NO and NO-independent mechanisms mediate ET$_B$ receptor buffering of ET-1-induced renal vasoconstriction in the rat

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Just, Armin, Andrea J. M. Olson, John R. Falck, and William J. Arendshorst. NO and NO-independent mechanisms mediate ET$_B$ receptor buffering of ET-1-induced renal vasoconstriction in the rat. Am J Physiol Regul Integr Comp Physiol 288: R1168–R1177, 2005. First published December 23, 2004; doi:10.1152/ajpregu.00550.2004.—Vascular endothelin (ET) type B (ET$_B$) receptors exert dilator and constrictor actions in a complex interaction with ET$_A$ receptors. We aimed to clarify the presence and relative importance of nitric oxide (NO) and other mechanisms underlying the dilator effects of ET$_B$ receptors in rat kidneys. Complete inhibition of NO production with N$	extsuperscript{G}$-nitro-L-arginine methyl ester (l-NNAME, 25 mg/kg iv) enhanced the renal vasoconstriction elicited by ET-1 injected into the renal artery from −15 to −30%. Additional infusion of the NO donor nitroprusside (NP) into the renal artery did not reverse this effect (−29%) but effectively buffered ANG II-mediated vasoconstriction. Similarly, ET-1 responses were enhanced after a smaller intrarenal dose of l-NNAME (−22 vs. −15%) and were unaffected by subsequent NP infusion (−21%). These results indicate that the responsiveness to ET-1 is buffered by ET$_B$ receptor-stimulated phasic release of NO, rather than its static mean level. Infusion of the ET$_B$ receptor antagonist BQ-788 into the renal artery further enhanced the ET-1 constrictor response to NP + l-NNAME (−92 vs. −49%), revealing an NO-independent dilator component. In controls, vasoconstriction to ET-1 was unaffected by vehicle (−27 vs. −20%) and markedly enhanced by BQ-788 (−70%). The same pattern was observed when indomethacin (Indo) was used to inhibit cyclooxygenase (−20% for control, −22% with Indo, and −56% with ET$_B$ antagonist) or methylsulfonyl-6-(2-propargyloxyphenyl)-hexanamide (MS-PPOH) or miconazole + Indo was used to inhibit epoxygenase alone (−10% for control, −11% with MS-PPOH, and −35% with ET$_B$ antagonist) or in combination (−14% for control, −20% with Indo + miconazole, and −43% with ET$_B$ antagonist). We conclude that phasic release of NO, but not its static level, mediates part of the dilator effect of ET$_B$ receptors and that an NO-independent mechanism, distinct from prostanooids and epoxyeicosatrienoic acids, perhaps ET$_B$ receptor clearance of ET-1, plays a major buffering role.

renal hemodynamics; endothelial cell; cyclooxygenase; epoxygenase; angiotensin II; dopamine; nitric oxide; endothelin

ENDOTHELIN (ET)-1 plays an important role in cardiovascular regulation, renal hemodynamics, and fluid and volume homeostasis (18). Although there is general agreement that ET-1 causes vasoconstriction in the renal as well as other vascular beds (18), this net response reflects a combination of constrictor effects of ET type A and B (ET$_A$ and ET$_B$) receptors on smooth muscle cells counteracted by the dilator influence of ET$_B$ receptors on endothelial cells. The pregglomerular resistance vessels have roughly an equal density of ET$_A$ and ET$_B$ receptors (12, 31). Recently, we and others observed in the renal microcirculation complex interactions between receptor subtypes, with the influence of ET$_B$ receptors varying between net constrictor or dilator, depending on whether ET$_A$ receptors are stimulated simultaneously (24, 27, 32). Selective ET$_B$ receptor stimulation produces renal vasoconstriction. On the other hand, the dilator action of ET$_B$ receptors modulates the magnitude of the overall constrictor effect of ET$_A$ + ET$_B$ receptors on smooth muscle cells. A strong dilator influence is indicated by marked enhancement of the ET-1-induced vasoconstriction during ET$_B$ receptor antagonism (5, 10, 15, 24, 27).

Evidence indicates that nitric oxide (NO) is released on ET$_B$ receptor activation (19, 20, 44). In vivo studies show that NO production attenuates the renal vasoconstrictor response to ET-1 (10, 15, 29, 30). In addition, prostanooids (13) and epoxygenase metabolites (22) may contribute to the dilator effect of ET$_B$ receptors.

Another potential mechanism by which ET$_B$ receptors counteract ET-mediated constriction is sequestration of ET and subsequent reduction of the amount available to activate ET$_A$ receptors. Evidence for such a clearance function derives from the rise in plasma levels (5, 39) and the marked reduction of pulmonary uptake of ET (5, 14) after inhibition of ET$_B$ receptors as well as reduction of plasma clearance in knockout mice missing one copy of the ET$_B$ receptor gene (3). However, plasma clearance is predominantly provided by the lungs (14), whereas the kidneys contribute −10–25% (14, 42). In addition, −50% of renal ET clearance seems to be independent of ET$_B$ receptors (14, 47). It is thus unclear whether and to what extent ET clearance may contribute to the buffering effect of ET$_B$ receptors in the kidney.

To our knowledge, only one previous in vivo study has combined ET$_B$ receptor antagonism with NO synthase (NOS) inhibition (32) to determine the relative contribution of NO and a possible NO-independent mechanism. In this and in vitro studies (10, 32, 45), it is important to appreciate that ET$_B$ receptor blockade had no additional effect after NOS inhibition. These findings suggest that the buffering action of ET$_B$ receptors is exclusively due to NO. However, results obtained from ex vivo preparations should be interpreted with caution. In vitro studies utilized hemoglobin-free perfusion solutions, which probably exaggerated the impact of NO (10, 45). In the sole in vivo study, NOS inhibition was combined with cyclooxygenase (COX) inhibition, so that the relative contributions of their metabolites could not be differentiated (32). Thus the
possible participation of a mechanism distinct from NO warrants further investigation, especially in the in vivo setting with an intact blood-perfused kidney.

The present study was conducted to test the hypothesis that ETB receptor-induced buffering of ET-1 responsiveness in the renal circulation is mediated by NO-dependent and NO-independent mechanisms. To this end, we used an in vivo model under well-defined euvoletic conditions and administered ET-1 and an ETB receptor antagonist directly into the renal artery to highlight local actions independent of systemic effects. To differentiate NO-dependent from NO-independent mechanisms of ETB receptor-mediated buffering, the effect of NOS inhibition alone was compared with that of additional ETB receptor antagonism. Because buffering effects of NO in response to transient stimulation of ETB receptors by bolus injection of ET-1 can only be mediated by transient release of NO, but not mean ambient levels of NO, NOS inhibition was combined with constant replacement of NO. To elucidate additional effects of static mean levels of NO, other experiments were conducted with variable levels of NO.

METHODS

Experiments were conducted on 53 male Sprague-Dawley rats (6–8 wk of age, 190–340 g body wt) from our local breeding colony in accordance with institutional guidelines for the care and use of research animals and were approved by the local Institutional Animal Care and Use Committee. The animals were fed a standard laboratory chow with free access to tap water and kept on a 12:12-h light-dark cycle. Surgical preparation and general methods were similar to those described previously (24).

Surgical Preparation

After induction of anesthesia by pentobarbital sodium (50–60 mg/kg body wt ip; Nembutal, Abbott, Chicago, IL), a rat was placed on a temperature-controlled table kept at 37°C. The depth of anesthesia was monitored by the response to ear or toe pinching. The left femoral artery was catheterized for measurement of arterial pressure, and two femoral venous catheters were used for infusion of volume replacement and injection of pentobarbital. The trachea was cannulated to facilitate respiration. Via a midline abdominal incision, the aorta and left renal artery were exposed. A catheter was inserted into the left common iliac artery and advanced until its tip faced the origin of the left renal artery and used for infusion into the renal artery. An ultrasound transit-time flow probe (model 1RB, Transonic, Ithaca, NY) was placed around the left renal artery and filled with ultrasonic coupling gel (Surgilube, Fugera, Melville, NY). Urine was drained from the bladder by gravity via a 23-gauge needle. Isonicotinic bovine serum albumin (4.75 g/dl) was infused initially at 50 µl/min to replace surgical losses (1.25 ml/100 g body wt) and then at 10 µl/min for maintenance. The renal artery catheter was perfused with normal saline at 5 µl/min. Additional doses of pentobarbital were given intravenously as required. All syringes and catheters in contact with peptides were pretreated with albumin solution (0.5 g/dl) to reduce surface adhesion. At least 60 min were allowed after surgery before the experiments were begun.

Measurements

Femoral arterial pressure (AP) was measured via a pressure transducer (Statham P23 DB) connected to the arterial catheter. Renal blood flow (RBF) was measured by a flowmeter (model T 420, Transonic; low-pass filter 40 Hz) connected to the flow probe. RBF values were corrected for zero offset determined at the end of an experiment after cardiac arrest. AP and RBF were recorded on a computer (Pentium III, DataTranslation analog-to-digital converter, and Labtech Notebook-Pro 10.1) at 100 Hz and stored at 1 Hz as consecutive mean values over 1-s periods. AP was also stored at 100 Hz for later determination of heart rate.

Protocols

The RBF response to bolus injections of 10 µl of 0.3 µM ET-1 into the renal artery was measured throughout an experiment. At 5 min after each injection, the renal arterial infusion rate was increased from 5 to 143 µl/min. A 10-µl bolus of ET-1 was then injected into the renal artery line through a microinjector valve (Chemirat, Valco Instruments, Houston, TX), and a new recording was started. The initial 20 s served as the baseline values of AP and RBF. At 10 min after ET-1 injection, the infusion rate was returned to 5 µl/min, and the recording was continued for another 20 min. At least 30 min were allowed for recovery after each ET-1 injection. In some experiments, the response to intrarenal injection of 10 µl of 0.4 µM ANG II was recorded for 5 min. The infusion rate was raised to 143 µl/min 30 s before injection and returned to 5 µl/min 5 min after injection. At least 5 min were allowed for recovery after an injection of ANG II.

Experimental Groups

Renal vasoconstrictor response to ET-1 before and during ETB receptor antagonism. To confirm the enhancing effects of ETB receptor antagonism on the renal vasoconstrictor response to ET-1 we observed previously (24), the effect of ET-1 was investigated during three consecutive periods: control, saline, and intrarenal infusion of an ETB receptor antagonist. The infusion of saline (32 µl/min iv) started 30 min after the first and 15 min before the second injection of ET-1 and continued for the remainder of the experiment. The ETB receptor antagonist BQ-788 (7 nmol/min in 1 ml/kg saline) was infused between 5 min before and 10 min after the third injection of ET-1. This dose of BQ-788 is known to exert maximal inhibitory effects in this experimental setup (24).

Effects of clamping NO levels before and during ETB receptor blockade. To investigate a possible role of NO in buffering of renal vasoconstrictor responses to ET-1 and/or in the effects of ETB receptor antagonism, NOS was inhibited by Nω-nitro-L-arginine methyl ester (l-NNAME, 25 mg/kg bolus iv in 1 ml/kg saline) 35 min after the first injection of ET-1. This dose of l-NNAME was chosen on the basis of our preliminary experiments and the literature to exceed the 10 mg/kg known to produce maximum effects on AP and RBF (2, 9, 28, 46). To minimize the vasoconstrictor and pressor effects of l-NNAME and to maintain constant ambient levels of NO, the NO donor nitroprusside (NP, 1 mg·kg⁻¹·min⁻¹) was infused intravenously. Temporarily reductions of NO levels and hypertensive responses were avoided by starting NP infusion 3–5 min before administration of l-NNAME. On the basis of preliminary experiments, the infusion rate of NP was initially set to 42 µl/min (42 µg·kg⁻¹·min⁻¹) and then reduced so that AP after l-NNAME was 90–100 mmHg. This infusion rate (24 ± 8 µl/min, 24 µg·kg⁻¹·min⁻¹) was maintained for the remainder of an experiment. At 20 min after l-NNAME, ET-1 was injected alone and 30 min later during intrarenal infusion of BQ-788 (7 µmol/min).

Effects of ET-1 and ETB receptor inhibition during NOS inhibition and NO-independent vasodilator. To investigate the role of ambient levels of NO, the renal vasoconstrictor effects of ET-1 alone and during ETB receptor blockade were assessed before and after l-NNAME as described above for NP, except vasodilatation was produced by the dopamine D1 receptor agonist fenoldopam (Fen). This agent was chosen, because it elicits vasodilatation by a pathway different from NO and acts preferentially on renal vessels (36), thus allowing more complete restoration of RBF without inducing compromising hypotension. At ±30 min after the first injection of ET-1, Fen (0.08 mg·kg⁻¹·min⁻¹) was infused at 32–42 µl/min (2.5–3.3 µg·kg⁻¹·min⁻¹ iv). After 3–5 min, l-NNAME was injected (25 mg/kg iv).

AJP-Regul Integr Comp Physiol • VOL. 288 • MAY 2005 • www.ajpregu.org
Time-control experiments. To ensure that the magnitude of ET-1 responses was stable over time, separate experiments were conducted similar to those described above for L-NAME + vasodilator agent, except saline, instead of BQ-788, was infused into the renal artery during the third period.

Dose testing for L-NAME. To test whether 25 mg/kg L-NAME was sufficient to completely inhibit NO production, experiments were conducted following the same protocol used for the time-control experiments, except an additional dose of 100 mg/kg L-NAME was injected 15 min before the third injection of ET-1.

Effect of ambient levels of NO on the renal vasoconstrictor response to ET-1. The purpose of these experiments was to determine a possible role of different mean levels of NO in buffering of renal vasoconstriction produced by ET-1. ET-1 was injected while intrarenal levels of NO were varied during NOS inhibition. As a control, 10 and 5 min before administration of ET-1, the response to ANG II was determined. Different levels of NO were induced by infusion of saline, NP (31 μg·kg⁻¹·min⁻¹), or Fen (3.1 μg·kg⁻¹·min⁻¹) into the renal artery at 143 μl/min beginning 5 min before the first injection of ANG II and ending 10 min after the injection of ET-1.

Effect of partial inhibition of NOS on the buffering effect of ambient levels of NO on the renal response to ET-1. These studies determined the buffering influence of more physiological mean levels of NO. NOS was partially inhibited by infusion of a smaller dose of L-NAME into the renal artery (38 μg·kg⁻¹·min⁻¹ for 2 min). ET-1 was injected before and 7–8 min after commencement of L-NAME infusion. After 30 min, NP was infused into the renal artery (16 μg·kg⁻¹·min⁻¹); 20 min later, ET-1 was injected again. For the sake of comparison, ANG II was injected 5 min before each injection of ET-1.

Effects of COX inhibition. Other experiments were conducted to assess a possible buffering role of COX metabolites. Responses to ET-1 were tested before and 25 min after intravenous injection of indomethacin (5 mg/kg in 1 ml/kg of 0.1% Na₂CO₃). After 40 min, ET-1 was injected during infusion of BQ-788 into the renal artery. As a positive control confirming the known buffering effect of COX metabolites on renal vasoconstrictor responses to ANG II, ANG II was injected into the renal artery 5 min before the first and second injections of ET-1, respectively.

Effects of combined inhibition of COX and epoxygenase. To investigate a possible role of other known vasodilator metabolites of arachidonic acid, ET-1 was given before and 25 min after epoxygenase inhibition (miconazole, 4 mg/kg + 4 mg·kg⁻¹·h⁻¹ iv in 1 ml/kg of 45% 2-hydroxypropyl-β-cyclodextrin) and indomethacin (5 mg/kg iv). This dose of miconazole was chosen to be an effective inhibitor on the basis of its estimated plasma concentration of 1–4 μM (33, 38). At 40 min after the second injection of ET-1, ET-1 was tested during intrarenal infusion of BQ-788 (7 n mole/min) and continued intravenous infusion of miconazole. In five of the six animals, additional bolus doses of miconazole (2 mg/kg) and indomethacin (2.5 mg/kg) were given ≥5 min before the last ET-1 injection to ensure complete inhibition. As a control, ANG II was injected 5 min before the first and second injections of ET-1.

Effects of epoxygenase inhibition. To confirm the results obtained with miconazole, other experiments using a similar protocol were conducted using the epoxygenase inhibitor methylsulfonyl-6-(2-propargyloxyphenyl)-hexanamide (MS-PPOH). To avoid a potential interference from possible constrictor metabolites of COX, MS-PPOH was given without indomethacin in four experiments. In two additional experiments, MS-PPOH was given with indomethacin. MS-PPOH was injected intravenously (20 mg/kg in 1 ml/kg saline with 2% DMSO) at the same point in time as the initial bolus of miconazole. This dose of MS-PPOH has been shown to maximally inhibit renal epoxyeicosatetraenoic acid (EET) production in rats without affecting production of 20-hydroxyeicosatetraenoic acid for ≥6 h after bolus application (7).

Drugs and Chemicals

ET-1 and BQ-788 were obtained from American Peptide (Vista, CA). Albumin, L-NAME, NP, Fen, indomethacin, miconazole, and hydroxypropylcyclodextrin were purchased from Sigma (St. Louis, MO). MS-PPOH was synthesized by J. R. Falck.

Data Analysis

The maximum RBF decrease after each injection was determined offline by custom-built software (A. Just) from the 1-Hz data after smoothing by sliding average over five values. The change was expressed as percentage of the baseline value. Baseline RBF and AP were determined from the average of the first 20 s of each recording immediately before injection. To obtain mean time courses, the original 1-Hz recordings (without smoothing) were averaged for each experimental period of all animals in a group. Heart rate was determined from the 100-Hz recording of AP offline. Values are means ± SE. Statistical significance among groups was tested by ANOVA in conjunction with the Holm-Sidak test or Tukey’s test for multiple comparisons (SigmaStat 3.00, SPSS, Chicago, IL). In the case of nonnormal distribution, data were transformed by square root before analysis. Baseline RBF values of the low-dose L-NAME group could not be transformed into normal distribution and were thus analyzed by the Kruskal-Wallis test on ranks.

RESULTS

The averaged time course of the RBF response to injection of ET-1 into the renal artery is shown in Fig. 1. Major effects are evident in maximum responses that are summarized in Figs. 2–6. During control conditions, ET-1 reduced RBF by ~23% at 128 ± 6 s (Figs. 1 and 2A). Recovery of RBF was slow, returning to 97 ± 8% of baseline by 30 min. The maximum constrictor response was reproducible and stable over time; a second injection of ET-1 produced a similar fall in RBF (~27 ± 6%; Fig. 2A, gray bar). ETβ receptor antagonism...
(BQ-788) markedly enhanced the ET-1-induced renal vasoconstriction to −70% (Fig. 2A, solid bar), indicating a strong net vasodilator-like influence of ETB receptors. Such buffering of the overall vasomotor response to ET-1 confirms previous findings from our laboratory (24) and others (5, 10, 15, 27). Similar results were obtained when the responses to ET-1 were analyzed in terms of absolute reduction of RBF integrated over the 10-min period after ET-1 injection displayed in Fig. 1 (“area under the curve,” in ml·min⁻¹·g kidney wt⁻¹·s): 519 ± 71 for control, 662 ± 93 for saline (P > 0.6 vs. control), and 1,161 ± 234 for ETB antagonist (P < 0.05 vs. control). Thus both methods of analysis provide similar results and changes.

Earlier studies of ET receptor actions in the renal vasculature employed NOS inhibition. We extended the protocol to include “clamping” to maintain NO levels constant. This maneuver, however, was ineffective in buffering the renal vasoconstriction produced by ET-1. The ET-1-induced decrease in RBF during l-NAME + NP (from −18 to −49%, P < 0.05; Figs. 1 and 2B) did not differ appreciably from that seen with l-NAME alone (−15 to −30%; Fig. 3A). This finding indicates that constant NO levels are considerably less effective than the buffering effect of ETB receptor-mediated phasic release of endogenous NO evident in the absence of NOS inhibition. Antagonism of ETB receptors further augmented the constrictor action of ET-1 to −92 ± 3% (P < 0.001 vs. control and l-NAME alone; Figs. 1 and 2B), indicating buffering by a mechanism distinct from NO. Similar results were obtained when the vasoconstrictor responses to ET-1 were analyzed as integrated reduction of RBF (in ml·min⁻¹·g kidney wt⁻¹·s): 561 ± 134 for control, 860 ± 92 for l-NAME (P = 0.08 vs. control), and 1,218 ± 88 for ETB antagonist (P < 0.01 vs. control and P < 0.05 vs. l-NAME).

In time-control experiments, saline replaced the ETB receptor antagonist during the third period. The ET-1 constrictor...
To determine whether NO was acting via generalized vasodilatation, the NO-independent dopamine D1 receptor agonist Fen was tested. The combination of NOS inhibition + Fen reduced RBF by 11% ($P > 0.2$) and increased AP by 22% ($P > 0.1$). The increased renal responses to ET-1 (−41 vs. −18%, $P < 0.01$; Fig. 2C) were similar to those during L-NAME + NP (Fig. 2B). The constriction to ET-1 was further augmented by ETB receptor blockade ($P < 0.05$; Fig. 2C). In time-control experiments, the RBF responses to ET-1 were −21% before and −49% and −45% during NOS inhibition + Fen (Fig. 2D), verifying reproducibility over time.

The similarity of the effects with either NP or Fen combined with NOS inhibition suggests that tonic mean levels of NO or cAMP/PKA are of minor importance in modifying ET responsiveness. However, because infused NO may not have reached endogenous levels, additional studies were conducted in which the dilator agents NP and Fen were infused directly into the renal artery. In these studies, baseline RBF was reduced ~60% by NOS inhibition ($P < 0.001$; Fig. 3C) and was increased almost twofold by NP or Fen (Fig. 3C; $P < 0.05$). As noted earlier, the constrictor response to ET-1 was enhanced by L-NAME (−30 ± 4 vs. −15 ± 2%, $P < 0.01$; Fig. 3A) and was unaffected by NP (−29 ± 3%, $P > 0.9$) or Fen (−36 ± 4%, $P > 0.2$). In contrast, the renal vasoconstriction elicited by ANG II during L-NAME (−31 ± 6%, $P < 0.05$) was completely reversed by NP (−18 ± 2%, $P < 0.05$), with a tendency for reversal by Fen (−24 ± 6%, $P > 0.1$; Fig. 3B).

In other studies, we partially inhibited NO production by infusing smaller amounts of L-NAME into the renal artery. By design, intrarenal L-NAME elicited minor changes in RBF (3.9 ml·min⁻¹·g⁻¹, $P > 0.2$; Fig. 4C). Interestingly, the constrictor response to ET-1 was consistently enhanced (−22% vs. −15%, $P < 0.01$; Fig. 4A) whereas a constant level of intrarenal NP restored RBF to the control level immediately before L-NAME (4.4 ml·min⁻¹·g⁻¹, $P > 0.2$; Fig. 4C), it was ineffective in attenuating the constrictor action of ET-1 ($P > 0.6$). In contrast, the enhancement of ANG II-induced constriction during NOS inhibition (−34 vs. −22%, $P < 0.001$) was completely reversed by NP (−15%, $P < 0.001$; Fig. 4B).

To test for possible NO-independent mechanisms, we evaluated the possible involvement of endothelium-derived dilator agents such as prostanoids or EETs on reactivity to ET-1 and ANG II in other animals. Indomethacin inhibition of COX enhanced the response to ANG II (from −29 to −44%, $P < 0.05$; Fig. 5B) but did not affect the response to ET-1 ($P > 0.6$; Fig. 5A). Similar to control experiments, subsequent antagonism of ETB receptors with BQ-788 enhanced the vasoconstriction produced by ET-1 to −56% (Fig. 5A), demonstrating an unabated dilator influence of the ETB receptors.

Similar results were obtained when miconazole inhibited epoxygenase in combination with indomethacin. The renal vasoconstrictor response to ET-1 was unaffected on the average by miconazole (−20 vs. −14%, $P > 0.2$; Fig. 6A) as was the response to antagonism of ETB receptors (−44%, $P < 0.001$; Fig. 6A). The mean effect of ANG II was also unaffected (−32% vs. −25%, $P > 0.2$; Fig. 6B).

Similar to miconazole, inhibition of epoxygenase by MS-PPOH did not affect the renal vasoconstrictor response to ET-1 (−11 vs. −10%, $P > 0.7$; Fig. 6C), nor did it affect the enhancement of the ET-1 response induced by ETB receptor agonists.

<table>
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<tr>
<th>Control</th>
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<td>Nitroprusside</td>
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### Fig. 3. Effect of NO synthase inhibition alone and in combination with the NO donor nitroprusside or the NO-independent vasodilator fenoldopam on renal vasoconstrictor responses to ET-1 and ANG II. A and B: maximum reduction of RBF to response to intrarenal injection of ET-1 (3 pmol) and ANG II (4 nmol), respectively. C: baseline RBF immediately before injection. Injections during control conditions (open bars) were always made first. After L-NAME (25 mg/kg), injections were made during additional infusion of saline vehicle (solid bars), nitroprusside (31 μg·kg⁻¹·min⁻¹, light gray bars), or fenoldopam (3.1 μg·kg⁻¹·min⁻¹, gray bars) into the renal artery in random order. Values are means ± SE; n = 8. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control. &P < 0.05; &&P < 0.01; &&&P < 0.001 vs. L-NAME + saline.
antagonism (35%, $P < 0.001$ vs. control and MS-PPOH; Fig. 6C). In these experiments, MS-PPOH was administered without indomethacin to minimize potential interactions between COX and epoxygenase pathways. However, the same results were obtained when MS-PPOH was given in combination with indomethacin in two additional experiments: −15.6% during control, −18.3% with MS-PPOH + indomethacin, and −52.5% with additional ETB antagonist.

Baseline hemodynamic values obtained before the first injection of ET-1 and baseline RBF before subsequent injections are given in Table 1. Initial baseline values did not differ among groups. Average hematocrit of all animals (42 ± 2%) body weight (264 ± 0.1 g), and weight of the left kidney (1.3 ± 0.1 g) were similar in the groups.

**DISCUSSION**

ETB receptors exert a strong net vasodilator-like influence on ET-1-induced renal vasoconstriction. Inhibition of renal ETB receptors markedly enhances the renal vasoconstriction produced by local administration of ET-1 (Fig. 2A), as we and others showed previously (5, 10, 15, 24, 27). Part of the buffering provided by ETB receptors is mediated by the vasodilator NO. Inhibition of NO production magnifies the renal vasoconstrictor response to intrarenal ET-1 (Figs. 1, 2, B–E, 3A, and 4A), an observation in accord with the literature in which systemic administration of ET was utilized (10, 15, 29, 30). We extend our knowledge of NO participation by demonstrating that phasic release of NO is more effective than constant ambient levels associated with constitutive release.

The present study provides convincing new information about the involvement of an NO-independent mechanism that accounts for approximately one-half of the ETB receptor buffering of the in vivo renal vasoconstrictor response to ET-1. The NO-independent second major mechanism is poorly characterized, although we show that it is independent of NOS activity by definition and that it does not appear to involve local production of typical endothelium-derived vasodilator agents such as prostaglandins or EETs produced by COX or epoxygenase metabolism. In this context, the second buffering mechanism might involve clearance of ET-1 by ETB receptors, acting to reduce local availability of ET-1 for ET constrictor receptors on smooth muscle cells. However, this mechanism was not investigated directly in the present protocols.

During inhibition of NO production, ETB receptors continue to offset significant renal vasoconstriction produced by ET-1 (Figs. 1 and 2, B and D). Marked enhancement of ET-1-induced vasoconstriction was observed consistently after ETB antagonism (35%, $P < 0.001$ vs. control and MS-PPOH; Fig. 6C). In these experiments, MS-PPOH was administered without indomethacin to minimize potential interactions between COX and epoxygenase pathways. However, the same results were obtained when MS-PPOH was given in combination with indomethacin in two additional experiments: −15.6% during control, −18.3% with MS-PPOH + indomethacin, and −52.5% with additional ETB antagonist.

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During inhibition of NO production, ETB receptors continue to offset significant renal vasoconstriction produced by ET-1 (Figs. 1 and 2, B and D). Marked enhancement of ET-1-induced vasoconstriction was observed consistently after ETB antagonism (35%, $P < 0.001$ vs. control and MS-PPOH; Fig. 6C). In these experiments, MS-PPOH was administered without indomethacin to minimize potential interactions between COX and epoxygenase pathways. However, the same results were obtained when MS-PPOH was given in combination with indomethacin in two additional experiments: −15.6% during control, −18.3% with MS-PPOH + indomethacin, and −52.5% with additional ETB antagonist.

Baseline hemodynamic values obtained before the first injection of ET-1 and baseline RBF before subsequent injections are given in Table 1. Initial baseline values did not differ among groups. Average hematocrit of all animals (42 ± 2%), body weight (264 ± 0.1 g), and weight of the left kidney (1.3 ± 0.1 g) were similar in the groups.
receptor antagonism during l-NAME treatment. This was the case whether receptor blockade was applied alone (Fig. 3A) or in combination with NP (Fig. 2B) or Fen (Fig. 2D). By design, NOS inhibition was completely inhibited, inasmuch as the dose of l-NAME (25 mg/kg) was two to three times that (~10 mg/kg) required to produce maximum increases in AP and renal vascular resistance (2, 9, 28, 46; unpublished observations). The assertion that maximum inhibition of NO production was reached is documented by our observation that elevation of l-NAME to approximately five-times-higher levels did not exert an additional effect on the responses to ET-1.

It is important to note that the present results are at variance with previous in vitro studies in which a similar protocol combining NOS and ETB receptor inhibition resulted in predominant NO mediation without involvement of a second mechanism (10, 45). The reasons for the discrepancy among results may be multiple. A major difference is in the experimental preparations. Both previous in vitro studies utilized isolated organs perfused with cell-free solutions devoid of hemoglobin, so the reduced NO-scavenging capacity in these preparations may have artificially magnified local NO gradients and effects. Another consideration is that the constant-flow perfusion system used in the previous isolated kidney study (10) may have augmented shear stress-induced NO release during constrictor responses and, thereby, exaggerated the apparent influence of NO. In addition, it should be recognized that if NO were the only mediator of the buffering effect of ETB receptors and if smooth muscle ETB receptors contribute to the constrictor effect of ET-1, then the inhibition of ETB receptors in the absence of endogenous NO should reduce the

Table 1. Baseline hemodynamic data

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>AP, mmHg</th>
<th>HR, beats/min</th>
<th>RBF, ml/min ( \cdot ) g(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Control</td>
<td>4</td>
<td>98±3</td>
<td>373±27</td>
<td>4.7±0.7 ( \text{Period 1} )</td>
</tr>
<tr>
<td>2) l-NAME + nitroprusside</td>
<td>4</td>
<td>96±2</td>
<td>351±5</td>
<td>5.8±0.5 ( \text{Period 2} )</td>
</tr>
<tr>
<td>3) l-NAME + nitroprusside (time control)</td>
<td>3</td>
<td>104±10</td>
<td>325±31</td>
<td>5.6±1.2 ( \text{Period 1} )</td>
</tr>
<tr>
<td>4) l-NAME + fenoldopam (time control)</td>
<td>4</td>
<td>94±2</td>
<td>327±11</td>
<td>4.4±0.3 ( \text{Period 3} )</td>
</tr>
<tr>
<td>5) l-NAME + fenoldopam (dose testing)</td>
<td>5</td>
<td>112±4</td>
<td>331±23</td>
<td>4.6±0.3 ( \text{Period 2} )</td>
</tr>
<tr>
<td>6) l-NAME + nitroprusside/fenoldopam</td>
<td>8</td>
<td>101±4</td>
<td>376±18</td>
<td>4.9±0.4 ( \text{Period 3} )</td>
</tr>
<tr>
<td>7) l-NAME + saline/nitroprusside/fenoldopam</td>
<td>6</td>
<td>103±3</td>
<td>307±6</td>
<td>4.7±0.5 ( \text{Period 2} )</td>
</tr>
<tr>
<td>8) l-NAME (low dose) + nitroprusside</td>
<td>5</td>
<td>102±3</td>
<td>351±26</td>
<td>4.9±0.5 ( \text{Period 3} )</td>
</tr>
<tr>
<td>9) Indomethacin</td>
<td>5</td>
<td>108±5</td>
<td>318±21</td>
<td>5.3±0.4 ( \text{Period 3} )</td>
</tr>
<tr>
<td>10) MS-PPOH + miconazole</td>
<td>6</td>
<td>103±3</td>
<td>348±5</td>
<td>5.1±0.5 ( \text{Period 2} )</td>
</tr>
</tbody>
</table>

Values are means ± SE. AP, arterial pressure; HR, heart rate; RBF, renal blood flow; periods 1, 2, and 3, baseline before 1st, 2nd, and 3rd injections of endothelin-1 (ET-1). All except groups 6 and 7 were treated with 3 consecutive injections of ET-1 during control conditions (period 1), intravenous application of the specified agents (period 2), and additional infusion of BQ-788 into the renal artery (period 3). In time-control and dose-response experiments (groups 3, 5, and 6), saline, instead of BQ-788, was infused in period 3. In group 7, ET-1 was injected once before and 3 times after N⁶-nitro-l-arginine methyl ester (l-NAME); injections after l-NAME in group 1 during infusion of saline, nitroprusside, or fenoldopam in random order. In group 7, ET-1 was injected during control conditions, l-NAME, or additional infusion of nitroprusside. All data were obtained immediately before injection of respective bolus of ET-1, i.e., in the presence of all agents listed above for the respective group and injection period but before ET-1. MS-PPOH, methylsulfonyl-6-(2-propargyloxophenyl)-hexanamide. *P < 0.01 vs. period 1; †P < 0.01 vs. period 2.
constrictor response to ET-1. However, this was observed in none of the previous studies. Wang et al. (45) reported a small additional effect of ET<sub>B</sub> receptor antagonism during NOS inhibition, which, however, did not reach statistical significance. An in vivo study concluded that NO and/or COX metabolites are the only mediators of ET<sub>B</sub>-induced buffering in the renal circulation (32). However, this conclusion was based on comparison of the direct vasoconstrictor effects produced by NOS inhibition vs. ET<sub>B</sub> antagonism, rather than responses to administered ET-1. Because the constrictor effect of NOS inhibition is mainly due to the elimination of NO, the contribution of endogenous ET-1 and, thus, the effect of ET<sub>B</sub> receptor inhibition may have been too small to be detected.

We provide new evidence for the renal circulation that this second, NO-independent mechanism is distinct from dilator-like agents such as prostanoiids or EETs. Neither indomethacin nor miconazole combined with indomethacin nor MS-PPOH alone or with indomethacin augments ET-1-induced vasoconstriction or prevents the enhancement due to ET<sub>B</sub> receptor antagonism (Figs. 5 and 6). In contrast, renal vasoconstrictor responses to ANG II were effectively buffered by vasodilator COX metabolites, confirming our previous studies (8) and demonstrating effective inhibition of COX in the present study. Because of possible interactions between COX and epoxygenase pathways, only epoxygenase was inhibited in other experiments using MS-PPOH, which is believed to exert less severe unspecific side effects on other heme-containing enzymes, intracellular calcium signaling, and ion channel activities than miconazole (41). In other experiments, MS-PPOH was combined with indomethacin, and similar negative results were obtained. Taken together, our results do not indicate involvement of endogenous NO in the buffering effect of ET<sub>B</sub> receptors in the renal circulation. To our knowledge, no other endothelium-derived vasodilator agent has been implicated as a mediator of the buffering effect of ET<sub>B</sub> receptors in the renal circulation. Potential candidates of known endothelium-derived vasodilator agents not typically seen in connection with ET<sub>B</sub> receptor stimulation include endothelium-derived hyperpolarizing factor, bradykinin, and calcitonin gene-related peptide (34). However, at least in renal microvessels, the majority of endothelium-derived hyperpolarizing factor-like activity seems to be mediated through EETs (4), thus making it an unlikely agent to explain our results with miconazole and MS-PPOH. Bradykinin and calcitonin gene-related peptide seem to be excluded, inasmuch as their action depends largely on NO production, and there is no evidence for release of these agents stimulated by ET<sub>B</sub> receptors (34). By default, it is reasonable to propose that the most likely explanation for the NO-independent buffering effect of ET<sub>B</sub> receptors in the renal microcirculation is clearance of ET.

NOS inhibition increases the renal vasoconstriction produced by ET-1 almost twofold, inasmuch as the decreases in RBF increased from 15–20% to 30–40% (Figs. 2, B–E, and 3A). Additional ET<sub>B</sub> receptor antagonism augmented the constrictor response to ET-1 during NOS inhibition, amounting to ~50% of the overall effect of combined blockade (Figs. 2, B and D). Thus it is reasonable to conclude that each of these distinct mechanisms, NO-dependent and NO-independent, contribute ~50% of the total buffering action of renal ET<sub>B</sub> receptors in vivo. In this regard, our results for the renal circulation highlight the contribution of a previously unrecognized NO-independent mechanism that plays an important role in the anticonstrictor action of ET<sub>B</sub> receptors.

To our knowledge, the “rescue” protocol utilizing NP infusion to replace the reduction in NO production during NOS inhibition has not been employed previously to evaluate the contribution of NO to ET<sub>B</sub>-mediated buffering in the renal or other vascular beds. Our results indicate that production of endogenous NO in response to bolus injection of ET-1 is effective, whereas administration of NP to cause a steady-state change in NO is not. Several sets of studies tested the influence of administered NO donor on restoration of RBF and on renal vascular reactivity to ET-1 during NOS inhibition. In our initial experiments, NP followed by l-NAME was given intravenously to fix NO levels at a stable level capable of countering roughly one-half of the RBF decrease in response to ET-1, thereby restoring RBF to ~75% of control. Under these conditions, ET produced twofold greater renal vasoconstriction than before NOS inhibition (Fig. 2), a finding consistent with removing a buffering effect of endogenous NO and little influence of exogenous NO. This was remarkable with regard to the buffering effect of NO on the renal vascular responsiveness to ANG II, which is thought to be predominantly due to static levels of NO, as shown by our (8) and other groups (21, 37). We therefore investigated the impact of various static levels of NO on the responsiveness to ET-1 and ANG II more directly. Roughly 10-fold higher renal NO levels were achieved by infusion of the same amount of NP into the left renal artery, and lower levels of NO were achieved by infusion of l-NAME alone. Nevertheless, there were no additional effects on restoration of RBF during NOS inhibition or on renal vasoconstriction produced by ET-1 (Fig. 3). In a third set of studies, a low dose of l-NAME was given intrarenally to partially attenuate NO production, as evidenced by minimal reductions in basal RBF. In this setting of relatively normal levels of synthesis, renal reactivity to ET-1 was again enhanced (Fig. 4), although the degree of constriction was less than that seen during complete inhibition of NOS (Figs. 2 and 3). Subsequent infusion of NP restored RBF to the control level immediately before l-NAME, indicating attainment of near-physiological levels of NO. Responsiveness to ET-1, however, was unaffected. Taken together, our data indicate that a range of stable mean levels of exogenous NO was ineffective in buffering renal vasoconstriction elicited by ET-1.

Our data advance the concept that phasic release of NO secondary to transient stimulation of ET<sub>B</sub> receptors plays a major role in buffering the ET-1 response, in contrast to the relatively weak influence of stable static levels of exogenous NO. Several explanations may account for the greater efficiency of phasic bursts of native NO. One is that the amount of local transient release of NO upon receptor activation is considerably greater than ambient levels due to constitutive production. Another possibility is that endogenous release of NO from endothelial cells favors a gradient of NO in the vessel wall, with the highest concentration presented to the innermost smooth muscle cells, a gradient magnified by surge release of NO. In addition, model calculations predict that bursts of NO more efficiently activate its target guanylyl cyclase because of differences between activation and deactivation kinetics (43).

We observed that the dopamine D<sub>1</sub> receptor agonist Fen, a vasodilator acting independently of NO (36), was ineffective in...
buffering ET-1-induced renal vasoconstriction when administered at a vasodilator dose that compensated for the reduced RBF associated with 1-NAME (Fig. 2C). On the other hand, continuous stimulation of cGMP/PKG signaling by Fen effectively buffered the renal reactivity to ANG II (Fig. 3), as we previously reported (8). The similarity of responses to ET-1 during infusion of the vasodilators NP and Fen, both of which reversed some of the vasoconstriction associated with NOS inhibition, indicates that the renal vascular constrictor tone per se is not a major determinant of renal vascular reactivity to ET-1.

An interesting and novel finding is that constant ambient levels of NO and vasodilator COX metabolites effectively buffered renal vasoconstrictor responses to ANG II but were ineffective against the renal vasoconstriction produced by ET-1. Such differential buffering efficiency is somewhat surprising with regard to the presumed common phospholipase C and cytosolic calcium signaling pathways associated with AT1 and ET1/ETB receptors, typical G protein-coupled receptors (11, 35). The varying effectiveness of signal modulation of target responsiveness indicates subtle but important differences in second-messenger pathways of the two peptides that are worthy of further investigation.

In conclusion, our results provide new information that advances our understanding of ET receptors and their mechanisms of action on the renal microcirculation in vivo. We present evidence that the vasodilator-like buffering action of ETB receptors is due to stimulated phasic release of NO and an ill-defined distinct NO-independent mechanism. The latter may represent clearance of ET, which reduces its availability to constrictor receptors. We found no evidence to implicate vasodilator metabolites of COX or epoxygenase metabolism, either alone or in combination.

Perspectives

The prominent buffering effects mediated by ETB receptors are important in attenuating ET-1-induced vasoconstriction in the renal and other circulatory beds. The relative importance of the two mechanisms, phasic release of NO and presumably clearance of ET, may contribute to differences of ET-1 responsiveness among vascular beds. For example, the contribution of NO may be greater in skeletal muscle circulation than in those beds that exhibit weak or nonexistent initial transient increases in blood flow in response to ET-1 (17, 40). Deterioration of ETB receptor-mediated buffering may be critical in the genesis and/or progression of vascular and renal disease. Abnormal ET function is likely in hypertension, congestive heart failure, chronic renal failure, atherosclerotic and other vasculopathies, pulmonary hypertension, and aging (1, 6). More specifically, exaggerated vascular reactivity to ET-1 has been described in diseases with endothelial dysfunction such as atherosclerosis (6, 26), diabetes mellitus (25), and genetic hypertension (16). A similar defect in the balance between vasoconstrictors and dilators has been noted by us (8) and others (23) in the young spontaneously hypertensive rat, causing a hyperresponsiveness of the renal circulation to ANG II in these animals.

REFERENCES