Maintenance of blood pressure in normotensive dogs by endothelin

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Münter, Klaus, Heimo Ehmke, and Michael Kirchenga$t. Maintenance of blood pressure in normotensive dogs by endothelin. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1022–H1027, 1999.—The role of endothelin (ET)-1 in blood pressure homeostasis and the interaction with the renin-angiotensin system (RAS) was investigated in normotensive conscious dogs. ET_A receptors were blocked by LU-135252 (1–30 mg/kg); trandolapril (2 mg/kg) or losartan (10 mg/kg) was used to inhibit the RAS. LU-135252 in oral doses of 3–30 mg/kg significantly reduced mean arterial pressure (MAP) by ~10 mmHg maximally, whereas trandolapril or losartan were without any effect. MAP reduction was more pronounced when LU-135252 was combined with either losartan (~15.5 ± 3.2 mmHg; 2 h postadministration; P < 0.05) or trandolapril (~30.9 ± 3.6 mmHg; P < 0.05). When endogenous nitric oxide (NO) generation was blocked but NO concomitantly infused, this synergistic effect on MAP was prevented. The data show that ET-1 contributes to the maintenance of blood pressure via ETA receptors. Furthermore, ET-1 and ANG II play a prominent role in the control of blood pressure by opposing the effects of NO. The pronounced blood pressure fall after combined blockade of ETA receptors and the RAS may be mediated by an enhanced release of NO.

METHODS

Male beagle dogs (n = 5–10/group) weighing 13–17 kg (BASF) were kept on standard diet (Herilam Hu-Expan 197, Eggersmann, Germany) with water ad libitum. At least 8 days before the study, the dogs were subjected to intravenous neuroleptic analgesia (combination of propionylpromazine and l-methadone), and a polyethylene catheter was implanted into the aorta abdominalis. Animal care and husbandry were in compliance with the EC directive 86/609. The study was approved by the local authorities (Bezirksregierung Neustadt).

All experiments (except for the ET-1 bolus experiments under pentobarbital sodium anesthesia) were made in conscious dogs lying quietly and connected to the recording instruments via extension cables. The experiments started between 7:30 and 9:00 in the morning, 16 h after the last feeding. Between administration of the different drugs a washout period of at least 7 days was kept. The following experimental protocols were used.

Protocol 1: Effect of LU-135252 on ET-1 plasma levels. LU-135252 was administered orally in a soft gelatin capsule in different dosages (1, 3, 10, or 30 mg/kg). Two hours later plasma samples were drawn from the aortic catheter for the determination of ET plasma levels by a commercially available sandwich ELISA (Biomedica, distributed by Immudiagnostics, Bensheim, Germany).

Protocol 2: Effect of intravenous ET-1 on mean arterial pressure. After intravenous administration of 0.75 nmol ET-1/kg to dogs that were anesthetized by a single intravenous administration of pentobarbital sodium (60 mg/kg, Narcoren, Sanofi), the effect on blood pressure was studied for 20 min.
Two hours before administration of ET-1, the dogs were orally treated with either placebo or LU-135252 (3, 10, or 30 mg/kg).

Protocol 3: Cardiovascular effects of LU-135252. In dose-effect studies, LU-135252 was administered orally in a soft gelatin capsule in different dosages (1, 3, 10, or 30 mg/kg). Cardiovascular parameters were recorded for 6 h after substance administration on a computer-based data acquisition system (M1®, Modular Instruments). Systolic and diastolic pressures (in mmHg) were measured in the abdominal artery by Statham transducer P23 Db; mean arterial pressure (MAP) was calculated automatically. The changes in MAP were expressed as relative changes from the initial values or as area under the curve (AUC, in mmHg x 360 min). Heart rate (HR, in beats/min), was calculated from the systolic pressure signal.

Protocol 4: Effect of combined ET and RAS blockade. Trandolapril (2 mg/kg; n = 10) or losartan (10 mg/kg; n = 5) was administered orally in a soft gelatin capsule or as film-coated tablet alone or in combination with LU-135252 (10 mg/kg), and the cardiovascular effects were studied as described above.

Protocol 5: Effect of clamping NO plasma levels. The NO synthase inhibitor N⁵-nitro-L-arginine (L-NNA; n = 5) was infused for 4 h at a rate of 30 µg·kg⁻¹·min⁻¹. In a second experiment, 1 h after the start of the L-NNA infusion, the NO donor S-nitroso-N-acetyl-penicillamine (SNAP; Sigma, Munich, Germany) was additionally infused at a rate of 5 µg·kg⁻¹·min⁻¹ (n = 5). This experimental setting (block of endogenous NO generation and supplementing NO by exogenous infusion) is called NO clamping. In a third experiment, 1 h after the start of the SNAP infusion (2 h after L-NNA), LU-135252 and trandolapril (10 and 2 mg/kg; n = 5) were orally administered.

Drugs and justification of dosage. Trandolapril was a gift from Hoechst-Marriott-Roussel; LU-135252 [2-(4,6-dimethylpyrimidin-2-yloxy)-3-methoxy-3,3-diphenyl-propionic acid] was synthesized at BASF (Ludwigshafen, Germany); losartan was purchased as Lorzaar from DuPont; L-NNA was from Sigma; and ET-1 was from Alexis (Grünenberg, Germany).

The extent of ACE inhibition by trandolapril was investigated in conscious dogs in challenge experiments with ANG I. Two hours after trandolapril administration, the blood pressure response evoked by an intravenous dose of 0.05 µg/kg ANG I was inhibited by 74 ± 6% (n = 6, mean ± SE).

The dose of losartan was chosen as described in Ref. 6. According to the pharmacokinetic data, 2 h after oral administration of 10 mg/kg Losartan a plasma concentration in the range of 0.3 µM should be achieved. This should correspond to half-maximal inhibition of ANG II response (6). The extent of ANG II receptor blockade was confirmed in conscious dogs in challenge experiments with ANG I. Two hours after losartan administration, the blood pressure response evoked by an intravenous dose of 0.05 µg/kg ANG I was inhibited by 56 ± 13% (n = 6, mean ± SE), which was not significantly different from the extent of ACE inhibition produced by trandolapril.

The dose of the selective ETₐ receptor antagonist LU-135252 (Kᵢ for ETₐ: 1.4 nM; Kᵢ for ETₐ: 184 nM; Ref. 21), which completely and specifically blocks ETₐ receptors, was determined according to experimental protocols 1 and 2 (described above). It has been shown that ETₐ receptors in the lung serve as clearance receptors for ET (14). Thus blockade of ETₐ receptors will increase levels of circulating ET. By experimental protocol 1 it was possible to detect at which dose of LU-135252 the selectivity for ETₐ receptors is lost and LU-135252 additionally blocks ETₐ receptors. From the results of the experiments of protocol 2 the extent of ETₐ blockade was estimated.

The dose of L-NNA was determined in challenge experiments with acetylcholine (2 µg/kg iv), which induced a short-lasting decrease in blood pressure when the NO system was intact. This effect was decreased by 64 ± 14% during blockade of NO generation by L-NNA (mean ± SE, n = 6).

Statistics. Arithmetic means and SE and their changes were evaluated (software RS/1, BBN, Munich, Germany; Microsoft Excel; SAS Research application, Cary, NC).

Significant differences (P < 0.05) between the values measured after drug administration and initial values were calculated by Student’s t-test for paired samples (two sided). Differences between individual groups were assessed by ANOVA for multiple comparisons with a general linear model. To characterize possible combination effects on blood pressure, AUC values were calculated and statistically evaluated as described in Ref. 15.

RESULTS

The influence of different doses of LU-135252 on plasma levels of ET is shown in Fig. 1. Neither placebo nor oral doses of 1, 3, or 10 mg/kg of LU-135252 resulted in any change in ET plasma levels. Only at a dose of 30 mg/kg did LU-135252 significantly increase ET plasma levels, from 3.9 ± 0.1 to 14.8 ± 2.8 fmol/ml.

The extent of the ET blockade was investigated by bolus injection of exogenous ET-1. After intravenous administration of ET-1 (0.75 nmol/kg), an immediate blood pressure decrease followed by a delayed and sustained blood pressure increase was observed (Fig. 2). Oral administration of LU-135252 at doses of 3, 10, and 30 mg/kg, 2 h before ET-1, resulted in a prolonged blood pressure increase. The blood pressure increase was partially (3 mg/kg) or totally (10 and 30 mg/kg) blocked by LU-135252 (Fig. 2).

Whereas no effect on MAP was seen in the placebo group, it slightly decreased after oral administration of LU-135252 at a dose of 1 mg/kg (Fig. 3). Increasing the dose of LU-135252 to 3, 10, and 30 mg/kg significantly reduced blood pressure from 1.5 or 2 h postadministration onward by ~10 mmHg (Fig. 3). Calculation of AUC values (Table 1) revealed that LU-135252 significantly decreased MAP at all doses higher than 3 mg/kg (not dose dependently) compared with placebo-treated control dogs. Because at 30 mg/kg LU-135252 also blocked...
ET\textsubscript{B} receptors (see Fig. 1) and ET\textsubscript{A} blockade was not complete after 3 mg/kg (see Fig. 2), we decided to use a dose of 10 mg/kg in all further experiments.

The AT\textsubscript{1} receptor antagonist losartan, at an oral dose of 10 mg/kg, affected neither blood pressure (Fig. 4 and Table 1) nor HR. Similarly, trandolapril (2 mg/kg orally) had no effect on MAP, but HR increased by 16 ± 4 beats/min 2 h postadministration (P < 0.05; data not shown).

These experiments showed that it was nearly impossible to drastically lower blood pressure in normotensive dogs by interfering with only one regulatory mechanism. Therefore, various drug combinations were employed. Losartan in combination with LU-135252 (10 and 10 mg/kg) induced a marked decrease in MAP from 97 ± 2 mmHg to 81 ± 2 mmHg (Fig. 4). This effect was significant over the entire observation period of 6 h. The effect of the combination was overadditive compared with values expected from the experiments with the individual substances (Table 1). HR was only slightly increased (statistically not significant).

An even more pronounced blood pressure lowering effect was observed after simultaneous ET\textsubscript{A} receptor blockade and ACE inhibition (Table 1 and Fig. 4). After combined administration of LU-135252 (10 mg/kg) and trandolapril (2 mg/kg), MAP was reduced from 96 ± 2 to 65 ± 3 mmHg (Fig. 4). This effect became significant after 1 h and lasted over the entire observation period of 6 h. The blood pressure decrease was overadditive compared with the respective individual substances. The fall in blood pressure was accompanied by an increase in heart rate by 36% (from 71 ± 4 to 96 ± 5 beats/min, P < 0.05; data not shown).

To clarify the underlying mechanism of this overadditive effect, experiments during blockade of the NO system were performed. During infusion of L-NNA, MAP increased from 100 ± 2 to 128 ± 3 mmHg and remained constant during the following 2 h (Fig. 5). In a second experiment, in addition to the L-NNA infusion, an infusion of the NO donor SNAP was started (NO clamping). With the use of this treatment regimen, normotensive conditions could again be achieved (98 ± 7 mmHg; Fig. 5), however, with simultaneous blockade

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**Fig. 2.** Changes in mean arterial pressure (MAP) after administration of an intravenous bolus of 0.75 nmol/Kg ET-1 in anesthetized dogs. Two hours before the bolus of ET-1 was given, the dogs were pretreated with placebo (A) or LU-135252 in doses of 3 (B), 10 (C), or 30 mg/kg (D). Data represent means ± SE; n = 3–11/group.

**Fig. 3.** Changes in MAP after oral administration of placebo or LU-135252 in doses of 1, 3, 10, and 30 mg/kg. Data represent means ± SE; n = 5–10/group. *P < 0.05 vs. baseline values (time = 0).
of endogenous NO generation. Under these conditions (normotension, blockade of NO generation), the combined blockade of ETA receptors and the RAS had only a minor impact on blood pressure (Fig. 5). Blood pressure dropped from 102 ± 4 to 88 ± 8 mmHg, which was not significantly different from the corresponding SNAP value without the combination.

**DISCUSSION**

Blood pressure is well controlled in conscious normotensive mammals. The control mechanisms include the autonomic reflexes, myogenic mechanisms, body fluid regulatory mechanisms, and vasoactive substances acting in an autocrine, paracrine, or endocrine way. The contribution of one component alone is not easily identified because other compensatory mechanisms ensure homeostasis. The aim of the present study was to investigate the contribution of ET and its interaction with the RAS to the blood pressure regulation in conscious normotensive dogs.

To identify a dose of LU-135252 that selectively blocks the ETA receptor subtype, dose-effect studies were performed. LU-135252 in oral doses from 3 to 30 mg/kg reduced blood pressure in normotensive dogs; the extent as well as duration of this effect was identical in all groups. Determination of ET plasma levels revealed that LU-135252 at a dose of 30 mg/kg increased circulating ET. This suggests that due to the high dose, LU-135252 lost its selectivity for ETA receptors and that ETB receptors were blocked, at least partially, as well. The ET challenge experiments showed a sustained blood pressure reduction after LU-135252 pretreatment (3, 10, and 30 mg/kg). We interpret this effect as follows. Because most of the accessible ETA receptors are blocked by LU-135252, the exogenously administered ET preferentially binds to ETB receptors whose activation causes the observed vasodilatation. Interestingly, this effect was not diminished after 30 mg/kg of LU-135252, even though this dose also blocks ETB receptors. This effect most likely can be explained by a competition problem: in the presence of low (picomolar range) circulating plasma levels of ET (i.e., without exogenous administration of ET-1), LU-135252 (at high plasma concentrations) will occupy both ETA and ETB receptors. In challenge experiments, however, plasma levels of ET-1 are immediately raised to the nanomolar range. At this high concentration, ET-1 may replace LU-135252 previously bound from the ETB receptor, especially at the endothelium, which is freely accessible to circulating ET-1. A displacement from ETA receptors is probably not possible because the affinity of LU-135252 to ETA receptors is more than 100-fold higher than to ETB receptors (21).

These dose-effect experiments further show that a comparable blood pressure reduction can be achieved in normotensive dogs by either selectively blocking ETA receptors (LU-135252: 3 and 10 mg/kg) or by blocking both receptor subtypes (LU-135252: 30 mg/kg). A possible reason for this effect may be that most resistance vessels are under a tonic control of ET-1. Accordingly, blockade of ETA receptors will lower vascular tone leading to vessel relaxation. Alternatively, the observed decrease in MAP may be mediated by endogenous ET, because blockade of ETA receptors may unmask the effects of binding of endogenous ET-1 to endothelial

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**Table 1. Changes (calculated as AUC) in MAP over 6 h after oral administration of different drugs**

<table>
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<th>Dose, mg/kg</th>
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<tr>
<td>Control</td>
<td>10</td>
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<td>2 + 10</td>
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Data represent means ± SE. AUC, area under the curve; MAP, mean arterial pressure. *P < 0.05 vs. control; †P < 0.05 vs. both respective individual substances.

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**Fig. 4. Effects of oral administration of losartan (10 mg/kg, n = 5; A), trandolapril (2 mg/kg, n = 5; B), losartan plus LU-135252 (10 and 10 mg/kg, n = 5; C), or trandolapril plus LU-135252 (2 and 10 mg/kg, n = 10; D) on MAP. Data represent means ± SE. *P < 0.05 vs. baseline values (time = 0).**

**Fig. 5. Contribution of endogenous nitric oxide generation to the hypotensive effect seen after combined blockade of ETA receptors and the renin-angiotensin system. In all experiments, N-nitro-L-arginine (L-NNA; 30 µg·kg⁻¹·min⁻¹) was continuously infused either alone (A) or in combination with S-nitroso-N-acetyl-penicillamine (SNAP; 3 µg·kg⁻¹·min⁻¹, starting after 1 h, B) or with SNAP (3 µg·kg⁻¹·min⁻¹, starting after 1 h) plus the combination of trandolapril and LU-135252 (2–10 mg/kg, administered after 2 h, C). Data represent means ± SE; n = 5/group. *P < 0.05 vs. L-NNA alone.**
ET\textsubscript{A} receptors, resulting in NO and/or prostacyclin release and subsequent vasodilatation. This latter explanation, however, is not likely because even high doses of LU-135252, which block both ET\textsubscript{A} and ET\textsubscript{B} receptors, caused a reduction of blood pressure. These results suggest that the ET\textsubscript{B} receptor does not seem to have a prominent function in blood pressure control in normotensive dogs. These findings are in line with the observation of Donckier et al. (12), who documented a small blood pressure lowering activity of the balanced ET\textsubscript{A}/ET\textsubscript{B} receptor antagonist bosentan (9) in normotensive dogs during anesthesia. However, the authors (12), as well as Teerlink et al. (27), concluded that ET-1 does not play any significant role in the maintenance of blood pressure in normotensive dogs.

It has been shown in our laboratory that ET inhibits (20) and blockade of ET\textsubscript{A} receptors stimulates renin secretion (H. Berthold, K. Münter, A. Just, H. R. Kirchheim, and H. Ehmkne, unpublished observations). Thus during LU-135252 treatment, plasma renin activity is increased, generating more ANG II, which may counteract the LU-135252-induced blood pressure reduction. Furthermore, because a number of interactions between the ET system and RAS have been shown previously in several species (1, 2, 8, 10, 11, 18, 19), we investigated whether a combined blockade of both systems might result in a larger reduction of blood pressure. Indeed, after combined blockade of ET\textsubscript{A} receptors (by LU-135252) and the RAS (by losartan or trandolapril), an enhanced effect on blood pressure, which was larger than the sum of the single effects, was observed. To address the possible role of NO in this hypotensive effect, experiments were repeated after NO synthase inhibition. Withdrawal of NO led to a hypertensive state in which vasoconstrictor systems, perhaps ET and ANG II, play a prominent role. When normal blood pressure was restored by supplementing NO exogenously with the NO donor SNAP, the combined blockade of ET and RAS had only minor effects on blood pressure. These experiments indicate that a significant part of the blood pressure lowering activity of the combination might be due to an enhanced release of NO, whose vasodilator activity is no longer opposed by ET-1 and ANG II. However, it cannot be excluded that under the conditions of NO clamping the vasculature is unresponsive in a nonspecific manner toward ET and RAS blockade.

Data of a recently published study by Donckier et al. (11) in hypertensive dogs showed that intravenous infusion of the balanced ET\textsubscript{A}/ET\textsubscript{B} receptor antagonist bosentan combined with ACE inhibition by enalaprilat produced an additive antihypertensive effect compared with that of the single substances. In contrast to their study, the present investigation demonstrates a significant contribution of ET-1 to the maintenance of MAP under normotensive conditions.

In conclusion, the present results show that ET-1 contributes to the maintenance of normal blood pressure by stimulating ET\textsubscript{A} receptors. Furthermore, the data suggest that ET-1 and ANG II play a prominent role in the control of blood pressure by opposing the effects of NO. The very large decrease of MAP after combined blockade of ET\textsubscript{A} receptors and the RAS may be mediated by an enhanced release of NO under these conditions.

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