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The selective angiotensin AT_1 receptor antagonist LR-B/081 potently inhibits drinking induced by central injection of angiotensin II in rats

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Abstract

LR-B/081, methyl-2-[[4-butyl-2-methyl-6-oxo-5-[[2′-(1*H*-tetrazol-5-yl) [1,1′-biphenyl]-4-yl] methyl]-1(6*H*)-pyrimidinyl] methyl]-3-thiophenecarboxylate, is a recently developed nonpeptide antagonist selective for angiotensin AT_1 receptors. The drug has been reported to be an insurmountable angiotensin AT_1 receptor antagonist endowed with long-lasting antihypertensive activity. A large body of evidence indicates that angiotensin AT_1 receptors mediate the dipsogenic action of angiotensin II in the central nervous system. The present study evaluated the ability of LR-B/081, in comparison with losartan and with its active metabolite EXP3174, to inhibit drinking induced by central injection of angiotensin II in water-sated rats. LR-B/081, in the dose range of 10–1000 pmol/rat, dose dependently inhibited the drinking response to angiotensin II, 10 pmol/rat. The ID_{50} of LR-B/081 was 25.9 pmol/rat, while that of losartan and EXP3174 was 357 and 3.9 pmol/rat, respectively. Therefore LR-B/081 was about 7 times less potent than EXP3174, but about 14 times more potent than the parent molecule losartan. LR-B/081 altered neither carbachol-induced water intake, nor 15% fat milk intake in rats, suggesting that its effect on angiotensin II-induced drinking is a behaviourally selective effect. These findings show that LR-B/081 potently inhibits central angiotensin AT_1 receptors involved in the control of body fluid homeostasis and suggest that this drug might be an interesting pharmacological tool to further investigate the role of the central renin-angiotensin system in physiological or pathological conditions.

Keywords: Angiotensin AT_1 receptor antagonist; Angiotensin II-induced drinking; Losartan; EXP3174; LR-B/081

1. Introduction

The octapeptide angiotensin II is known to play an essential role in the homeostasis of body fluids and Na^+ balance and in the control of blood pressure (Fitzsimons, 1986; Johnson et al., 1986; Phillips, 1987; Quadri et al., 1993; Timmermans et al., 1993; Wada et al., 1994). Therefore, great effort has been devoted to the development of nonpeptide angiotensin II receptor antagonists as potential antihypertensive agents. S-8307 and S-8308 were the first nonpeptide antagonists developed, but they were weak (Furukawa et al., 1982). Losartan (DuP753) was the first potent and highly selective angiotensin AT_1 receptor antagonist (Carini and Duncia, 1988).

Two distinct populations of specific angiotensin II binding sites have been demonstrated using structurally different nonpeptide ligands, such as losartan and PD 123177 (Blankley et al., 1991; Chiu et al., 1989, 1990; Whitebread et al., 1989). The angiotensin II binding sites that have affinity for losartan have been classified as AT_1 and those which bind PD 123177 as AT_2 (Bumpus et al., 1991). Both receptor subtypes are found in the periphery and in the central nervous system (Chang and Lotti, 1990a,b).

Autoradiographic studies have shown that the two receptor subtypes are not uniformly distributed in the rat brain (Rowe et al., 1991; Wamsley et al., 1990). The angiotensin AT_1 receptors are present in high density in the area postrema, subfornical organ, median preoptic nucleus and organum vasculosum of the lamina terminalis, whereas angiotensin AT_2 receptors are either found in very low density or are absent in these sites (Rowe et al., 1991; Steckelings et al., 1992; Tsutsumi and Saavedra, 1991). All of these brain regions are involved in the dipsogenic action of angiotensin II (Phillips, 1987). Accordingly, further studies have pro-

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vided evidence that angiotensin AT_1 receptors mediate angiotensin II-induced drinking (Fregley and Rowland, 1991; Beresford and Fitzsimons, 1992; Chow et al., 1992; Dourish et al., 1992; Rowland et al., 1992; Cooney and Fitzsimons, 1993; Sakai et al., 1994).

Recently a new potent and selective nonpeptide angiotensin AT_1 receptor antagonist, LR-B/081, has been discovered. The drug has been reported to be endowed with the following properties (Subissi et al., 1994; Renzetti et al., 1994): (a) High affinity for angiotensin AT_1 receptors in rat adrenal cortex membranes (about 10 times higher than that of losartan). (b) Negligible affinity for angiotensin AT_2 receptors in bovine cerebellum membranes. (c) Insurmountable antagonism at angiotensin AT_1 receptors in rabbit aorta strips. In this regard the drug behaves like EXP3174, an active metabolite of losartan (Wong et al., 1990), but differs from losartan, which is a surmountable angiotensin AT_1 receptor antagonist (Wong and Timmermans, 1991; Wienen et al., 1992). (d) LR-B/081 exerts a long-lasting antihypertensive effect (at least 24 h) following both oral and parenteral administration.

The present study was aimed at evaluating the ability of LR-B/081 to inhibit drinking induced by intracerebroventricular (i.c.v.) injection of angiotensin II in rats, which is known to be mediated by central angiotensin AT_1 receptors. The potency of the drug was compared to that of losartan and of its active metabolite EXP3174, which, like the parent molecule, is a selective angiotensin AT_1 receptor antagonist (Wong et al., 1990).

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River, Calco, Italy), weighing 325–350 g at the beginning of the experiments, were employed. They were individually housed in metal cages in a room with a 12 h light/dark cycle and controlled temperature and humidity ($20 \pm 2°$ C, 45–55%). They had free access to food pellets (4RF18, Mucedola, Settimo Milanese, Italy) and tap water.

2.2. Surgery

The animals were anesthetized with ketamine HCl (100 mg/kg body weight) and acepromazine (1.37 mg/kg body weight) and were injected with a prophylactic dose of gentamycin (10 mg/0.2 ml intramuscularly) before surgery. Using a stereotaxic instrument to reach the lateral ventricle, a guide cannula was implanted and cemented with dental acrylic cement to the skull, where three stainless-steel screws had been installed. The following coordinates were used for the guide cannula: AP = 1 mm behind the bregma, L = 2 mm from the sagittal suture, V = 2 mm from the surface of the skull. Intracerebroventricular injections were made by means of a stainless-steel injector temporarily inserted into the guide cannula and protruding 2.5 mm beyond the cannula tip.

2.3. Drugs

LR-B/081, methyl-2-[[4 butyl-2-methyl-6-oxo-5-[[2'-(1*H*-tetrazol-5-yl) [1,1'-biphenyl]-4-yl] methyl]-1(6*H*)-pyrimidinyl] methyl]-3-thiophenecarboxylate, was a gift of Lusofarmaco, Milan, Italy; losartan (Dup753) and its active metabolite EXP3174 were both gifts of DuPont Merck, Research and Development, Wilmington, DE, USA; Ile^5-angiotensin II was purchased from Novabiochem-Inalco, Milan, Italy; carbachol was purchased from Sigma Italia, Milan, Italy.

The angiotensin AT_1 receptor antagonists were dissolved as follows: 10 mg of each drug was dissolved in 0.19 ml of dimethyl sulphoxide (DMSO) and 1.81 ml of NaOH 0.01 N. The final solution was prepared by adding distilled water and bringing it to pH 7.8.

2.4. Angiotensin II-induced drinking

Angiotensin II was given by pulse i.c.v. injection at a dose of 10 pmol/rat in a volume of 1 μl.

Five minutes before angiotensin II injection, rats received an i.c.v. pretreatment with either LR-B/081, losartan, EXP3174 or vehicle (controls). The doses used were 10, 25, 50, 100 and 1000 pmol/rat for LR-B/081; 10, 100, 250, 500 and 1000 pmol/rat for losartan; and 1, 10, 50, 100 and 1000 pmol/rat for EXP3174. Injection volume was 1 μl. Water intake was recorded 15 and 30 min after angiotensin II injection.

To avoid possible alterations of the dipsogenic response to angiotensin II caused by previous injections of antagonists, the experiment was carried out according to a between-subjects design, in which each animal received a single dose of a single antagonist. Two days later a further injection of angiotensin II was given to evaluate whether antagonist administration might have a long-term effect.

2.5. Carbachol-induced drinking

Cholinergic agents are known to evoke drinking in the rat, through neurochemical mechanisms independent from those which mediate angiotensin II-induced drinking (Fitzsimons, 1979). Therefore, to assess the behavioural selectivity of the inhibitory effect of LR-B/081 on angiotensin II-induced water intake, the drug was tested on drinking induced by i.c.v. injection of carbachol, 300 ng/rat, dissolved in 1 μl of isotonic saline. LR-B/081 was given 5 min before carbachol.

Water intake was measured 15 and 30 min after carbachol injection. The experiment was carried out according to a within-subject design at intervals of 3–4 days, since the previous experiment had shown that LR-B/081 does not evoke long-term effects.

2.6. Intake of 15% fat milk

The behavioural selectivity of the effect of LR-B/081 on ingestive behaviour was also assessed by evaluating the effect of the antagonist on the intake of 15% fat milk. This highly caloric fluid diet has a composition similar to that of rat milk. Results of several experiments indicate that the feeding mechanisms control the ingestion of this diet (Bruno et al., 1980; Bruno, 1981; Massi et al., 1986). The 15% fat milk was made up by mixing whole milk and cooking cream containing 22% fat.

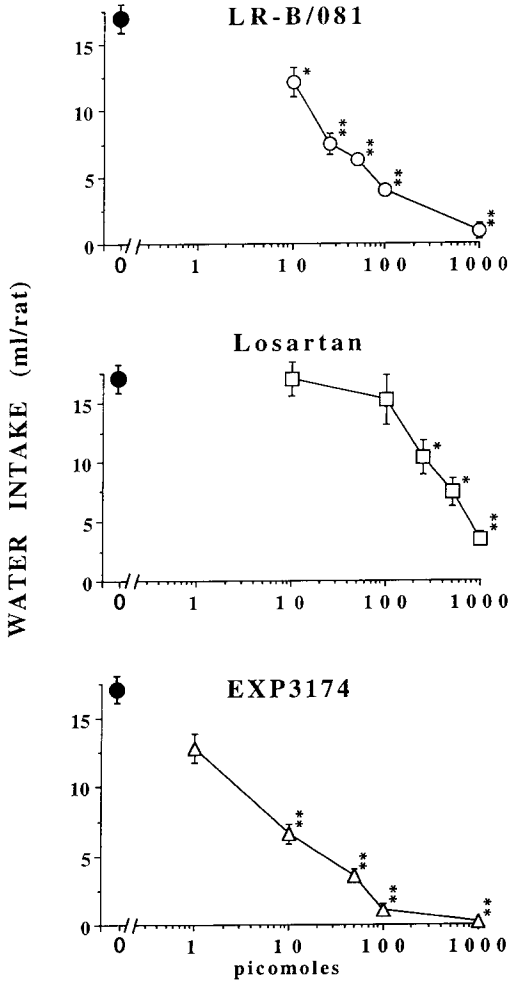

Fig. 1. Effect of i.c.v. injections of different doses (pmol/rat) of LR-B/081, losartan, EXP3174 or vehicle (0) on water intake induced by i.c.v. injection of angiotensin II, 10 pmol/rat. Data are reported as the 15 min water intake mean ± S.E.M. of 6–8 rats for LR-B/081, 6 rats for losartan, 6–8 rats for EXP3174 and 6 rats for vehicle. $^{*}P < 0.05$; $^{**}P < 0.001$.

Water- and food-sated rats were familiarized with a 2 h access (10:00 a.m.–12:00 p.m.) to the liquid diet. When the intake was stable enough, LR-B/081 or its vehicle (in a volume of 1 μl) was given by i.c.v. injection 5 min before access to the diet and the subsequent 15% fat milk intake was measured during a 2 h period. The doses of LR-B/081 used were 10, 100 and 1000 pmol/rat. The experiment was carried out according to a within-subject design at intervals of 3–4 days.

2.7. Validation of cannula placement

Before experiments, behavioural validation of i.c.v. cannula placement was done by evaluating the drinking response to i.c.v. injection of angiotensin II, 10 ng/rat. Only animals showing a drinking response of at least 9 ml/rat were included in the experimental groups.

After completion of experiments, 1 μl of black India ink was i.c.v. injected just before the rat was killed and ink diffusion into the ventricular space was evaluated.

2.8. Statistics

Data are presented as means ± S.E.M. The statistical analysis of angiotensin II-induced water intake data was performed by 'split-plot' multifactorial analysis of variance. The statistical analysis of carbachol-induced water intake and of 15% fat milk intake was performed by 'repeated measurements' multifactorial analysis of variance. Planned pairwise comparisons were carried out by means of t-tests, and statistical significance was set at $P < 0.05$.

The ID_{50} of the antagonists was determined according to Snedecor et al. (1967).

3. Results

3.1. Angiotensin II-induced drinking

Following pretreatment with vehicle, angiotensin II induced copious drinking after a latency of a few seconds. In the first 15 min after injection control rats drank 16.9 ± 1.9 ml of water. Only occasionally did drinking occur in the second 15 min period of observation, so that the cumulative 30 min water intake was 17.9 ± 2.1 ml.

LR-B / 081

In preliminary experiments, in which LR-B/081 was injected without angiotensin II, the drug never elicited water intake, not even in response to the highest dose tested, 1000 pmol/rat.

As shown in Fig. 1, in the dose range of 10–1000 pmol/rat, LR-B/081 produced a clear-cut inhibition of angiotensin II-induced drinking. The 30 min cumu-

lative water intake was only slightly superior to that measured at 15 min (less than 1 ml). The analysis of variance showed significant treatment effect ($F(5,32) = 30.93$; $P < 0.0001$), and time effect ($F(1,32) = 5.57$; $P < 0.05$), but no significant treatment-time interaction.

The inhibitory effect was dose-related and statistically significant from the dose of 10 pmol/rat. The ID_{50} of LR-B/081, calculated from the 15 min intake data, was 25.9 pmol/rat (confidence limits 16.8–36.9) (Table 1). The inhibitory effect of LR-B/081 was not accompanied by macroscopic behavioural alterations.

The dipsogenic response to angiotensin II (10 pmol/rat) 2 days after injection of the different doses employed of LR-B/081 was essentially identical to that of animals which never received LR-B/081 (data not shown).

Losartan

As shown in Fig. 1, losartan inhibited angiotensin II-induced drinking. The 30 min cumulative water intake was only slightly superior to that measured at 15 min (less than 1 ml). The analysis of variance showed significant treatment effect ($F(5,30) = 13.54$; $P < 0.0001$) and time effect ($F(1,30) = 7.25$; $P < 0.05$), even though the difference between the 15 and 30 min intake was modest. The analysis of variance did not show a significant treatment-time interaction.

The inhibitory effect of losartan was dose-related and statistically significant from the dose of 250 pmol/rat. The ID_{50} of losartan, calculated from the 15 min intake data, was 357 pmol/rat (confidence limits 215–705) (Table 1). The inhibitory effect of losartan was not accompanied by macroscopic behavioural alterations.

The dipsogenic response to angiotensin II (10 pmol/rat) 2 days after injection of the different doses employed of losartan was essentially identical to that of animals which never received losartan (data not shown).

EXP3174

As shown in Fig. 1, EXP3174 also inhibited angiotensin II-induced drinking. The 30 min cumulative water intake was only slightly superior to that measured at 15 min (less than 1 ml). The analysis of variance showed significant treatment effect ($F(5,36) = 46.22$; $P < 0.0001$) and time effect ($F(1,36) = 6.99$; $P < 0.05$), even though the difference between the 15 and 30 min intake was modest. The analysis of variance did not show a significant treatment-time interaction.

Table 1
ID_{50} for the inhibition of drinking induced by i.c.v. angiotensin II (10 pmol/rat)

	ID_{50} (pmol/rat)	Confidence limits (pmol/rat)
LR-B/081	25.9	16.8– 36.9
Losartan	357	215 –705
EXP3174	3.9	1.7– 7.1

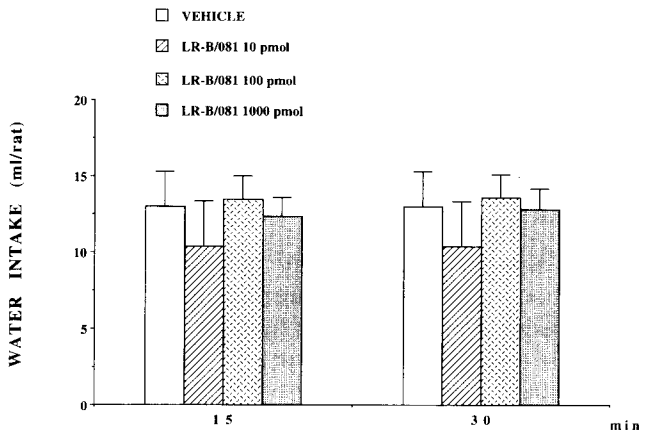

Fig. 2. Effect of i.c.v. injections of different doses (pmol/rat) of LR-B/081 or of its vehicle on water intake induced by i.c.v. injection of carbachol, 300 ng/rat. Data are reported as the 15 min water intake mean ± S.E.M. of 7 rats. Difference from controls (vehicle) was never statistically significant.

The effect was dose-related and statistically significant from the dose of 10 pmol/rat. The ID_{50} of EXP3174, calculated from the 15 min intake data, was 3.9 pmol/rat (confidence limits 1.7–7.1) (Table 1). The inhibitory effect of EXP3174 was not accompanied by macroscopic behavioural alterations.

The dipsogenic response to angiotensin II (10 pmol/rat) 2 days after the injection of the different doses employed for EXP3174 was essentially identical to that of rats which never received EXP3174 (data not shown).

3.2. Carbachol-induced drinking

The i.c.v. injection of 300 ng/rat of carbachol elicited a prompt dipsogenic effect similar to that of angiotensin II both for the amount of water taken and for the time course of the effect, which lasted essentially for 15 min.

As shown in Fig. 2, LR-B/081 in the dose range of 10–1000 pmol/rat did not modify carbachol-induced water intake.

3.3. Intake of 15% fat milk

As soon as the animals were given access to 15% fat milk they started to drink it after a latency of a few seconds. During the first 15 min the amount of 15% fat milk consumed by controls (18.0 ± 1.4 ml) was even larger than that of water in response to angiotensin II.

As shown in Fig. 3, LR-B/081 (10–1000 pmol/rat) did not modify the intake of 15% fat milk. The analysis

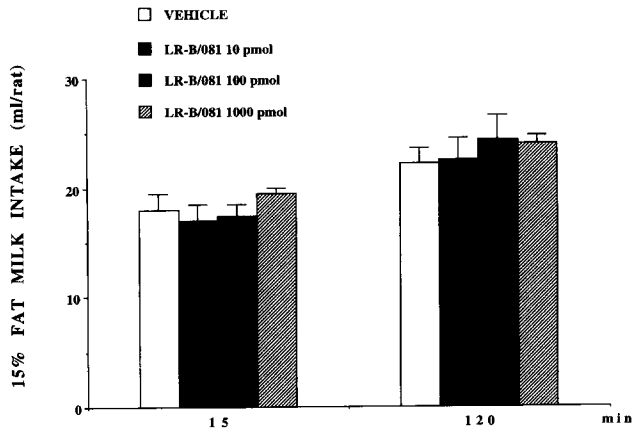

Fig. 3. Effect of i.c.v. injections of different doses (pmol/rat) of LR-B/081 or of its vehicle on cumulative intake of 15% fat milk. Data are reported as mean ± S.E.M. of 9 rats. Difference from controls (vehicle) was never statistically significant.

of variance revealed neither a significant treatment effect, nor a significant treatment-time interaction, but did evidence a significant time effect ($F(3,24) = 21.81$; $P < 0.0001$).

4. Discussion

The results of the present study show that central injections of the recently discovered angiotensin AT_1 receptor antagonist LR-B/081 (Subissi et al., 1994; Renzetti et al., 1994) potently inhibit drinking induced by i.c.v. angiotensin II in the rat.

In comparison to the other angiotensin AT_1 receptor antagonists tested, LR-B/081 proved to be about 14 times more potent that losartan, and 7 times weaker than EXP3174 in blocking angiotensin II-induced drinking. In this context, it is important to note that since losartan was injected just a few minutes before angiotensin II, the antidipsogenic potency measured for the drug is likely that relative to the molecule of losartan per se, in the absence of major conversion to its active metabolite EXP3174.

The doses of LR-B/081, which almost completely blocked the dipsogenic response of angiotensin II, altered neither the drinking response to carbachol nor the 2 h intake of 15% fat milk in food- and water-sated animals. Furthermore, central injection of LR-B/081, as well as that of losartan or EXP3174, was not followed by macroscopic behavioural alterations. These findings provide clear evidence that the antidipsogenic effect of LR-B/081 on angiotensin II-induced drinking, like that of losartan (Fregley and Rowland, 1991; Polidori et al., 1991; Beresford and Fitzsimons, 1992; Chow et al., 1992), is not due to general impairment of the rat ingestive behaviour, but is a behaviourally selective effect.

Recently it has been shown that the selective angiotensin AT_2 receptor antagonist PD 123319 inhibits carbachol-induced water intake, as well as drinking in response to other dipsogenic determinants, leading to the suggestion that angiotensin AT_2 receptors might play a role in a final common thirst pathway or signal (Rowland and Fregley, 1993). In our experiments LR-B/081 did not alter carbachol-induced water intake, suggesting that the compound is devoid of action at angiotensin AT_2 receptors, thus confirming its nature of selective angiotensin AT_1 receptor antagonist (Renzetti et al., 1994). Moreover, it is interesting to emphasize that even at the highest dose tested (1000 pmol/rat) LR-B/081 never evoked drinking, thus confirming that the drug lacks agonistic action on central angiotensin II receptors.

LR-B/081, either in vitro or peripherally administered, has been reported to be an insurmountable and long-acting angiotensin AT_1 receptor antagonist, thus raising the question whether it might evoke prolonged effects following acute administration. The dipsogenic action of angiotensin II 2 days after the i.c.v. injection of LR-B/081 was identical to that measured before LR-B/081 treatment, suggesting that a single injection of the drug does not produce long-term alterations of the central angiotensinergic mechanisms. These results are similar to those obtained with losartan and with its active metabolite EXP3174.

In conclusion, the findings of the present study provide functional evidence that LR-B/081 exerts a potent and selective antagonism at central angiotensin AT_1 receptors involved in the central actions of angiotensin II on body fluid homeostasis. These findings are in keeping with the results of binding studies on brain cell membranes and extend the results of peripheral functional studies (Renzetti et al., 1994; Subissi et al., 1994). Experiments are under way to evaluate the ability of the drug to cross the blood-brain barrier following peripheral administration.

Pharmacological studies have already raised interest in LR-B/081 as a potential antihypertensive agent. The present findings, showing its potent antagonism at central angiotensin AT_1 receptors, indicate that LR-B/081 might be an interesting pharmacological tool to further investigate the role of the brain renin-angiotensin system in physiological and pathological conditions.

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