Nonpeptide Angiotensin II Antagonist Losartan Inhibits Thromboxane A2-Induced Contractions in Canine Coronary Arteries1

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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 vasconstriction 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; Moriguchi 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 (Olshtein et al., 1992; Cachofeiro et al., 1995). However, it has been noted that in this same hypertensive model the peptide Ang II antagonist saralasin 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; Olshtein 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 Olshtein 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 imidazoline/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-{(3-hydroxy-1-octenyl)-2-oxacyclo [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 prostan- 
din 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 sus- 
pended 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-ethylenediami- 
netetraacetic 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 expo- 
sure to 40 mmol/l KCl. In some rings, the endothelium was denuded 
gently mechanical rubbing with a stainless steel wire. Isometric 
tension of vascular rings was measured continuously with force-

displacement transducer (FTO3, 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 acetyl- 
choline) and absence of acetylcholine-induced relaxation in vessels after 
mechanical denudation of the vascular endothelium.

Experimental protocol. Control cumulative concentration-con- 
tactile 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 then the concentration-response curves for 
U46619 were then repeated. To determine whether losartan inter- 
acts with other vasoconstrictors, concentration-response curves with 
PDGF (10 and 20 ng/ml) and KCl (10–80 mmol/l) were also con- 
structed in the absence and presence of losartan (10–8 mol/l) in 
isolated coronary vascular rings. Phenylephrine, arginine vasopres- 
sin 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 another nonpeptide Ang II receptor subtype 
agonists interact on the TxA2/PGH2 receptors in coronary vessels, 
the selective Ang II AT1 receptor antagonist CV11974, an active form 
of TCV-116 (Brummer 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 Sar1Thr3-Ang II (10–6 mol/l) 
was also tested. The cyclo-oxygenase 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, l-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 l-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 gener- 
sely supplied by Parke-Davis Inc. (Ann Arbor, MI) and CV-11974 
was from Takeda Chemical Industries, LTD. (Osaka, Japan). l-
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 calcu-
lated with the equation Kd = [B] 46 [ΔI/ΔI<sub>0</sub>]<sup>−1</sup>, where [B] is 
the concentration of the antagonist and [ΔI] and [ΔI<sub>0</sub>] 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 statisti-
cally 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 concen-
tration-response curve of U46619 to the right in a concentra-
tion-dependent manner without a change in the maximum 
constriction. The EC50 of U46619 (10.2 ± 0.9 nmol/l) was 

![Fig. 1](https://i.imgur.com/XYZ123.png)
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 $0.5 \pm 0.1$ and $0.8 \pm 0.1 \mu$mol/l in the presence of $1$ and $10 \mu$mol/l of losartan, respectively. Losartan at $10^{-4}$ M concentration completely reversed the constriction of $10 \mathrm{nM}$ 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 [$EC_{50} 10.6 \pm 0.9$ (control) vs. $199.1 \pm 17.7$ and $617.3 \pm 36.8 \mathrm{nmol/l}$ at $0.01$ and $0.1 \mu$mol/l of SQ29548, respectively] without changing the maximum contraction (fig. 2B). Pretreatment with $1 \mu$mol/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 $0.1 \mu$mol/l SQ29548 was 31-fold greater than losartan at equimolar concentrations [$EC_{50} 19.7 \pm 2.8$ (losartan) vs. $617.3 \pm 63.8$ (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 to U46619 at $1 \mu$mol/l of losartan (10 $\mu$mol/l) blocked the TxA2/PGH2 receptor agonist PGF2α-induced dose-dependent vasoconstriction (EC$_{50}$ 23.4 ± 3.4 vs. 205.1 ± 23.5 nmol/l; maximal contraction, 5.0 ± 0.2 vs. 4.9 ± 0.3 g without and with losartan, n = 2). Effects of losartan on the production of vasoactive prostaglandins and nitric oxide. Copreincubation of $10 \mu$mol/l of the cyclo-oxygenase inhibitor indomethacin with $1 \mu$mol/l of losartan for $30$ min did not significantly shift the concentration-response curve of U46619 as compared with pretreatment with $1 \mu$mol/l of losartan alone (fig. 6A). On the other hand, U46619-preconstricted rings (10 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$^{-4}$ mol/l), abolished the relaxation induction by losartan (fig. 6B). There were no differences in the IC$_{50}$ of losartan-induced vasodilation [1.4 ± 0.2 (intact) vs. 1.3 ± 0.4 (denuded) and 1.1 ± 0.2 (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$^{-5}$ 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 AT$_1$ antagonist, inhibits TxA$_2$ 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 cyclo-oxygenase 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 antagonist possessing no intrinsic agonist effects. Losartan 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 in vivo active metabolite of losartan, is approximately 10- to 15-fold more potent than losartan and has a longer plasma half-life (Wong et al., 1990b). Evidence suggests that EXP3174 makes a major contribution to the long-lasting blood-pressure-lowering effects of losartan (Wong et al., 1990b; Tallant and Ferrario, 1996).

Several studies indicate that Ang II blockade may not fully account for the long-term antihypertensive and antiproliferative actions of losartan. Ohlstein et al. (1992) reported a recovery of the pressor responses to both Ang I and Ang II during chronic therapy with losartan. More recent studies indicate that several non-Ang II-related actions of losartan may be involved in its prolonged blood-pressure-lowering effects. Those studies suggested that losartan may stimulate the production of vasodilator prostaglandins and nitric oxide (Jaiswal et al., 1991; Cachofeiro et al., 1995), interact with alpha-1 receptor (Maeso et al., 1995), and block the tachykinin (Picard et al., 1995), imidazoline/guanidinium (Li et al., 1996) and Txa2 receptors (Liu et al., 1992; Bertolino et al., 1994; Corriu et al., 1995). In agreement with those observations, we showed that the blocking actions of losartan on the Txa2 receptor were quite specific, because administration of either the cyclo-oxygenase inhibitor, indomethacin, or a nitric oxide synthase inhibitor did not eliminate the antagonistic effect of losartan on the U46619-induced vasoconstriction. These results suggest that the competitive inhibitory effects of losartan on the Txa2 receptor are not mediated by stimulation of vasodilators, prostaglandins or nitric oxide. Furthermore, losartan did not inhibit coronary artery vasoconstriction induced with PDGF, KCl, phenylephrine and vasopressin. In the rat brain neither losartan nor EXP3174 at 10 µmol/l were shown to be potent antagonists at the imidazoline/guanidinium receptor sites (Li et al., 1996). Taken together, these findings are consistent with a specific effect of losartan on the Txa2/PGH2 receptor as reported in previous studies (Liu et al., 1992; Bertolino et al., 1994; Corriu et al., 1995).

The structural requirements necessary for antagonism of the Txa2/PGH2 receptor are different than those involved in antagonism of the AT1 receptor, because we demonstrated that losartan, but not another AT1 antagonist, CV11974, or the nonselective angioteinsin II antagonist Sar1Thr8-Ang II were effective in blocking U46619-induced vasoconstrictor responses. In agreement with other studies demonstrating that EXP3174 is more potent than losartan at the AT1 receptor (Timmermans et al., 1993; Wong et al., 1990b), our study did prove that EXP3174 is a more potent antagonist than losartan for U46619-induced contraction of canine coronary artery rings. These findings are consistent with studies which demonstrated that the metabolite acts as a selective, noncompetitive receptor antagonist which dissociates from its receptor slowly and is more potent than losartan (Sacchindis et al., 1993; Wong et al., 1990b). With use of rat denuded aorta and small mesenteric vessels, Corriu et al. (1995) showed that losartan had an inhibiting effect on U46619-induced contractile response. In their studies, however, EXP3174 at 3, 10, and 30 µmol/l was without effect, which suggests that the structural requirements for this antagonistic action may be different for the Txa2/PGH2 receptor in the rat.

Both losartan and EXP3174 have a benzylimidazole 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 Txa2/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 al.).
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 μM. 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; Cachoferio et al., 1995; DeGraaff et al., 1993). In the rat circu-
cation, the concentration of losartan was estimated to reach approximately 250 \mu M/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 \mu M/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 vasocostructor and platelet aggregating actions of TxA2.

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References


