Nonpeptide Angiotensin II Antagonist Losartan Inhibits Thromboxane A2-Induced Contractions in Canine Coronary Arteries

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ABSTRACT

We investigated the selectivity of a nonpeptide angiotensin II AT1 receptor antagonist losartan for the vascular thromboxane A2 (TxA2)/prostaglandin endoperoxide (PGH2) receptor in canine coronary arteries. Isometric tension was measured in canine coronary artery rings suspended in organ chambers perfused with 95% O2/5% CO2. The TxA2 analog, U46619, produced dose-dependent vasoconstriction in coronary rings (EC50, 10.6 ± 0.9 nmol/l). Pretreatment with losartan (10-8-10-5 mol/l) inhibited the contractile response of U46619 and shifted the concentration-response curve to the right in dose-dependent manner. The EC50 of U46619 was increased 3- and 13-fold in the presence of both 1 and 10 μmol/l of losartan without a change in maximal contraction. The selective TxA2/PGH2 receptor antagonist SQ29548 blocked U46619-induced contraction with greater potency than losartan in isolated coronary arteries. The active metabolite of losartan EXP3174 at 1 μmol/l did competitively block U46619-induced contractions in canine coronary rings. In contrast, the contractile responses produced by U46619 were unaffected by exposure to the nonpeptide AT1 receptor antagonist CV11974, the AT2 receptor antagonist PD123319 or the nonselective peptide angiotensin II antagonist Sar1-Thr8-Ang II, each at 1 μmol/l concentration. These data indicate that losartan and its active metabolite EXP3174 are antagonists to the TxA2/PGH2 receptor in canine coronary arteries. The antagonistic effect of losartan and EXP3174 on the vascular TxA2/PGH2 receptor may contribute to the long-term blood pressure-lowering effects of angiotensin antagonists in hypertension.

The renin-angiotensin system has been well recognized as an important contributor to the pathogenesis of hypertension, cardiac hypertrophy and vascular disease (Ferrario et al., 1994). Losartan is a potent nonpeptide, selective Ang II AT1 receptor antagonist, which produces concentration-dependent inhibition of Ang II-induced vasoconstriction in vivo and in vitro and displaces 125I-Ang II binding in radioligand binding studies (Timmermans et al., 1993; Liu, 1993; Rhaleb et al., 1991). Losartan reduces blood pressure in human hypertensive subjects, as well as in animal models of hypertension, such as renal hypertensive rats, SHR and transgenic hypertensive rats (Townsend and Ford, 1996; Wong et al., 1990c; Moriguichi et al., 1994). Acute administration of losartan is highly effective in lowering blood pressure in SHR, a genetic model of hypertension in which plasma renin is not elevated (Ohtstein et al., 1992; Cachofeiro et al., 1995). However, it has been noted that in this same hypertensive model the peptide Ang II antagonist sarafasin is not as effective as losartan, and angiotensin converting enzyme inhibitors are only modestly effective after acute administration (Pals et al., 1971; Sweet et al., 1981; Ohtstein et al., 1992). Recent studies suggest that the long-lasting antihypertensive effect of losartan may not be caused solely by Ang II receptor blockade. This was illustrated by Ohtstein et al. (1992) who showed that 48 h after the administration of losartan, blood pressure was still reduced in the presence of a normal pressor response to Ang I or II. Furthermore, Cachofeiro et al. (1995) reported that nitric oxide and prostaglandin production are involved in the prolonged blood pressure-lowering effects of losartan in SHR. Losartan has also been shown to attenuate catecholamine-induced contractions in aortic rings of SHR in an endothelium-dependent manner, stimulate vasodilatory prostaglandin release in cultured cells and at high doses bind to imidazole/guanidinium receptors in rat forebrain cardiovascular areas (Maeso et al., 1995; Jaiswal et al., 1991; Li et al., 1996).

Recent studies have shown that losartan interacts with the TxA2/PGH2 receptor in human platelets and inhibits the TxA2 analog U46619-induced platelet aggregation and pul-

ABBREVIATIONS: TxA2, thromboxane A2; PGH2, prostaglandin H2; PGF2α, prostaglandin F2α; KCl, potassium chloride; SHR, spontaneously hypertensive rat; PDGF, platelet-derived growth factor; l-NAME, N”-nitro-l-arginine methyl ester; U 46619, 5-heptenoic-7-[6-(3-hydroxy-1-octenyl)-2-oxabicyc[2.2.1]hept-5-yl]-2-oxabicyc[2.2.1]hept-5-yl] acid; Ang II, angiotensin II.
monary hypertension in rats (Liu et al., 1992; Bertolino et al., 1994). Furthermore, TxA2 and PGH2 are involved in Ang II-dependent hypertension by stimulating contraction of vascular smooth muscle by a common receptor (Lin et al., 1991, 1994). In the present study, we further investigated whether losartan and its active metabolite EXP3174 interact with the TxA2/PGH2 receptor and stimulate production of prostaglandin and nitric oxide in isolated canine coronary arteries.

Materials and Methods

After approval by the Institutional Animal Care and Use Committee, 16 male dogs (15–25 kg b.w.) were anesthetized with ketamine (15 mg/kg i.m.) and 2% halothane inhalation; the dogs were then sacrificed with sodium pentobarbital (50 mg/kg i.v.). The heart was harvested immediately and immersed on ice-cold modified Krebs-Henseleit buffer, whereas the left anterior descending coronary artery was carefully dissected free of fat and adhering connective tissues. The coronary artery was cut into 3-mm-long rings and suspended in organ chambers containing Krebs-Henseleit solution of the following composition (in mmol/l): NaCl, 118.3; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.2; KH2PO4, 1.2; NaHCO3, 25; CaNa-ethylenediaminetetraacetic acid, 0.026; and glucose, 11. The Krebs' solution was aerated with 95% O2 and 5% CO2 at 37°C (pH 7.4) and the rings were allowed to equilibrate for 60 min at 1 g initial resting tension. Basic tension was increased individually in a step-by-step fashion until the optimal length-tension relationship was obtained by repeated exposure to 40 mmol/l KCl. In some rings, the endothelium was denuded by gentle mechanical rubbing with a stainless steel wire. Isometric tension of vascular rings was measured continuously with force-displacement transducer (FT03, Grass Instruments Co., Quincy, MA) connected to a Grass polygraph. The integrity of functional endothelium of vascular rings was confirmed by the presence of acetylcholine-induced relaxation in preconstricted rings with 10–8 mol/l U46619 (more than 90% relaxation at 10–7 mol/l of acetylcholine) and absence of acetylcholine-induced relaxation in vessels after mechanical denudation of the vascular endothelium.

Experimental protocol. Control cumulative concentration-contractile response curves for TxA2 analog U46619 (10–10–3 × 10–6 mol/l) were generated after 1 h equilibration in intact quiescent rings. Losartan (10–8–10–5 mol/l) was used to pretreat the coronary artery rings for 30 min, and the concentration-response curves for U46619 were then repeated. To determine whether losartan interacts with other vasoconstrictors, concentration-response curves with PDGF (10 and 20 ng/ml) and KCl (10–8 mmol/l) were also constructed in the absence and presence of losartan (10–5 mol/l) in isolated coronary vascular rings. Phenylphrine, arginine vasopressin and PGF2α (each at concentrations ranging from 10–10 to 10–6 mol/l) were also tested. In addition, to compare the potency and selectivity of losartan on TxA2 receptor in coronary arteries, the potent, selective TxA2/PGH2 receptor antagonist SQ29548 (Ogletree et al., 1985) was used to pretreat the tissues for 30 min, and then concentration-response curves for U46619 were determined.

To evaluate whether other nonpeptide Ang II receptor subtype antagonists interact on the TxA2/PGH2 receptors in coronary vessels, the selective Ang II AT1 receptor antagonist CV11974, an active form of TCV-116 (Brunner et al., 1994), the Ang II AT2 receptor antagonist PD123319 and the active metabolite of losartan, EXP3174 (all at 10–6 mol/l) were chosen to pretreat the rings for 30 min, and then concentration-response curves for U46619 were generated. The non-selective peptide Ang II antagonist Sar1Thr8-Ang II (10–6 mol/l) was also tested. The cyclooxygenase inhibitor indomethacin (10–5 mol/l) combined with losartan (10–6 mol/l) for copretreatment of vascular rings was used to ascertain whether the production of vasoactive prostaglandins is involved in the interaction of losartan with U46619 in the isolated coronary arteries. In addition, to test whether the interaction of losartan with U46619 is related to the release of nitric oxide in vasculature, rings were pretreated with the nitric oxide synthase inhibitor, t-NAME (10–4 mol/l). The vascular rings were then preconstricted with either 40 mmol/l of KCl or 10 mmol/l of U46619 to reach a similar degree of stable contraction, and then losartan (10–10–10–5 mol/l) was cumulatively added to organ chambers. The antagonists tested had no effects on the basal vascular tone, except a minimal constriction induced by t-NAME. Each ring was used only once for the antagonist study. A 60-min incubation was allowed between observations.

Chemicals. Losartan and EXP3174 were generous gifts from DuPont/Merck Company (Wilmington, DE). PD 123319 was generously supplied by Parke-Davis Inc. (Ann Arbor, MI) and CV-11974 was from Takeda Chemical Industries, LTD. (Osaka, Japan). t-NAME and SQ29548 were purchased from Research Biochemicals International (Natick, MA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Indomethacin and CV-11974 were dissolved in 0.2 N Na2CO3 solution and diluted with Krebs' buffer. U46619 was prepared as stock in ethanol and diluted with Krebs' buffer. The concentrations of drugs reported are at final concentration in organ chambers.

Data and statistical analysis. The concentration of U46619 and losartan causing 50% of the maximal contraction (EC50) and the maximal relaxation (IC50) were calculated with use of a nonlinear regression sigmoid curve fitting program of PRISM (Graphpad Inc., San Diego, CA). The apparent dissociation constant (Kd) was calculated with the equation 

\[ K_d = \frac{[A]}{[A]} - 1 \]

where [B] is the concentration of the antagonist and [A] and [A'] are the EC50 values obtained in each artery before and after the addition of antagonist, respectively (Corriu et al., 1995). Data were expressed as mean ± S.E.M. One-way analysis of variance followed by Newman-Keuls multiple comparisons and Student's t test for paired observations was used for statistical evaluation. P < .05 was considered statistically significant.

Results

Effects of losartan on U46619-induced contraction in coronary artery rings. The TxA2 analog, U46619, caused concentration-dependent contractions in canine coronary artery rings. Figure 1 illustrates a typical response in which addition of 10–6 M losartan reversed the contraction of U46619 and pretreatment with losartan shifted the concentration-response curve of U46619 to the right in a concentration-dependent manner without a change in the maximum constriction. The EC50 of U46619 (10.2 ± 0.9 nmol/l) was

**Fig. 1.** Typical dose-response contractions of U46619 during control conditions and after pretreatment with 10–6 mol/l losartan. Figure in upper left-hand corner shows a contractile response to 10–8 mol/l U46619, which is reversed by the addition of losartan (10–6 mol/l).
shifted 3- and 13-fold by preincubation with 1 and 10 μmol/l of losartan, respectively (P < .001 as compared with control) (fig. 2A, table 1). The apparent dissociation constant \( K_d \) averaged 0.5 ± 0.1 and 0.8 ± 0.1 μmol/l in the presence of 1 and 10 μmol/l of losartan, respectively. Losartan at 10⁻⁴ M concentration completely reversed the constriction of 10 nM U46619 (Li, P., Ferrario, C. M. and Brosnihan, K. B., unpublished observations). The potent, selective TxA₂/PGH₂ receptor antagonist SQ29548 markedly shifted the concentration-response curves of U46619 to the right \([EC_{50} 10.6 ± 0.9 \text{ (control) vs. } 199.1 ± 17.7 \text{ and } 617.3 ± 63.8 \text{ nmol/l at } 0.01 \text{ and } 0.1 \text{ μmol/l of SQ29548, respectively}]\) without changing the maximum contraction (fig. 2B). Pretreatment with 1 μmol/l of SQ29548 abolished the contractile responses of U46619 at the concentrations tested (Li, P., Ferrario, C. M. and Brosnihan, K. B., unpublished observations). The potency of antagonism of 0.1 μmol/l SQ29548 was 31-fold greater than losartan at equimolar concentrations \([EC_{50} 19.7 ± 2.8 \text{ (losartan) vs. } 617.3 ± 63.8 \text{ (SQ29548) nmol/l}]\).

Selectivity of nonpeptide Ang II antagonists on the TxA₂/PGH₂ receptor. Pretreatment with the Ang II AT₁ receptor antagonist CV11974, the active form of TCV-116, or the AT₂ antagonist PD123319 for 30 min. did not change the concentration-response curve of U46619 at 1 μmol/l \([EC_{50} 10.6 ± 0.9 \text{ (control) vs. } 12.6 ± 1.7 \text{ (CV11974) and } 12.9 ± 0.7 \text{ (PD123319) nmol/l, } P > .05 \text{ as compared with control} (\text{fig. 3, table 2})]. \)

In contrast, 1 μmol/l EXP3174, the active metabolite of losartan at the AT₁ receptor, significantly shifted the concentration-response curve of U46619 to the right (P < .001 as compared with control). In addition, EXP3174 was more potent than losartan \((EC_{50} 33.4 ± 4.6 \text{ vs. } 66.9 ± 9.2 \text{ nmol/l, losartan vs. EXP3174, } P < .01)\). None of the nonpeptide Ang II receptor antagonists changed the maximum constriction response to U46619 (table 2). In addition, the nonselective peptide Ang II receptor antagonist Sar¹Thr³-Ang II at 1 μmol/l did not affect the U46619-induced contraction of coronary arteries.

Specificity of losartan for vasoconstrictor-induced contraction in coronary arteries. Losartan pretreatment (1 μmol/l) did not affect the coronary vasoconstrictor responses to either PDGF (fig. 4) or the smooth muscle cell depolarizing agent KCl (fig. 5). Phenylephrine and arginine vasopressin showed minimal contractile responses in canine coronary arteries, and losartan did not change these contractile effects. However, in preliminary studies, losartan \((10⁻³ \text{ mol/l})\) blocked the TxA₂/PGH₂ receptor agonist PGF₂α, induced dose-dependent vasoconstriction \((EC_{50} 23.4 ± 3.4 \text{ vs. } 205.1 ± 23.5 \text{ nmol/l; maximal contraction, } 5.0 ± 0.2 \text{ vs. } 4.9 ± 0.3 \text{ g without and with losartan, } n = 2)\).

Effects of losartan on the production of vasoactive prostaglandins and nitric oxide. Copreincubation of 10 μmol/l of the cyclo-oxygenase inhibitor indomethacin with 1 μmol/l of losartan for 30 min did not significantly shift the concentration-response curve of U46619 as compared with pretreatment with 1 μmol/l of losartan alone (fig. 6A). On the other hand, U46619-preconstricted rings \((10 \text{ nmol/l})\) were dilated by losartan in a dose-dependent manner. Neither removal of the endothelium nor pretreatment with NO synthase inhibitor, L-NAME \((10⁻⁴ \text{ mol/l})\), ablated the relaxation induced by losartan (fig. 6B). There were no differences in the IC₅₀ of losartan-induced vasodilation \([1.4 ± 0.2 \text{ (intact) vs. } 1.3 ± 0.4 \text{ (denuded) and } 1.1 ± 0.2 \text{ (L-NAME treated) μmol/l, } P > .05]\). On the other hand, in intact coronary rings preconstricted with 40 mmol/l KCl, losartan \((10⁻¹₀⁻¹⁰⁻⁵ \text{ mol/l})\) had no vasodilatory effects on the vascular rings (Li, P., Ferrario, C. M. and Brosnihan, K. B., unpublished observations).

Discussion

Losartan, the nonpeptide Ang II AT₁ antagonist, inhibits TxA₂ analog U46619-induced contractions of canine coronary arteries without changing the maximal contractile response. These findings are consistent with losartan acting as a competitive antagonist of the TxA₂/PGH₂ receptor in canine coronary arteries. The active metabolite of losartan, EXP3174, also antagonized the TxA₂/PGH₂ receptor-mediated contractions in coronary arteries. In contrast, two other AT₁ receptor antagonists (CV11974 and Sarthran) and an AT₂ antagonist (PD123319) did not interact with the TxA₂/PGH₂ receptor in coronary vessels. The significant shift in the dose-response curve produced by losartan was not mediated by release of either prostaglandins or nitric oxide from the coronary arteries, because the responses were not altered by preincubation with inhibitors of cyclooxygenase or nitric oxide synthase. These data suggest that both losartan and its active metabolite EXP3174 bind to both the AT₁ and the TxA₂/PGH₂ receptors, a finding that calls attention to a separate and
possibly complementary action of losartan potassium on proatherogenic events which result from platelet aggregation and plaque rupture (Berliner et al., 1995).

In contrast to saralasin, the first characterized Ang II peptide antagonist (Pettinger et al., 1975), losartan is an orally active, nonpeptide AT₁ receptor antagonist possessing no intrinsic agonist effects. Losartan potassium blocks most of the known Ang II-induced vasoconstrictor, dipsogenic, aldosterone and catecholamine stimulatory responses (Timmermans et al., 1995; Chiu et al., 1991). In addition, losartan possesses significant antihypertensive activity in most species studied (Wong et al., 1990a; Ohlstein et al., 1992; Moriguchi et al., 1994). Preclinical research showed that losartan reverses cardiac hypertrophy and vascular hyperplasia (Dostal and Baker, 1992; Dahlof, 1993). EXP3174, the vascular hyperplasia (Dostal and Baker, 1992; Dahlof, 1993).

TABLE 1

| EC50 values of U46,619 in various concentrations of losartan-treated canine coronary rings (nmol/l) |
|----------------------------------|-----------------|
| Control                          | Losartan        |
|                                  | 10⁻⁶ mol/l      | 10⁻⁷ mol/l      | 10⁻⁶ mol/l      | 10⁻⁵ mol/l      |
| 10.6 ± 0.9                       | 16.7 ± 2.4      | 19.7 ± 2.8      | 33.4 ± 4.6*     | 139.6 ± 13.9*   |

* P < .001 compared with control.

![Fig. 3. Average dose-response contraction curve to U46619 during control (n = 12 dogs) and after pretreatment with the Ang II AT₁ receptor antagonist CV11974, the AT₂ antagonist PD123319, the active metabolite of losartan EXP3174 and the nonselective peptide Ang II receptor antagonist Sar¹Thr⁵-Ang II. All Ang II antagonists were used at 10⁻⁷ mol/l concentration. (n = 3–6 dogs). Values are mean ± S.E.M.](image)

Both losartan and EXP3174 have a benzylimidazol moiety, with EXP3174 differing from losartan only by its being a diacidic metabolite of losartan. CV11974 is also a biphenyl tetrazole; however, the imidazole ring is fused to another heterocyclic ring which possesses a carboxylic acid at position 7. Our studies suggest that the extra phenyl ring of CV11974 either reduces or prevents the binding of this AT₁ antagonist at the AT₁/PGH₂ receptor. Thus, our studies indicate that differences in the structure-activity of AT₁ receptor antagonists determine the ability of biphenyl tetrazole to bind to the TXA₂ receptor.

Recent studies have linked TXA₂ and PGH₂ to mechanisms associated with renin-dependent and angiotensin-induced hypertension. The selective antagonist of the TXA₂/PGH₂ receptor SQ29548 was reported to elicit a 20 mm Hg decrease in blood pressure in rats with either aortic coarctation-induced hypertension or Ang II-induced hypertension (Lin et
In these experiments, administration of an angiotensin antagonist reduced blood pressure by 60 mm Hg. This suggests that in high renin models of hypertension, TxA2 contributes about 30% to the elevation of blood pressure. An increase in the renal excretion of TxA2 and its production in blood vessels was found in several models of hypertension, including renal hypertension and SHR (Lin et al., 1994; Konieczkowski et al., 1983). In addition, it has been shown that TxA2 and PGH2 are endothelium-derived contracting factors and that these factors contribute to the evolution of hypertension in SHR (Dai et al., 1992; Kato et al., 1990).

In our study, losartan blocked the TxA2/PGH2 receptor of canine coronary arteries as a competitive antagonist with \( K_B \) values similar to those determined by Corriu et al. (1995) in rat aorta and mesenteric arteries. This indicates that the apparent affinity of losartan for the TxA2 receptor was approximately 1000-fold lower than that for the AT1 receptor (Rhaleb et al., 1991). Our studies agree with observations by Liu and colleagues (1992), who reported that losartan was a competitive ligand at human platelet TxA2 receptor with a \( K_d \) value of 9.6 \( \mu \)M. An effect of losartan on TxA2 receptor cannot be completely excluded after in vivo administration of a large concentration (Wong et al., 1990a; Ohlstein et al., 1992; Caçalo et al., 1995; DeGraaff et al., 1993). In the rat circu-

<table>
<thead>
<tr>
<th>Control</th>
<th>Losartan (1 ( \mu )M)</th>
<th>CV11974 (1 ( \mu )M)</th>
<th>PD123319 (1 ( \mu )M)</th>
<th>EXP3174 (1 ( \mu )M)</th>
<th>Sar1Thr8-Ang II (1 ( \mu )M)</th>
</tr>
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<tbody>
<tr>
<td>EC50 (nM)</td>
<td>10.6 ( \pm ) 0.9</td>
<td>33.4 ( \pm ) 4.6*</td>
<td>12.6 ( \pm ) 1.7</td>
<td>12.9 ( \pm ) 0.7</td>
<td>66.9 ( \pm ) 9.2†</td>
</tr>
<tr>
<td>Maximal contraction (g)</td>
<td>6.2 ( \pm ) 0.2</td>
<td>6.2 ( \pm ) 0.7</td>
<td>7.2 ( \pm ) 0.7</td>
<td>6.1 ( \pm ) 0.6</td>
<td>5.5 ( \pm ) 0.4</td>
</tr>
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* \( P < .001 \) compared with control.
† \( P < .01 \) compared with losartan-treated group.

**Fig. 4.** Pretreatment with losartan (10 \( \times \) 6 mol/l) did not affect the coronary constrictor responses to PDGF (10 and 20 ng/ml). Values are mean \( \pm \) S.E.M. Studies were conducted in four dogs.

**Fig. 5.** Pretreatment with losartan (10 \( \times \) 6 mol/l) did not affect the coronary constrictor response to KCl (10– 80 mmol/l). Maximal contraction was 5.69 \( \pm \) 0.26 vs. 6.1 \( \pm \) 0.28 g, control vs. losartan treated. Values are mean \( \pm \) S.E.M. Studies were conducted in six dogs.

**Fig. 6.** (A) Effect of copretreatment with losartan and indomethacin on U46,619 dose-response curve. There is no difference in coronary constrictor responses to U46619 (10 \( \times \) 10–10–10 \( \times \) 6 mol/l) when copretreated with losartan (10 \( \times \) 6 mol/l) and the cyclo-oxygenase inhibitor indomethacin (10 \( \times \) 3 mol/l) as compared with pretreatment with losartan (10 \( \times \) 6 mol/l) alone. (B) Effect of endothelium denudation (denuded ED) or treatment with L-NAME on losartan dilation of coronary vessels preconstricted with 10 nM U46619. Studies were conducted in six to eight dogs. Values are mean \( \pm \) S.E.M.

**TABLE 2**

Effects of Ang II antagonists on the U46,619-induced contraction in canine coronary arteries
loration, the concentration of losartan was estimated to reach approximately 250 μmol/l after a 10 mg/kg i.v. injection (Liu et al., 1992; Corriu et al., 1995). In humans, the concentration of losartan and its active metabolite EXP3174 was 250–550 ng/ml and 500–800 ng/ml (approximately 1 μmol/l) after an oral therapeutic dose of losartan (80–120 mg oral dose) (Munafò et al., 1992). From both the rat and the human studies, we estimate that the circulating values obtained by others after losartan administration are consistent with the concentration range of the binding and functional inhibition constants at the TXA2/PGH2 receptor. These findings suggest that the antagonistic effect of losartan and its active metabolite EXP3174 on the vascular TXA2/PGH2 receptor may contribute to the prolonged blood pressure reduction during long-term antihypertensive treatment. Thus losartan with its actions on the TXA2/PGH2 receptor may have therapeutic advantages over a more selective AT1 angiotensin receptor antagonist in preventing the vasoconstrictor and platelet aggregating actions of TXA2.

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References