Differential Effect of Chronic Antihypertensive Treatment on Vascular Smooth Muscle Cell Phenotype in Spontaneously Hypertensive Rats

Rosario Bravo, Beatriz Somoza, Mariano Ruiz-Gayo, Carmen González, Luis Miguel Ruilope, Maria Soledad Fernández-Alfonso

Abstract—The aim of this study was to investigate the effect of chronic losartan or captopril on vascular smooth muscle cell (VSMC) phenotype and vascular function in spontaneously hypertensive rats. Male 12-week-old rats were treated for 16 weeks with losartan (15 mg/kg per day) or captopril (60 mg/kg per day) in their drinking water. Systolic blood pressure, measured by the tail-cuff method, was reduced $\approx 40$ mm Hg in both treatment groups compared with a nontreated control group. Cell structure and proliferation studies were performed in VSMCs obtained from rat carotid arteries. Cells from the losartan-treated group showed a significant reduction in size, total protein content, and nucleus number, as well as proliferation after stimulation with 10% fetal calf serum and an increased percentage of cells in the G1 phase compared with the control and captopril-treated groups. Functional studies were performed in isolated carotid arteries from these groups. Contractions elicited by 75 mmol/L KCl or $10^{-7}$ mol/L norepinephrine and relaxations elicited by acetylcholine were similar in all groups. Concentration-response curves to angiotensin I or angiotensin II ($10^{-10}$ to $3 \times 10^{-7}$ mol/L) were almost abolished in the losartan-treated group and were not modified by preincubation with the angiotensin type 2 receptor antagonist PD 123,319. These results suggest that long-term losartan treatment significantly changes VSMC phenotype and proliferative status, apparently unrelated to blood pressure lowering or to endothelial function improvements. (Hypertension. 2001;37:e4-e10.)

Key Words: losartan $\square$ captopril $\square$ muscle, smooth, vascular $\square$ cell cycle $\square$ hypertrophy

In the hypertensive state, a number of adaptive changes occur in blood vessels. In addition to endothelial dysfunction and extracellular matrix increase, the main structural alteration of the vascular wall in hypertension involves the medial layer. Spontaneously hypertensive rats (SHR) show an increase in the relation media:lumen because of medial hypertrophy/hyperplasia. Hypertrophy of vascular smooth muscle cells (VSMCs) is accompanied by an increase in DNA ploidy, with up to 40% of the cellular population in SHR being polyploid. It seems to be an incomplete response of the cell to growth factors caused by mechanisms that were not completely understood until now. The cell is able to increase its mass and DNA content, but it is unable to divide.

Structural abnormalities may play an important role in the development and maintenance of hypertension, because they may amplify the vasoconstrictor action of several local factors at the vascular wall. The objective of antihypertensive treatment is to improve vascular function, induce a regression of medial hypertrophy, and reduce the number of polyploid cells.

Angiotensin (Ang) II is now being regarded as a causal factor in the development of hypertension. It is a vasoconstrictor and trophic factor that mediates contractile and proliferative actions mainly by stimulating the AT1 receptor. There are 2 different pharmacological ways of interfering with Ang II–mediated actions: ACE inhibitors (ACEI) inhibit Ang II generation, whereas angiotensin type 1 (AT1) receptor antagonists block the binding of the peptide to its receptor. Both pharmacological interventions have been successfully introduced in the treatment of hypertension and have been shown to have a beneficial effect on vascular structure and function. They attenuate endothelial dysfunction and enhance NO release from vascular endothelium, which may contribute to the reduction of vascular structural alterations.

Despite the large amount of work regarding the beneficial effects of ACE inhibition and AT1 receptor blockade on...
vascular structure, there are few studies assessing the effect of those treatments on VSMC hypertrophy and proliferative impairment. In the present study, we have investigated the effect of chronic losartan or captopril on VSMC phenotype in SHR. For this purpose, we first cultured VSMCs from carotid arteries of treated and control rats, and we analyzed VSMC size, total protein content, cell cycle, and serum-induced proliferation. Second, we analyzed the effect of chronic treatment on endothelial function and vascular contractility in response to Ang I and Ang II in rat carotid arteries. This approach may help to determine whether there is a link among treatment, structure, and function.

Methods

Animals and Treatment

Twelve-week-old male SHR weighing 250 to 300 g were obtained from Charles River France (France). Rats were housed in groups of 4, under controlled dark-light cycles (12 h/12 h) and temperature conditions, with food (A.04, Panlab) and water available ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee according to the guidelines of the European Community for the ethical care of experimental animals.

Animals were randomly grouped (n=12) to receive either losartan (15 mg/kg per day), captopril (60 mg/kg per day), or tap water (control group) for 16 weeks. Drugs were dissolved daily in tap water, at a final concentration calculated as a function of body weight and the volume of water consumed the day before. Systolic blood pressure (SBP) was measured by the tail-cuff method (Narco Biosystems) before the beginning of treatment and every 2 weeks during treatment until week 10. The average of 3 measurements was taken as initial mean SBP. After treatment, rats were weighed, anesthetized with pentobarbital (50 mg/kg), and killed by exsanguination through cardiac puncture.

Culture of Rat Carotid VSMCs

VSMCs were prepared from common carotid arteries. Briefly, carotid arteries were carefully isolated and placed in a physiological solution ([in mmol/L]: NaCl 115, KCl 4.6, CaCl2 2.5, NaHCO3 25, KH2PO4 1.2, MgSO4 1.2, EDTA 0.01, and glucose 11) at 37°C. All experiments were performed in presence of 5×10^-3 mol/L dexamethasone to avoid induction of NO synthase. Each vascular ring (3 mm in length) was suspended on 2 intraluminal parallel wires, introduced into an organ bath containing physiological solution S at 37°C, and connected to a Pidem strain gauge for isometric tension recording. All segments were given an optimal resting tension of 2 g, which was readjusted every 15 minutes during a 90-minute equilibration period.

At the beginning of the experiment, vessels were exposed to 75 mmol/L KCl to check their functional integrity. Thereafter, vessels were precontracted with 10^-7 mol/L norepinephrine (NE), and a concentration-response curve to acetylcholine (ACh, 10^-6 to 10^-3 mol/L) was performed to functionally evaluate the presence or absence of endothelium. Thereafter, concentration-response curves to Ang I and Ang II were performed (10^-10 to 3×10^-7 mol/L).

Reagents

Ang I, Ang II, fluorescein, and propidium iodide were obtained from Sigma; PD 123,319, from RBI. Tissue culture media and supplements were purchased from Gibco. Drugs were dissolved in distilled water. [3H]methylthymidine was obtained from Amersham; Kinesis 50, from Bio Rad.

Analysis of Data

Comparisons among groups were made by 1-way ANOVA. Post hoc comparisons were made by the Newman-Keuls test. Statistical significance was set at P<0.05.

Results

Effect of Treatment on SBP and Left Ventricular Hypertrophy

A significant decrease of ~40 mm Hg, which was equipotent for both losartan and captopril, was observed for SBP 2 weeks after the beginning of treatment and during the next 10 weeks (data not shown). Body weights were not affected by the antihypertensive treatment (control, 438±16 g; losartan, 449±10 g; and captopril, 413±5 g).

The ratio between left ventricular weight and body weight was significantly reduced by treatment (control, 2.9±0.1 mg/g; losartan, 2.5±0.005 mg/g, P<0.05; and captopril, 2.4±0.07 mg/g, P<0.05), whereas right ventricular weight (control, 0.5±0.03 mg/g; losartan, 0.48±0.03 mg/g; and
captopril, 0.47±0.04 mg/g) was unaffected. A correlation between systolic blood pressure and left ventricular weight/body weight could be established ($r=0.736$, $P<0.001$).

Cell Structure and Proliferation

VSMCs in culture were used only until passage 5. At higher passages, cells began to slowly lose their phenotypic differences compared with cells of the untreated group (data not shown).

Relative cell size and total protein content were determined in VSMCs in culture by flow cytometry. Both parameters were significantly reduced in the losartan-treated group compared with those in the control or captopril-treated groups (Figure 1). VSMCs from the control and captopril-treated rats had 2 nuclei, whereas cells from the losartan-treated rats had only 1 nucleus (Figure 2).

DNA synthesis was measured by the incorporation of [3H]methylthymidine 24 and 48 h after the addition of 10% FCS. Incorporation of [3H]methylthymidine significantly increased with time in both the control and treated groups (Figure 3a). Protein content after 10% FCS stimulation increased significantly with time in the losartan-treated group but not in the control or captopril groups (Figure 3b). When both responses were compared, only VSMCs from the losartan-treated group showed a parallel increase in [3H]methylthymidine incorporation and protein content, indicating proliferation to 10% FCS.

Cell cycle analysis of VSMCs from the losartan-treated group revealed a significant increase of the number of cells in the G1 phase, as well as a significant decrease of cells in the S phase, compared with the untreated group (Figure 4). No changes were observed in the G2+M phase.

Functional Studies

Contractions to 75 mmol/L KCl or to $10^{-7}$ mol/L NE were analyzed to test functional behavior of the vessel. Responses to both contractile agents were similar in all groups (KCl: control, 932±41 mg; losartan, 989±240 mg; and captopril, 916±32 mg; n=50 to 51; NE: control, 691±26 mg; losartan, 630±28 mg; and captopril, 640±29 mg; n=49 to 51).

The functional integrity of the endothelium was assessed with acetylcholine. The concentration-response curve to acetylcholine ($10^{-9}$ to $10^{-4}$ mol/L) induced a relaxation, which was significantly greater in both treated groups at a concentration of $10^{-6}$ mol/L (Figure 5a).

![Figure 1](image1.png)

**Figure 1.** Effect of chronic losartan or captopril on relative cell size (a) and protein content (b) determined by flow cytometry of rat carotid VSMCs in culture. CO indicates control; L, losartan; and C, captopril. Results are expressed as mean±SEM, n=4 measurements (4×100,000 cells). *$P<0.05$ vs control.

![Figure 2](image2.png)

**Figure 2.** Photomicrographs from immunohistochemical staining with anti-α-actin antiserum of VSMCs from untreated (a), captopril-treated (b), and losartan-treated (c) SHR. Arrows indicate polyploid cells.
Vascular reactivity in response to Ang I and Ang II (10^{-10} to 3 \times 10^{-7} \text{ mol/L}) was analyzed (Figure 5b and 5c). The concentration-response curves were started at 10^{-10} \text{ mol/L} to avoid desensitization at higher concentrations of the peptides. Both peptides elicited a contraction maximum at 10^{-7} \text{ mol/L}, and tone was lost at higher concentrations. Ang I-- and Ang II--induced contractions in the captopril segments were similar to those elicited in the control group. Contractions elicited by either Ang I or Ang II were almost abolished in the losartan group compared with the nontreated group.

To analyze the putative role of AT_{1} receptors on the reduced contractions induced by Ang II in the losartan-treated group, segments were preincubated with 10^{-7} \text{ mol/L} PD 123,319 for 30 minutes. No differences could be observed in the group treated with losartan (Figure 5c) or the untreated or captopril-treated groups (data not shown).

**Discussion**

The major finding of the present study is that long-term losartan treatment results in a significant change in VSMC phenotype and proliferative status. This effect does not seem to be related to lowering of blood pressure or improvement of endothelial function, because it was not observed in VSMCs of the captopril-treated group.

VSMCs obtained from the carotid artery from the untreated group showed hypertrophy and polyploidy, according to previous results documented in SHR. Hypertrophy and polyploidy are found preferentially in conduit arteries, whereas hyperplasia and remodeling are found mainly in small arteries and arterioles. VSMCs from the losartan-treated rats had only 1 nucleus and exhibited proliferation in response to growth stimuli, whereas VSMCs obtained from the captopril group had a phenotype similar to that of cells from the untreated SHR. It is well known that VSMCs undergo phenotypic modulation in culture, changing from a contractile to a secretory phenotype. It is unlikely that differences observed between control and losartan-treated cells in this study can be attributed to a differential phenotypic modulation in vitro. We used cells only at low passages to discriminate between differences caused by treatment or phenotypic modulation. In addition, we observed that differences in structure and growth responses were maintained for a number of passages and were then slowly lost during culture for higher passages. The changes observed in this work seemed to occur, therefore, in the rat during treatment.

Similar findings regarding vascular structure have been described after a 2-week treatment with losartan, in which reduction of both aortic and tail artery weight and medial thickness could be observed. No changes in vascular hypertrophy were detected, however, after chronic treatment with subantihypertensive doses of ACEI. To the contrary, at antihypertensive doses of ACEI, structural alterations in different vascular beds were reduced. In the present study, both antihypertensive treatments reduced left ventricular weight in an equipotent way, and a correlation could be observed with changes in systolic blood pressure, as expected.

These observations suggest a differential effect between ACEI and AT_{1} receptor antagonists on cardiovascular struc-
ture, which might be a consequence of their different molecular mechanisms of action. The competitive blockade of the AT$_1$ receptor by AT$_1$ antagonists leaves the AT$_2$ receptor unopposed. Under these circumstances, a number of indirect observations suggest an AT$_2$ receptor–mediated NO release. Long-term blockade of AT$_1$ receptors with losartan leads to increased cGMP levels in the aorta of SHR rats, which may be secondary to an elevation of plasma Ang II levels and a stimulation of AT$_2$ receptors. Seyedi et al suggested that Ang II, by acting on AT$_2$ receptors, may activate local kinin release, leading to the increase of NO production. Likewise, Gohlke et al recently demonstrated that the activation of AT$_2$ receptors by Ang II results in a bradykinin-dependent stimulation of NO release in the rat aorta, with the subsequent increase in cGMP levels.

During ACE inhibition there is an alternative cleavage of Ang I by neutral endopeptidase (EC 3.4.24.11), which yields Ang-(1-7). This peptide elicits a concentration-dependent vasodilatation in porcine and canine coronary arteries, which seems to be mediated by NO release, probably by an intermediate release or accumulation of kinins through a receptor characterized as non-AT$_1$–non-AT$_2$. In addition, ACE inhibitors increase the half-life of bradykinin (BK) by inhibiting its degradation. BK is a vasodilator peptide, which releases NO from the endothelium after stimulation of the B$_2$ receptor.

It can be argued that the increase in half-life of BK caused by captopril treatment might be responsible for VSMC phenotype. It has generally been assumed that BK had an antiproliferative effect, based on its ability to release NO from the endothelium. It was recently reported, however, that BK stimulates proliferation of VSMCs. During treatment with captopril, VSMCs would be in an environment of higher BK concentrations, submitted to 2 opposed actions of the peptide, in a direct proliferative action on VSMCs and an indirect antiproliferative action caused by stimulation of NO release. These opposed actions might result in the lack of benefit of ACEI treatment on VSMC phenotype. In contrast, in the losartan-treated animals, the proliferative action of Ang II is blocked, and there is a subsequent increase in NO availability. Both actions could contribute to the antiproliferative effect of Ang II antagonism.

It is interesting to note that left ventricular weight/body weight is reduced in both treated groups, although BK has been demonstrated to induce proliferation of cardiomyocytes. This might indicate, once again, that left ventricular hypertrophy depends on blood pressure levels at a higher extent than on local growth factors. To the contrary, vascular hypertrophy seems to be mediated mainly by local growth modulators and less by blood pressure levels.

The second interesting finding of this work is the persistent effect elicited by long-term losartan treatment on contractility in response to Ang I and Ang II, as well as on VSMC phenotype. The decrease in vascular responsiveness observed with the peptides, but not with NE or KCl, suggests that it might be a direct consequence of the specific blockade of the AT$_1$ receptor. Like our results, part of the hypotensive effect induced by a long-term or even a shorter treatment with losartan persisted for a long time after the withdrawal of
treatment. A long-lasting inhibition of the potentiating effect of Ang II on sympathetic nerve function has been also described. A loss of effect of Ang II would be consistent with a downregulation of AT$_1$ receptors, which is rather unexpected from a chronic treatment with a receptor antagonist but has been previously described. This might be because of the increase in NO availability, which has been shown to downregulate AT$_1$ receptor expression and number.

We tested the hypothesis that the diminished contractile effect of the peptides in the losartan-treated group could be caused by stimulation of AT$_2$ receptors. Gohlike et al demonstrated that a specific AT$_2$ receptor antagonist, PD 123,319, blocked the effect of Ang II alone and Ang II plus losartan. The lack of effect of PD 123,319 in this work suggests that AT$_2$ receptors are not involved in this persistent inhibition of contractions observed with the peptides.

The active metabolite of losartan, EXP 3174, a noncompetitive antagonist, has a slow dissociation kinetic from AT$_1$ receptors and theoretically could account for the result obtained in the contractility studies but probably not the persistent change in phenotype of VSMCs in culture.

Another possibility would be an eventual partial agonist character of losartan or of a losartan metabolite. Most of the work in this field shows a lack of agonist character of losartan, although some partial agonism has been detected in vitro experiments in rat isolated glomeruli and in human mesangial cells.

In conclusion, the present study demonstrates that chronic treatment with losartan or captopril exerts a beneficial action on vascular functional alterations observed in SHR. The same treatment conditions, however, have a differential effect on VSMC phenotype and proliferative status. This seems to be independent of blood pressure levels but might be related to the mechanism in the inhibitory action of the renin-angiotensin system exerted by ACE inhibitors and AT$_1$ receptor antagonists.

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