

## Comparative Study of the Cardiovascular Effects of Losartan in Normal and in Water- and Salt-Depleted Sheep

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**ABSTRACT.** The cardiovascular effects of losartan, a non-peptidic angiotensin II (ANG II) receptor antagonist, were studied in sheep. Eight normotensive, conscious sheep were tested twice: first under normal conditions and second when subjected to water and electrolytic depletion (furosemide 5 mg/kg twice a day for 3 days). Intravenous injection of 30 mg/kg losartan lowered the mean arterial blood pressure (MABP) in both control and water- and electrolyte-depleted sheep alike. The maximal decrease in MABP was significantly greater in diuretic-treated sheep than in controls ( $20.0 \pm 2.7$  vs  $9.3 \pm 1.1$  mmHg) and occurred earlier ( $8.0 \pm 3.3$  min vs  $12.1 \pm 2.9$  min). The decrease in blood pressure was associated with tachycardia in both controls and diuretic-treated sheep ( $+5.5 \pm 1.8$  vs  $+11.3 \pm 3.9$  beats/min). The vasopressor response to  $0.1 \mu\text{g/kg}$  ANG II administered 30 min after losartan was completely antagonized. Two hours after losartan administration, MABP was on the increase in all animals and ANG II receptor blockade was partially obliterated in control sheep. The more marked cardiovascular effects recorded in diuretic-treated sheep as compared to control animals were associated with an increased activation of the renin-angiotensin system (plasma renin concentration:  $6.51 \pm 1.33$  vs  $1.42 \pm 0.37$  ng angiotensin I/ml/hr). **KEY WORDS:** angiotensin II, blood pressure, furosemide, losartan, sheep.

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The recent introduction of non-peptidic angiotensin II (ANG II) receptor antagonists has provided an important new tool to explore in different species the function of the renin-angiotensin system (RAS) under various conditions. RAS blockade by ANG II type 1 receptor (AT1 receptor) antagonist losartan (DuP 753) induces a hypotensive action whose magnitude and duration depend on 1) the specific oxidative metabolism of losartan and 2) RAS activity.

Characterization of losartan activity in animals has shown that metabolism played an important role in its *in vivo* activity [17]. The antihypertensive effect of losartan has been partially attributed to its active carboxylic acid metabolite Exp 3174 [26]. The action of this major losartan metabolite is stronger and more durable than that of losartan itself [17]. The metabolic formation of Exp 3174 varies between species [18]: it has been shown that very little Exp 3174 was formed in dogs and monkeys [3, 4] whereas the metabolite was readily produced in rats and humans [4, 12]. Thus, the duration of hypotensive activity of losartan in monkeys [10] or in dogs [9] was markedly shorter than in rats [23, 25]. Consequently, ANG II inhibition *in vivo* was less durable in dogs [4] and monkeys [7], whereas it lasted longer in rats and humans [12, 25].

Moreover, it has been shown that the extent to which losartan lowered MABP was linked to the individual RAS activation status of the subject animal [18]. RAS activity in normotensive animals was stimulated by sodium or volumic depletion or by a decrease in blood pressure [28]. It appeared that there was a relation between basal plasma renin activity (PRA) and the acute hypotensive effect of losartan in rats [23]. Thus, losartan did not lower blood pressure acutely in conscious normotensive rats [23] whereas in sodium-depleted rats with high renin levels, intravenous (i. v.) losar-

tan administration significantly lowered arterial pressure [27]. Likewise, losartan acutely altered basal blood pressure in sodium-depleted dogs [9, 13] and monkeys [7].

To our knowledge, no studies have explored the hypotensive effect of losartan in non pregnant normotensive ewes under varying water and electrolytic conditions. In the present work, conscious normotensive sheep were used to comparatively study the cardiovascular effects of losartan on controls and on sodium and water-depleted individuals. The experiments were so designed as to investigate the acute arterial blood pressure lowering effect of losartan, heart rate changes and angiotensin II blockade in the sheep subjected to diuretic treatment associated with water deprivation.

### MATERIALS AND METHODS

**Animals:** Eight adult Ile de France ewes weighing 50 to 70 kg provided by local breeders were used in this study. Except during the experimental protocol, the animals were housed in individual cages in accordance with European experimental procedures and maintained at  $19 \pm 1^\circ\text{C}$ . One week acclimatization was observed before the study. Hay and water were provided *ad libitum*. Food and water were withheld for 24-hr before surgery.

**Surgical procedures:** Two single lumen catheters (14 g, 20 cm length; Leader-flex, Vygon, Ecouen, France) were inserted through jugular vein and carotid artery according to Seldinger's method under anaesthesia (sodium thiopental 15 mg/kg i.v.). The venous catheter permitted blood sampling and drug infusion. The arterial catheter provided a route for measuring MABP. A post-operative 5-day recovery period was granted before testing. Systemic administration of 15 mg/kg amoxicillin (Clamoxyl® suspension, Beecham, St

Brieuc, France) was maintained for 4 days after the implantation procedure.

**Biochemical tests:** Five ml venous blood sample was collected on lithium heparinized tube (Sarstedt, Nümbrecht, Germany). Haematocrit was measured using the capillary tube method (Micro-haematocrit reader, Hawksley, Great Britain). Plasma was separated by centrifugation ( $2000 \times g$ , 6 min) for the determination of biochemical parameters. Osmotic pressure was measured using a digital osmometer (Roebeling, Giessen, Germany). Total protein concentration was determined with a refractometer (Atago, Japan). Plasma sodium, potassium and chloride concentrations were measured using an ion-specific electrode system (Vetlyte, Idexx, Westbrook, U.S.A.). Additionally, 5 ml venous blood were placed in an iced EDTA tube (Sarstedt) for plasma renin concentration (PRC) determination. The sample was promptly centrifuged at  $4^{\circ}\text{C}$  and the plasma was then frozen at  $-20^{\circ}\text{C}$  until time for analysis. PRC was measured by radioimmunoassay according to the method described by Stockigt *et al.* [19]. Plasma was incubated with an excess of angiotensinogen using sheep plasma obtained 48 hr after nephrectomy. PRC was expressed as ng of angiotensin I (ANG I) formed per ml and per hour. The sensitivity of the method was below  $0.2 \text{ ng ANG I/ml/hr}$ .

**Drugs used:** The following drugs were used: Losartan potassium salt (Dupont Merck Pharmaceutical Company, Wilmington, DE, U.S.A.) was dissolved on the day of the experiment in 5 ml sterile isotonic saline. A  $1 \mu\text{g}/\mu\text{l}$  synthetic ANG II solution (Sigma-Aldrich Chimie, L'Isle d'Abeau Chesnes, France) was prepared by dilution in 0.01% bovine serum albumin solution and stored at  $-20^{\circ}\text{C}$ . On the day of the experiment,  $0.1 \mu\text{g/kg}$  ANG II was taken after thawing and added to 1 ml sterile isotonic saline for i.v. administration. Furosemide (Dimazon®, Distrivet, Paris, France) was a  $50 \text{ mg/ml}$  solution. Epinephrine  $0.25 \text{ mg/ml}$  (Aguettant, Lyon, France) was diluted 1:10 with sterile isotonic saline. All the drugs were administered through the venous catheter over a period of 10–15 sec. The catheter was immediately flushed with 5 ml sterile heparinized isotonic saline ( $33 \text{ UI heparin/ml}$ ).

**Blood pressure recording:** The arterial catheter was connected to a strain-gauge pressure transducer P1000B (Narco Biosystems Inc., Houston, Texas, U.S.A.) positioned at the level of the heart and coupled to a polygraph (Physiograph, Narco Biosystems Inc., Houston, Texas, U.S.A.). Commencing 45 min before losartan i.v. administration, blood pressure curve was continuously monitored. MABP and heart rate were computed using a software programme developed in Labview 4.1 language (National Instruments, Austin, Texas, U.S.A.).

**Experimental protocol:** The eight ewes were studied on two separate occasions 4 days apart. Two similar losartan tests were performed on each ewe, one under control conditions, the other after 3 days of diuretic treatment (furosemide  $5 \text{ mg/kg}$  i.v. twice a day) and 20-hr total water deprivation. The experiments were performed under quiet, isolated conditions with the ewes being conscious and standing in a study

cart. They were weighed before testing.

Immediately before recordings were performed, blood samples were drawn from the venous catheter for biochemical assays. A 30 min equilibration period was started during which 5 ml sterile isotonic saline were administered to control the lack of pressor response to vehicle injection. Immediately after,  $0.5 \mu\text{g/kg}$  i.v. epinephrine were administered to 5 of the 8 ewes, and the maximal MABP increase was noted. During the same equilibration period, a bolus injection of ANG II ( $0.1 \mu\text{g/kg}$ ) was administered in every sheep and the maximal blood pressure increase was also noted. The 15-min control period began after the ANG II-induced hemodynamic alterations had disappeared (typically within 3 to 5 min). A  $30 \text{ mg/kg}$  losartan bolus was administered at the end of the control period and recordings went on for 120 min. To evaluate cardiovascular response to losartan administration, MABP was averaged over 5-min periods from 15 min before losartan injection to 30 min after. Then, MABP was averaged each 10 min up to 120 min after losartan. Baseline MABP was computed over the 15-min control period. MABP was also determined through a 15-min monitoring period following losartan injection (15 min post-losartan) and its difference with baseline was noted. The lowest MABP recorded after losartan was noted as well as its timing.

The ANG II bolus administration ( $0.1 \mu\text{g/kg}$ ) was repeated 30 and 120 min after losartan and the maximal MABP increase was used to determine the extent of AT1 receptor blockade. A second epinephrine bolus ( $0.5 \mu\text{g/kg}$ ) was administered 35 min after losartan and the rise in MABP was noted.

Heart rate was averaged over the 15-min control period (baseline) and over the 15-min post-losartan period.

**Statistical analyses:** Data are presented as means  $\pm$  standard error of the mean (S.E.M.). Wilcoxon signed-rank test (Statview, SAS Institute Inc., Cary, North Carolina, U.S.A.) was used for statistical evaluation. The significance threshold was set at 0.05 to reject the null hypothesis.

## RESULTS

**Water and electrolyte status:** After 3 days of diuretic treatment and 20-hr water deprivation the sheep exhibited changes in water and electrolytic status (Table 1). Comparing the data between control and diuretic-treated sheep revealed a significant body weight decrease after diuretic treatment ( $-4 \pm 0.8 \text{ kg}$ ) ( $p < 0.05$ ) and no significant haematocrit, total protein concentration, sodium and osmotic pressure differences. However, potassium ( $p < 0.05$ ) and chloride ( $p < 0.05$ ) concentrations were significantly lower after diuretic treatment.

**Plasma renin concentration:** A significant increase ( $p < 0.05$ ) in PRC was noted after water depletion ( $6.51 \pm 1.33$  diuretic-treated sheep vs  $1.42 \pm 0.37 \text{ ng ANG I/ml/hr}$  controls).

**Blood pressure response to vasoconstrictors:** Figure 1 illustrates MABP effect of ANG II and epinephrine administration before and after losartan in the two hydration states.

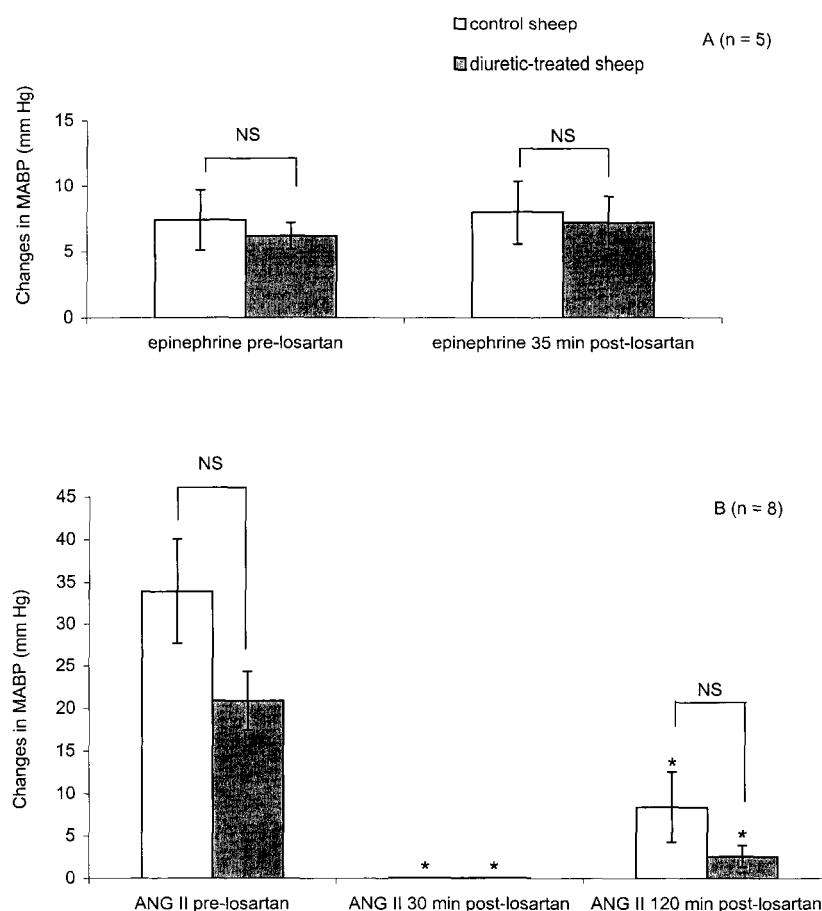


Fig. 1. Changes in mean arterial blood pressure (MABP) after  $0.5 \mu\text{g/kg}$  i.v. epinephrine (A) and after  $0.1 \mu\text{g/kg}$  i.v. angiotensin II (ANG II) (B). Values expressed as mean  $\pm$  S.E.M. \*: Significant difference compared to pre-losartan values ( $p < 0.05$ ). NS: non significant difference between diuretic-treated sheep and control sheep.

Table 1. Biochemical characteristics in control and diuretic-treated sheep

| Parameters                  | Control         | Diuretic          |
|-----------------------------|-----------------|-------------------|
| body weight (kg)            | $60.7 \pm 3.7$  | $56.7 \pm 3.3^*$  |
| haematocrit (%)             | $29.3 \pm 2.7$  | $31.5 \pm 3.4$    |
| total plasma proteins (g/l) | $67.8 \pm 1.8$  | $71.5 \pm 2.3$    |
| plasma [Na] (mEq/l)         | $150.8 \pm 0.7$ | $147.3 \pm 1.9$   |
| plasma [K] (mEq/l)          | $4.6 \pm 0.2$   | $3.8 \pm 0.2^*$   |
| plasma [Cl] (mEq/l)         | $112.9 \pm 0.8$ | $99.4 \pm 0.8^*$  |
| osmotic pressure (mOsm/l)   | $300.5 \pm 1.4$ | $295.8 \pm 4.1$   |
| PRC (ng ANG I/ml/hr)        | $1.42 \pm 0.37$ | $6.51 \pm 1.33^*$ |

Values expressed as mean  $\pm$  S.E.M. (n=8). PRC: plasma renin concentration. \*: Significant difference between control and diuretic-treated sheep ( $p < 0.05$ ).

The administration of  $0.5 \mu\text{g/kg}$  epinephrine increased MABP in control and diuretic-treated sheep alike and its effect was not altered by losartan. Before losartan, i.v. administration of  $0.1 \mu\text{g/kg}$  ANG II increased blood pressure more in controls ( $+33.9 \pm 6.2$  mmHg) than in diuretic-treated sheep ( $+23.1 \pm 3.2$  mm Hg). However, the difference did not

reach significance. The same bolus injection 30 min after losartan administration produced no effect. Two hours after losartan treatment, the hypertensive effect of ANG II partially reappeared in both groups. Injection of 5 ml isotonic saline during the equilibration period never modified blood pressure in the sheep (data not shown).

**Cardiovascular effects of losartan administration:** Short-term changes in MABP and heart rate after losartan administration are reported in Table 2. Figure 2 illustrates the evolution of MABP during the losartan test, in control and diuretic-treated sheep. Baseline MABP was slightly lower in diuretic-treated sheep ( $90.9 \pm 2.6$  mmHg) than in controls ( $94.9 \pm 4.1$  mmHg) but the difference didn't reach significance. Losartan significantly decreased ( $p < 0.05$ ) MABP within the first five minutes in both groups. The maximal decrease in blood pressure was  $9.3 \pm 1.1$  mmHg (9.8% reduction in MABP) in control sheep and  $20.0 \pm 2.7$  mmHg (22.0% reduction in MABP) in diuretic-treated sheep with a significant inter-group difference ( $p < 0.05$ ). The maximal blood pressure-lowering effect of losartan occurred earlier ( $p < 0.05$ ) in diuretic-treated sheep ( $8.0 \pm 3.3$  min) than in con-

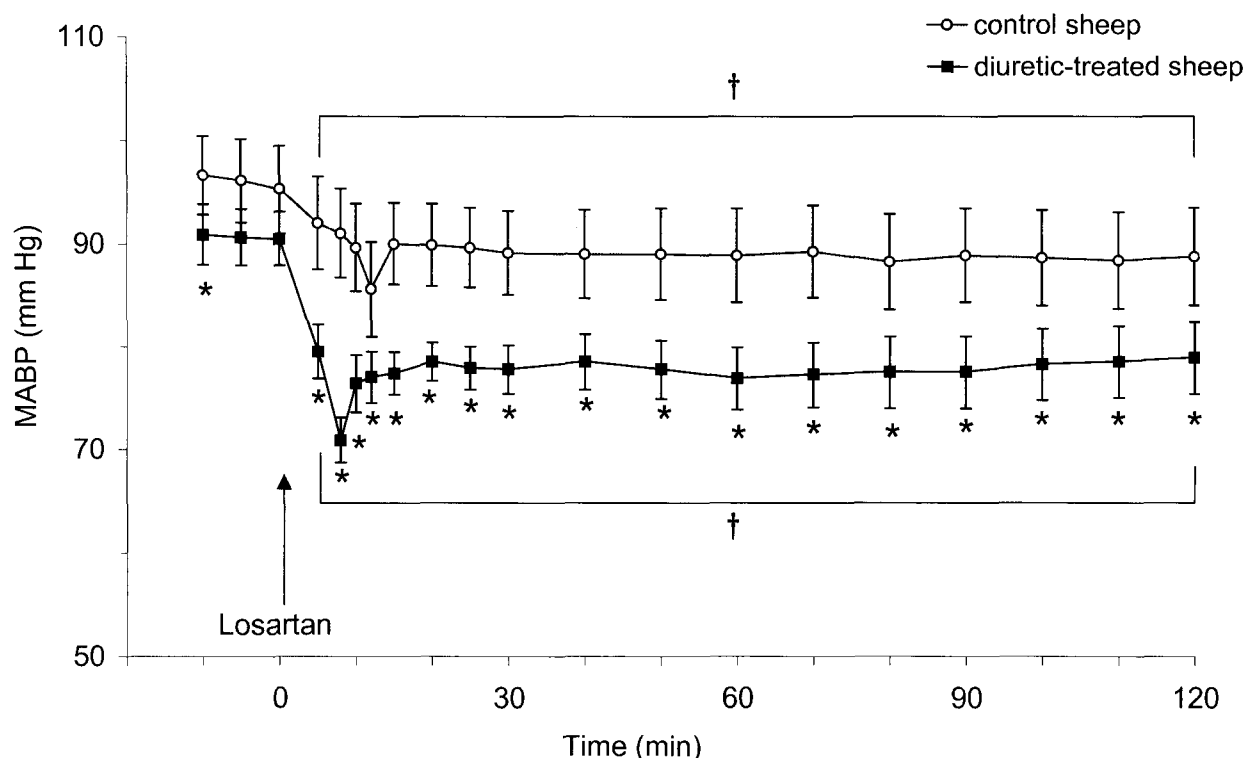


Fig. 2. Evolution in mean arterial blood pressure (MABP) during losartan test in control and diuretic-treated sheep. Values expressed as mean  $\pm$  S.E.M. ( $n=8$ ). \*: Significant difference compared to control sheep on different time points ( $p<0.05$ ). †: Significant difference compared to baseline values ( $p<0.05$ ).

Table 2. Cardiovascular effects of losartan administration (30 mg/kg i.v.) in control and diuretic-treated sheep

| Parameters                          | Control                      | Diuretic                     |
|-------------------------------------|------------------------------|------------------------------|
| Mean arterial blood pressure (mmHg) |                              |                              |
| Baseline <sup>a</sup>               | 94.9 $\pm$ 4.1               | 90.9 $\pm$ 2.6               |
| 15 min post-losartan <sup>b</sup>   | 90.6 $\pm$ 4.4 <sup>†</sup>  | 78.0 $\pm$ 2.3 <sup>*†</sup> |
| Change                              | -4.3 $\pm$ 0.7               | -12.9 $\pm$ 2.6 <sup>*</sup> |
| Heart rate (beats/min)              |                              |                              |
| Baseline <sup>a</sup>               | 96.0 $\pm$ 4.3               | 104.9 $\pm$ 5.3              |
| 15 min post-losartan <sup>b</sup>   | 101.5 $\pm$ 3.5 <sup>†</sup> | 116.1 $\pm$ 4.5 <sup>†</sup> |
| Change                              | +5.5 $\pm$ 1.8               | +11.3 $\pm$ 3.9              |

Values expressed as mean  $\pm$  S.E.M. ( $n=8$ ). a) The baseline values were computed over the 15-min control period. b) Values computed over the 15-min period immediately following losartan injection. †: Significant change from baseline values ( $p<0.05$ ). \*: Significant difference between control and diuretic-treated sheep ( $p<0.05$ ).

controls ( $12.1 \pm 2.9$  min). This inter-group difference was confirmed by examining the blood pressure decrease over 15 min after losartan administration (Table 2). Then, MABP slowly rose again, as 120 min after losartan, it did not return to pre-treatment baseline values either in diuretic-treated sheep or in controls (13.1% vs 5.8% reduction in MABP). Before losartan, the 15 min averaged heart rate was not significantly higher in diuretic-treated sheep ( $104.9 \pm 5.3$  beats/min) than in controls ( $96.0 \pm 4.3$  beat/min). Losartan induced a significant ( $p<0.05$ ) heart rate increase which

did not significantly differ among control ( $+5.5 \pm 1.8$  beats/min) and diuretic-treated sheep ( $+11.3 \pm 3.9$  beats/min).

## DISCUSSION

**Validation of water and electrolyte depletion:** Diuretic treatment and water restriction induced dehydration as proven by the 4-kg weight loss, although it did not come out from changes in total plasma protein content or haematocrit. Plasma sodium and osmotic pressure were not significantly decreased since as the sodium decreased, water was drawn, through an osmotic effect, from extracellular space into the cells. Nevertheless, the significant fall in plasma potassium and chloride substantiated the salidiuretic effect of furosemide. Water and electrolyte depletion did not significantly alter baseline MABP. Combining 3-day diuretic treatment and 20-hr water deprivation in sheep produced water and electrolytic depletion propitious to RAS activation [16].

**Validation of ANG II antagonism:** The dose of ANG II used ( $0.1 \mu\text{g/kg}$ ) was lower than used by Chan *et al.* [2] in the dog, where a bolus injection of  $0.4 \mu\text{g/kg}$  ANG II i.v. was repeated to substantiate the effective ANG II receptor antagonism. It was chosen with reference to a study conducted in man, where a 30-mmHg increase in systolic blood pressure after ANG II was used to control the efficacy of AT1 receptor blockade after administration of an antagonist

[15]. The resultant increase in MABP induced by ANG II was lower, although non significant, in diuretic-treated sheep than in controls. This was likely to reflect a down regulation of AT1 receptors secondary to the diuretic-induced activation of the RAS. ANG II receptor blockade occurred early in sheep (100% blockade at 30 min post-losartan), similarly to the dog, where the blockade of ANG II-induced vasopressor response after a single 3 mg/kg i.v. dose of losartan reached 89% after 5 min and only 11% after 240 min [4]. In control sheep, the ANG II receptor blockade significantly declined at 120 min after administration as MABP changes ( $+8.4 \pm 4.1$  mmHg) were observed after ANG II. Nevertheless, diuretic-treated sheep exhibited no significant decrease in ANG II receptor blockade at 120 min.

**Blood pressure effect of losartan:** In this study, we observed an unexpectedly significant decrease in blood pressure in the control sheep after 30 mg/kg i.v. losartan. This effect could not be attributed to the vehicle because of the lack of blood pressure effect of 5 ml isotonic saline i.v. RAS inhibition under normal or salt-loaded condition was generally considered to have a negligible effect on blood pressure [14]. For example, no significant changes in MABP were noted after losartan oral administration to normal human volunteers [5]. Neither changes in MABP, cardiac output nor systemic vascular resistance were observed during the infusion of 100  $\mu$ g/kg/min of losartan in normal salt anesthetized dog [2]. On the contrary, in states of RAS activation such as induced by furosemide in rats [24] or renovascular hypertension in cynomolgus monkeys [10] or in spontaneously hypertensive rats [23], losartan acutely and dose-dependently decreased MABP and the hypotensive effect was related to PRA.

Nevertheless, more recent studies in normoreninemic hypertensive conscious rats have shown that intravenous injection of EXP 3174 also reduced blood pressure but the fall was moderate and slow [1]. Besides, it has been shown that in conscious normal monkeys, 10 mg/kg i.v. losartan significantly decreased MABP [7]. Likewise, according to a study involving anesthetized, normotensive, normoreninemic rabbits, intravenous administration of losartan induced a 9% reduction in blood pressure [8]. The hypotensive effect should there be explained by anaesthesia. Indeed, according to Basso *et al.* [1], anaesthesia and acute surgical manipulations increased renin release and the hypotensive effect of intravenous injection of Exp 3174 was higher in anesthetized than in conscious animals.

The blood pressure-lowering effect that we observed in conscious, normotensive control sheep may be related to the high dose of losartan used i.v. Thus, Chan *et al.* [2] showed that high dose of ANG II receptor antagonists may induce systemic response whereas low doses did not induce any cardiovascular changes. Some authors had envisaged that the blood pressure lowering effect of losartan given to normotensive animals could be the result of a non-specific effect of losartan [6, 11, 21]. In our study, a non-specific effect of losartan on adrenergic receptors was excluded since losartan did not affect the epinephrine induced-increase in MABP. In

addition, since the time course of MABP after losartan was similar to that of the responses to ANG II injections, the hypothesis that the above reported effects were due to AT1 receptor blockade was reinforced. This was also strengthened by the observation that the MABP lowering efficacy of losartan was greater in diuretic-treated sheep in which the RAS was of greater importance in the maintenance of blood pressure.

In sheep, a rapid drop in MABP was observed after losartan administration, reaching a nadir within 15 min. Then, MABP did not remain constant for the remainder of the 2-hr recording period. Indeed, 120 min after losartan administration, MABP was still lower than baseline but the hypotensive effect was below the maximal blood pressure-lowering effect. This precocious decrease in losartan hypotensive effect was concomitant with the exogenous ANG II-induced vasopressor response recovery at 120 min. The rapid onset of the hypotensive effect of losartan in sheep was probably due to the high doses given and the administration route chosen. Nevertheless, it was not conceivable that sheep received oral doses of losartan because of their large stomachs and strong microbial fermentation. In others species, the hypotensive effect was also observed very rapidly after i.v. dose. In sodium-deplete anesthetized dogs, the mean arterial blood pressure response after a single dose of DuP 753 administered intravenously was rapid (maximum within five min) and biphasic (return to control values at 1–2 hr and a further decline at 3–4 hr [9]).

Moreover, the metabolic formation of the long-acting EXP 3174 participates to the duration of the blood pressure-lowering effect of losartan [22]. In rats and in man, losartan is readily converted into EXP 3174 whereas in dogs, losartan is not converted into an active metabolite to any significant extent and in monkeys the primary route of metabolism is not oxydative [4, 20]. It could be postulated that losartan produces no or few metabolite Exp 3174 in sheep, as in dogs and monkeys, resulting in a rapid ANG II receptor blockade which declines 2 hr after the i.v. administration. This hypothesis need to be tested.

**Heart rate effect of losartan:** In most species, losartan did not affect heart rate [17]. Thus, despite a decrease in blood pressure by losartan, no reflex tachycardia was observed in renal hypertensive rats [23, 25] or primates [10]. In sodium-depleted anesthetized dogs, bradycardia was observed over the dose range [9]. Apparently, the absence of reflex tachycardia is an effect commonly observed with RAS blockers. It may be caused, according to Basso *et al.* [1] by enhanced vagal tone or reduction of the sympathetic baroreceptor response to the fall in blood pressure induced by RAS inhibition. In most studies where no changes in heart rate were noted, losartan was orally administered. Thus, it may also be speculated that tachycardia did not occur because of the slow decrease in blood pressure after oral losartan administration.

In our experiment, i.v. administration of losartan significantly increased heart rate. A similar increase was recorded in the rabbit after ANG II receptor blockade with losartan i.v. [8] and in conscious, salt-depleted beagle dog after con-

tinuous infusion of losartan [13]. Some reflex tachycardia was also observed in rats after an ANG II antagonist was administered intravenously [27]. Losartan is likely to induce a heart reflex acceleration in sheep because of the rapid onset of the hypotensive effect after intravenous administration of losartan (within 12.1 and 8.0 min post-dose in control and diuretic groups, respectively).

To conclude, the data presented here show that 30 mg/kg losartan administered intravenously produce a rapid decrease in blood pressure with heart rate acceleration either in normal or in water- and salt-depleted sheep. The blockade of the hypertensive response to 0.1  $\mu$ g/kg i.v. ANG II is rapid but declines within 2-hr after losartan administration.

The magnitude of the blood pressure-lowering effect of losartan is rather modest in control sheep and much greater in diuretic-treated sheep, either when examining the maximal MABP decrease or the effects within 15 min or at 120 min. In addition, the hypotensive response occurs earlier in diuretic-treated sheep compared to controls. This difference in blood pressure-lowering effect of losartan could be attributable to RAS activation. The administration of furosemide (5 mg/kg twice a day, 3 days) associated to 20-hr water deprivation induces in the sheep a short-term dehydration. It provides an interesting normotensive animal model with an activated RAS.

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