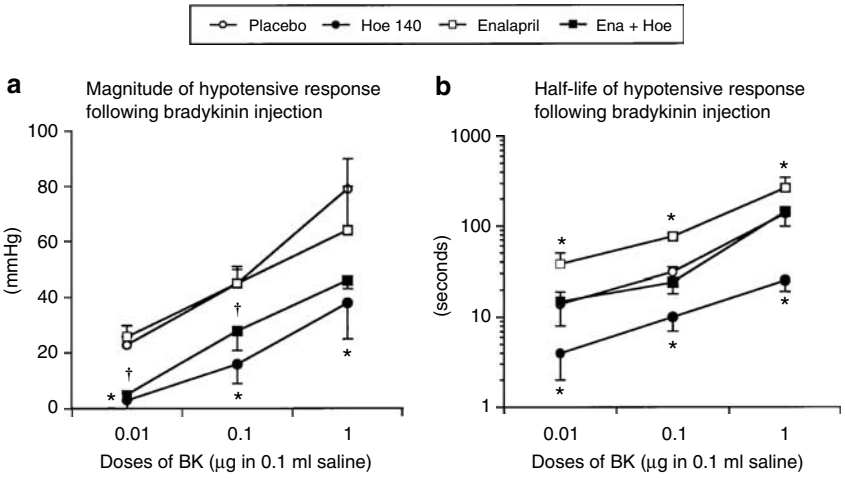

Figure 2 Blockade of kinin B_2 receptors in anesthetized rat after 4 weeks of treatment. Magnitude (a) and half-life (b) of the hypotension induced by various doses (0.01, 0.10 and 1.0 μg dissolved in 0.1 ml saline) of intra-arterial BK bolus injection. Mean ± s.e.m.; $n = 4$–7 per group. *Significantly different ($P < 0.05$) from placebo group. †Significantly different ($P < 0.05$) from enalapril group.

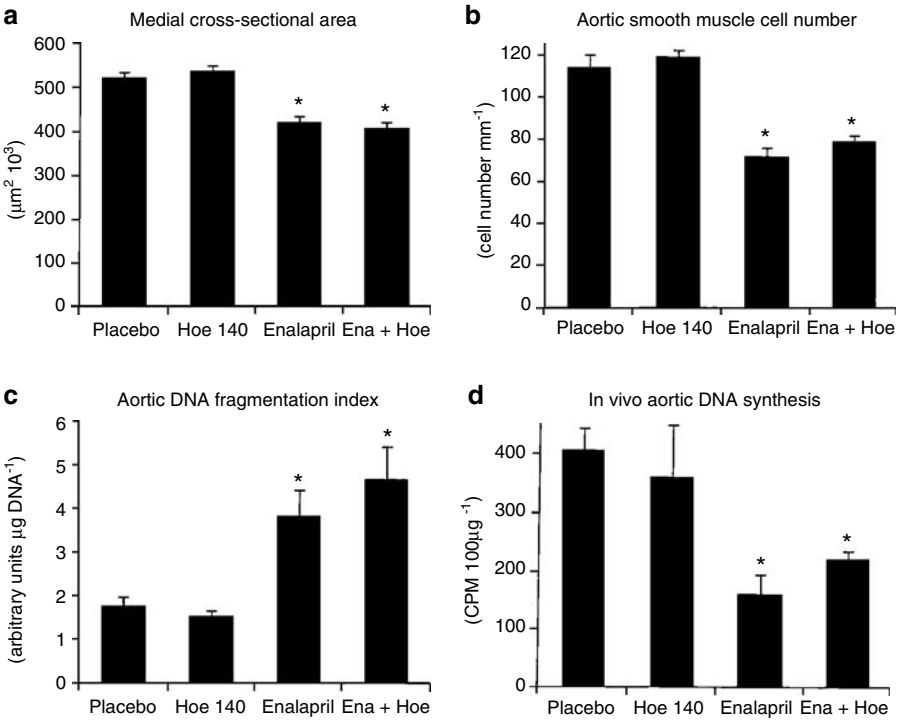

Figure 3 Enalapril induces aortic hypertrophy regression and SMC apoptosis even in the presence of a kinin B_2 receptor antagonist. (a) Aortic medial mass, indicative of aortic hypertrophy, evaluated quantitatively by image analysis of the vessel medial cross-sectional area (CSA). (b) Aortic hyperplasia evaluated using the three-dimensional disector method and presented as SMC number per unit length. WKY rats showed 85 ± 6 SMC μm^{-1}, $P < 0.0001$ vs control SHR. (c) DNA fragmentation index was obtained after quantification of DNA ladders as those shown in Figure 4. (d) Aortic SMC DNA synthesis. Mean $\pm$ s.e.m.; $n = 9$–10 per group. $n = 6$ per group for DNA synthesis, see Methods. *Significantly different ($P < 0.05$) from the placebo group.

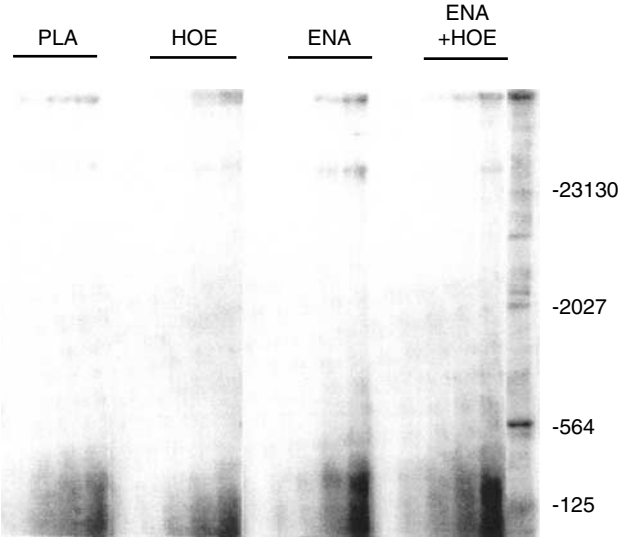

Figure 4 Enalapril induces aortic DNA fragmentation even with the coadministration of Hoe 140. DNA ladders were obtained with DNA extracted from the aorta of SHR treated for 4 weeks with placebo (PLA), enalapril alone (ENA), Hoe 140 alone (Hoe) or a combination of both treatments (ENA + Hoe). DNA free 3′OH ends were nick end labeled with dUTP-[P^{32}] using terminal deoxynucleotidyl transferase. Increasing amounts of DNA (0.05–0.4 μg) were fractionated by electrophoresis on 1.5% agarose gel.

Functional and expression evidence of kinin B_1 receptor induction

We evaluated the functional response of SHR aorta when stimulated with a kinin B_1 receptor agonist (dBK) after 0–14 days of enalapril treatment. dBK elicited a contraction response significantly higher after 8–9 days of treatment compared to control animals, and it was back to control values after 14 days of therapy (Figure 5a). dBK-induced contractions were significantly reduced in the presence of [Leu^8]dBK (10 μM), a competitive B_1 receptor antagonist (Figure 5b), but were not affected by the presence of indomethacine (not shown). Autoradiograms show the distribution of total and nonspecific B_1 and B_2 receptor-binding sites in the wall of aortae from SHR and SHR treated for 8–11 days with enalapril (Figure 6a). The densities of specific B_1 and B_2 receptor-binding sites were, respectively, 1.51 ± 0.08 and 1.06 ± 0.37 fmol mg^{-1} tissue in the aorta of untreated SHR. These values were significantly increased for both B_1 (1.5-fold, $P < 0.01$) and B_2 receptors (2.3-fold, $P < 0.01$) in the aorta of SHR treated with enalapril for 8–11 days (Figure 6a, b).

Cardiac remodeling

Parameters evaluated in vascular tissue were also evaluated in cardiac tissue to gain insight into the role of kinin B_2 receptors in enalapril-induced cardiac hypertrophy regression. As shown in Table 1, enalapril treatment induced a significant 25% reduction in left-ventricular mass. This was accompanied by a significant reduction in left-ventricular DNA content. After 4 weeks of therapy, no difference was seen in left-ventricular DNA fragmentation index between any groups. However, DNA synthesis, evaluated by *in vivo* [^{3}H]-thymidine incorporation in the left ventricle, was significantly reduced by 28% within the group treated with enalapril. Hoe 140 given alone or in combination with enalapril affected none of these measures.

The right ventricle showed no changes with treatment in any of the parameters evaluated (Table 1).

Discussion

The kallikrein–kinin system is underexpressed in various forms of hypertension (Carretero *et al*., 1993). Many beneficial effects of therapy with an ACEi have been linked directly to the elevation of kinins circulating levels (Gohlke *et al*., 1997; Heusch *et al*., 1997). Moreover, inhibition of cell growth by kinins involves endothelial NO production and subsequent generation of cyclic GMP in target cells (Pollman *et al*., 1996; Ishigai *et al*., 1997; Ritchie *et al*., 1998). Several lines of evidence suggest, however, that the implication of kinins in the antihypertrophic actions of ACEi depends on the experimental model. In normotensive rats submitted to arterial injury, inhibition of neointima formation by enalapril is reversed by

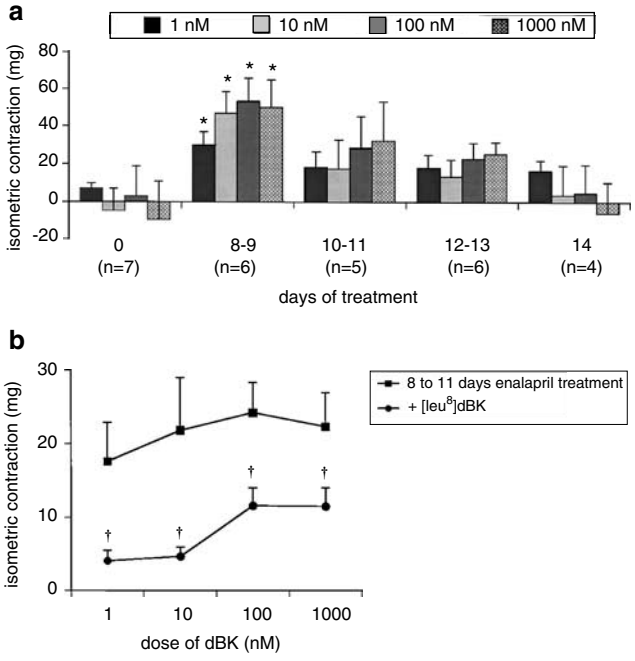

Figure 5 Enalapril treatment transiently increased aortic contraction elicited by a kinin B_1 receptor agonist. (a) Isometric contractions elicited by increasing doses of dBK are expressed in mg. Aortic strips were isolated from SHR after the different time period indicated. (b) Vasoactive responses to dBK between 8 and 13 days ($n=9$) were significantly reduced by the addition of Leu^8-dBK before stimulation. *Significantly different ($P<0.05$) from untreated rats. †Significantly different ($P<0.05$) from responses observed in the absence of leucine^8-dBK.

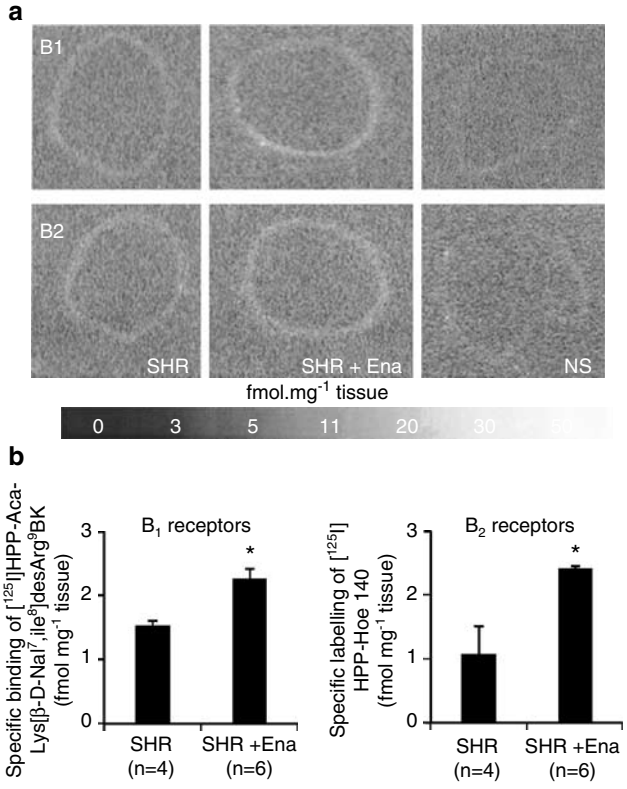

Figure 6 Increased expression of kinin B_1 and B_2 receptors after enalapril treatment. (a) Autoradiograms showing total binding sites of [^{125}I]HPP-Aca-Lys[β-D-Nal^7,Ile^8]des-Arg^{9}BK (for B_1 receptor) and [^{125}I]HPP-Hoe 140 (for B_2 receptor) in aorta sections taken from control SHR or SHR treated with enalapril for 8–11 days. Nonspecific binding (NS) in the presence of 1 μM unlabelled radioligand is shown in the right panels. (b) Quantification of the specific binding sites in the aortae of SHR and SHR treated with enalapril for 8–11 days. *Significantly different ($P<0.01$) from SHR.

Table 1 Cardiac hypertrophy following SHR treatment with enalapril, Hoe 140 or a combination of both drugs for 4 weeks

	Treatment (n)			
	Placebo (9)	*Hoe 140 (10)*	*Enalapril (9)*	*Ena+Hoe (10)*
Left ventricle				
Left-ventricular: body weight ratio (mg g^{-1} 10^{-3})	2.64±0.04	2.58±0.05	1.97±0.06*	1.93±0.06*
DNA content μg g^{-1} BW^{-1}	4.30±0.15	4.42±0.15	3.08±0.35*	3.58±0.26*
DNA synthesis (CPM 100 μg^{-1})	1071±138	989±71	774±93*	654±74*
DNA fragmentation index arbitrary units μg^{-1} DNA^{-1}	1.68±0.26	1.59±0.25	1.38±0.19	1.61±0.29
Right ventricle				
Right-ventricular: body weight ratio (mg g^{-1} 10^{-3})	0.51±0.04	0.51±0.04	0.50±0.05	0.50±0.06
DNA content μg g^{-1} BW^{-1}	0.73±0.07	0.75±0.08	0.71±0.08	0.77±0.08
DNA synthesis (CPM 100 μg^{-1})	645±117	600±98	604±97	567±90
DNA fragmentation index arbitrary units μg^{-1} DNA^{-1}	ND	ND	ND	ND

Values are mean±s.e.m. ND: not determined. $n=6$ for DNA synthesis, see Methods. *Significantly different ($P<0.05$) from placebo group.

Hoe 140 (Farhy *et al*., 1993). In young SHR, prevention of aortic hypertrophy development by quinapril is blocked by Hoe 140 (Benetos *et al*., 1997). In rats with aortic banding, prevention of left-ventricular hypertrophy (LVH) development by an ACEi was blocked by Hoe 140 (Linz & Schölkens, 1992). However, in the same model, B_2 receptor antagonism could not block the regression of LVH induced by an ACEi. Likewise, in adult SHR, regression of LVH by perindopril was not blocked by Hoe 140 (Black *et al*., 1996). Kinins, therefore, seem more involved in the antihypertrophic actions of ACEi during prevention than regression of structural modifications.

One hypothesis for the induction of apoptosis with ACEi is the decrease in angiotensin II receptor stimulation due to inhibition of angiotensin II formation. We recently reported, however, that combined blockade of AT1 and AT2 receptors does not induce apoptosis in the SHR aorta (Tea *et al*., 2000), suggesting an alternative mechanism possibly *via* increased kinin half-life and receptor stimulation. A significant new finding of the present study is that SMC apoptosis and regression of vascular hypertrophy induced within 4 weeks of enalapril treatment in SHR are not dependent on kinin B_2 receptor activation. Transient induction of aortic SMC apoptosis by enalapril occurs around the second week of treatment (deBlois *et al*., 1997; Tea *et al*., 2000). In order to evaluate the cumulative effect on SMC deletion, we examined the SHR aorta after 4 weeks of treatment. The depletion of medial SMC and the elevated internucleosomal DNA fragmentation index in the aorta suggest effective induction of SMC apoptosis within the first 4 weeks of treatment with enalapril with or without Hoe 140. To validate the effective blockade of kinin B_2 receptors, we examined BK-induced hypotensive effects in a subset of rats anesthetized at the end of the 4-week treatment period. As compared to control rats, the amplitude of the hypotensive response to an intra-arterial BK bolus was significantly attenuated in all rats treated with Hoe 140. Moreover, the half-life of the hypotensive response to BK showed a significant increase in rats treated with enalapril, and this effect was completely prevented in rats cotreated with Hoe 140, therefore validating the effectiveness of Hoe 140.

[^{3}H]-thymidine incorporation into DNA, an indicator of DNA synthesis *in vivo*, was also reduced by enalapril, independently of the presence of the B_2 receptor antagonist. Attenuation of [^{3}H]-thymidine incorporation can be interpreted as an inhibition of DNA synthesis, since we previously showed that aortic tissue uptake of [^{3}H]-thymidine is unaffected by enalapril (deBlois *et al*., 1997). Hoe 140 alone did not affect SMC DNA synthesis. Together, these results suggest that B_2 receptors are not involved in aortic growth regulation within a 4-week period in adult SHR. We reported that SMC in the aorta of neonatal SHR exhibit an abnormally high turnover with a dysregulation of cell death, leading to increased aortic DNA content. Treatment with enalapril normalizes SMC turnover mainly *via* the inhibition of SMC growth (Thorin-Trescases *et al*., 2001).

Several studies showed that kinins are involved in the reduction of blood pressure by ACEi in renovascular models of hypertension (e.g. aortic banding, two-kidney 1-clip rat), but not in genetic models (Bao *et al*., 1992; Zhu *et al*., 1997; Sharma *et al*., 1998). In the present model, the current evidence suggests a strong pressure-independent component in the induction of SMC apoptosis during onset regression of aortic mass by various antihypertensive agents (deBlois *et al*., 1997; Tea *et al*., 2000). It is therefore unlikely that failure of Hoe 140 to prevent SMC apoptosis is related to its lack of effect on enalapril antihypertensive actions.

We recently showed that a 4-week treatment with either valsartan or enalapril reduced LVH and abolished fibroblast hyperplasia without affecting cardiomyocyte number in SHR (Der Sarkissian *et al*., 2003). Transient fibroblast apoptosis with either AT_1 antagonist or ACEi was not affected by AT_2 receptor blockade (Der Sarkissian, S., unpublished observation). This suggests a possible role for a downregulation of AT_1 receptor signaling in cardiac apoptosis induction in SHR treated with renin–angiotensin system antagonists, unlike in aortic SMC. Since enalapril had no effect on cardiac remodeling in the right ventricle but reduced hypertrophy in the left ventricle, these effects could include a pressure-dependent component. The observation of a reduced left-ventricular DNA content and DNA synthesis after 4 weeks of enalapril treatment with no apparent DNA fragmentation at this time point suggests that a transient cardiac apoptosis occurred earlier in the treatment period.

The mechanisms underlying the proapoptotic effect of enalapril in the SHR aorta remain elusive. Although constitutive B_2 receptors are primarily involved in mediating the actions of kinin under physiological conditions (Bhoola *et al*., 1992), it is possible that changes evaluated here result from the stimulation of the inducible B_1 receptor for kinins. Expression of kinin B_1 receptor can be induced by various inflammatory stimuli (Marceau *et al*., 1998). Upregulation of B_1 receptor expression occurs in the brain (Emanueli *et al*., 1999) and hypothalamus (Qadri *et al*., 2002) in SHR with established hypertension. In rabbits, Marceau *et al*. (1999) reported that treatment of rabbits with an ACEi does not regulate B_1 receptors. In normotensive rats, however, convincing evidence for the induction of functional renal B_1 receptors during ACEi treatment has been reported recently (Marin-Castano *et al*., 2002). We recently reported an upregulation of B_1 receptors in the lamina I of the thoracic spinal cord after 4 weeks of therapy with an ACEi, and a marked decrease after 8 and 16 weeks of therapy (Ongali *et al*., 2003). Thus, the induction of B_1 receptors with ACEi treatment is controversial and may depend on the species or duration of treatment. Consistent with this, our functional evidence suggest that sensitization to a B_1 receptor agonist gradually decreases to baseline values by 14 days of enalapril treatment in the SHR aorta. The time course of B_1 receptor sensitization is compatible with the previous observation that SMC apoptosis is triggered between 7 and 14 days of enalapril treatment (deBlois *et al*., 1997; Tea *et al*., 2000). One group reported the direct stimulation of B_1 receptor by enalaprilat (Ignjatovic *et al*., 2002), while this observation could not be reproduced by others (Fortin *et al*., 2003). Kinin B_1 receptor mediates inhibition of neointima formation after balloon angioplasty in rat artery (Agata *et al*., 2000) and inhibits platelet-derived growth factor-stimulated mitogenesis in cultured SMC (Dixon & Dennis, 1997). A molecular mechanism for B_1 receptor-mediated apoptosis remains to be defined, but may be analogous to the recently described inhibition of cell growth by B_2 receptor *via* release of NO (Dhaunsi *et al*., 1997) or the stimulated activity of protein tyrosine phosphatase (Cellier *et al*., 2003). Alternatively, SMC apoptosis induction by enalapril may involve *N*-acetyl-seryl-

aspartyl-lysyl-proline (Ac-SDKP), a natural inhibitor of pluripotent hematopoietic stem cell proliferation, which is hydrolyzed exclusively by ACE and which inhibits the proliferation of cardiac fibroblasts *in vivo* and *in vitro* (Rhaleb *et al*., 2001a, b).

In summary, we used five complementary parameters to rule out the implication of the kinin B_2 receptor in SMC apoptosis induction and vascular hyperplasia regression induced by enalapril in SHR. In contrast, preliminary data identify the induction of B_1 receptors as a candidate mechanism. Further studies will be required to assess the molecular mechanisms of this SMC apoptosis induction participating in hypertrophy regression.

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