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Synergistic interaction between enalapril, L-arginine and tetrahydrobiopterin in smooth muscle cell apoptosis and aortic remodeling induction in SHR

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1 Smooth muscle cell (SMC) apoptosis occurs at the onset of enalapril-induced regression of aortic hypertrophy in SHR. A potential mechanism is the correction of endothelial dysfunction (ED) leading to reduced production of reactive oxygen species and enhanced bioavailability of nitric oxide (NO), a potent apoptosis inducer. Stimulants of NO include the precursor L-arginine and the NO synthase cofactor tetrahydrobiopterin (BH_4), which correct ED in several models.

2 The objective was to examine the relationships between ED and the cell growth/death balance during vascular remodeling induced by enalapril in SHR.

3 SHR, 10-week-old, received enalapril (ENA: 30 mg.kg^{-1}.day^{-1} p.o.) for 1 or 2 weeks, or a co-treatment of L-arginine (2.0 g.kg^{-1}.day^{-1} p.o.) and BH_4 (5.4 mg.kg^{-1}.day^{-1} i.p. twice daily) administered alone (group: LB) or in combination with enalapril (ENA + LB) for 1 week. Controls received vehicle.

4 After 1 week, ED was completely corrected with LB but not affected significantly by ENA, whereas both treatments failed to induce SMC apoptosis or aortic remodeling. The correction of ED and the induction of SMC apoptosis (3.3-fold increase in TUNEL labeling) required 2 weeks of ENA treatment. The combination of LB with ENA for 1 week, however, was additive for the reduction of SMC proliferation, and synergistic for the induction of apoptosis and regression of vascular hypertrophy. These interactions were independent of blood pressure regulation.

5 Our results suggest that the correction of ED is not sufficient to induce SMC apoptosis and vascular remodeling, although it facilitates these responses during enalapril treatment.
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Abbreviations: BH_4, tetrahydrobiopterin; ED, endothelial dysfunction; NO, nitric oxide; SHR, spontaneously hypertensive rat; SMC, smooth muscle cell

Introduction

Hypertension is associated with endothelial dysfunction (ED), which is characterized by an increased production of reactive oxygen species (ROS) and a decreased bioavailability and output of nitric oxide (NO) by the NO synthase (NOS) enzyme. This impairs vasorelaxation (Tschudi *et al*., 1996; Crabos *et al*., 1997; Boulanger, 1999) and more importantly, in the present context, vascular mass regulation by NO.

Tetrahydrobiopterin (BH_4), an essential cofactor of NOS, plays a key role in endothelial homeostasis (Heitzer *et al*., 2000; Ueda *et al*., 2000) and in the generation of NO (Nathan, 1992). Other than direct antioxidant mechanisms attributed to BH_4 (Verhaar *et al*., 1998), the administration of this cofactor normalizes eNOS function in spontaneously hypertensive rat (SHR) vasculature, whereas inhibition of its synthesis induces endothelial NOS (eNOS) dysfunction in isolated vessels (Cosentino & Katusic, 1995; Cosentino *et al*., 1998; Cosentino & Luscher, 1999; Katusic, 2001; Yang *et al*., 2003).

The SHR is a model of primary hypertension in which an imbalance between cell growth and apoptosis favors increased cardiovascular mass and DNA content, which represent underlying causes of pathologic remodeling in neonatal and adult animals (Walter & Hamet, 1986; Hamet, 1995; Moreau *et al*., 1997). Altered cardiac tissue ACE activity (Diez *et al*., 1997) and ED (Cosentino *et al*., 1998) also represent common features of this model.

According to several studies, aortic and cardiac eNOS dysfunction in SHR is corrected following long-term treatment with certain classes of antihypertensive drugs such as angiotensin pathway blockers (Rubanyi *et al*., 1993; Tschudi *et al*., 1994; Linz *et al*., 1999), but not some vasodilators (Clozel *et al*., 1990). Interestingly, our group has previously reported that ACE inhibitors, AT_1 antagonists, but not hydralazine, induce a transient wave of apoptosis in cardiac fibroblasts and aortic SMC leading to cardiovascular hypertrophy regression in SHR (deBlois *et al*., 1997; Tea *et al*., 1999; 2000; Der Sarkissian *et al*., 2003). It therefore appears that antihypertensive drugs known to normalize endothelial function also share the ability to induce apoptosis during

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regression of cardiovascular hypertrophy. Moreover, *in vitro* studies have demonstrated that ROS such as superoxide (O_2^-) increase cell growth or survival in SMC and fibroblasts (Li *et al*., 1997; 1999), whereas NO elicits the opposite effect by inducing apoptosis in these cells via cGMP-dependent and -independent pathways (Garg & Hassid, 1989; Fukuo *et al*., 1996; Shin *et al*., 1996; Khan *et al*., 1997). Overall, this suggests that the balance between ROS and NO, an indicator of endothelial functionality, constitutes a key determinant in the control of cell fate.

Based on these observations, we postulated that normalization of ED may be a key determinant of apoptosis regulation and cardiovascular DNA content in the ACE inhibitor enalapril treated SHR. To promote endothelial function, we have designed a therapy in which L-arginine, the precursor molecule of NO, and BH_4 are supplemented. Our results demonstrate that the correction of endothelial dysfunction in the SHR is permissive to apoptosis stimulation and provides a precocious and synergistic interaction with enalapril in regulating SMC apoptosis leading to vascular remodeling.

Methods

Animal procedures

In all, eighty-seven 10-week-old male SHR were purchased (Charles-River, St-Constant, QC, Canada) and housed for at least 10 days before the initiation of the study. Food and water were administered *ad libitum*.

Since our preliminary data indicated that treatment with L-arginine or BH_4 administered alone for 1 week failed to modulate vascular growth, apoptosis or remodeling in animals treated with placebo or enalapril, we postulated that their combination may be more efficient. Thus, the co-administration of L-arginine and BH_4 termed 'NO-enhancing therapy' was examined alone or in combination with enalapril. Rats ($n = 5$–11 per group) were treated for 1 or 2 weeks with the ACE inhibitor enalapril (ENA: 30 mg.kg^{-1}.day^{-1} p.o.; Sigma Chemicals, St Louis, MO, U.S.A.), the NO-enhancing therapy (LB): L-arginine (2 g.kg^{-1}.day^{-1} p.o., Sigma Chemicals, St Louis, MO, U.S.A.) + (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4: cumulative dose 10.8 mg.kg^{-1}.day^{-1}; Schircks Laboratories, Jona, Switzerland) dissolved in saline and given fractionally in two i.p. injections per day, or a combination of both treatments (ENA + LB). Control rats received vehicle according to the same administration schedule as treated animals. Moreover, subsets of SHR were treated with L-arginine and BH_4 separately. Systolic blood pressure was determined in conscious restrained rats by the tail-cuff method as we described previously (deBlois *et al*., 1997). Rats were anesthetized with a single i.m. injection of a mixture of ketamine (80 mg.kg^{-1}; MTC Pharmaceuticals, Cambridge, ON, Canada), xylazine (4 mg.kg^{-1}; Bayer, Etobicoke, ON, Canada), and acepromazine (2 mg.kg^{-1}; Ayerst, Montreal, QC, Canada). In order to evaluate vascular DNA synthesis *in vivo*, a subgroup of rats received a single i.v. bolus of [^{3}H]-thymidine (0.5 mCi.kg^{-1}; New England Nuclear, Mississaugua, ON, Canada) after induction of anesthesia at 1.5 h prior to sacrifice. Death was induced by exsanguination by draining *via* the jugular vein. The thoracic aorta was isolated, cleaned of adherent tissue and a vascular segment (3 mm) between the third and fourth intercostal arteries was fixed in 4% paraformaldehyde and later paraffin-embedded for *in situ* histological studies. In a subgroup of rats, a segment was placed in cold Krebs solution for immediate vasoreactivity studies. The rest of the aortic media was denuded of endothelium, snap-frozen and pulverized in liquid nitrogen and stored at −80°C until further processing for DNA internucleosomal fragmentation (hallmark feature of apoptosis), synthesis and content measurements, as described previously (deBlois *et al*., 1997). All animal manipulations were conducted according to institutional guidelines.

Vasoreactivity

Freshly isolated aortic rings were placed in organ chambers filled with oxygenated (95% O_2 – 5% CO_2) 37°C Krebs solution (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 were measured using isometric force transducers (Harvard Apparatus, Montreal, QC, Canada), and a computerized data-acquisition system (Biopac System, Harvard Apparatus, Montreal, QC, Canada). Tissues were subjected to $1.0 \times g$ of tension and instrinsic contractility was assessed by stimulating tissues with KCl (70 mM) as previously established (Lemay *et al*., 2000a, b). Indomethacin (10 μM; Sigma Chemicals, St Louis, MO, U.S.A.) was added in order to block vasoactive prostaglandins. At 5 min prior to assay, segments of aortic rings from each rat were incubated with catalase (1200 units.ml^{-1}; Roche, Indianapolis, IN, U.S.A.) or superoxide dismutase (SOD: 150 units.ml^{-1}; Sigma Chemicals, St Louis, MO, U.S.A.) for hydrogen peroxide (H_2O_2) and superoxide (O_2^-) reduction, respectively. Next, tissues were precontracted with phenylephrine (PE; 0.3 mM; Sigma Chemicals, St Louis, MO, U.S.A.) and endothelium-dependent vasorelaxations were measured by exposing tissues to cumulative concentrations of acetylcholine (Ach: 1–1000 nM range). Relaxations were expressed as a percentage of the PE-induced plateau of contraction. Following acetylcholine, a subset of the relaxed tissues were challenged with the NOS inhibitor N^G-nitro-L-arginine-methylester (L-NAME; 1–300 μM; Calbiochem, San Diego, CA, U.S.A.). Finally, tissues were contracted again with phenylephrine (0.3 mM) and stimulated with sodium nitroprusside (1 nM–100 μM; Sigma Chemicals).

Histology

Paraffin-embedded aortic segments were cut into 5 μm sections, deparaffinized and stained with hematoxylin (Sigma Chemicals, St Louis, MO, U.S.A.). Arterial sections were captured at ×40 magnification and digitized for medial cross-sectional area (CSA) measurements (deBlois *et al*., 1997; Tea *et al*., 2000) using the NIH Image 1.61 program (http://rsb.info.nih.gov/nih-image/).

SMC nuclei showing evidence of internucleosomal DNA fragmentation were detected *in situ* using a fluorescent terminal deoxynucleotidyl transferase (tdt)-mediated dUTP-biotin nick end labeling (TUNEL) assay using biotin-16-dUTP (Roche, Indianapolis, IN, U.S.A.) and FITC-labeled Extravidin (Sigma Chemicals, St Louis, MO, U.S.A.) as tracers and propidium iodide as a counterstain as described previously (Der Sarkissian *et al*., 2003). Negative controls had water instead of tdt.

Image analysis of TUNEL-positive nuclei was performed in a blinded manner using a fluorescence microscope with appropriate filters. Every TUNEL-positive SMC nuclei per cross section was counted.

Statistical analysis

Values are presented as mean ± s.e.m. Data from treated groups were compared with those of control group by two-way analysis of variance (ANOVA) and unpaired Student's *t*-test with Bonferroni/Dunn correction for multiple comparisons. $P<0.05$ was considered statistically significant.

Results

Pretreatment values of body weight were 250 ± 2 g ($n=83$). All rats gained weight during the experiment, but final values of body weight were similar between all groups (not shown). High blood pressure was significantly reduced by 6 days of treatment with enalapril alone or in combination with LB as compared to controls, whereas LB alone had no significant effect *versus* controls (Figure 1).

Correction of endothelial dysfunction in SHR aortic rings precontracted with phenylephrine was measured by the catalase-resistant components of vasorelaxations in response to increasing cumulative concentrations of Ach in the presence of indomethacin. The contractile response to KCl or phenylephrine (0.80 ± 0.10 and 1.16 ± 0.12 g, respectively) was not significantly different between placebo and treated groups (not shown). In control animals, the vasorelaxation curve of the catalase group (strictly NO-mediated relaxation) was significantly shifted towards the right as compared to the vehicle group (NO and H_2O_2-mediated relaxations), suggesting endogenous ROS production in these tissues (Figure 2a). This effect of catalase was still present after 1 week but not 2 weeks of enalapril treatment (Figures 2b and c), suggesting correction of ED at this later time point. However, a 1-week treatment with LB alone or combined with enalapril abolished the sensitivity to catalase, suggesting the early correction of ED with the 'NO-enhancing therapy' (Figures 2d and e). Incubation of aortic rings with SOD had no significant effect on vasorelaxations. Following relaxation with Ach, rings incubated in the absence of catalase or SOD were immediately stimulated with L-NAME. This elicited a dose-dependent increase in vascular tone (Figure 3). A two-way ANOVA revealed that contractile responses to L-NAME were significantly enhanced in tissues from rats treated for 1 week with LB in combination with enalapril. Finally, relaxations in response to sodium nitroprusside were maximal in all groups and unaffected by treatment (not shown). These data suggest that Ach-induced vasorelaxations were attributable to NO synthase activity.

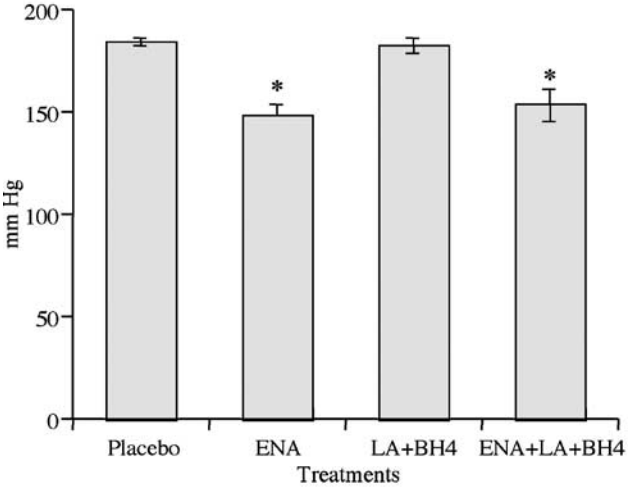

Figure 1 Changes in systolic blood pressure in untreated (Placebo) and SHR treated for 1 week with enalapril (ENA), L-arginine + BH4 (LB), or a combination of both (ENA + LB). *Significantly different ($P<0.05$) from Placebo group ($n=6$ per group).

As we have previously reported, there was no significant change in TUNEL labeling in SHR treated with enalapril for 1 week as compared to controls. However, in SHR treated with enalapril for 2 weeks and consistent with the time course of correction of ED, TUNEL labeling in the aortic medial segment revealed a significant 3.3-fold increase in the percentage of apoptotic nuclei (Figure 4c). Moreover, whereas a 1-week treatment with the ED correcting LB therapy did not significantly increase the levels of internucleosomal DNA fragments in extracted DNA, its combination with enalapril stimulated a 9.7-fold increase in fragmentation values (*versus* controls: 0.53 ± 0.18 arbitrary units.μg^{-1} DNA; Figure 5a). This synergistic interaction of treatments on apoptosis induction was corroborated by values obtained from TUNEL labeling. Co-treatment of enalapril and LB caused a significant 4.6-fold increase in the percentage of TUNEL-positive nuclei in the aortic media, whereas enalapril or LB applied separately had no effect (Figure 6). Incorporation of [^{3}H]-thymidine into aortic DNA was significantly reduced by enalapril and LB (by 27% and 19%, respectively). However, the combination of enalapril and LB elicited an additive 51% decrease of DNA synthesis (Figure 5b). At this early time point (1 week), DNA content in the aortic media was not significantly affected by the treatments with enalapril and LB applied alone or in combination *versus* controls (1.48 ± 0.33 μg.mm^{-1}). Treatment with enalapril or LB alone did not affect aortic CSA, whereas the combination of enalapril and LB elicited a 13% decrease indicative of hypertrophy regression (Figure 5c).

Discussion

Vascular oxidative stress due to dysregulation of endothelial function contributes to the pathophysiology of cardiovascular diseases including hypertension. Loss of homeostatic balance between cellular growth and death remains at the basis of morbidity such as hypertrophy and atherosclerosis. Potential underlying mechanisms are provided by a growing body of evidence demonstrating that while H_2O_2 and ROS increase cell growth or survival in cell types such as SMC and fibroblasts (Li *et al*., 1997; 1999), NO exerts the opposite effect in those cells (Shin *et al*., 1996) and induces apoptosis by cGMP-dependent and -independent pathways (Garg & Hassid, 1989; Fukuo *et al*., 1996; Shin *et al*., 1996; Khan *et al*., 1997). Therefore, it seems that ED favors cardiovascular growth, while its restoration favors regression of hypertrophy.

In a recent study, we demonstrated that apoptosis is stimulated in aortic SMC of SHR treated with the angiotensin

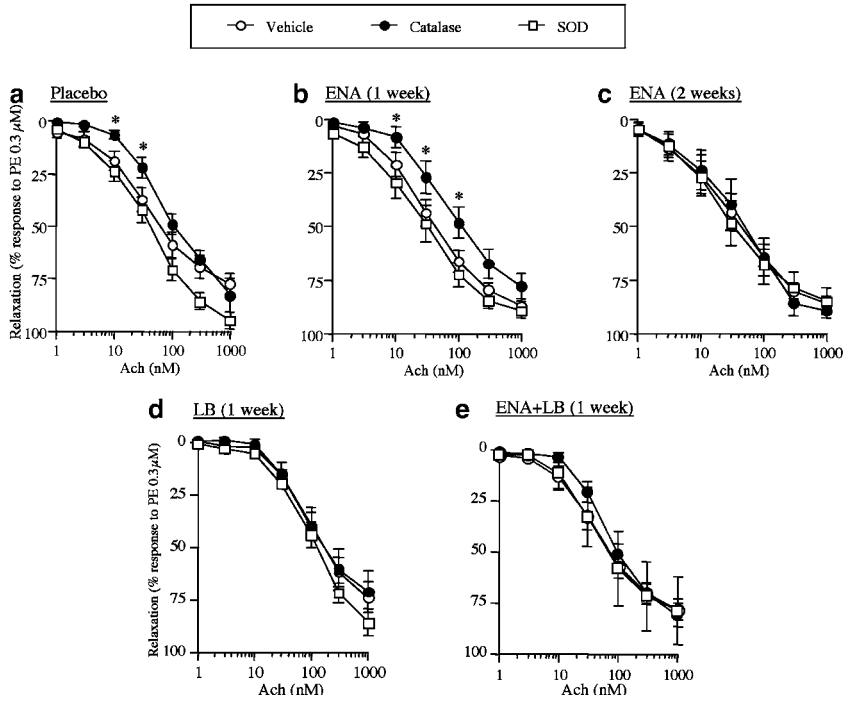

Figure 2 Endothelium-dependant acetylcholine-(Ach) induced vasorelaxations in vehicle, catalase or SOD pre-incubated aortic rings of (a) untreated controls, SHR treated with (b) enalapril (ENA) for 1 week, (c) ENA for 2 weeks, (d) L-arginine + BH4 (LB) for 1 week, (e) or a combination of both ENA + LB for 1 week. Relaxations are expressed by the percentage response to phenylephrine (PE) pre-contraction. *Significantly different ($P<0.05$) from vehicle group ($n=6$–12 per group).

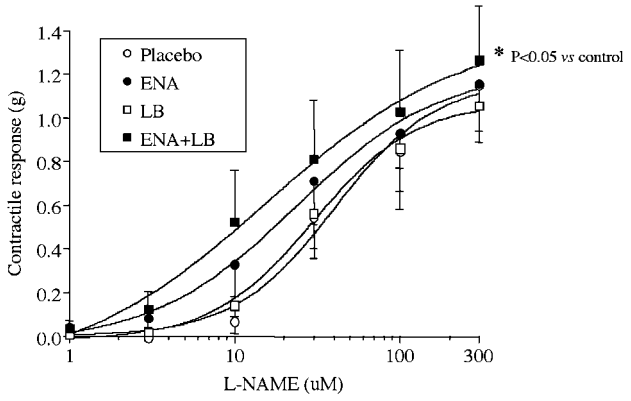

Figure 3 L-NAME induced vascular contractile response in vehicle, catalase or SOD pre-incubated aortic rings of untreated (Placebo) and SHR treated for 1 week with enalapril (ENA), L-arginine + BH4 (LB), or a combination of both (ENA + LB). *Significantly different ($P<0.05$) from Placebo ($n=6$–12 per group).

AT_1 receptor antagonist valsartan following an AT_2-dependant mechanism, since co-treatment with PD123319, a selective AT_2 receptor antagonist, completely abolished valsartan's proapoptotic effect. However, unlike the combination of valsartan and PD123319, attenuation of signaling to both receptor subtypes with the ACE inhibitor enalapril is effective in stimulating apoptosis in aortic SMC (Tea *et al*., 2000). Since ACE inhibitors correct ED and the restoration of this function could contribute to the regression of vascular mass, we decided to investigate the implication of the correction of ED in enalapril's proapoptotic ability.

In order to examine the relationship between correction of ED and vascular remodeling, we tested various drug combinations to enhance vascular NO output. In preliminary experiments, the administration of L-arginine or BH_4 alone did not elicit vascular remodeling within 1 week. This was seen in animals receiving placebo or enalapril. Thus, we tested these drugs in combination in animals treated with or without enalapril for 1 week.

Administration of L-arginine and BH_4 completely corrected ED, as interpreted by the loss of H_2O_2-mediated vasorelaxations. Co-administration of enalapril did not alter L-arginine and BH_4's combined effect on restoration of endothelial function. Interestingly, L-NAME-induced contractions were significantly potentiated only in animals treated with the combination of L-arginine, BH_4 and enalapril, that is, the drug combination that induced SMC apoptosis and vascular remodeling. Moreover, since evidences show that arterial cGMP production is higher in SHR than in normotensive Wistar-Kyoto rats (Qiu *et al*., 1998), our data suggest increased vascular NOS activity and/or improved cGMP coupling in animals receiving tri-therapy.

Suppression of SMC DNA synthesis was observed in aortic extracts from SHR treated with enalapril and with L-arginine + BH_4 for 1 week, whereas the combination of both treatments had an additive effect on DNA synthesis reduction, suggesting the activation of common pathways. Consistent with our previous observations (deBlois *et al*., 1997), enalapril induced SMC apoptosis after 2 but not 1 week of administration, and aortic apoptosis correlated in time with the correction of ED during enalapril treatment. Moreover, although a 1-week treatment with L-arginine + BH_4 alone corrected ED, this treatment had no effect on aortic apoptosis

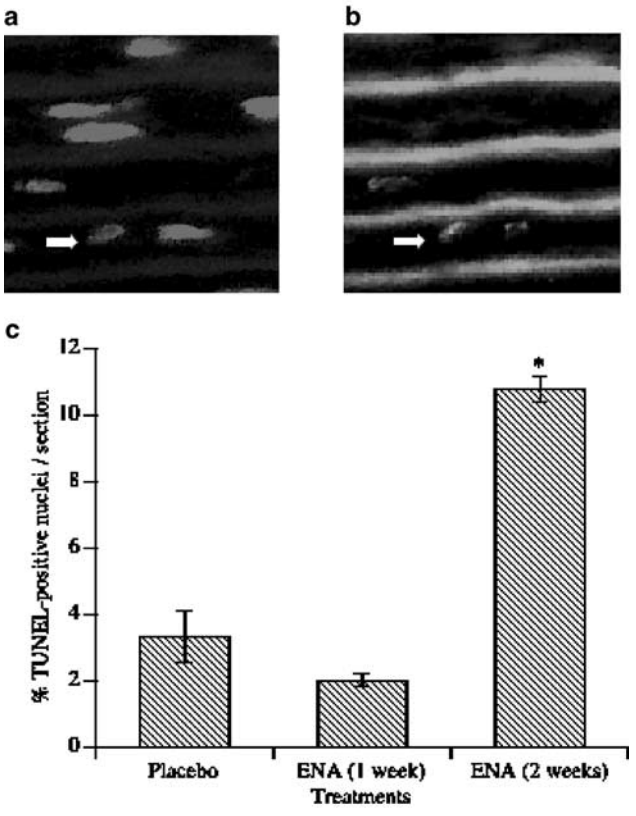

Figure 4 Photomicrographs taken in the aortic media of SHR showing (a) total SMC nuclei stained with propidium iodide, (b) TUNEL-positive nuclei in the same field visualized under an FITC filter. (c) Changes in the number of TUNEL-positive SMC nuclei per cross section of the aortic media in SHR treated with placebo or enalapril (ENA) for 1 or 2 weeks. *Significantly different ($P<0.05$) from Placebo group ($n=5$ per group).

measured by oligosomal DNA fragmentation and TUNEL assays. Interestingly, the combination of both enalapril and L-arginine + BH_4 treatments had a synergistic interaction on SMC apoptosis stimulation independently of blood pressure regulation since L-arginine + BH_4 treatment had no hemodynamic effect. In all, these results suggest that the correction of ED is associated with the induction of SMC apoptosis; however, since correction of ED alone with L-arginine + BH_4 did not induce SMC death, the data suggest that correction of ED is not sufficient although it may be permissive for SMC apoptosis induction during regression of vascular hypertrophy with ACE inhibitors.

The synergistic response in apoptosis observed with tritherapy may reflect the cumulative proapoptotic and antigrowth effects of NO-dependent and -independent pathways. In fact, NO is a known inducer of apoptosis in cultured SMC, whereas AT_1 receptors for AngII elicit antiapoptotic effects in those cells (Pollman *et al*., 1996). Moreover, since cardiovascular diseases are often associated with a decreased bioavailability of NO and increased levels of AngII (Gibbons, 1995), our results support the notion that the balance between NO and AngII plays an important role in the modulation of vascular structure.

The important role of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system in ED and vascular hypertrophy in hypertension has been well established.

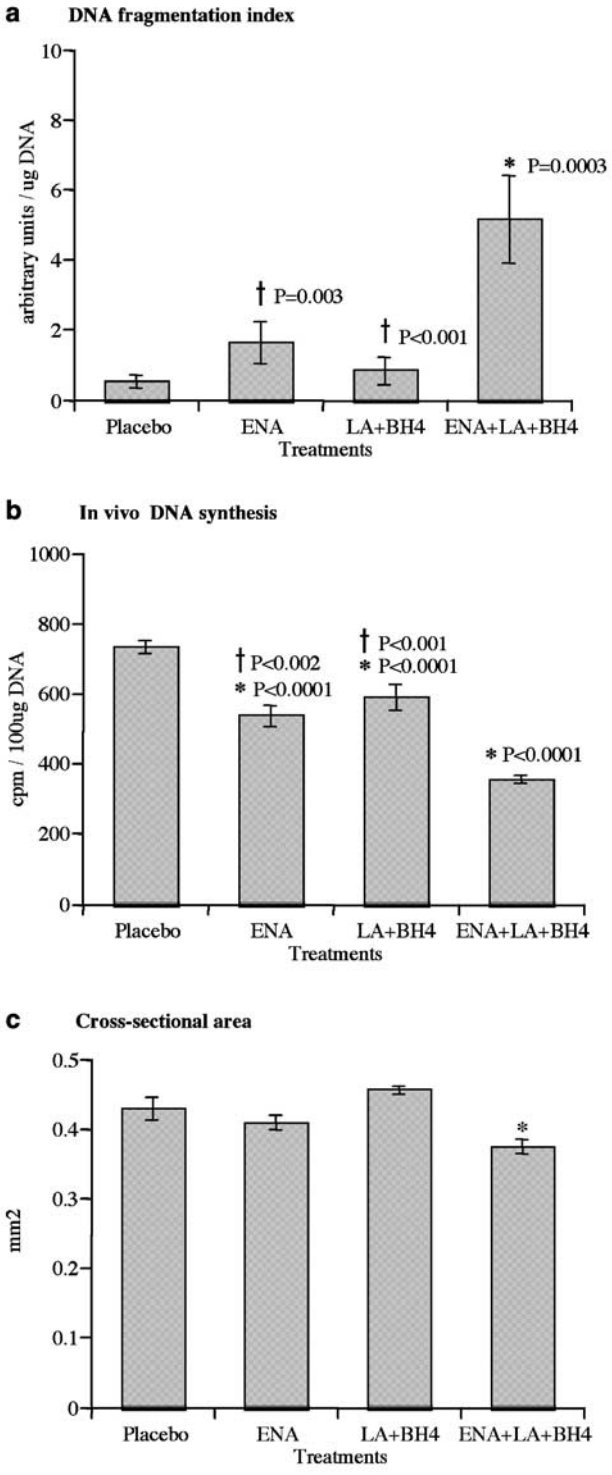

Figure 5 Changes in (a) internucleosomal DNA fragmentation ($n=6$ per group), (b) DNA synthesis ($n=6$–12 per group), (c) medial cross-sectional area in the aorta ($n=12$ per group) of untreated (Placebo) and SHR treated for 1 week with enalapril (ENA), L-arginine + BH4 (LB), or a combination of both (ENA + LB). *Significantly different ($P<0.05$) from Placebo group. †Significantly different ($P<0.05$) from ENA + LB group.

Infusion of AngII *in vivo* doubles the production of O_2^- in the rat aorta *via* the activation of the NADPH oxidase by a mechanism dependant of AT_1 receptors but independently of

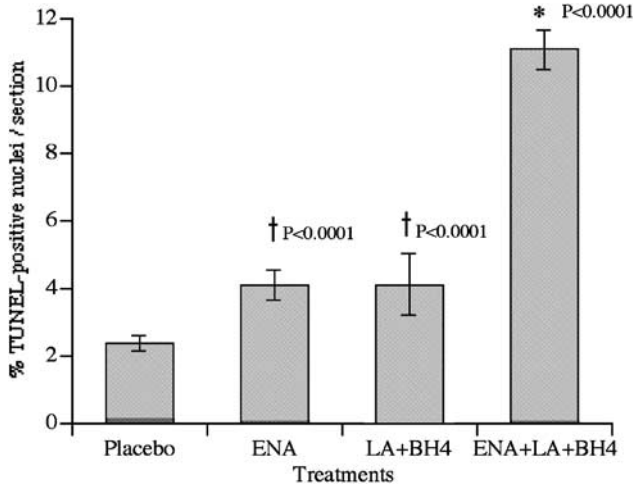

Figure 6 Changes in the percentage of TUNEL-positive SMC nuclei per cross section in the aortic media of untreated (Placebo) and SHR treated for 1 week with enalapril (ENA), L-arginine + BH4 (LB), or a combination of both (ENA + LB). *Significantly different ($P<0.05$) from Placebo group. †Significantly different ($P<0.05$) from ENA + LB group ($n=6$ per group).

pressure elevation (Laursen *et al*., 1997). Hence, the increase of NO bioavailability for apoptosis stimulation in our model could be due to a decrease in deleterious ROS production by blunting of the NADPH oxidase pathway by enalapril. Also, the pressure decrease by enalapril could potentially contribute to the increase in NO bioavailibility by reduction of sheer stress and ROS. However, this effect is considered marginal in this context since a 1-week treatment with enalapril although effective at decreasing high blood pressure failed to produce an apoptotic response.

The kinin/NO pathway is an interesting candidate pathway for apoptosis induction in this model. ACE inhibitors decrease kinin degradation which promote the release of NO *via* the activation of endothelial B_1 and B_2 receptors (Fukuo *et al*., 1996; Pollman *et al*., 1996; Berthiaume *et al*., 1997). Activation of the kinin/NO pathway reduces cardiovascular mass during ACE inhibition (Linz & Scholkens, 1992; Wollert *et al*., 1997), decreases excessive deleterious AngII production (Boulanger, 1999) and prevents kinin B_2 receptor desensitization (Marcic *et al*., 1999; Minshall *et al*., 1997). However, recent studies from our laboratory have demonstrated that the induction of apoptosis and the regression of aortic hypertrophy induced by 4 weeks of treatment with enalapril is unaffected by the B_2 receptor antagonist HOE 140 (Duguay *et al*., 2004). Unlike the B_2 receptor normally expressed in abundance in mammalian tissues and mediating most of the physiological actions of kinins, the inductible B_1 receptor is generally expressed in pathological conditions and during inflammation (Marceau & Bachvarov, 1998). However, recent studies have reported the activation of the B_1 receptor by ACE inhibitors in normotensive rats (Marin-Castano *et al*., 2002), and an interesting direct stimulation of the B_1 receptor by ACE inhibitors such as enalaprilat (Ignjatovic *et al*., 2002a, b). Taken together, these observations argue for the possible implication of the kinin B_1 receptor in mediating enalapril's NO output increase in our studied model.

The cumulative effects of both enhanced DNA synthesis reduction and synergistic interaction on apoptosis stimulation with the combination of both enalapril, L-arginine + BH_4 treatments was associated with a significant reduction of aortic mass. Consistent with our previous observations (deBlois *et al*., 1997), DNA content was not yet reduced at the early time of 1 week of enalapril treatment. It is tempting to speculate that the modulation of SMC growth and apoptosis by the combined treatment with LB and enalapril would result in regression of SMC number at later time point.

Endothelial dysfunction causes vascular complications in hypertension. Normalization of endothelial function by an 'NO-enhancing therapy', as we describe or by chronic supplementation of folates that increase BH_4 availability (Verhaar *et al*., 1998; 1999), could constitute an important goal for antihypertensive therapy. The synergistic interaction of our 'NO-enhancing therapy' on enalapril's ability in SMC apoptosis induction during vascular hypertrophy regression reveals possible new avenues for rapid therapeutic intervention in cardiovascular remodeling.

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