British Journal of Pharmacology (2005) 144, 755–760
www.nature.com/bjp

Evidence for a functional cardiac interaction between losartan and angiotensin-(1–7) receptors revealed by orthostatic tilting test in rats

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1 Studies have shown that the angiotensin II (Ang II) AT_1 receptor antagonist, losartan, accentuates the orthostatic hypotensive response in anesthetized rats, and there is evidence indicating that this effect is not exclusively mediated by AT_1 receptors.

2 We investigated whether the pronounced orthostatic cardiovascular response observed in losartan-treated rats involves an interference with angiotensin-(1–7) (Ang-(1–7)) receptors.

3 Urethane-anesthetized rats were submitted to orthostatic stress (90° head-up tilt for 2 min). Intravenous injection of losartan (1 mg kg^{-1}, $n=9$) significantly accentuated the decrease in mean arterial pressure (MAP) induced by head-up tilt (-33 ± 6% after losartan vs -15 ± 8% control tilt). This effect was accompanied by a significant bradycardia (-8 ± 3% after losartan vs -3 ± 3% control tilt). Another AT_1 antagonist, candesartan, did not potentiate the decrease of MAP and did not change the cardiac response induced by head-up tilt. Strikingly, administration of the Ang-(1–7) antagonist, A-779 (10 nmol kg^{-1}, $n=5$), totally reversed the bradicardiac effect caused by losartan and this effect was accompanied by a tendency towards attenuation of the hypotensive response caused by losartan.

4 These findings indicate that the marked orthostatic cardiovascular response is specific for losartan, and that it may be due, in part, to an interaction of this antagonist with Ang-(1–7) receptors, probably at the cardiac level.
British Journal of Pharmacology (2005) **144,** 755–760. doi:10.1038/sj.bjp.0706039
Published online 31 January 2005

Keywords: Ang antagonists; ang receptors; tilt test; cardiovascular system

Abbreviations: Ang II, angiotensin II; Ang-(1–7), angiotensin-(1–7)

Introduction

Previous studies have shown that, in the rat model of orthostatic challenge, or tilting test, the angiotensin II (Ang II) AT_1 receptor antagonist, losartan, produces a pronounced orthostatic hypotensive response (Ohlstein *et al*., 1992; Hashimoto *et al*., 1999). The same effect is not observed with other Ang II AT_1 receptor antagonists (Ohlstein *et al*., 1992; Hashimoto *et al*., 1999), or even with converting enzyme inhibitors (Ohlstein *et al*., 1992). This suggests that, at least peripherally, the orthostatic hypotensive effect caused by losartan is beyond antagonism of Ang II AT_1 receptor blockade. However, the mechanisms involved in mediating the orthostatic hypotensive effect of losartan in rats were not investigated in these previous studies (Ohlstein *et al*., 1992; Hashimoto *et al*., 1999).

During the last decade, studies using the selective angiotensin-(1–7) (Ang-(1–7)) antagonist D-Ala^7-Ang-(1–7) (A-779) (Santos *et al*., 1994) provided pharmacological evidence for an Ang-(1–7) receptor distinct from the classical Ang II receptors AT_1 and AT_2 (Fontes *et al*., 1994; Ferrario *et al*., 1998; Santos *et al*., 2000; Heringer-Walther *et al*., 2001). These findings were recently corroborated by the identification of an Ang-(1–7) receptor, the G protein-coupled receptor Mas (Santos *et al*., 2003). Von Bohlen Und Halbach *et al*. (2000) reported that an interaction between this Ang-(1–7) receptor and Ang II AT_1 receptors exists. In addition, several previous studies have shown that, under certain conditions, Ang-(1–7) actions can be blocked by relatively low doses of losartan (Santos *et al*., 2000). Furthermore, reports suggesting a previously unsuspected nonspecificity for losartan provided additional arguments to the hypothesis that Ang-(1–7) receptors, in the kidney (Chansel *et al*., 1994; Gironacci *et al*., 1999) and perhaps in the heart (Gironacci *et al*., 1994), are sensitive to losartan.

Therefore, the aim of the present study was to investigate whether the previously reported orthostatic hypotension caused by losartan involves AT_1 receptor-independent mechanisms and specifically interaction with Ang-(1–7) receptors. For this purpose, the effects of losartan alone on the cardiovascular response to orthostatic tilt were compared with another AT_1 antagonist, candesartan, and with that obtained in the presence of blockade of Ang-(1–7) receptors.

Methods

Experiments were performed on male Wistar rats (~300 g) bred at the animal facilities of the Biological Sciences Institute

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Published online 31 January 2005

(CEBIO, UFMG, Belo Horizonte, MG, Brazil). All experiments were carried out in accordance with the guidelines established by our local Institutional animal Welfare Committee. Animals were housed individually and permitted free access to food and water. Efforts were made to avoid any unnecessary distress to the animals. On the day of the experiments, rats were anesthetized (urethane, 1200–1400 mg kg^{-1} i.p.) and the adequacy of anesthesia was verified by the absence of a withdrawal response to nociceptive stimulation of a hind paw. Supplemental doses of urethane (100 mg kg^{-1} i.v.) were administered if necessary. A tracheotomy was performed and polyethylene catheters were placed into a femoral vein and a femoral artery for infusion of drugs and recording of cardiovascular parameters, respectively. At this stage, a stable baseline arterial pressure and heart rate (HR) was helpful to indicate an adequate level of anesthesia and, as urethane is an anesthetic of long duration of action, this condition was maintained throughout the experimental period (maximum 3 h). To reduce the cardiovascular variability among rats caused by a possible head movement and consequent interference of vestibulosympathetic reflex (Yates *et al*., 1993; Kerman *et al*., 2000) during the tilt procedure, the head was carefully placed in a stereotaxic frame (David Kopf Instruments, CA, U.S.A.) with the tooth bar fixed 11 mm below the level of the interaural line. To avoid undesirable body movements during the tilting procedure, an adjustable saddle fixed to the stereotaxic frame supported the animals. Mean arterial pressure (MAP) and HR were continuously recorded with a transducer connected to a data acquisition system (Power Lab, ADInstruments). The pressure transducer was maintained at the level of the heart so that tilting does not influence blood pressure measurement. Changes in blood pressure from pretilt levels were monitored continuously.

Experimental procedure

The tilting test (a test of postural hypotension) was conducted by raising the head side of a tilt board from a horizontal position to a 90° head-up position within approximately 1 s. Rats were subjected to a 90° head-up tilt for 2 min. Then, they were returned to the horizontal position (in approximately 1 s), after which the cardiovascular parameters were allowed to stabilize.

Baseline BP and HR levels were monitored over 30 min. Then, rats received an initial i.v. injection of vehicle (0.9% NaCl, 1 ml kg^{-1}) and a control tilt was performed. At the end of the control tilt, a recovery period of 15 min was waited; after that, the rats were then injected once (i.v.) with one of five different treatments: either vehicle (0.1 ml kg^{-1}; $n=5$), candesartan (1 mg kg^{-1}; $n=5$), losartan (1 mg kg^{-1}; $n=9$), losartan (1 mg kg^{-1}) plus A-779 (Ang-(1–7) antagonist; 10 nmol kg^{-1}; $n=5$) or A-779 alone (10 nmol kg^{-1}; $n=8$). Tilting was repeated at 60 and 120 min after each treatment (designated tilt 2 and 3, respectively). Changes in blood pressure and HR from pretilt levels were monitored continuously.

Losartan (Merck, NJ, U.S.A.), candesartan (Takeda Chemical Industries, Osaka, Japan) and A-779 (BACHEM, CA, U.S.A.) were dissolved in sterile saline (NaCl 0.9%) and administered in a volume of 0.1 ml kg^{-1}.

Data analysis

The basal values of MAP and HR were measured as the average values of these variables over the 5-min period immediately preceding the tilting tests as described above. Owing to the marked fall in MAP caused by i.v. injections of AT_1 antagonists, the effects of treatment on cardiovascular reactivity to the tilting test were expressed as the percent change from basal values shown in Table 1. Comparisons between control tilt responses and tilt after treatment were determined by the paired Student's *t*-test. Differences at $P<0.05$ were considered to be statistically significant. All values are presented as mean ± s.e.

Results

Basal levels of MAP and HR obtained before the control tilting test for all different groups of treatment are presented in Table 1. No significant differences in the baseline values of cardiovascular variables among groups were found.

In the saline-treated group (Figure 1a), head-up tilt induced a progressive fall in MAP, with onset immediately after the beginning of the test. The mean maximum fall in MAP during the 2-min tilting period was 18 ± 1 mmHg ($P<0.01$ vs baseline levels). Immediately after the end of tilting period, there was a gradual increase in MAP back to the baseline levels (within 60 s). No significant difference in the pattern of blood pressure response was observed when the test was repeated at 60 or 120 min after the control tilt. In the saline-treated group, head-up tilt caused no changes in baseline HR levels. The same pattern of HR response was observed when tilting was performed 60 or 120 min after the control tilt (Figure 1a).

Intravenous injections of losartan resulted in a large decrease in baseline blood pressure levels and the magnitude

Table 1 Absolute basal values of MAP and HR before and 60 or 120 min after intravenous injections of different compounds

		Before		*60* min		*120* min	
Treatment	n	*MAP* (mmHg)	*HR* (b.p.m.)	*MAP* (mmHg)	*HR* (b.p.m.)	*MAP* (mmHg)	*HR* (b.p.m.)
Vehicle	5	105 ± 6	383 ± 29	103 ± 7	393 ± 20	100 ± 6	394 ± 21
Losartan	9	94 ± 5	375 ± 8	71 ± 4**	352 ± 8*,†	73 ± 4**	354 ± 6††
Candesartan	5	94 ± 7	388 ± 16	63 ± 9**	391 ± 18	61 ± 7**	403 ± 16
Losartan + A-779	5	90 ± 4	389 ± 28	67 ± 5**	393 ± 29#	69 ± 9*	398 ± 26#
A-779	8	88 ± 4	350 ± 14	78 ± 2*	339 ± 15	65 ± 4**	334 ± 13

The basal values of MAP and HR presented were measured as the average values of these variables over the 5-min period immediately preceding the control tilt, tilt 2 or tilt 3. Values are mean ± s.e.m. MAP: mean arterial pressure; HR: heart rate. *$P<0.05$ vs before; **$P<0.01$ vs before; †$P<0.05$ losartan vs candesartan; ††$P<0.01$ losartan vs candesartan; #$P<0.05$ losartan vs losartan + A-779.

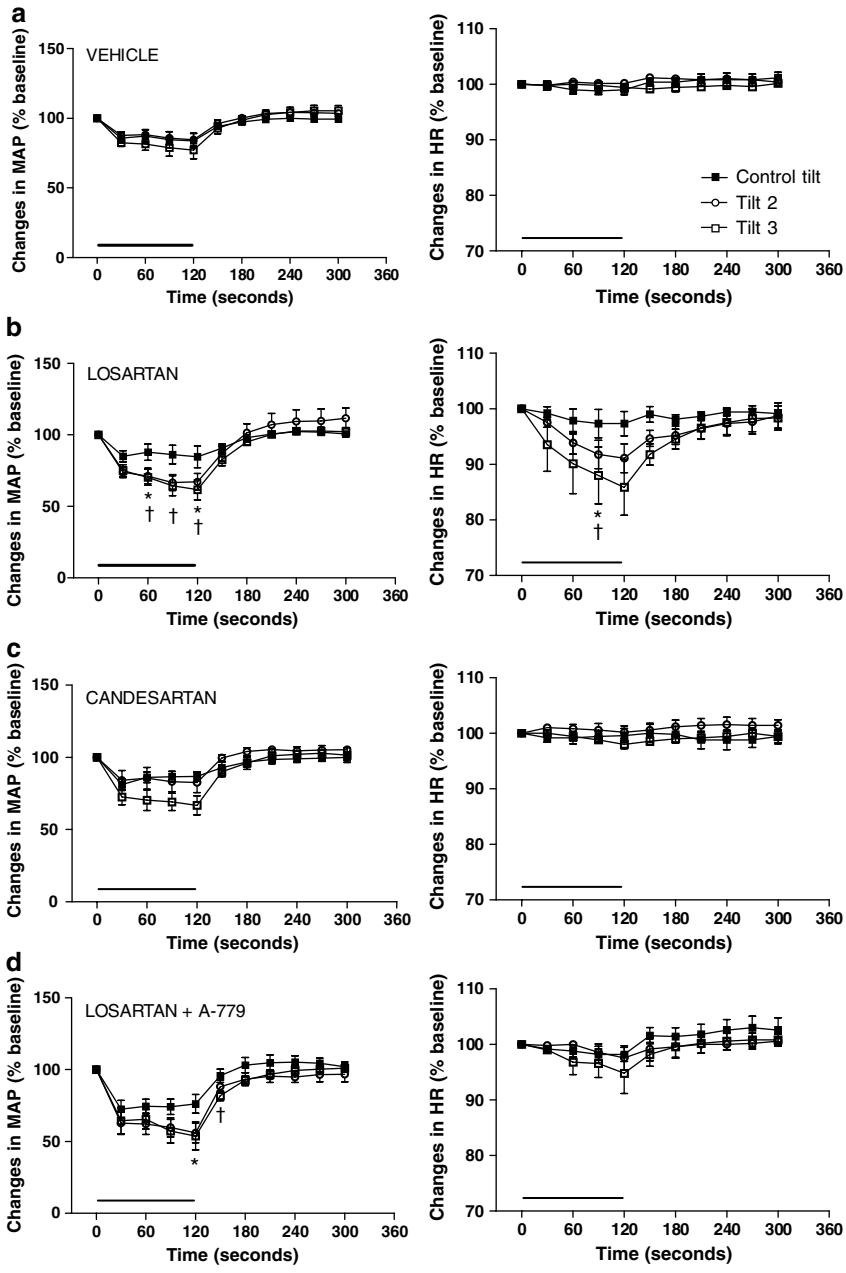

Figure 1 Changes in MAP and HR to 90° head-up tilt, before any treatment (control tilt) and at 60 and 120 min (designated tilt 2 and 3, respectively) after intravenous injection of: panel a, vehicle (0.9% NaCl, 1 ml kg^{-1}, $n=5$); panel b, losartan (1 mg kg^{-1}, $n=9$); panel c, candesartan (1 mg kg^{-1}, $n=5$); panel d, losartan + A-779 (losartan, 1 mg kg^{-1}; A-779 10 nmol kg^{-1}; $n=5$). Percent changes were calculated from pre-tilt basal values at the respective times as shown in Table 1. Bar indicates the duration of the maneuver (0–120 s). *$P<0.05$ tilt 2 vs control tilt; †$P<0.05$ tilt 3 vs control tilt (paired t-test).

of the effect was comparable to that observed after candesartan injection (Table 1). Losartan also produced a fall in baseline HR that reached statistical significance 60 min after administration. No significant changes in baseline HR were observed after candesartan treatment (Table 1). Addition of A-779 to losartan did not alter the magnitude of the fall in arterial pressure observed after losartan alone (Table 1). However, the fall in baseline HR by losartan was not observed when A-779 was also administered.

Losartan caused a significant potentiation of the decrease in MAP induced by head-up tilt. The mean maximal decrease in MAP was $-33\pm6\%$, which was much greater than that observed in the control tilt ($-15\pm8\%$, $P<0.05$). In particular, this effect was accompanied by an accentuated bradycardia ($-8\pm3\%$ after losartan vs $-3\pm3\%$ control tilt; $P<0.05$) (Figure 1b). In contrast, candesartan did not potentiate the decrease of MAP and did not change the cardiac response induced by head-up tilt (Figure 1c). The hypotensive effect caused by losartan showed a tendency towards attenuation when this antagonist was administered together with A-779. However, the attenuation was partial and small, since the hypotensive response was still significant for tilt 2, and tilt 3 when compared to the control tilt (Figure 1d). Strikingly however, A-779 totally reversed the losartan-induced brady-

cardia ($-1.4\pm1\%$ losartan + A-779 vs $-1.8\pm1\%$ control; $P=0.73$) (Figure 1d). Administration of A-779 alone caused a significant reduction in baseline blood pressure (Table 1), but did not change the tilt response (mean maximum changes: $-16\pm3\%$ control vs $-22\pm6\%$ after A-779; $-1\pm1\%$ control vs $-1\pm1\%$ after A-779).

Discussion

The results of the present study indicate that the orthostatic hypotension caused by losartan is related to an altered cardiac response, which was completely blocked by the Ang-(1–7) receptor antagonist, implicating Ang-(1–7) receptors in these effects. Therefore, this study confirms and extends previous findings showing that losartan treatment accentuates the orthostatic hypotensive response observed during the head-up tilt test in anesthetized rats.

Animal models have been largely used to clarify different aspects of cardiovascular compensatory mechanisms in response to passive tilt (Stella & Zanchetti, 1977; Stella *et al*., 1978; Gotoh *et al*., 1987; Golin *et al*., 1988). The present experiments were performed in anesthetized animals because this model has the advantage of minimizing the cardiovascular variability observed in conscious animals, caused by stress that may interfere with the central mechanisms involved in the reflex cardiovascular changes (Golin *et al*., 1991; Fontes *et al*., 2001; Dampney *et al*., 2002). In addition, urethane anesthesia is more suitable for experiments in which autonomic reflex compensatory changes are involved (Shimokawa *et al*., 1998), such as tilting experiments (Miki *et al*., 1989).

The vehicle-treated series of experiments (Figure 1a) demonstrate that successive tilt maneuvers, 60 or 120 min after the control tilt, were characterized by reproducible cardiovascular responses, similar to that observed in the control tilt and featured by a quite stable pattern of HR. Therefore, this model is appropriate to evaluate drug-related interference in postural cardiovascular compensatory mechanisms.

In this study, treatment with candesartan did not alter the cardiovascular reactivity to orthostatic tilt. Candesartan is a highly selective AT_1 antagonist, 48 times more potent than losartan (Shibouta *et al*., 1993). Therefore, our study supports previous findings showing that the orthostatic effect caused by losartan is beyond antagonism of Ang II AT_1 receptor blockade (Ohlstein *et al*., 1992; Hashimoto *et al*., 1999). Previous reports of nonspecificity of losartan for AT_1 receptors provided arguments to the hypothesis that Ang-(1–7) receptors, in the kidney (Chansel *et al*., 1994; Gironacci *et al*., 1999) and perhaps in the heart (Gironacci *et al*., 1994), are sensitive to losartan. Regarding the latter, a possible interaction at cardiac level is suggested in view of our experiments in which losartan treatment clearly altered the cardiac response normally observed during the control tilt maneuver, causing an accentuated bradycardia. This result suggests that the hypotensive response observed during the tilt maneuver after losartan treatment was largely due to an altered cardiac reactivity that was normalized by Ang-(1–7) receptor blockade.

It is possible that losartan could be interfering with a cardiac chronotropic regulatory action mediated by Ang-(1–7). Gironacci *et al*. (1994) found that losartan entirely prevented the increases in the electric stimulation-induced release of [3H]norepinephrine caused by Ang-(1–7) in the rat atria, suggesting a neuromodulatory and facilitatory action for this heptapeptide on the cardiac sympathetic neurotransmission (Gironacci *et al*., 1994). In addition, in a model of simulated ventricular ischemia and reperfusion, losartan display antiarrhythmic properties and this activity was independent of AT_1 receptor blockade (Thomas *et al*., 1996). Interestingly, Ang-(1–7) also displays antiarrythmogenic properties in reperfused isolated rat hearts and this effect appears to be mediated by a specific Ang-(1–7) receptor, although the effects of Ang-(1–7) in the presence of losartan or other Ang antagonists in this model have not been evaluated (Ferreira *et al*., 2001). Clearly, further studies will be needed to determine the mechanism involved.

In a very recent study, Collister & Hendel (2003) demonstrated that the chronic hypotensive effects of losartan in normal rats is attenuated by the Ang-(1–7) antagonist, A-779. In the same study, it was reported that rats chronically treated with losartan plus A-779 displayed a higher level of baseline HR than rats treated with losartan alone (Collister & Hendel, 2003). Interestingly, we also found in the present study that losartan alone reduced the baseline HR and this effect was abolished by treatment with A-779. Taken together, the results of the present and previous studies (Ferreira *et al*., 2001; Collister & Hendel, 2003) suggest that losartan might be interfering in a chronotropic regulatory action mediated by Ang-(1–7) receptors. In this regard, there is pharmacological evidence for the presence of a selective Ang-(1–7) receptor in the heart (Ferreira *et al*., 2001) and coronary arteries (Porsti *et al*., 1994). In addition, immunoreactivity for Ang-(1–7) was recently detected in cardiac myocites (Averill *et al*., 2003). Indeed, the recently characterized Ang-(1–7) receptor, Mas (Santos *et al*., 2003), is expressed in the rodent heart (Metzger *et al*., 1995). However, in a recent study, Sampaio *et al*. (2003) found that infusion of Ang-(1–7) does not alter the baseline HR. This finding argues against our proposal of a possible chronotropic effect mediated by Ang-(1–7), which says that losartan could be interfering. Firstly, it is important to point out that in the Sampaio *et al*. (2003) study, the Ang-(1–7) infusion effects on cardiovascular variables were evaluated for a short term in anesthetized rats under basal conditions. Our study suggests an acute action of losartan on Ang-(1–7) receptors in a model of cardiovascular stress (tilt). As the simplest explanation, it may be that such interactions are negligible under basal conditions, and only become visible and effective when the cardiovascular system is stressed even mildly as in tilting. Secondly, apart from this possibility, a previous study performed in conscious rats demonstrating that long-term infusion of Ang-(1–7) produces a small but significant sustained bradycardia (Braga *et al*., 2002), gives additional support to our proposal. However, the understanding of the functional contribution of Ang-(1–7) on cardiac function, and the exact mechanism of the interaction between losartan and Ang-(1–7) receptors, seems complex and requires further investigation.

Other mechanisms may be involved in the bradicardic effect of losartan, for example, a central action. In this regard, it is known that losartan can cross the blood–brain barrier (Li *et al*., 1993; Polidori *et al*., 1996) or act at the area postrema, a circumventricular organ lacking a blood–brain barrier (Johnson & Gross, 1993). Also, losartan may act the rostral ventrolateral medulla, a key region involved in the tonic and

reflex control of the sympathetic output (Tagawa *et al*., 2000) and where a high density of angiotensin receptors is present (Song *et al*., 1992). We are currently investigating this possibility.

The present study adds to growing evidence that suggests the involvement of Ang-(1–7) receptors in cardiac regulation both in normal (Gironacci *et al*., 1994; Porsti *et al*., 1994; Brosnihan *et al*., 1996) or pathological conditions (Ferreira *et al*., 2001; Loot *et al*., 2002; Zisman *et al*., 2003). In addition, our results suggest that losartan might interfere with Ang-(1–7) receptors in the regulation of cardiac chronotropism, which probably accounts for the orthostatic hypotension observed in losartan, but not in candesartan, treated rats. These observations provide insights of possible clinical relevance. Considerable evidence, albeit indirect, suggests that losartan is effective at suppressing complex ventricular arrhythmias, a common cause of death in patients with chronic heart failure, but the mechanism contributing to this antiarrhythmogenic property is yet undefined (Gavras & Gavras, 2000). Therefore, we could speculate that, part of this antiarrhythmogenic property of losartan could be mediated by an interaction with Ang-(1–7) receptors. This seems to be an interesting possibility, since cardioprotective effects were recently described for Ang-(1–7) (Santos *et al*., 2004). Clearly, future studies are necessary to unravel the mechanism and other consequences involved in the interaction between losartan and Ang-(1–7) receptors on the cardiac function.

This work was supported by PRONEX and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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(Received September 9, 2004
Accepted October 4, 2004)