Nitric oxide and NO-independent mechanisms mediate ETB receptor buffering of ET-1-induced renal vasoconstriction in the rat

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Running head: Renal ETB receptor buffering by NO and non-NO mechanisms

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Abstract

Vascular ETB receptors exert both dilator and constrictor actions in a complex interaction with ETA receptors. The aim of this study was to clarify the presence and relative importance of nitric oxide and other possible mechanisms underlying the dilator effects of ETB receptors in the rat kidney. Complete inhibition of NO production (L-NAME, 25 mg/kg, iv) enhanced the renal vasoconstriction elicited by endothelin-1 (ET-1) injected into the renal artery from -15 to -30%. Counteraction of the L-NAME-induced vasoconstriction by infusion of the NO-donor nitroprusside (NP) into the renal artery did not reverse this effect (+NP=-29%), but nevertheless effectively buffered Ang II-mediated renal vasoconstriction. Similarly, renal vasoconstrictor responses to ET-1 were enhanced after a smaller dose of L-NAME administered into the renal artery (-22 vs. -15%) and unaffected by subsequent infusion of a vasodilator dose of NP (-21%). These results indicate that the responsiveness to ET-1 is buffered by endothelial ETB receptor stimulated phasic release of NO rather than the static mean ambient NO level. In other experiments, intrarenal infusion of ETB-receptor antagonist BQ788 further enhanced the constrictor response to ET-1 seen during NP + L-NAME (-92 vs. -49%), revealing a NO-independent dilator component. In controls, the vasoconstriction to ET-1 was unaffected by vehicle (-27 vs. -20%) and markedly enhanced during ETB receptor antagonism (-70%). The same pattern of ET-1 responses was observed when indomethacin was given to inhibit cyclooxygenase (control=-20%, indo=-22%, +ETB-antagonist=-56%) or MS-PPOH or Miconazole+indomethacin to inhibit epoxygenase alone (control=-10%, MSPPOH=-11%, +ETB-antag.=-35%) or in combination (control=-14%, indo+mico=-20%, +ETB-antag.=-43%). We conclude that phasic release of endogenous NO, but not the static ambient level, mediates part of the dilator effect of ETB receptors. In addition, playing a major buffering role is a NO-independent mechanism, perhaps reflecting clearance of ET by ETB receptors, that is distinct from prostanoids and epoxyeicosatrienoic acids.

Keywords: renal hemodynamics, endothelial cell, cyclooxygenase, epoxygenase, angiotensin II, dopamine
**Introduction**

Endothelin-1 (ET-1) plays an important role in cardiovascular regulation, renal hemodynamics, as well as 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 ETA and ETB receptors on smooth muscle cells counteracted by the dilator influence of ETB receptors on endothelial cells. The preglomerular resistance vessels have roughly an equal density of ETA and ETB receptors (12; 31). Recently, we and others observed in the renal microcirculation complex interactions between receptor subtypes with the influence of ETB receptors varying between net constrictor or dilator depending on whether or not ETA receptors are stimulated simultaneously (24; 27; 32). Selective ETB receptor stimulation produces renal vasoconstriction. On the other hand, the dilator action of endothelial ETB receptors modulates the magnitude of the overall constrictor effect of ETA + ETB receptors on smooth muscle cells. A strong dilator influence is indicated by marked enhancement of the ET-1-induced vasoconstriction during ETB receptor antagonism (5; 10; 15; 24; 27).

Evidence indicates that nitric oxide (NO) is released upon ETB 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, prostanoids (13) and epoxygenase metabolites (22) may contribute to the dilator effect of endothelial ETB receptors.

Another potential mechanism by which ETB receptors counteract ET-mediated constriction is by sequestration of ET and subsequent reduction of the amount available to activate ETA 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 ETB receptors as well as the reduction of plasma clearance in knockout mice missing one copy of the ETB receptor gene (3). However, plasma clearance is predominantly provided by the lungs (14), whereas the kidneys contribute about 10-25% (14; 42). In addition, almost 50% of renal ET clearance seems to be independent of ET
receptors (14; 47). It is thus unclear whether and to what extent ET clearance may contribute to the buffering effect of ETB receptors in the kidney.

To our knowledge, only one previous in vivo study has combined ETB 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 ETB receptor blockade had no additional effect after NOS-inhibition. These findings suggest that the buffering action of ETB 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 that 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 both 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 to 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 weeks 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. The animals were fed a standard lab chow with free access to tap water and kept on a 12 h: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 (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 to the left renal artery and used for infusion into the renal artery. An ultrasound transit-time flowprobe (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. Isoncotic 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), followed by a maintenance rate of 10 µl/min. The renal artery catheter was perfused with normal saline at 5 µl/min. Additional doses of pentobarbital were given iv 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 starting the experiments.

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 (T 420, Transonic, low-pass filter 40 Hz) connected to the flowprobe. 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 A/D converter + 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 endothelin-1 (ET-1, 10 µl x 0.3 µM) into the renal artery was measured throughout an experiment. 5 min before 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 infusion line through a microinjector valve (Cheminert, Valco Instruments, Houston, TX) and a new recording was started. The initial 20 s served as the baseline values of AP and RBF. 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 angiotensin II (Ang II, 10 µl x 0.4 µM) was recorded for 5 min. The infusion rate was raised to 143 µl/min 30 s before and returned to 5 µl/min 5 min after injection. At least 5 min were allowed for recovery after an injection of Ang II. For schematic diagrams of drugs administered in the experimental groups, see Figs. 2 - 6.

**Experimental groups:**

Renal vasoconstrictor response to ET-1 before and during ETB receptor inhibition: 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 iv infusion of saline infusion (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 BQ788 (7 nmol/min in 143 µl/min saline) was infused between 5 min before and 10 min after the third injection of ET-1. This dose of BQ788 is known to exert maximal inhibitory effects in this experimental setup (24).

Effects of clamping of 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, NO-synthase (NOS) was inhibited by Nω-nitro-L-arginine methyl ester (L-
NAME, 25 mg/kg bolus iv in 1 ml/kg saline) 35 min after the first injection of ET-1. This dose of L-
NAME was chosen based on 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-NAME and to maintain constant ambient levels of NO, the NO-donor NP
(1 mg/kg/ml) was infused iv. Temporary reductions of NO levels and hypertensive responses were
avoided by starting NP 3-5 min preceding administration of L-NAME. Based on preliminary
experiments, the infusion rate of NP was initially set to 42 µl/min (42 µg/kg/min) and then reduced so
that AP after L-NAME was 90-100 mmHg. This infusion rate (24±8 µl/min, 24 µg/kg/min) was
maintained for the remainder of an experiment. 20 min after L-NAME, ET-1 was injected alone and
30 min later during intrarenal infusion of BQ788 (7 nmol/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-NAME identical to the experiments
described above for NP, except that 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 least 30 min after the first injection
of ET-1, FEN (0.08 mg/kg/ml) was infused at 32-42 µl/min (2.5-3.3 µg/kg/min, iv). 3-5 min later, L-
NAME was injected (25 mg/kg iv).

**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 that saline was infused into the renal artery instead of BQ788 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 as for the time-control
experiments, except that 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. To this end, 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 either saline, NP (31 \( \mu g/kg/min \)), or FEN (3.1 \( \mu g/kg/min \)) into the renal artery at 143 \( \mu l/min \) beginning 5 min before the first injection of Ang II and ending 10 min after the injection of ET-1.

The influence 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 \( \mu g/kg/min \) for 2 min). ET-1 was injected before and 7-8 min after commencement of L-NAME infusion. 30 min later, NP was infused into the renal artery (16 \( \mu g/kg/min \)); another 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 injection of indomethacin (5 mg/kg in 1 ml/kg 0.1% Na\(_2\)CO\(_3\), iv). 40 min later ET-1 was injected during infusion of BQ788 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 injection 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/h iv in 1 ml/kg 45% 2-hydroxypropyl-\( \beta \)-cyclodextrin (HBCD)) and indomethacin (5 mg/kg iv). This dose of miconazole was chosen to be an effective inhibitor based on its estimated plasma concentration of 1-4 \( \mu M \) (33; 38). 40 min after the second injection of ET-1, ET-1 was tested during intrarenal infusion of BQ788 (7 nmol/min) and
continued iv 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 at least 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 injection of ET-1, respectively.

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 together with indomethacin. MS-PPOH was injected iv (20 mg/kg in 1 ml/kg saline with 2 % DMSO) at the same time point as described for the initial bolus of miconazole. This dose of MS-PPOH has been shown to maximally inhibit renal EET production in rats without affecting production of 20-HETE for a duration of at least 6 hours after bolus application (7).

Drugs and Chemicals: ET-1 and BQ-788 were obtained from American Peptide Comp. (Vista, CA). Albumin, L-NAME, NP, FEN, indomethacin, miconazole, and hydroxypropyl-cyclodextrin were from Sigma (St Louis, MO). MS-PPOH was synthesized by JRF.

Data analysis: The maximum RBF decrease following each injection was determined off-line by custom-built software (AJ) from the 1 Hz data after smoothing by sliding average over 5 values. The change was expressed as percent 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 off-line. Data are expressed as mean ± SE. Statistical significance among groups was tested by ANOVA in conjunction with Holm-Sidak or Tukey test for multiple comparisons (SigmaStat 3.00, SPSS, Chicago, IL). In case of non-normal 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 Kruskal-Wallis test on ranks. \( p < 0.05 \) was considered statistically significant.

**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 subsequent figures. 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, hatched bar). ETB receptor antagonism (BQ788) 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 lab (24) and others (5; 10; 15; 27). Similar results were obtained when the responses to ET-1 are 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”): Control 519±71, saline 662±93 (\( p > 0.6 \) vs. control), ETB-antagonist 1161±234 ((ml/min)/(gKW)*min) (\( p < 0.05 \) vs. control). Thus, both methods of analysis provide similar results and changes.

Earlier studies of ET receptor actions in the renal vasculature have 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 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 (control
561±134, L-NAME 860±92 (P=0.08 vs. control), ETB-antagonist 1218±88 ((ml/min)/gKW)*min (p<0.01 vs. control, p<0.05 vs. L-NAME).

In time-control experiments, saline replaced the ETB-receptor antagonist during the third period. The ET-1 constrictor responses during the two saline periods were virtually identical (-56 and –55%, Fig. 2C), indicating reproducibility over time and that the effect of ETB receptor antagonism (Fig. 2B) cannot be ascribed to time effects or a progressive decrease of baseline RBF with repeated injections of ET-1.

Additional experiments were conducted to establish that near maximal inhibition of NO-production was achieved by 25 mg/kg L-NAME. Before the third injection of ET-1, a 4-times larger dose of L-NAME was administered. In these experiments, the renal vasoconstrictor response to ET-1 became two-fold greater following the standard dose of L-NAME (25 mg/kg) from -21±5 to -42±10 %, where it remained (-40±9 %) after the higher L-NAME dose. Thus, L-NAME was sufficient to achieve complete inhibition of NO-production in our studies.

To determine whether NO was acting via generalized vasodilation, 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 suggest 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 about 60 % by NOS inhibition (p<0.001, Fig. 3C), and was increased almost two-fold by either 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 either 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 infusion of smaller amounts of L-NAME into the renal artery. By design, intrarenal L-NAME elicited minor changes in RBF (3.9 vs. 4.4 (ml/min)/g, 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)/g, 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 endothelial-derived dilator agents such as prostanoids or epoxyeicosatrienoic acids (EET's) on reactivity to ET-1 and Ang II in other animals. Indomethacin inhibition of cyclooxygenase (COX) enhanced the response to Ang II (-29 to -44 %, p<0.05, Fig. 5B), but it did not affect that 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 epoxyxygenase 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 epoxyxygenase 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 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 (control -15.6 %, MS-PPOH+indomethacin -18.3 %, +ETB-antagonist -52.5 %).

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\( \pm \)2 %), body weight (264\( \pm \)8 g), and weight of the left kidney (1.3\( \pm \)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 have shown 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, 2B - E, 3A, and 4A), an observation in accord with the literature that utilized systemic administration of ET (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 a 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 endothelial-derived vasodilator agents such as prostaglandins or EET’s produced by COX or epoxygenase metabolism. In this context, the second buffering mechanism might involve clearance of ET-1 by endothelial 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 vasoconstrictor produced by ET-1 (Figs. 1, 2B and 2D). Marked enhancement of ET-1-induced vasoconstriction was observed consistently following ETB receptor antagonism during L-NAME treatment. This was the case whether receptor blockade was applied alone (Fig. 3A) or in combination with either NP (Fig. 2B) or FEN (Fig. 2D). By design, NOS inhibition was completely inhibited as the employed dose of L-NAME (25 mg/kg) was two- to three-times that (~ 10 mg/kg) required to produce maximum increases in arterial pressure and renal vascular resistance (2; 9; 28; 46) (unpublished obs.). The assertion that maximum inhibition of NO-production was reached is documented by our observation that elevation of L-NAME levels to approximately five-times higher levels did not exert any 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 using a similar protocol combining NOS and ETB-receptor inhibition which had reported 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 that 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 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 ETB-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 ETB-induced
buffering in the renal circulation (32). However, this conclusion was based on comparison of the direct vasoconstrictor effects produced by NOS-inhibition vs. ETB-antagonism rather than responses to administered ET-1. Since 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 ETB-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 prostanoids 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 ETB 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 that is believed to exert less unspecific affection of 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 prostanoids or EET’s, either alone or in combination, in the buffering effect of ETB-receptors in the renal circulation,. To our knowledge, no other endothelial-derived vasodilator agent has been implicated as a mediator of the buffering effect of endothelial ETB receptors in the renal circulation. Potential candidates of known endothelial-derived vasodilator agents not typically seen in connection with ETB receptor stimulation include endothelial-derived hyperpolarizing factor (EDHF), bradykinin and CGRP (34). However, at least in renal microvessels, the majority of EDHF-like activity seems to be mediated through EET’s (4), thus making it an unlikely agent to explain our results using miconazole and MS-PPOH. Bradykinin and CGRP seem to be excluded as their action depends largely on NO production and there is no evidence for release stimulated by ETB receptors (34). By default, it is reasonable to propose that the most likely explanation for the NO-independent buffering
effect of ETB receptors in the renal microcirculation is clearance of ET.

NOS inhibition increases the renal vasoconstriction produced by ET-1 almost two-fold as the decreases in RBF increased from -15-20 to -30-40% (Figs. 2B - E, and 3A). Additional ETB receptor antagonism augmented the constrictor response to ET-1 during NOS-inhibition, amounting to approximately 50% of the overall effect of combined blockade (Figs. 2B and 2D). Thus, it is reasonable to conclude that each of these distinct mechanisms, the NO-dependent and the NO-independent, contribute approximately 50% of the total buffering action of renal endothelial ETB 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 anti-constrictor action of ETB 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 ETB-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 were given iv to fix NO levels at a stable level capable of counteracting roughly one-half of the RBF decrease to L-NAME and thereby restoring RBF to about 75% of control. Under these conditions, ET produced two-fold 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 ten-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 by 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 NO 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 & 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 ETB 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 D1 receptor agonist FEN, a vasodilator acting independent 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 L-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 have 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, indicate 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 ETA/ETB receptors, typical G-protein coupled receptors (11; 35). Although not clear, the varying effectiveness of signal modulation of target responsiveness indicates subtle but important differences in second messenger pathways of the two peptides, which 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 endothelial 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 that 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 to nonexistent initial transient increases in blood flow 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 hyper-responsiveness of the renal circulation to Ang II in these animals.

**Acknowledgements**

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References


10. **D'Orleans-Juste P, Claing A, Telemaque S, Maurice MC, Yano M and Gratton JP.** Block of endothelin-1-induced release of thromboxane A2 from the guinea pig lung and nitric oxide from...


Figure Legends

Figure 1. Time courses of renal blood flow (RBF) responses to ET-1 during inhibition of NO production and ETB receptors.

ET-1 (3 pmol) was injected into the renal artery during control conditions (ET-1 control, small circles), during inhibition of NO-synthase and infusion of NO-donor nitroprusside (ET-1 + L-NAME + nitroprusside, large open circles) and during ETB receptor antagonism (ET-1 + LNAME + nitroprusside + ETB-inhibition (BQ788), large filled circles) in anesthetized rats. Mean ± SE, n=4. In addition, the response to ET-1 in the presence of ETB receptor antagonism without L-NAME from a different group is shown by the broken line (n=4); SE is omitted in this group for clarity.

Figure 2. Effect of inhibition of NO production and additional ETB receptor antagonism on the renal vasoconstrictor response to ET-1.

Maximum reduction of renal blood flow (RBF) produced by intrarenal injection of ET-1 (3 pmol). A: Response to ET-1 during control conditions (open bar), during intravenous infusion of saline vehicle (hatched bar), and during infusion of ETB-antagonist BQ-788 (7 nmol/min) into the renal artery (solid bar, n=4). B: RBF response to ET-1 during control (open bar), during inhibition of NO production by L-NAME (25 mg/kg) combined with iv infusion of NO-donor nitroprusside (80 nmol/kg/min, hatched bar), and during additional intrarenal infusion of BQ-788 (solid bar, n=4). C: Response to ET-1 in separate control experiments identical to those in Fig. 2B except that saline replaced BQ-788 (n=3). D: Response to ET-1 during control (open bar), during L-NAME combined with iv. infusion of the vasodilator fenoldopam, a dopamine D1 agonist (8.7 nmol/kg/min, hatched bar), and during additional intrarenal infusion of BQ-788 (solid bar, n=4). E: RBF response to ET-1 in separate control experiments (n=4) identical to those in Fig. 2C except that saline replaced BQ-788 (n=4). Mean ± SE. *: p<0.05; **: p<0.01; ***: p<0.001 vs. respective control. ¤: p<0.05, ¤¤: p<0.01 vs. period of ETB inhibition.
Figure 3: Effect of NO-synthase inhibition alone and in combination with NO-donor nitroprusside or NO-independent vasodilator fenoldopam on the renal vasoconstrictor responses to ET-1 and angiotensin II.

Maximum reduction of renal blood flow (RBF) in response to intrarenal injection of A: ET-1 (3 pmol) or B: Angiotensin II (Ang II, 4 nmol). 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/min, hatched bars) or fenoldopam (3.1 µg/kg/min, striped bars) into the renal artery in random order. Mean ± 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.

Figure 4: Effect of partial NO-synthase inhibition alone and in combination with nitroprusside on the renal vasoconstrictor responses to ET-1 and Ang II.

Maximum reduction of renal blood flow (RBF) to intrarenal injection of A: ET-1 (3 pmol) or B: Ang II (4 nmol). C: baseline RBF immediately before injection. Injections were made during control conditions (open bars), after L-NAME (38 µg/kg/min for 2 min, solid bars), and during subsequent infusion of nitroprusside (16 µg/kg/min, hatched bars). The narrow bar in C denotes baseline RBF immediately before L-NAME. Mean ± SE, n=6. *: p<0.05; **: p<0.01; ***: p<0.001 vs. control; &&&: p<0.001 vs. L-NAME; +++: p<0.001 vs. immediately before L-NAME (narrow bar) by paired t-test; ##: p<0.01 vs. L-NAME by paired t-test.
Figure 5. Effect of inhibition of cyclooxygenase and ETB receptors on the renal vasoconstrictor responses to ET-1 and Ang II.

Maximum reduction of RBF elicited by intrarenal injection of A: ET-1 (3 pmol, n=5) or B: Ang II (4 µmol, n=4). Injections were made during control conditions (open bars) and during inhibition of cyclooxygenase by indomethacin (5 mg/kg iv, hatched bars). The response to ET-1 was also investigated during subsequent intrarenal infusion of ETB-antagonist BQ-788 (7 nmol/min, solid bar). Mean ±SE. *: p<0.05; ***: p<0.001 vs. respective control.

Figure 6. Effect of inhibition of epoxygenase and ETB receptor blockade on the renal vasoconstrictor responses to ET-1 and angiotensin II with and without COX-inhibition.

Maximum reduction of RBF produced by intrarenal injection of A and C: ET-1 (3 pmol) or B and D: Angiotensin II (Ang II, 4 µmol). Panels A and B: Injections were made during control conditions (open bars) and during inhibition of epoxygenase (miconazole, 4 mg/kg + 4 mg/kg/h iv, hatched bars) combined with inhibition of cyclooxygenase by indomethacin (5 mg/kg iv). The response to ET-1 was also investigated during additional intrarenal infusion of ETB-antagonist BQ-788 (7 nmol/min, solid bar). Panels C and D: Injections were made during control (open bars), during inhibition of epoxygenase by MS-PPOH (20 mg/kg, hatched bars) and during additional ETB-antagonism (solid bar). Mean ± SE, n=6. ***: p<0.001 vs. respective control.
Table 1. Baseline Hemodynamic Data

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>AP-1 mmHg</th>
<th>HR-1 bpm</th>
<th>RBF-1 (ml/min)/g</th>
<th>RBF-2 (ml/min)/g</th>
<th>RBF-3 (ml/min)/g</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Control</td>
<td>98±3</td>
<td>373±27</td>
<td>4.7±0.7</td>
<td>4.4±0.3</td>
<td>3.2±0.4</td>
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<tr>
<td>2) L-NAME + Nitroprusside</td>
<td>96±2</td>
<td>351±5</td>
<td>5.8±0.5</td>
<td>3.9±0.3</td>
<td>2.8±0.3* &amp;</td>
<td>4</td>
</tr>
<tr>
<td>3) L-NAME + Nitroprusside - Time control</td>
<td>104±10</td>
<td>325±31</td>
<td>5.6±1.2</td>
<td>4.3±0.5</td>
<td>3.8±0.3</td>
<td>3</td>
</tr>
<tr>
<td>4) L-NAME + Fenoldopam</td>
<td>94±2</td>
<td>328±7</td>
<td>4.4±0.3</td>
<td>3.9±0.4</td>
<td>2.0±0.2* &amp;</td>
<td>4</td>
</tr>
<tr>
<td>5) L-NAME + Fenoldopam - Time control</td>
<td>94±2</td>
<td>327±11</td>
<td>3.8±0.2</td>
<td>3.3±0.2</td>
<td>2.0±0.2* &amp;</td>
<td>4</td>
</tr>
<tr>
<td>6) L-NAME + Nitroprusside, dose-testing</td>
<td>112±4</td>
<td>331±23</td>
<td>4.6±0.3</td>
<td>3.9±0.3</td>
<td>4.3±0.5</td>
<td>5</td>
</tr>
<tr>
<td>7) L-NAME + Saline/Nitroprusside/Fenoldopam</td>
<td>101±4</td>
<td>376±18</td>
<td>4.9±0.4</td>
<td>---</td>
<td>---</td>
<td>8</td>
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<tr>
<td>8) L-NAME low dose + Nitroprusside</td>
<td>103±3</td>
<td>307±6</td>
<td>4.7±0.5</td>
<td>3.9±0.5</td>
<td>4.1±0.5</td>
<td>6</td>
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<tr>
<td>9) Indomethacin</td>
<td>102±3</td>
<td>351±26</td>
<td>4.9±0.5</td>
<td>4.0±0.4</td>
<td>3.0±0.4*</td>
<td>5</td>
</tr>
<tr>
<td>10) Indomethacin + Miconazole</td>
<td>108±5</td>
<td>318±21</td>
<td>5.3±0.4</td>
<td>4.0±0.3</td>
<td>4.1±0.6</td>
<td>6</td>
</tr>
<tr>
<td>11) MS-PPOH</td>
<td>103±2</td>
<td>348±5</td>
<td>5.1±0.5</td>
<td>4.7±0.5</td>
<td>3.7±0.3</td>
<td>4</td>
</tr>
</tbody>
</table>

AP-1: arterial pressure; HR-1: heart rate; RBF-1 - 3: renal blood flow at baseline before the first, second, and third injection of ET-1. *: p<0.01 vs. RBF-1, &: p<0.01 vs. RBF-2. All groups except #6 and #7 consisted of three consecutive injections of ET-1 during control conditions (suffix -1), iv application of the specified agents (suffix -2), and additional infusion of BQ788 into the renal artery (suffix -3). In time control and dose-response experiments (#3, #5, and #6), saline was infused in the third period instead of BQ788 (suffix -3 for groups #3, #5, and #6). In group #7 ET-1 was injected once before and three times after L-NAME; the injections after L-NAME in this group were made during infusion of either 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 in the table were obtained immediately before injection of the 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.
Figure 1

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ETB buffering by NO and non-NO

RBF (% change) vs Time (min)
Figure 2
Just, Olson, Falck, Arendshorst
ETB buffering by NO and non-NO
Figure 3
Just, Olson, Falck, Arendshorst
ETB buffering by NO and non-NO
Figure 4

Just, Olson, Falck, Arendshorst
ETB buffering by NO and non-NO
Figure 5
Just, Olson, Falck, Arendshorst
ETB buffering by NO and non-NO
Figure 6

Just, Olson, Falck, Arendshorst

ETB buffering by NO and non-NO

A

Miconazole + Indomethacin

Saline | Saline | ETB-inhib.

ET-1 | ET-1 | ET-1

B

Ang II | Ang II

C

MS-PPOH

Saline | Saline | ETB-inhib

ET-1 | ET-1 | ET-1

D

Ang II | Ang II

***

-100 -90 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50 60 70 80 90 100

RBF (% change)