Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 516 (2005) 253 – 259

www.elsevier.com/locate/ejphar

Angiotensin-converting enzyme inhibition and angiotensin AT_1-receptor antagonism equally improve endothelial vasodilator function in L-NAME-induced hypertensive rats

Vito De Gennaro Colonna[a,*], Antonello Rigamonti[a], Simona Fioretti[a], Sara Bonomo[a], Barbara Manfredi[a], Paolo Ferrario[a], Mauro Bianchi[a], Ferruccio Berti[a], Eugenio E. Muller[a], Giuseppe Rossoni[b]

[a]*Department of Pharmacology, Chemoterapy and Medical Toxicology, University of Milan, Via Vanvitelli 32, 20129 Milan, Italy*
[b]*Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy*

Received 1 July 2004; received in revised form 27 January 2005; accepted 2 February 2005

Abstract

Male Sprague–Dawley rats given N^{ω}-nitro-L-arginine methyl ester (L-NAME) in drinking water for 8 weeks showed: (1) a clear-cut increase in systolic blood pressure; (2) a consistent decrease of endothelial-cell nitric oxide synthase (eNOS) gene expression in aortic tissue; (3) a marked reduction of plasma nitrite/nitrate concentrations; (4) a reduction of the relaxant activity of acetylcholine (ACh, from 10^{-10} to 10^{-4} M) on norepinephrine-precontracted aortic rings (reduction by 48 ± 5%); (5) a marked decrease (−58%) of the basal release of 6-keto-prostaglandin F1α (6-keto-PGF1α) from aortic rings. In L-NAME-treated rats, administration in the last 4 weeks of either the angiotensin-converting enzyme (ACE) inhibitor enalapril (10 mg/kg/day in tap water) or the angiotensin AT_1-receptor antagonist losartan (10 mg/kg/day in tap water) decreased systolic blood pressure levels, completely restored eNOS mRNA levels in aortic tissue and plasma nitrite/nitrate levels, and allowed a consistent recovery of both the relaxant activity of acetylcholine and the generation of 6-keto-PGF1α. Coadministration of icatibant, a bradykinin B_2-receptor antagonist (200 μg/kg/day), with enalapril blunted the stimulatory effect of the ACE inhibitor on eNOS mRNA expression, circulating levels of nitrite/nitrate, the relaxant activity of ACh and the release of 6-keto-PGF1α in L-NAME-treated rats. The generation of 6-keto-PGF1α from aortic rings was also decreased in rats coadministered icatibant with losartan. These findings indicate that (1) the ACE inhibitor enalapril and the angiotensin AT_1-receptor blocker losartan are equally effective to reverse NAME-induced endothelial dysfunction; (2) the beneficial effect of enalapril on the endothelial vasodilator function in L-NAME-treated rats is mediated by bradykinin B_2-receptor activation; and (3) the enhanced endothelial generation of prostacyclin induced by losartan in L-NAME rats is also mediated by bradykinin B_2-receptor activation.

Keywords: Endothelial dysfunction; NO (nitric oxide); Hypertension; Bradykinin; Angiotensin-converting enzyme inhibitor; AT_1-receptor antagonist

1. Introduction

In a previous paper from our group (De Gennaro Colonna et al., 2002), angiotensin-converting enzyme inhibitors (enalapril and quinapril) proved capable to reverse endothelial dysfunction in rats treated with the NOS inhibitor L-NAME. In fact, in these animals, 2 weeks administration of either enalapril or the cognate drug quinapril decreased systolic blood pressure, completely restored aortic eNOS mRNA levels and allowed a consistent recovery of both the relaxant activity of acetylcholine and the generation of 6-keto-PGF1α in the aortic tissue. Angiotensin-converting enzyme, also known as kininase II, is able to catabolize bradykinin (BK) to inactive fragments (Erdos, 1975). Therefore, a potentiation of bradykinin effects through stimulation of the bradykinin

* Corresponding author. Tel.: +39 2 503 17017; fax: +39 2 503 17011.
E-mail address: vito.colonna@unimi.it (V. De Gennaro Colonna).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.04.004

B_2-receptor subtype has been implicated in the beneficial cardiovascular effects of angiotensin-converting enzyme inhibitors (Bao et al., 1992; Linz and Scholkens, 1992; Bouaziz et al., 1994).

In the present study we evaluate the contribution of the bradykinin B_2-receptor activation to the beneficial effects exerted by angiotensin-converting enzyme inhibitors on endothelial function of L-NAME-treated rats.

To this purpose, rats made hypertensive by chronic L-NAME were simultaneously treated with enalapril and the bradykinin B_2-receptor antagonist icatibant and compared with animals administered the ACE inhibitor alone. At the same time we tested the ability of losartan, the prototype of angiotensin AT_1-receptor antagonists, a class of drugs become popular and alternative to ACE inhibitors for treatment of hypertension, to reverse the endothelial dysfunction due to chronic NOS inhibition.

2. Materials and methods

2.1. Animals and treatments

Adult male Sprague–Dawley rats (200–250 g body weight) were purchased from Charles River Italia (Calco, Lecco, Italy) and were housed under controlled conditions (22±2 °C, 65% humidity and artificial light from 0600 to 2000 h).

Five groups of 12 rats each were studied (protocol 1). In four of the groups, hypertension was induced by administration of the NOS inhibitor L-NAME (60 mg/kg body wt./day) in drinking water for 8 weeks. The fifth group received normal tap water throughout all the experiment (control group). Animals made hypertensive were then treated during the last fourth weeks of treatment with: 1, enalapril (10 mg/kg/day in tap water) (L-NAME+enalapril); 2, losartan (10 mg/kg/day in tap water) (L-NAME+losartan); 3, enalapril (same dose as in group 1)+icatibant (200 μg/kg/day subcutaneously) (L-NAME+enalapril+icatibant); 4, L-NAME+vehicle (L-NAME). Basally and every 2 weeks during the 8 weeks of follow-up, systolic blood pressure was estimated by the tail-cuff method (BP recorder, mod. 58000, U. Basile, Comerio, Varese, Italy) at the same time of the day between 1300 and 1500 h. At the end of the 4-week period of drug administration, all rats were killed by cervical dislocation. From each animal, a segment of the thoracic aorta was rapidly removed for determination of eNOS mRNA levels and for evaluation of the endothelial vasodilator function (relaxant effects of acetylcholine) and the basal release of 6-keto-PGF1α, the stable metabolite of prostacyclin (PGI_2). Furthermore blood samples were collected for plasma nitrite/nitrate determinations.

An additional experiment (protocol 2) was performed to investigate possible dependence of PGI_2 generation by losartan on bradykinin B_2-receptor activation. For this purpose four experimental groups (L-NAME—60 mg/kg body wt./day; L-NAME+losartan—losartan 10 mg/kg/day in tap water; L-NAME+losartan+vicatibant—icatibant 200 μg/kg/day subcutaneously) of 7 rats each were studied. The experimental design of protocol 2 was the same as described in protocol 1. At the end of the 4-week period of drug administration rats were sacrificed and a segment of the thoracic aorta was removed for the evaluation of the basal release of 6-keto-PGF1α.

The experimental protocol was approved by the Review Committee of the Department of Pharmacology and met the Italia guidelines for use of laboratory animals, which conform with the European Communities directive of November 1986 (86/609/EEC).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR): aortic eNOS mRNA

Total RNA was isolated from aortic tissues by the single-step acid guanidium–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). RT-PCR was prepared by standard methods with 1 μg of total RNA. First-strand cDNA was synthesized with oligo dt and Molony murine leukemia virus reverse transcriptase (GIBCO, Milano, Italy). Reverse transcription was performed at 37 °C for 50 min followed by an initial denaturation at 70 °C for 15 min.

PCR amplification was then performed with synthetic gene-specific primers (GENENCO, Firenze, Italy) for eNOS (forward primer, 5′-TGCACCCTTCCGGGGATTCT-3′; reverse primer, 5′-GGATCCCTGGAAAAGGCGGT-3′; product length, 189 bp).

Amplification was performed with 35 cycles of denaturation (95 °C for 30 s), annealing (62 °C for 30 s) and extension (72 °C for 30 s). Parallel amplification of rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was performed. The reaction was linear to 35 cycles with use of the ethidium bromide detection method. PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and were visualized by ultra-violet-induced fluorescence. The intensity of each band was quantified using a densitometer. The resulting densities of the eNOS bands were expressed relative to the corresponding densities of the GAPDH bands from the same RNA sample (Hattori et al., 1997; Kobayashi et al., 1999).

2.3. Plasma nitrite/nitrate concentrations

The nitric oxide radical has a short half-life, and thus, the plasma concentrations of NO stable end products, nitrite plus nitrate, were used as an index of vascular nitric oxide production. Combined plasma nitrite and nitrate concentrations were measured using a commercially available colorimetric kit according to the well-recognized method based on the use of Griess reagent (Green et al., 1982).

2.4. Isolated aortic rings

2.4.1. Relaxant effects of acetylcholine and sodium nitroprusside

Segments of thoracic aorta obtained from the five experimental groups of rats (protocol 1) were cleaned of adherent connective tissue in Krebs–Henseleit solution and cut into rings (3–5 mm in length). The rings were carefully handled to avoid damage to the inner surface and suspended in organ bath chambers (10 ml) containing Krebs–Henseleit solution of the following composition (mM): NaCl, 118; KCl, 2.8; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 5.5.

The medium was gassed with a mixture of CO_2 (5%) and O_2 (95%) and maintained at 37 °C (pH 7.4). Tissues were connected via silk sutures to force-displacement transducers (model 7004; U. Basile, Comerio, Varese, Italy), and changes in isometric force were displayed on a Gemini chart recorder (model 7070; U. Basile). All rings were gradually stretched to a basal resting tension of 1–1.5 g,

which was maintained throughout the experiment; the preparations were allowed to equilibrate for 90 min. To evaluate maximal contraction, the tissues were depolarized with potassium chloride (KCl; 60 mM) and washed with Krebs–Henseleit solution. After 30 min, the aortic rings were contracted by norepinephrine (3×10^{-6} M), and when the contractile response was stabilized (steady-state phase, 12–15 min), relaxation was evaluated by cumulative addition of acetylcholine (from 10^{-10} to 10^{-4} M). The direct relaxant effect of the NO-donor sodium nitroprusside (cumulative concentrations 10^{-9} to 10^{-5} M) was also recorded.

2.4.2. 6-Keto-PGF$_{1\alpha}$ release

After a suitable period of equilibration, the basal prostacyclin-releasing capacity of the aortic rings was measured by evaluating the concentration of 6-keto-PGF1α, the stable metabolite of prostacyclin, in 1 ml of the bathing fluid after 20 min of incubation. The rate of release of 6-keto-PGF1α was determined according to the enzyme immunoassay method (detection limit, 3 pg/ml) described by Pradelles et al. (1985), and was expressed as pg/mg wet tissue (pg/mg wt.).

2.5. Statistical analysis

Differences of data among groups in individual experiments were analyzed for statistical significance by one-way analysis of variance (ANOVA), followed by the Bonferroni test. A value of $P<0.05$ was considered significant.

2.6. Drugs

The following drugs were used: N^G-monomethyl-L-arginine, norepinephrine chloride and acetylcholine chloride (Sigma, St. Louis, MO, USA); icatibant (kindly provided by Aventis Pharma Deutschland GmbH and Jerini AG); enalapril maleate, losartan potassium (Merck Sharp and Dohme, New Jersey, USA); sodium nitroprusside (Merck, Darmstadt, Germany). Colorimetric assay kit for nitrite/nitrate (cat. no. 780001) determination was obtained from Cayman Chemical (Ann Arbor, MI, USA).

3. Results

3.1. Systolic blood pressure

Rats drinking water supplemented with L-NAME exhibited a progressive increase in systolic blood pressure from the second week on, reaching the value of 216±3 mm Hg at week 8 (Table 1). Enalapril or losartan in the drinking water significantly, and similarly, decreased systolic blood pressure (140±2 mm Hg, L-NAME+enalapril; 142±3 mm Hg, L-NAME+losartan; $P<0.05$ vs. L-NAME alone) (Table 1). Coadministration of icatibant with enalapril reversed the antihypertensive effect of the angiotensin-converting enzyme inhibitor (200±4 mm Hg, L-NAME+enalapril+icatibant; $P<0.05$ vs. L-NAME+enalapril).

3.2. RT-PCR for aortic eNOS mRNA

In aortic tissue from L-NAME-treated rats, the levels of eNOS mRNA were significantly decreased as compared to those of control rats (−30%, $P<0.05$; Fig. 1). Treatment of these rats with enalapril or losartan completely restored aortic eNOS mRNA (Fig. 1). In aortic tissue from rats treated with enalapril+icatibant, eNOS mRNA was significantly decreased as compared to corresponding values from rats treated with enalapril alone (−34%, $P<0.05$; Fig. 1).

3.3. Plasma nitrite/nitrate concentrations

Plasma nitrite/nitrate levels from the different experimental groups (protocol 1) is reported in Table 2. In L-NAME-treated rats plasma nitrite/nitrate concentrations were significantly reduced (~50%; $P<0.01$) as compared to levels measured in control rats (Table 2). Treatment of L-NAME rats with enalapril or losartan similarly restored circulating concentrations of nitrite/nitrate (Table 2). Coadministration of icatibant with enalapril (L-NAME+enalapril+icatibant group) blunted plasma levels of nitric oxide end products ($P<0.01$ vs. L-NAME+enalapril) (Table 2).

3.4. Isolated aortic rings

3.4.1. Relaxant effect of acetylcholine

In this set of experiments, the contractions caused by norepinephrine (3×10^{-6} M) in aortic tissue prepared from untreated animals (1.85±0.21 g over the resting tension), L-NAME (2.17±0.32 g), L-NAME+enalapril (2.05±0.18 g), L-NAME+losartan (1.82±0.15 g), L-NAME+enalapril+icatibant-treated rats (1.97±0.31 g) were not significantly different. Exposure to cumulative concentrations of acetylcholine (from 10^{-10} to 10^{-4} M) resulted in a marked relaxation (94±6%, maximal relaxant effect), expressed as percentage of norepinephrine-induced contraction of the aortic rings from control rats. In contrast, the sensitivity to acetylcholine of the aortic rings from rats given L-NAME alone was significantly reduced, corresponding only to 48±5% (Fig. 2). When aortic tissues from rats given L-

Table 1
Time-related changes in systolic blood pressure (mm Hg) in rats treated with L-NAME, L-NAME+enalapril, L-NAME+losartan, L-NAME+enalapril+icatibant (ICA) and in control rats

Group	Control	L-NAME	L-NAME+enalapril	L-NAME+losartan	L-NAME+enalapril+ICA
Basal	128.5±1.7	129.6±1.6	129.2±1.9	131.0±1.5	129.4±1.8
Second week	133.0±1.8	167.1±1.7[a]	168.3±2.0[a]	170.4±2.0[a]	168.5±2.0[a]
Fourth week	131.3±2.0	179.3±2.0[a]	180.0±2.5[a]	181.8±2.4[a]	182.7±2.3[a]
Sixth week	132.2±2.3	194.2±2.0[a]	167.5±2.2[a,b]	165.8±2.6[a,b]	184.7±2.4[a,b,c]
Eight week	133.8±1.9	216.7±3.3[a]	140.4±2.7[a,b]	142.1±3.5[a,b]	200.5±4.1[a,b,c]

Data are mean±S.E.M. of 12 animals.
[a] $P<0.05$ vs. Control.
[b] $P<0.05$ vs. L-NAME.
[c] $P<0.05$ vs. L-NAME+enalapril, L-NAME+losartan.

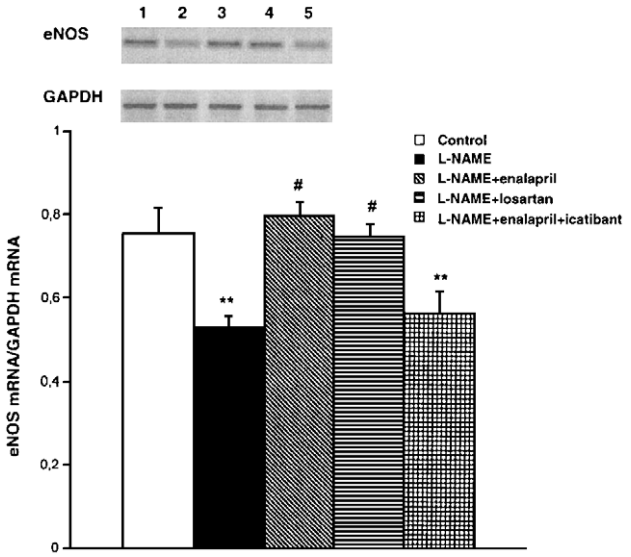

Fig. 1. eNOS mRNA expression in aortas from rats given L-NAME, L-NAME+enalapril, L-NAME+losartan, L-NAME+enalapril+icatibant and in control rats (protocol 1). Total RNA was assayed by RT-PCR with gene-specific primers for eNOS and GAPDH. Values are expressed as means±S.E.M. of five determinations. $^{**}P<0.05$ vs. control; $^{\#}P<0.05$ vs. L-NAME. Top panels are representative of typical RT-PCR bands. Lane 1, control rats; lane 2, L-NAME-treated rats; lane 3, L-NAME+enalapril-treated rats; lane 4, L-NAME+losartan-treated rats; lane 5, L-NAME+enalapril+icatibant-treated rats.

NAME+enalapril or L-NAME+losartan were challenged with acetylcholine, the vasodilation was almost fully restored, the maximal relaxant effect being 84±6% and 81±8%, respectively (Fig. 2). Aortic rings from rats given L-NAME+enalapril+icatibant exposed to cumulative concentrations of acetylcholine showed a decreased vasodilation not significantly different from that obtained in aortic tissues from rats treated with L-NAME alone (58±4%) (Fig. 2).

Exposure of the aortic rings precontracted with norepinephrine to cumulative concentrations (from 10^{-10} to 10^{-5} M) of the nitrovasodilator sodium nitroprusside produced dose–response curves that were almost superimposable for all the experimental groups (data not shown).

3.4.2. 6-Keto-PGF1α

The spontaneous release of 6-keto-PGF1α into the bathing fluid of the aortic rings from the different experimental groups (protocol 1) is reported in Fig. 3. Aortic segments of control rats released

Table 2
Plasma nitrite/nitrate concentrations in rats treated with L-NAME, L-NAME+enalapril, L-NAME+losartan, L-NAME+enalapril+icatibant and in control rats

Group	Nitrite/nitrate (μM/l)
Control	27.5±2.0
L-NAME	14.1±2.1[a]
L-NAME+enalapril	25.3±2.5[b]
L-NAME+losartan	25.0±2.7[b]
L-NAME+enalapril+icatibant	15.3±1.8[a,c]

Data are mean±S.E.M. of 8 determinations.
[a] $P<0.01$ vs. Control.
[b] $P<0.01$ vs. L-NAME.
[c] $P<0.01$ vs. L-NAME+enalapril, L-NAME±losartan.

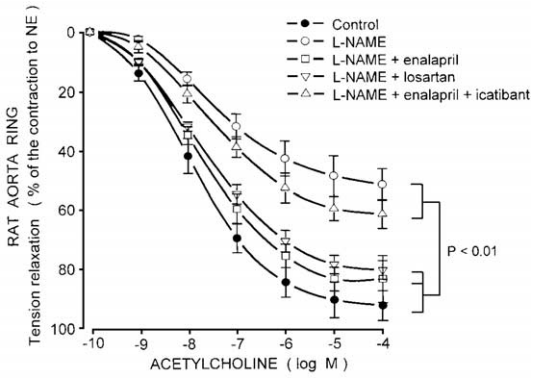

Fig. 2. Cumulative dose–response curves of acetylcholine in norepinephrine-precontracted aortic rings prepared from rats given L-NAME, L-NAME+enalapril, L-NAME+losartan, L-NAME+enalapril+icatibant and in control rats (protocol 1). Points represent the mean values, and vertical bars, the S.E.M. of seven determinations. Statistical differences related to acetylcholine-induced maximal relaxation are: L-NAME and L-NAME+enalapril+icatibant, $P<0.01$ vs. control; L-NAME+enalapril and L-NAME+losartan, $P<0.01$ vs. L-NAME.

consistent amounts of the prostanoid (122±11 pg/mg wt.), whereas the corresponding segments from rats given L-NAME alone exhibited a marked reduction in the release of 6-keto-PGF1α (51±4 pg/mg wt.) ($P<0.01$ vs. control). In the organ bath of the aortic rings from rats given L-NAME+enalapril or L-NAME+losartan, the release of the prostacyclin metabolite was significantly increased (105±8 and 100±7 pg/mg wt., respectively; $P<0.01$ vs. L-NAME alone) (Fig. 3). Aortic rings of rats treated with L-NAME+enalapril+icatibant showed a significant decrease in the release of 6-keto-PGF1α (63±7 pg/mg wt.) which was not significantly different from that found in rats treated with L-NAME alone (Fig. 3).

Table 3 reports the spontaneous release of 6-keto-PGF1α into the bathing fluid from the aortis rings of the experimental groups

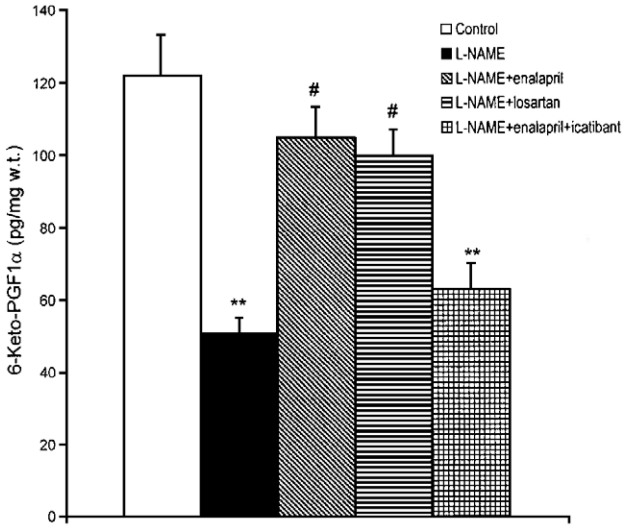

Fig. 3. Unstimulated release of 6-keto-prostacyclin (PGF1α) in 20 min from isolated aortic rings from rats treated with L-NAME, L-NAME+enalapril, L-NAME+losartan, L-NAME+enalapril+icatibant and control rats (protocol 1). Values are expressed as means±S.E.M. of seven determinations. $^{**}P<0.01$ vs. control; $^{\#}P<0.01$ vs. L-NAME.

Table 3
Unstimulated release of 6-keto-prostacyclin (PGF1α) in 20 min from isolated aortic rings from rats treated with L-NAME, L-NAME+losartan, L-NAME+losartan+icatibant and control rats (protocol 2)

Group	6-Keto-PGF1α (pg/mg wt.)
Control	115.4±7.5
L-NAME	48.1±5.1[a]
L-NAME+losartan	100.6±5.8[b]
L-NAME+losartan+icatibant	58.7±3.0[c]

Data are mean±S.E.M. of 7 determinations.
[a] $P<0.01$ vs. Control.
[b] $P<0.01$ vs. L-NAME.
[c] $P<0.01$ vs. L-NAME+losartan.

used in protocol 2. Similarly as in protocol 1, aortic rings of L-NAME-treated rats showed a significant reduction in the release of the prostanoid, an effect reversed by in vivo chronic administration of losartan (Table 3). Coadministration of icatibant with losartan (L-NAME+losartan+icatibant group) caused a significant decrease in the release of 6-keto-PGF1α ($P<0.01$ vs. L-NAME+losartan) to levels similar to that measured in rats treated with L-NAME alone (Table 3).

4. Discussion

The present study demonstrates that: (1) the beneficial effect of angiotensin-converting enzyme inhibitors on the endothelial vasodilator function of L-NAME-treated rats is mediated by bradykinin B_2-receptor; (2) the angiotensin AT_1-receptor antagonist losartan is as effective as the ACE inhibitor enalapril to reverse the endothelial vasodilator dysfunction in the present rat model of chronic NO inhibition; (3) also the beneficial effect of losartan on NAME-induced endothelial dysfunction is, at least as regards endothelial PGI_2 generation, mediated by bradykinin B_2-receptor activation.

Rats chronically treated with L-NAME showed: a clear-cut increase in systolic blood pressure; a significant decrease of eNOS gene expression in aortic tissue; a marked reduction of plasma nitrite/nitrate levels, nitric oxide end products; a reduction of the relaxant activity of acetylcholine on norepinephrine-precontracted aortic rings and a marked decrease of the basal release of 6-keto-PGF1α from aortic rings. In L-NAME-treated rats, administration of the angiotensin-converting enzyme inhibitor enalapril in the last fourth weeks of treatment reversed all these markers of endothelial dysfunction. Thus, the present data confirm our previous report on the beneficial effect of angiotensin-converting enzyme inhibitors on NAME-induced endothelial dysfunction (De Gennaro Colonna et al., 2002).

Coadministration of the bradykinin B_2-receptor antagonist icatibant with enalapril prevented the beneficial effect of the angiotensin-converting enzyme inhibitor on endothelial function of L-NAME rats. In fact, as compared to rats treated with enalapril alone, rats administered enalapril+icatibant showed a significant increase in systolic blood pressure and a marked decrease of (1) aortic eNOS mRNA levels, (2) plasma nitrite/nitrate concentrations, (3) the vasodilator responses of the aortic tissues to acetylcholine and (4) the basal release of 6-keto-PGF1α from the aortic rings—all values not significantly different from those registered in L-NAME rats. These data clearly demonstrate that bradykinin through B_2-receptor activation mediates the beneficial effects exerted by angiotensin-converting enzyme inhibitors on the endothelial vasodilator function in a model of chronic NO inhibition. Namely, the increase in bradykinin concentration resulting from inhibition of its breakdown by angiotensin-converting enzyme inhibitors would increase NOS activity and NO synthesis via stimulation of the bradykinin B_2-receptor. Simultaneous increase of both aortic eNOS mRNA expression and plasma concentrations of nitric oxide end products in L-NAME+enalapril and L-NAME+losartan-treated rats (present study) is indicative of an enhanced eNOS activity caused by both drug treatments. Furthermore, the ability of these drugs to enhance NO synthesis in our experimental model of chronic NOS inhibition bespeaks an incomplete blockade of the NO-synthase by L-NAME, at least at the dose of the NOS inhibitor used in this study (60 mg/kg, body wt./day). According to this view, Kobayashi et al. (2000) demonstrated that in rats treated with L-NAME for 10 weeks (at the same dose used in our experiments), the ACE inhibitor imidapril, chronically administered for the last 4 weeks of L-NAME treatment (as in our study), was able to significantly increase eNOS mRNA in the left ventricle of the heart.

To our knowledge, no other reports are available on the effects of bradykinin B_2-receptor blockade on the endothelial function of L-NAME+ACE inhibitor-treated rats. In another animal model, namely spontaneously hypertensive rats, the angiotensin-converting enzyme inhibitor ramipril or perindopril was capable to increase aortic cGMP content (index for NO formation), an effect completely abolished by long-term blockade of bradykinin B_2-receptors with HOE 140 (former name for icatibant) (Gohlke et al., 1993). In this vein, previous reports had demonstrated that angiotensin-converting enzyme inhibition increased the formation of NO in cultured endothelial cells (indirectly assessed by measurement of intracellular cGMP) (Wiemer et al., 1991) and that bradykinin B_2-receptor blockade by icatibant suppressed cGMP accumulation (Wiemer et al., 1991; Busse et al., 1993).

More recently, angiotensin-converting enzyme inhibitors were found to increase blood bradykinin level (Su et al., 1999) and exert acute vasodilator effects (reduction of mean aortic pressure) (Barbe et al., 1996; Su et al., 1998) in dogs with experimental heart failure induced by ventricular pacing (a condition in which the endothelial vasodilator function is blunted). Pretreatment of these animals with HOE 140 markedly reduced the vasodilator effect of ACE inhibitors (Barbe et al., 1996; Su et al., 1998).

In humans, quinaprilat acutely increased flow-dependent dilation of the radial artery in healthy volunteers, an effect

that was completely abolished after coinfusion of icatibant (Hornig et al., 1997). Our present results broaden these findings and demonstrate that angiotensin-converting enzyme inhibitors reverse the endothelial vasodilator dysfunction in the L-NAME model of chronic NO inhibition through bradykinin and B_2-receptor activation.

A noteworthy aspect of our study was the ability of enalapril to increase the release of 6-keto-PGF1α from the aortic rings of L-NAME-treated rats. Also this effect was completely reversed by coadministration of the bradykinin B_2-receptor antagonist icatibant.

Stimulation of endothelial prostacyclin release by angiotensin-converting enzyme inhibitors in the L-NAME model of hypertension has been previously shown by our group (De Gennaro Colonna et al., 2002). Present data demonstrate that this action was mediated by bradykinin B_2-receptor activation. In agreement with this conclusion are previous observations by Wiemer et al. (1991) showing the ability of ramiprilat to increase the formation of prostacyclin in cultured human and bovine endothelial cells, an effect abolished by bradykinin B_2-receptor blockade with HOE 140. The contribution of the enhanced prostanoid production to the improvement of endothelial vasodilator function in L-NAME+enalapril-treated rats does not escape attention. According to this view, preliminary results obtained by our group in rats treated with L-NAME (for 8 weeks), coadministered enalapril (10 mg/kg/day) and indomethacin (5 mg/kg/day) for the last 4 weeks of the experiment, showed the ability of the cyclooxygenase inhibitor to significantly reduce the hypotensive effect of enalapril and the release of 6-keto-PGF1α from the aortic tissues (data not shown). In keeping with our observations, Guazzi et al. (1998) demonstrated the ability of aspirin (administered at a dose of 300 mg/day) to attenuate the antihypertensive action of enalapril in hypertensive patients, an effect likely mediated through prostaglandin inhibition.

Another interesting finding of the present study is that the angiotensin AT_1-receptor antagonist losartan improved the endothelial vasodilator dysfunction in L-NAME-treated rats at a similar extent as the ACE inhibitor enalapril. All the markers of endothelial function studied (eNOS mRNA levels, plasma nitrite/nitrate concentrations, the relaxant activity of acetylcholine on aortic rings and the basal release of 6-keto-PGF1α) were restored by treatment of L-NAME rats with losartan for 4 weeks. At present no other reports regarding the ability of angiotensin AT_1-receptor antagonists to reverse endothelial dysfunction in the L-NAME model of hypertension are available, although the capacity of these drugs to improve the endothelial vasodilator function have been ascertained in other animal models and in humans. In stroke-prone spontaneously hypertensive rats, Gohlke et al. (1996) demonstrated that long-term treatment with losartan improved cardiac function and metabolism and increased aortic cGMP content. In dogs (Krishnankutty et al., 1993), losartan acutely injected into the coronary circulation induced vasodilator responses greater in magnitude than those evoked by the angiotensin-converting enzyme inhibitor enalaprilat. The vasodilator responses to losartan were partly endothelium-dependent as they were partially inhibited by L-NAME (Krishnankutty et al., 1993). Another report (Higashi et al., 2000), in agreement to our data, has shown the ability of angiotensin AT_1-receptor antagonists to stimulate NOS activity. In fact, a 6-week treatment of Goldblatt hypertensive rats with TCV-116 (an angiotensin AT_1-receptor antagonist), at a subdepressor dose, proved capable to significantly increase eNOS mRNA and protein levels in the left ventricle. Finally, in humans (Hornig et al., 2003), intrabrachial infusion with the angiotensin AT_1-receptor antagonist candesartan improved flow-dependent, endothelium mediated vasodilatation in patients with coronary artery disease, an effect consistent with previous observations in peripheral artery disease (Prasad et al., 2000) and diabetes (Cheetam et al., 2000).

The positive effect of losartan on the endothelial vasodilator function of L-NAME rats (present study) was likely related both to an enhanced endothelial NO production, as shown by the ability of losartan to increase the eNOS mRNA in the aortic tissue and circulating levels of nitrite/nitrate, and to the increased release of 6-keto-PGF1α from the aortic rings induced by the drug.

The underlying mechanism(s) mediating these effects are an interesting matter of debate. In fact, a growing body of evidence indicates that angiotensin AT_1-receptor antagonists, via stimulation of angiotensin AT_2 receptors, may activate the bradykinin-NO cascade (Searles and Harrison, 1999). In this vein it is known that administration of angiotensin AT_1-receptor antagonists increases angiotensin II plasma levels (Campbell et al., 1995) which, in turn, may stimulate the AT_2 receptor (Gohlke et al., 1998). In addition, angiotensin AT_2 receptor activation, in spontaneously hypertensive rats, proved capable to increase vascular cGMP levels (Gohlke et al., 1998), an effect inhibited by bradykinin B_2- and angiotensin AT_2-receptor blockade (Gohlke et al., 1998).

In this vein, in humans (Hornig et al., 2003), the flow-dependent endothelium mediated vasodilatation induced in patients with coronary heart disease by candesartan was inhibited by coinfusion with icatibant. Our data showing that losartan was capable to increase aortic eNOS mRNA levels, plasma nitrite/nitrate concentrations and endothelial prostacyclin release from aortic rings of L-NAME-treated rats is in keeping with the concept that angiotensin AT_1-receptor antagonists, via AT_2 receptor activation, may increase bradykinin formation and thus stimulate NO and prostacyclin production.

This view is strengthened by our results (protocol 2) obtained in L-NAME hypertensive rats chronically treated with losartan and the bradykinin B_2-receptor antagonist icatibant. In this experimental group, the endothelial release of 6-keto-PGF1α was blunted as compared with that of the

group treated with losartan alone, indicating that the angiotensin AT_1-receptor blocker increased endothelial prostacyclin release through bradykinin and B_2-receptor activation.

In conclusion, our present work demonstrates that the ability of angiotensin-converting enzyme inhibitors to restore endothelial vasodilator function in L-NAME hypertensive rats is mediated by bradykinin B_2-receptor activation. The angiotensin AT_1-receptor antagonist losartan proved as affective as the ACE inhibitor enalapril to reverse the endothelial vasodilator dysfunction in L-NAME-treated rats, improving all parameters of endothelial function studied (aortic eNOS mRNA levels, plasma nitrite/nitrate concentrations, the relaxant activity of acetylcholine in precontracted aortic rings and the basal release of 6-keto-PGF1α). Finally, similarly to the results obtained with the angiotensin-converting enzyme inhibitor, also the ability of the angiotensin AT_1-receptor antagonist losartan to increase endothelial prostacyclin release was found to be mediated by bradykinin B_2-receptor activation.

Acknowledgments

This work was supported in part by a grant from the Italian Ministry of University and Scientific Research (COFIN 2003; prot. 2003070823_04) to G.R.

References

Bao, G., Gohlke, P., Qadri, F., Unger, T., 1992. Chronic kinin receptor blockade attenuates the antihypertensive effect of ramipril. Hypertension 20, 74–79.

Barbe, F., Su, J.B., Guyene, T.T., Crozatier, B., Menard, J., Hittinger, L., 1996. Bradykinin pathway is involved in acute hemodynamic effects of enalaprilat in dogs with heart failure. Am. J. Physiol. 270, H1985–H1992.

Bouaziz, H., Joulin, Y., Safar, M., Benetos, A., 1994. Effects of bradykinin B_2 receptor antagonism on the hypotensive effects of ACE inhibition. Br. J. Pharmacol. 113, 717–722.

Busse, R., Fleming, I., Hecker, M., 1993. Endothelium-derived bradykinin: implications for angiotensin-converting enzyme-inhibitor therapy. J. Cardiovasc. Pharmacol. 22 (Suppl. 5), S31–S36.

Campbell, D.J., Kladis, A., Valentijn, A.J., 1995. Effects of losartan on angiotensin and bradykinin peptides and angiotensin-converting enzyme. J. Cardiovasc. Pharmacol. 26, 233–240.

Cheetam, C., Collis, J., O'Driscoll, G., Stanton, K., Taylor, R., Green, D., 2000. Losartan, an angiotensin type 1 receptor antagonist, improves endothelial function in non-insulin-dependent diabetes. J. Am. Coll. Cardiol. 36, 1461–1466.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

De Gennaro Colonna, V., Rossoni, G., Rigamonti, A.E., Bonomo, S., Manfredi, B., Berti, F., Muller, E.E., 2002. Enalapril and quinapril improve endothelial vasodilator function and aortic eNOS gene expression in L-NAME-treated rats. Eur. J. Pharmacol. 450, 61–66.

Erdos, E.G., 1975. Angiotensin I converting enzyme. Circ. Res. 36, 247–255.

Gohlke, P., Lamberty, V., Kuwer, I., Bartenbach, S., Schnell, A., Linz, W., Scholkens, B.A., Wiemer, G., Unger, T., 1993. Long-term low-dose angiotensin converting inhibitor treatment increases vascular cyclic guanosine 3′,5′-monophosphate. Hypertension 22, 682–687.

Gohlke, P., Linz, W., Scholkens, B.A., Wiemer, G., Unger, T., 1996. Cardiac and vascular effects of long-term losartan treatment in stroke-prone spontaneously hypertensive rats. Hypertension 28, 397–402.

Gohlke, P., Pees, C., Unger, T., 1998. AT_2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. Hypertension 31, 349–355.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and ^{15}N nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Guazzi, M.D., Campodonico, J., Celeste, F., Guazzi, M., Santambrogio, G., Rossi, M., Trabattoni, D., Alimento, M., 1998. Antihypertensive efficacy of angiotensin converting enzyme inhibition and aspirin counteraction. Clin. Pharmacol. Ther. 63, 79–86.

Hattori, Y., Akimoto, K., Murakami, Y., Kasai, K., 1997. Pyrrolidine dithiocarbamate inhibits cytokine-induced VCAM-1 gene expression in rat cardiac myocytes. Mol. Cell. Biochem. 177, 177–181.

Higashi, T., Kobayashi, N., Hara, K., Shirataki, H., Matsuoka, H., 2000. Effects of angiotensin II type 1 receptor antagonist on nitric oxide synthase expression and myocardial remodelling in Goldblatt hypertensive rats. J. Cardiovasc. Pharmacol. 35, 564–571.

Hornig, B., Kohler, C., Drexler, H., 1997. Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. Circulation 95, 1115–1118.

Hornig, B., Kohler, C., Schlink, D., Tatge, H., Drexler, H., 2003. AT_1-receptor antagonism improves endothelial function in coronary artery disease by a bradykinin/B_2-receptor-dependent mechanism. Hypertension 41, 1092–1095.

Kobayashi, N., Kobayashi, K., Hara, K., Higashi, T., Yanaka, H., Yagi, S., Matsuoka, H., 1999. Benidipine stimulates nitric oxide synthase and improves coronary circulation in hypertensive rats. Am. J. Hypertens. 12, 483–491.

Kobayashi, N., Kazuoyoshi, H., Watanabe, S., Higashi, T., Matsuoka, H., 2000. Effect of imidapril on myocardial remodeling in L-NAME-induced hypertensive rats is associated with gene expression of NOS and ACE mRNA. Am. J. Hypertens. 13, 199–207.

Krishnankutty, S., MacGregor, J.S., Gupta, M., Barbant, S.D., Redberg, R., Yock, P.G., Chatterjee, K., 1993. Effect of selective angiotensin II receptor antagonism and angiotensin converting enzyme inhibition on the coronary vasculature in vivo. Circulation 87, 931–938.

Linz, W., Scholkens, B.A., 1992. A specific B_2-bradykinin receptor antagonist HOE 140 abolishes the antypertrophic effect of ramipril. Br. J. Pharmacol. 105, 771–772.

Pradelles, P., Grassi, J., MacLouf, J., 1985. Enzyme immunoassays of eicosanoids using acetylcholine esterase as label: an alternative to radioimmunoassay. Anal. Chem. 57, 1170–1173.

Prasad, A., Tupas-Habib, T., Schenke, W.H., Mincemoyer, R., Panza, J.A., Waclawin, M.A., Ellahham, S., Quyyumi, A.A., 2000. Acute and chronic angiotensin-1 receptor antagonism reverses endothelial dysfunction in atherosclerosis. Circulation 101, 2349–2354.

Searles, C.D., Harrison, D.G., 1999. The interaction of nitric oxide, bradykinin, and angiotensin II type 2 receptor: lessons learned from transgenic mice. J. Clin. Invest. 104, 1013–1014.

Su, J.B., Barbe, F., Remi, H., Guyene, T.T., Crozatier, B., Hittinger, L., 1998. Preserved vasodilator effect of bradykinin in dogs with heart failure. Circulation 98, 2911–2918.

Su, J.B., Barbe, F., Crozatier, B., Campbell, D.J., Hittinger, L., 1999. Increased bradykinin levels accompany the hemodynamic response to acute inhibition of angiotensin-converting enzyme in dogs with heart failure. J. Cardiovasc. Pharmacol. 34, 700–710.

Wiemer, G., Scholkens, B.A., Becker, R.H.A., Busse, R., 1991. Ramiprilat enhances endothelial autacoid formation by inhibiting breakdown of endothelium-derived bradykinin. Hypertension 18, 558–563.