Olmesartan Improves Endothelin-Induced Hypertension and Oxidative Stress in Rats

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Abstract

Recent studies have indicated that both endothelin (ET) and angiotensin (Ang) II stimulate oxidative stress, which contributes to the development of hypertension. Here, we examined the effects of Ang II type 1 (AT_1) receptor blockade on reactive oxygen species (ROS) formation in ET-dependent hypertension. Chronic ET-1 infusion (2.5 pmol/kg/min, i.v., $n=7$) into rats for 14 days increased systolic blood pressure from 113±1 to 141±2 mmHg. ET-1-infused rats showed greater plasma renin activity (8.1±0.8 Ang I/ml/h), and greater Ang I (122±28 fmol/ml) and Ang II levels (94±13 fmol/ml) than vehicle (0.9% NaCl)-infused rats (3.1±0.6 Ang I/ml/h, 45±8 and 47±7 fmol/ml, respectively, $n=6$). Angiotensin converting enzyme and AT_1 receptor expression in aortic tissues were similar between the vehicle- and ET-1-infused rats. Vascular superoxide anion (O_2^-) production and plasma thiobarbituric acid-reactive substance (TBARS) levels were greater in ET-1-infused rats (27±1 counts per minutes [CPM]/mg dry tissue weight and 8.9±0.8 μmol/l, respectively) than vehicle-infused rats (16±1 CPM/mg and 5.1±0.1 μmol/l, respectively). The ET-1-induced hypertension was prevented by simultaneous treatment with a new AT_1 receptor antagonist, olmesartan (0.01% in chow, 117±5 mmHg, $n=7$), or hydralazine (15 mg/kg/day in drinking water, 118±4 mmHg, $n=6$). Olmesartan prevented ET-1-induced increases in vascular O_2^- production (15±1 CPM/mg) and plasma TBARS (5.0±0.1μmol/l). Vascular O_2^- production and plasma TBARS were also decreased by hydralazine (21±1 CPM/mg and 7.0±0.3 μmol/l, respectively), but these levels were significantly higher than in vehicle-infused rats. These data suggest that ET-dependent hypertension is associated with augmentation of Ang II levels and ROS formation. The combined effects of the elevations in circulating ET-1 and Ang II, as well as the associated ROS production, may contribute to the development of hypertension induced by chronic ET-1 infusion.

Keywords

endothelin; angiotensin II; angiotensin II type 1 receptor; reactive oxygen species; olmesartan

Introduction

The renin-angiotensin system (RAS) and the endothelin (ET) system entail potent vasopressor mechanisms that contribute to the development of hypertension and cardiovascular injury (1, 2). Many studies have indicated that angiotensin (Ang) II exerts its hypertensive effects *via* interaction with the ET system (1,3-6). For example, it has been shown that ET mediates some of the vasoconstrictor actions of Ang II in the peripheral vasculature (3). Furthermore, ET

treatment augments the pressor effects of an acutely administered subpressor dose of Ang II (4). The hypertension associated with chronic infusion of Ang II can be markedly attenuated by ET_A receptor blockade (5,6). Although these observations indicate potential interactions between the RAS and the ET system, there has been little investigation into the specific role of the RAS in the development of ET-dependent hypertension. In hypertensive uremic rats, ET-1 levels in blood vessels and glomeruli were reduced by AT_1 receptor blockade with losartan (7,8). It was also shown that losartan reduced constrictor responses to ET-1 in aortic rings from spontaneously hypertensive rats (9). Chronic infusion of a subpressor dose of Ang II in combination with a subpressor dose of ET-1 induced hypertension, indicating a synergistic interaction between Ang II and ET-1 (10). In addition, treatment with an angiotensin converting enzyme (ACE) inhibitor attenuated the reductions in renal blood flow and urinary sodium excretion induced by acute administration of ET-1 (11). Similarly, the hypertension induced by chronic infusion of ET-1 was prevented by an ACE inhibitor (12).

Ang II stimulates superoxide anion (O_2^-) production through NAD(P)H oxidase-dependent pathways (13,14), and this may at least partially contribute to the development of Ang II-dependent hypertension (13,15). Recent studies have also indicated a role for ET in reactive oxygen species (ROS) formation (16-21). *In vitro* studies have shown that ET-1 increases ROS formation in endothelial cells (16), vascular smooth muscle cells (17) and rat aortic rings (18). Treatment with ET antagonists has been shown to reduce ROS levels in streptozotocin-induced diabetic rats (19) and hypertensive rats (18,20). Li *et al.* (18,21) showed that both the ET-1 level and the vascular NAD(P)H oxidase activity are increased in deoxycorticosterone acetate (DOCA)-salt hypertensive rats, and that NAD(P)H oxidase-derived O_2^- augments ET-1-induced venous vasoconstriction in these animals. Recent studies also have shown that thiobarbituric acid-reactive substance (TBARS) levels in renal tissues and urinary 8-isoprostaglandin $F_{2\alpha}$ excretion are significantly increased in ET-induced hypertensive rats, and that treatment with tempol, a superoxide dismutase mimetic, normalizes the hypertension and these oxidative stress markers in these animals (17). These observations indicate that the formation of ROS plays a critical role in mediating the hypertension induced by chronic elevations in ET.

The aim of the present study was to determine whether the RAS was involved in the development of ET-dependent hypertension and ROS formation. Therefore, we investigated the status of oxidative stress and the RAS, including the plasma renin activity (PRA), Ang I, Ang II and angiotensinogen (AGT) levels as well as vascular ACE and AT_1 receptor expression in ET-1-infused hypertensive rats. We also examined the effects of olmesartan, a potent new AT_1 receptor antagonist (22,23), on blood pressure and oxidative stress in ET-1-infused rats.

Methods

Animal Preparation

All surgical and experimental procedures were performed according to the guidelines for the care and use of animals established by the Kagawa Medical University. Male Sprague-Dawley rats (Clea Japan, Tokyo, Japan), 5-6 weeks of age, were selected at random to receive a continuous infusion of ET-1 (*n*=7) or vehicle (0.9% NaCl, *n*=6). ET-1 (Peptide Institute Inc., Minoh, Japan) was dissolved in 0.9% NaCl and infused into the jugular vein at a rate of 2.5 pmol/kg/min for a period of 14 days. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the vascular catheter (PE-60) was tunneled subcutaneously to an osmotic minipump (model 2ML2; Alza Co., Cupertino, USA) implanted subcutaneously at the dorsum of the neck. ET-1 was also infused into rats treated with a new AT_1 receptor antagonist, olmesartan (CS-866; Sankyo Co. Ltd., Tokyo, Japan; 0.01% in chow, approximately 6 mg/kg/ day, *n*=7), or a nonspecific antihypertensive agent, hydralazine (Wako Co., Osaka, Japan; 15

mg/kg/day in drinking water, *n*=6). The doses of ET-1, olmesartan and hydralazine were determined based on previous studies in rats (24-26).

Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan) at 0, 7 and 14 days. Blood and tissue samples were harvested at the end of the experimental periods. After decapitation, the trunk blood was collected into chilled tubes containing an inhibitor mixture (5 mmol/l EDTA+20μmol/l enalaprilat+1.25 mmol/l 1,10-*o*-phenanthroline+10μmol/l pepstatin A), and processed for measurements of the plasma Ang I and Ang II concentrations as well as the AGT protein level (27-29). Blood was also collected into chilled tubes containing 5 mmol/l EDTA for measuring PRA (28,29) and TBARS (28,30). The aorta was quickly removed followed by the perivascular tissues. Five millimeter ring segments of the aorta were used to measure the O_2^- production (15,28), and the remaining aorta samples were snap-frozen in liquid nitrogen and stored at -80 °C until processing for protein extraction.

Plasma AGT Levels and the Expression of ACE and AT_1 Receptors in Aortic Tissues

Plasma AGT protein levels were determined by Western blotting analysis as described previously (27,28). Briefly, protein samples were separated by 8% polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was incubated with the primary antibody (sheep anti-rat AGT, 1:5,000), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (donkey anti-sheep IgG, 1:30,000; Sigma Chemical Co., St. Louis, USA). All values were normalized by arbitrarily setting the integrated densitometric values (IDV) of vehicle-infused rats to 1.0.

The levels of ACE and AT_1 receptor protein expression in aortic tissues were determined by Western blotting analyses (28,29). Briefly, protein samples were separated by 8% polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was reacted with a polyclonal anti-ACE antibody (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, USA) or a polyclonal anti-AT_1 receptor antibody (1:1,000; Santa Cruz Biotechnology Inc.), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (for ACE: donkey anti-goat immunoglobulin G [IgG], 1:1,000, Santa Cruz Biotechnology Inc.; for AT_1 receptors: goat anti-rabbit IgG, 1:4,000, Cell Signaling Technology, Beverly, USA). To check for equal loading, membranes were re-probed with an antibody against *β*-actin. All values were normalized by arbitrarily setting the IDV of vehicle-infused rats to 1.0.

Measurement of Vascular O_2^- Production

O_2^- production in aortic segments was determined with the use of lucigenin chemiluminescence, as described previously (15,28). Briefly, 5-mm aortic rings were placed in bicarbonate buffer for 30 min at 37 °C. After the equilibration, the rings were rinsed with prewarmed (37 °C) modified Krebs-HEPES buffer. Rings were placed in 1 ml Krebs-HEPES buffer containing lucigenin (20μmol/l) and equilibrated in the dark for 10 min at 37 °C. The chemiluminescence was then recorded every 30 s for 15 min with a luminescence reader (BLR-301; Aloka Co., Ltd., Tokyo, Japan). Thereafter, a cell-permeant, nonenzymatic O_2^- scavenger, Tiron (20 mmol/l) was added to quench the O_2^--dependent chemiluminescence (31), and 18 more cycles were read. The final 10 values, which appeared to be maximally reduced, were averaged. The differences between the values obtained before and after Tiron addition were calculated and defined as the Tiron-quenchable lucigenin chemiluminescence. Lucigenin chemiluminescence was expressed as counts per minute (CPM) per milligram of dry tissue weight.

Analytical Procedures

We determined the degree of lipid peroxidation using a biochemical assay of the TBARS level in plasma as described previously (28,30). PRA, Ang I and Ang II levels were measured by radioimmunoassays as previously reported (27-29).

Statistical Analysis

The values are presented the as means±SEM. Statistical comparisons of differences were performed using one way or two way ANOVA for repeated measures combined with Newman-Keuls *post hoc* test. Values of *p*<0.05 were considered statistically significant.

Results

Blood Pressure

The temporal profiles of SBP are depicted in Fig. 1. SBP was identical among the 4 groups at the beginning of the protocol. SBP was significantly elevated in ET-1-infused rats at week 2 compared with that in vehicle-infused rats (141±2 *vs.* 113±1 mmHg). Concurrent administration of olmesartan or hydralazine prevented ET-1-induced hypertension (117±5 mmHg and 118±4 mmHg, respectively).

The RAS in ET-Induced Hypertensive Rats

Consistent with previous studies (27), Western blotting analysis of the plasma proteins using a specific anti-AGT polyclonal antibody showed 2 bands at 52 and 64 kDa (slightly glycosylated and highly glycosylated forms of circulating AGT), with the greatest abundance at 52 kDa. Figure 2 shows a representative Western blot of plasma AGT. Densitometric analysis of the immunoreactive bands showed that ET-1 infusion had no effect on the expression of plasma AGT. On the other hand, concurrent administration of olmesartan to ET-1-infused rats significantly increased both forms of plasma AGT protein compared with the levels in vehicle-infused rats (1.7±0.2-, 1.8±0.2- and 1.5±0.2-fold for 52 kDa, 64 kDa and total AGT, respectively). Similarly, treatment with hydralazine also increased both forms of plasma AGT protein (1.5±0.2-, 1.6±0.1- and 1.6±0.1-fold for 52 kDa, 64 kDa and total AGT, respectively).

Compared with vehicle-infused rats, PRA was significantly increased in ET-1-infused rats (3.1±0.6 *vs.* 8.1±0.8 Ang I/ml/h). Treatment with olmesartan caused a further increase in PRA in ET-1-infused rats (16.6±0.8 Ang I/ml/h). On the other hand, concurrent administration of hydralazine to ET-1-infused rats did not alter PRA (10.7±1.7 Ang I/ml/h, Fig. 3A). In ET-1-infused rats, the plasma Ang I level was significantly elevated compared with that in vehicle-infused rats (122±28 *vs.* 45±8 fmol/ml, Fig. 3B). Similarly, the plasma Ang II levels was higher in ET-1-infused rats (94±13 fmol/ml) than in vehicle-infused rats (47±7 fmol/ml, Fig. 3C). Concurrent administration of olmesartan to ET-1-infused rats further increased the plasma Ang I and Ang II levels (471±70 and 354±42 fmol/ml, respectively). The plasma Ang I and Ang II levels in hydralazine-treated ET-1-infused rats (153±20 and 118±13 fmol/ml, respectively) were higher than those in vehicle-infused rats, but these levels were not different from those in rats treated with ET-1 alone.

Western blotting analyses showed that ACE and AT_1 receptor expression levels in aortic tissue were similar between vehicle- and ET-1-infused rats. Furthermore, neither olmesartan nor hydralazine altered vascular ACE or AT_1 receptor expression in ET-1-infused rats (Fig. 4).

Plasma TBARS Levels

ET-1-infused rats showed a significantly higher plasma TBARS level compared with vehicle-infused rats (8.9±0.8 *vs.* 5.1±0.1μmol/l, Fig. 5A). Concurrent administration of olmesartan

prevented the increase in the plasma TBARS levels in ET-infused rats ($5.0\pm0.1\mu$mol/l). On the other hand, hydralazine did not alter the plasma TBARS level in ET-infused rats ($7.0\pm0.3\mu$mol/l), but this level was significantly higher than that in vehicle-infused rats. Furthermore, the TBARS level in hydralazine-treated ET-1-infused rats was significantly higher than that in olmesartan-treated ET-1-infused rats ($p<0.05$).

Vascular O_2^- Production

Lucigenin chemiluminescence from aortic segments of vehicle-infused rats averaged 16 ± 1 CPM/mg dry tissue weight. Compared with vehicle-infused rats, ET-1-infused rats showed higher lucigenin chemiluminescence (27 ± 1 CPM/mg dry tissue weight, $p<0.05$, Fig. 5B). Olmesartan prevented the ET-1-induced increase in lucigenin chemiluminescence (15 ± 1 CPM/mg dry tissue weight). Similarly, hydralazine treatment of ET-1-infused rats also decreased lucigenin chemiluminescence (21 ± 2 CPM/mg dry tissue weight, $n=6$). However, this level was significantly higher than that in vehicle-infused rats ($p<0.05$). Furthermore, lucigenin chemiluminescence from aortic segments of hydralazine-treated ET-1-infused rats was significantly higher than that of olmesartan-treated ET-1-infused rats ($p<0.05$).

Figure 5C shows the Tiron-quenchable lucigenin chemiluminescence from aortic segments. Compared with vehicle infused rats, ET-1-infused rats showed higher Tiron-quenchable lucigenin chemiluminescence (5.5 ± 0.3 *vs*. 18.9 ± 1.1 CPM/mg dry tissue weight, $p<0.05$). The increased Tironquenchable lucigenin chemiluminescence in ET-1-infused rats was normalized by treatment with olmesartan (5.1 ± 0.1 CPM/mg dry tissue weight). Similarly, hydralazine administration to ET-1-infused rats decreased the Tiron-quenchable lucigenin chemiluminescence (10.2 ± 0.6 CPM/mg dry tissue weight, $n=6$). On the basis of group comparisons, however, this level was significantly higher than those in vehicle-infused rats and olmesartan-treated ET-1-infused rats ($p<0.05$, respectively).

Discussion

In vitro and *in vivo* studies have indicated that Ang II is a potent stimulator of ET-1 production (1,32,33). It has been shown that Ang II can turn on the transcription of the ppET-1 gene in vascular smooth muscle cells (32), and that chronic administration of Ang II increased renal ET-1 production in rats (33). On the other hand, the effects of the ET system on the RAS activity remain unclear. Matsumura *et al*. (34) showed that ET-1 decreased renin secretion from kidney cortical slices. However, ET-1 had no consistent effects on basal renin secretion from juxtaglomerular cells (35). *In vivo* studies have also shown that acute infusion of ET-1 significantly decreased (36) or significantly increased (37) renin secretion. In the present study, we investigated the status of the RAS in rats treated with chronic ET-1 infusion. The results showed that the plasma Ang II level was significantly increased by chronic infusion of ET-1, while the vascular ACE and AT_1 receptor expression remained constant. Elmarakbly *et al*. (38) reported that AT_1 receptor blockade with candesartan had no effect on the increase in blood pressure induced by acute infusion of ET-1. However, the present study has shown that a new AT_1 receptor antagonist, olmesartan (22,23), prevents the hypertension induced by chronic infusion of ET-1. These observations suggest that the RAS system is maintained or activated when ET-1 is infused chronically. Since AT_1 receptor blockade prevents ET-induced hypertension, activation of the RAS might be involved, at least in part, in the development of ET-dependent hypertension.

We observed that PRA was significantly increased in ET-1-infused hypertensive rats. These results are consistent with previous observations that acute hypertension induced by systemic infusion of ET is associated with an elevation in PRA (37). We also found that the plasma AGT levels were maintained in ET-1-infused hypertensive rats. These data indicate that increases in PRA, rather than increases in the AGT level, are responsible for the elevated Ang II level

in ET-1-induced hypertensive rats. However, the mechanisms by which chronic infusion of ET-1 increases PRA remain to be elucidated. Acute administration of ET-1 constricts the renal afferent arterioles (39,40), and decreases the renal blood flow (11) and glomerular filtration rate (38). Furthermore, significantly increased renal vascular resistance has been observed in ET-1-infused hypertensive rats (17). Therefore, the possibility exists that sustained renal vasoconstriction, a decrease in the glomerular filtration rate, or the subsequent renal damage induced by chronic elevation of the ET-1 level contributes to the increases in renin release. Other possibilities cannot be ruled out and need to be examined further.

Recent studies have indicated that ET-induced vasoconstriction may be mediated, at least partially, by increased ROS production (17-21). The present study showed that vascular O_2^- production and the plasma TBARS level were significantly increased in ET-1-infused hypertensive rats. These observations are in accordance with those of previous studies showing that the TBARS content in the kidney and urinary excretion of 8-isoprostaglandin $F_{2\alpha}$ were increased in ET-1-infused hypertensive rats (17). However, the mechanisms by which ET-1 stimulates ROS production are not clear. It is well known that Ang II stimulates O_2^- production through NAD(P)H oxidase activation (13,14). Recently, it has also been shown that ET-1 can stimulate O_2^- production *via* the NAD(P)H oxidase pathway (18,21). Furthermore, ET-1 increases O_2^- production and the expression of gp91phox, an essential component of NAD(P)H oxidase, in endothelial cells (16). ET-1 is a potent stimulator of protein kinase C (PKC) (40), which is also involved in NAD(P)H oxidase activity and O_2^- production (41). Therefore, it is also possible that ET-1 activates NAD(P)H oxidase through a PKC-dependent pathway. In the present study, we have demonstrated that ET-dependent hypertension is associated with augmentation of Ang II and ROS levels. Interestingly, AT_1 receptor blockade with olmesartan prevents the ET-1-induced hypertension and ROS formation. Although multiple mechanisms may contribute to the ET-induced ROS formation, it can be speculated that the augmented Ang II level also participates in the ROS formation induced by chronic infusion of ET.

In the present study, we also examined the effects of a non-specific vasodilator, hydralazine. We observed that treatment with hydralazine did not alter the plasma TBARS level, but did significantly decrease vascular O_2^- in ET-1-infused rats. This observation is consistent with previous observations that hydralazine did not change the plasma TBARS level but did reduce NAD(P)H oxidase-mediated vascular O_2^- production in Ang II-infused rats (42). These data raise two possibilities: first, hydralazine has antioxidant properties; and second, reductions in the ROS levels are a consequence of the antihypertensive effect of hydralazine. The possibility of antioxidative effects of hydralazine cannot be addressed from the present data. However, the vascular O_2^- level may not be simply changed by alterations in blood pressure, since previous studies have shown that vascular O_2^- release was not altered in norepinephrine-infused hypertensive rats (43).

In conclusion, the present study demonstrates that ET-1-induced hypertension is associated with augmentation of Ang II and ROS levels in rats. In addition, AT_1 receptor blockade with olmesartan prevents the development of ET-1-induced hypertension and oxidative stress. These data suggest that the combined effects of the elevations in circulating ET-1 and Ang II levels, as well as the associated ROS production, contribute to the development of ET-induced hypertension.

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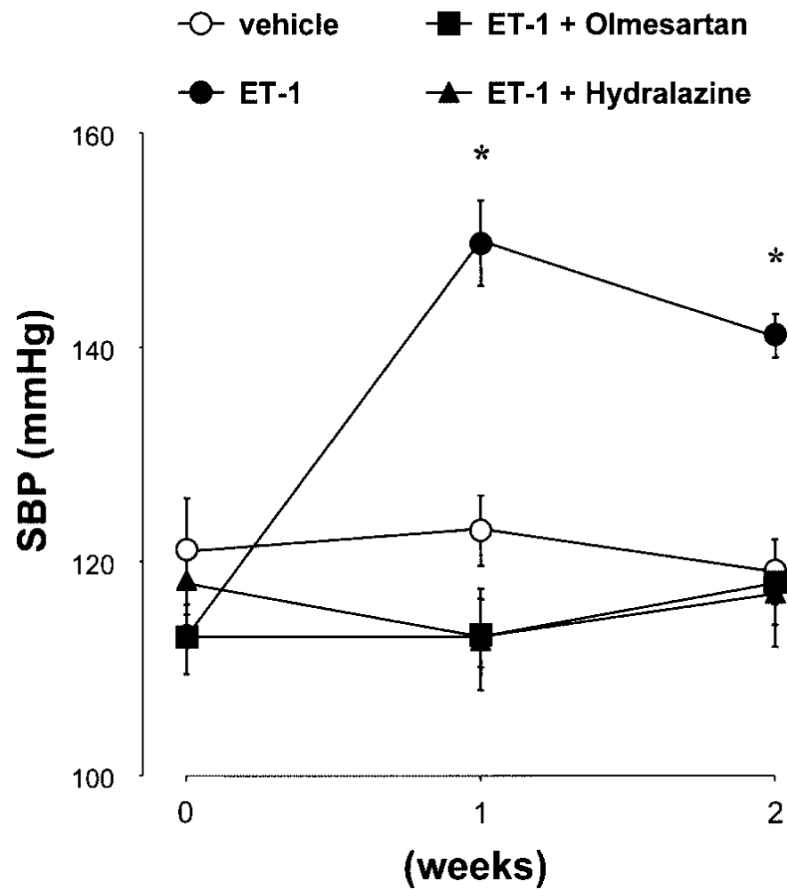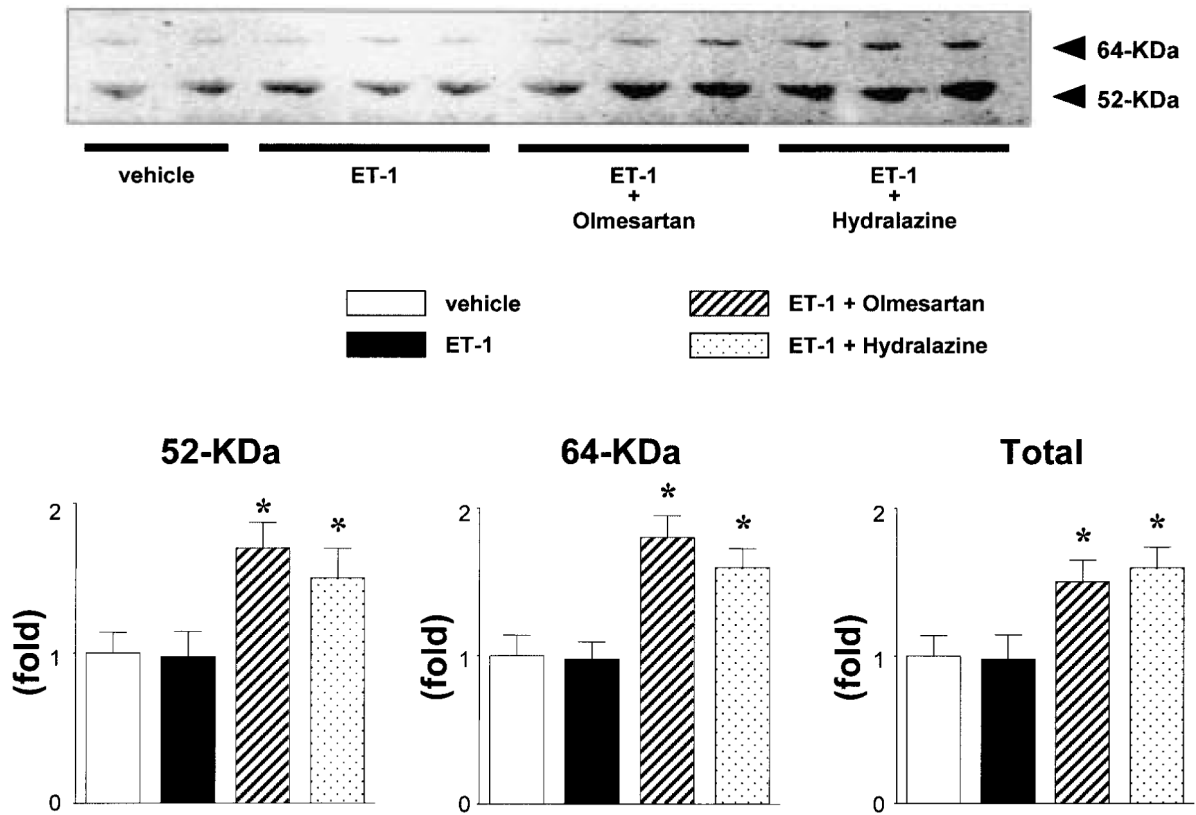
Fig. 1.
Temporal profiles of systolic blood pressure (SBP). SBP was similar among the 4 groups at the beginning of the protocol. Endothelin (ET)-1 infusion for 2 weeks significantly increased SBP. Concurrent administration of olmesartan or hydralazine prevented the development of hypertension in ET-1-infused rats. *p<0.05 vs. week 0.

Fig. 2.
Western blotting analysis of plasma angiotensinogen (AGT) expression. Densitometric analysis of the immunoreactive bands shows that chronic endothelin (ET)-1 infusion had no effect on the expression of plasma AGT. Concurrent administration of olmesartan or hydralazine to ET-1-infused rats significantly increased the expression of both forms of plasma AGT. All values were normalized by arbitrarily setting the densitometry of vehicle-treated rats to 1.0, as described in the Materials and Methods. *p<0.05 vs. vehicle-infused rats.
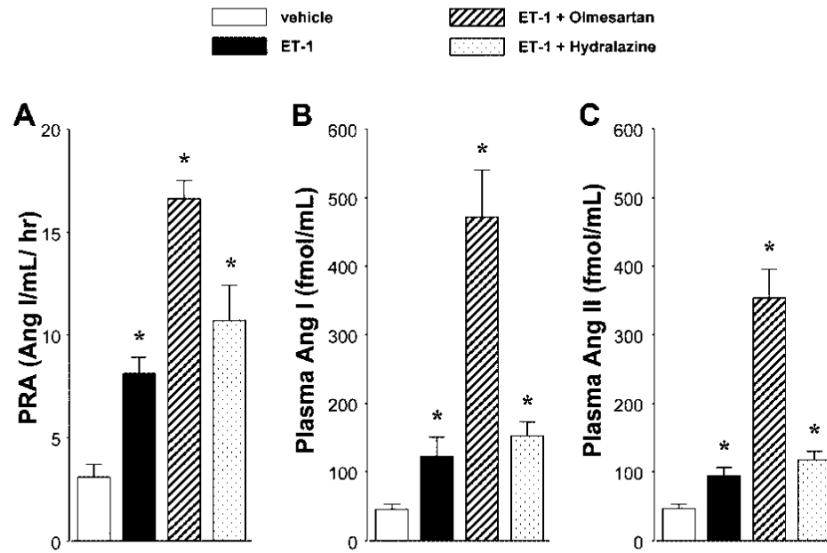
Fig. 3.
A: Plasma renin activity (PRA), B: plasma angiotensin I (Ang I) and C: plasma angiotensin II (Ang II) levels are shown. PRA, plasma Ang I and Ang II levels were significantly elevated in endothelin (ET)-1-infused hypertensive rats. Concurrent administration of olmesartan to ET-1-infused rats further increased these levels. PRA, Ang I and Ang II levels were significantly higher in hydralazine-treated ET-1-infused rats than in vehicle-infused rats, but these levels were not different from those in rats treated with ET-1 alone. *$p < 0.05$ vs. vehicle-infused rats.
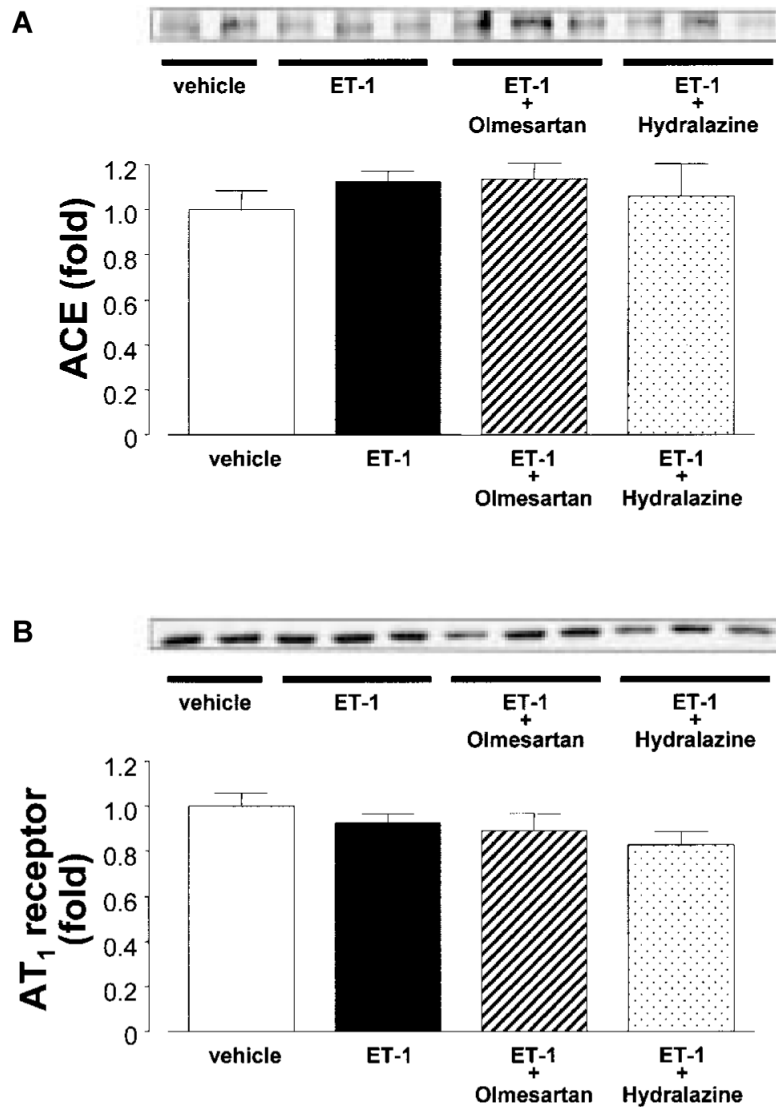
Fig. 4.
A: Western blotting analyses for A: protein expression of angiotensin converting enzyme (ACE) and B: AT_1 receptors in aortic tissues. The expression of ACE and AT_1 receptor in aortic tissues were similar between vehicle- and ET-1-infused rats. Furthermore, neither olmesartan nor hydralazine altered vascular ACE and AT_1 receptor expression in ET-1-infused rats. To check for equal loading, membranes were re-probed with an antibody against β-actin. All values were normalized by arbitrarily setting the densitometry of vehicle-treated rats to 1.0, as described in the Materials and Methods.
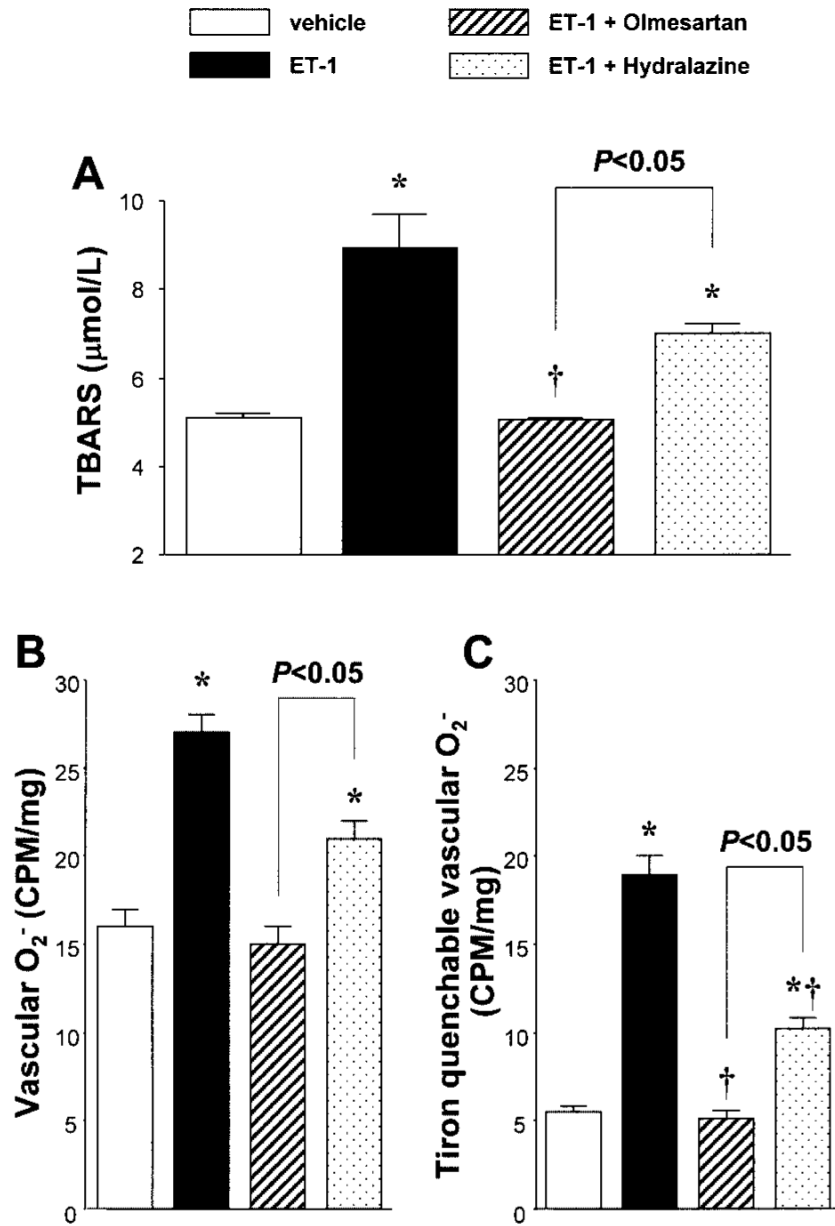
Fig. 5.
A: Plasma thiobarbituric acid-reactive substance (TBARS) levels. B: Vascular superoxide anion (O_2^-) production assessed by lucigenin chemiluminescence in aortic segments. C: The differences between the values obtained before and after Tiron (20 mmol/l) addition were calculated and defined as the Tiron-quenchable lucigenin chemiluminescence. Lucigenin chemiluminescence was expressed as counts per minute (CPM) per milligram of dry tissue weight. *p<0.05 vs. vehicle-infused rats. [†]p<0.05: endothelin (ET)-1-infused rats vs. ET-1-infused rats+olmesartan or hydralazine.