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AT_2 antagonist-sensitive potentiation of angiotensin II-induced constriction by NO blockade and its dependence on endothelium and P450 eicosanoids in rat renal vasculature

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1 We showed earlier that NO inhibition caused a left-shift and augmented E_{max} of the concentration-response curve of AT_1-mediated (angiotensin II)-induced vasoconstrictions (AII-VC) in the rat kidney. The 0.01–0.1 nM AII-VC unmasked by the potentiating effect of NO inhibition, were sensitive not only to AT_1 (L158809), but also to AT_2 receptor (PD123319) antagonists. We now demonstrate the role of endothelium and eicosanoids in the NO-masked AT_1/AT_2-mediated component of the AII-VC in isolated indomethacin-perfused kidneys of the rat.

2 L-NAME increased 0.1 nM AII-VC 7.2 fold. Pretreatment of the kidneys with factor VIII antibody/complement or with the detergent CHAPS to damage endothelium, decreased carbachol-induced vasodilatation and blunted by 60 and 30% respectively, the enhancement of AII-VC during NO inhibition.

3 L-NAME also increased 3 μM noradrenaline (NA)-induced vasoconstriction (NA-VC) 8.1 fold. In contrast to AII-VC, endothelium damage was without effect on the enhancement of NA-VC by L-NAME, suggesting a dominant role of endothelium-derived NO in the enhancement of NA-VC.

4 During NO inhibition, ETYA (2 μM; an inhibitor of all arachidonic acid derived pathways) and α-naphtoflavone (10 μM; an inhibitor of the cytochrome P450 isozymes), decreased by 85% the 0.1 nM AII-VC.

5 In conclusion, during NO inhibition, the AT_1-mediated constriction to low concentrations of AII, which is sensitive to AT_2 antagonists, depends on intact endothelium, and can be blocked by inhibition of eicosanoid synthesis. The results suggest that the AII-mediated vasoconstriction through AT_1 receptors is potentiated in the absence of NO, by the release of eicosanoids from the endothelium through AT_2 receptors.

Keywords: Isolated perfused kidney; vascular resistance; NO-synthase; receptors; arachidonic acid metabolism; cytochrome P450

Introduction

The release of endothelial vasoactive factors exerts direct vasorelaxing or vasoconstricting effects. In addition, it is now widely accepted that the suppression of these factors enhances or blunts the pressor effect of any vasoconstrictor currently active in the renovascular system (reviewed by Navar *et al.*, 1996). Thus, many studies have shown that endogenous nitric oxide (NO) attenuates the renal effect of angiotensin II (AII) (Adachi *et al.*, 1996; Parekh *et al.*, 1996). In other respects, the dominant AII receptor subtype (AT) in the adult kidney vasculature has been proven to be AT_1 and all of the major actions of AII on renal haemodynamics appear to be mediated primarily by activation of the AT_1 receptor (Navar *et al.*, 1996). However, a few AT_2 receptors have also been detected in large vessels of various species (reviewed by Navar *et al.*, 1996; Zhuo *et al.*, 1996). The participation of AT_2 receptors in pressure natriuresis has been demonstrated (Lo *et al.*, 1995). Moreover, using a microdialysis technique it has recently been demonstrated that AT_2 mediates the accumulation of NO in the renal interstitial fluid (Siragy & Carey, 1997) leading to an increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP; Siragy & Carey, 1996).

We recently demonstrated (Muller *et al.*, 1997) that the renal AII-induced vasoconstriction, which was antagonized only by AT_1 antagonists in the presence of endogenous NO, became sensitive to both AT_1 and AT_2 antagonists when endogenous NO was inhibited. While AT_1 antagonists abolished all AII-induced vasoconstrictions, AT_2 antagonists inhibited only the L-NAME-induced leftwards shift of the concentration-response curve without affecting L-NAME-induced E_{max} increase. For instance, under conditions in which NO synthesis was blocked, AT_2 antagonists blunted by 65% the vasoconstriction induced by 0.1 nM AII and had no effect on the vasoconstriction induced by a maximal effective concentration of 10 nM AII. Therefore, during NO synthesis inhibition, AT_2 mediates the potentiation of AT_1-mediated vasoconstrictions.

In these earlier studies, the mechanism by which endogenous NO inhibits the AT_2-sensitive potentiation of AII-induced vasoconstriction has not been explored. The inhibition of NO production could enable the release of other endogenous vasoconstrictors in response to AII. For instance, there are several studies demonstrating that AII stimulates the endothelial release of endothelin (ET) (Emori *et al.*, 1991; Imai *et al.*, 1992; Kohno *et al.*, 1992; Navar *et al.*, 1996), as well as that of metabolites of arachidonic acid (Bottari *et al.*, 1993; Douglas & Hopfer, 1994; Navar *et al.*, 1996). Among the arachidonic acid metabolites, cyclo-oxygenase (COX)-derived vasoconstrictors such as thromboxane A_2 are ruled out as our previous findings were obtained in the presence of indomethacin (Muller *et al.*, 1997).

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The present studies were undertaken to extent our previous findings by testing whether the endothelium and endothelium-derived ET or arachidonic acid metabolites, originating from lipoxygenase and cytochrome P450 enzyme (CytP450) pathways, are involved in the AT_2-sensitive potentiation of AII-induced constrictions in the renal vasculature. The rat isolated kidney perfused at constant flow, in an open circuit in the presence of indomethacin, was again used as an experimental model.

Methods

Preparation of the rat isolated kidney

Male Wistar rats, weighing 170 to 220 g, with free access to standard food and water, were anaesthetized by intraperitoneal injection of sodium pentobarbitone (65 mg kg^{-1}) and used for the preparation of the isolated perfused kidney exactly as described previously (Muller *et al*., 1997). Briefly, the right kidney was perfused in an open single-pass circuit through the superior mesenteric artery. Perfusion was started *in situ* immediately after the suprarenal aorta had been tied, therefore protecting the kidney from an ischaemia. Heparin (1000 u) was injected into the left femoral artery. Perfusion pressure was monitored through the infrarenal aorta. While perfused, the kidney was excised from animal body and transferred onto a heated holder. The kidney preparations were rendered non-filtered by tying the ureter. The composition of the basic perfusion solution consisted of a commercially available Krebs-Ringer-gelatin-lactate solution used as plasma substitute in hypovolaemic patients (Plasmion), supplemented extemporaneously with 1.5 mM Ca^{2+}, 11 mM glucose, 18 mM sodium bicarbonate and 10 μM indomethacin (Muller *et al*., 1997). This medium is designated as 'control perfusate'. The perfusate was routinely thermostated at 37°C and was continuously filtered through a 1.2 μm filter and gassed with 95% O_2-5% CO_2. The pH was 7.39–7.46 in the prewarmed, preoxygenated medium.

Experimental protocols

The vasoconstrictor responses to AII and NA were measured as perfusion pressure changes under constant perfusion flow conditions exactly as described before (Muller *et al*., 1997). The perfusion flows (expressed in ml min^{-1}) were adjusted during a 60 min equilibration period to achieve a common pressure baseline of 90 mmHg; thereafter, the flow thus adjusted was maintained constant. The resulting vascular resistance at the end of the equilibration period was expressed for 1 g kidney weight (in mmHg min g ml^{-1}) and the left kidney was used as a weight basis for calculation. As indicated in Figure 1, the kidney preparations were perfused with control perfusate or with control perfusate containing throughout L-NAME, AT or ET-receptor antagonists or inhibitors of the arachidonic acid metabolizing enzymes, alone or in combination. The kidneys in which the renovascular endothelium had been damaged were pretreated with either CHAPS 0.2% during 30 s or 1/1000-diluted factor VIII-related antigen antibody (F8Ab) and 2% complement (Cpl) for 10 min, immediately after the perfusion was started and the kidney placed on its holder. Intact and endothelium-damaged renal preparations were perfused throughout. After the initial 60 min equilibration period, a single concentration of either AII (0.1 or 10 nM) or NA (3 μM) was infused into the perfusate through an automatically pushed syringe, over a fixed period of 6 min, with an infusion rate of 0.5 ml min^{-1} representing 4–8% of the perfusate flow. AII and NA were dissolved in an aliquot of current perfusion medium. Pressure values measured during AII or NA infusion were corrected for the 0.5 ml min^{-1} overflow pressures (usually 4–7 mmHg) induced by the push-syringe. The concentration of infused constrictor was adjusted to obtain the required final concentration in renal artery perfusate.

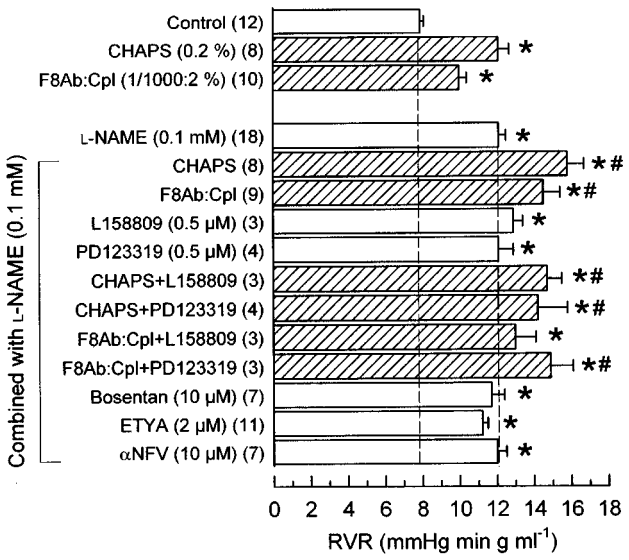

Figure 1 Renal vascular resistance (RVR) in intact kidneys (open columns) or in kidneys which had been pretreated with either CHAPS (0.2%) for 30 s or F8Ab:Cpl (1/1000 : 2%) for 10 min to damage endothelium (hatched columns). All other indicated drugs were present throughout the experiments. The perfusion flows were adjusted during an initial 60 min equilibration period to achieve a common perfusion pressure of 89.9 ± 0.2 mmHg (n = 110). RVR was measured after equilibration and before administration of AII or NA. The left kidney was used as a weight basis for calculations and was 0.79 ± 0.05 g (n = 110). The results are expressed as means ± s.e.means for the number of kidney preparations shown in parentheses. *P < 0.05 *vs* control perfusate; #P < 0.05 *vs* L-NAME perfusate.

In order to evaluate the degree of endothelium damage by CHAPS and F8Ab:Cpl, we measured carbachol (10 μM)-induced vasodilatation on phenylephrine-induced tone in two series of 4 rats. For that purpose, after the initial 60 min equilibration period, phenylephrine was injected for 15 s every 2 min to induce repetitive and transient vasoconstrictions of about 100 mmHg. Phenylephrine constrictions were measured on 3 consecutive peaks in the absence and presence of carbachol, before and after endothelium damage.

Drugs

Carbachol, indomethacin, N^G-nitro-L-nitro-L-arginine methyl ester (L-NAME), noradrenaline (NA), phenylephrine, 3-[3-cholamidopropyl)-1-propane-sulphonate (CHAPS), 7,8-benzoflavone (α-naphtoflavone, αNFV), rabbit anti-human factor VIII-related antigen (F8Ab), guinea-pig complement (Cpl) and carbachol were obtained from Sigma (St Louis, MO, U.S.A.). Plasmion was purchased from Rhône-Poulenc Rörer Laboratories (Antony, France). Angiotensin II (AII) was obtained from Neosystem Laboratory (Strasbourg, France), 5, 8, 11, 14-eicosatetraynoic acid (ETYA) from Calbiochem (La Jolla, CA, U.S.A.), PD123319 (S(+)-1-[[4-Dimethylamino)-3-methylphenyl]methyl] - 5 - (diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c] pyridine-6-carboxylic acid ditrifluoroacetate) from Research Biochemicals International (Natrick, U.S.A.) and L158809 (5,7-Dimethyl-2-ethyl-3-[[2′-(1H-tetrazol-5-yl)[1,1′-bi-

phenyl]-4-yl]methyl]-3H-imidazo[4,5-b]pyridine monohydrate) from Merck (Rahway, U.S.A.). Bosentan was kindly provided by Dr Clozel (Pharmacia Division Preclinical Research, Hoffman-Laroche, Basel, Switzerland).

Analysis of data

A computerized data acquisition system continuously collected pressure and flow values at 1 Hz throughout the experiment. Consecutive blocks of 15 data points were averaged to obtain 4 measurements per min of flow and pressure. The vasoconstrictor effects of AII and NA were expressed as the area under the curve over the 6 min of drug infusion. This way of presentation integrates both the speed at which pressure increases and the maximum responses to the vasoconstrictors. All values presented are expressed as means ± s.e.mean. ANOVA was performed on the absolute values of the various parameters. Differences were considered significant for $P<0.05$. When the ANOVA detected a significant effect, comparisons between individual means were based on the Student-Newman-Keuls test.

Results

Effect of perfusate composition on RVR

The effect of the various drugs on renal vascular resistance (RVR) are shown in Figure 1. Perfusion in the presence of L-NAME increased RVR by 50% as compared to control-perfused kidneys (12.1 ± 0.4 *vs* 7.9 ± 0.2). Pretreatment with CHAPS and F8Ab:Cpl to damage endothelium also increased RVR as compared to untreated control-perfused kidneys, CHAPS having a higher effect than F8Ab:Cpl (12.1 ± 0.6 *vs* 10.0 ± 0.4). In addition, in kidneys pretreated with CHAPS or F8Ab:Cpl, RVR was further increased by subsequent perfusion in the presence of L-NAME. This effect tended to be higher in F8Ab:Cpl-pretreated kidneys (14.5 ± 0.9 *vs* 10.0 ± 0.4) than in CHAPS-pretreated kidneys (15.8 ± 0.9 *vs* 12.1 ± 0.6). On the other hand, the addition of AT antagonists, an ET antagonist or blockers of arachidonic acid metabolism had no effect on RVR.

Effect of endothelium damage on AII- and NA-induced vasoconstrictions

Two different approaches were employed to damage endothelium: (i) pretreatment for 30 s with the detergent CHAPS (10.2%) (Bhardwaj & Moore, 1989; King & Brenner, 1991), and (ii) pretreatment for 10 min with an endothelial-targeted, complement fixing antiserum, F8Ab:Cpl (1/1000 : 2%) (Juncos *et al*., 1994). Evidence for endothelium damage by pretreatment with CHAPS is given by the marked reduction of the vasodilatation induced by 10 μM carbachol in phenylephrine-constricted kidney preparations (Figure 2). Thus, carbachol blunted the vasoconstriction induced by 3 μM phenylephrine-by 55 ± 7% (103 ± 9 *vs* 44 ± 6 mmHg) and 8 ± 10% (85 ± 15 *vs* 75 ± 9 mmHg) before and after treatment with CHAPS, respectively ($P<0.05$; $n=4$). In a second series of rats, carbachol reduced the phenylephrine-induced vasoconstriction by 78 ± 11% (98 ± 9 *vs* 24 ± 14 mmHg) and 57 ± 14% (66 ± 9 *vs* 31 ± 12 mmHg) before and after treatment with F8Ab:Cpl, respectively ($P<0.05$; $n=4$) (Figure 2). Thus, the efficiency of F8Ab:Cpl in damaging endothelium was one third of the efficiency of CHAPS.

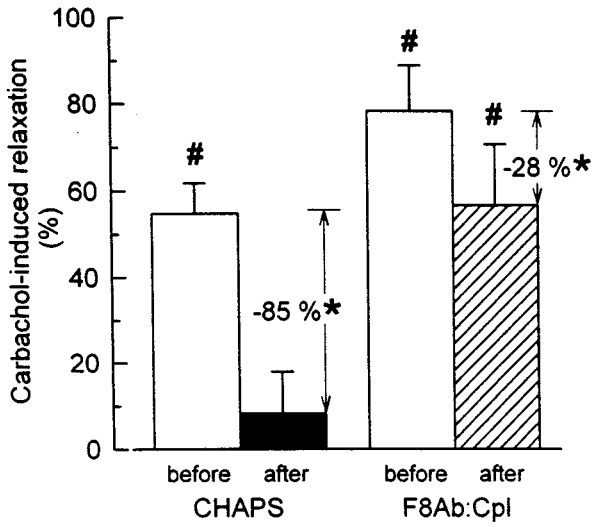

Figure 2 Effect of endothelium damage on carbachol-induced vasodilatation in the isolated perfused kidney preconstricted with 3 μM phenylephrine. To damage endothelium, kidneys were pretreated with either 0.2% CHAPS for 30 s or with F8Ab:Cpl (1/1000 : 2%) for 10 min, as indicated. The open columns represent the vasodilatation induced in kidneys with intact endothelium, the solid and the hatched columns represent the vasodilatation induced in endothelium-damaged kidneys pretreated with CHAPS or F8Ab:Cpl, respectively. Values are means ± s.e.means for 4 kidney preparations. *$P<0.05$ intact *vs* endothelium-damaged kidneys. #$P<0.05$, significant carbachol-induced vasodilatation.

Renovascular endothelium was damaged in order to determine whether this would mimic the potentiating effect of NO-synthase inhibition demonstrated earlier (Muller *et al*., 1997). For that purpose we explored the effect of endothelium damage on the vasoconstriction induced by 0.1 nM AII, a concentration at which the potentiating effect of L-NAME and the inhibitory effect of AT_2 antagonist were the highest in our previous studies (Muller *et al*., 1997). Since evidence has also been presented to show that endothelial-derived NO attenuates the effects of other vasoconstrictors such as NA (Cocks & Angus, 1983; Parekh *et al*., 1996), we also investigated the effect of endothelium damage on the renal vasoconstriction induced by 3 μM NA.

In intact kidneys perfused with control perfusate, 0.1 nM AII and 3 μM NA induced low vasoconstrictions which were strongly and similarly enhanced in L-NAME-perfused kidneys (Figure 3). Endothelium damage consistently reduced the speed of AII-induced pressure increase and/or the maximum effect of AII, whether kidneys were perfused in absence or presence of L-NAME. By contrast, endothelium damage mimicked part of the enhancing effect of L-NAME on the NA-induced vasoconstriction.

These observations have been quantified in Figure 4, in which the vasoconstrictor effects of AII and NA have been represented as areas under the curve over the 6 min period of drug infusion. L-NAME unmasked strong responses, giving comparable relative increases of 7.2 and 8.1 times the control vasoconstrictions induced by AII and NA, respectively. Unexpectedly, treatment with CHAPS or F8Ab:Cpl abolished the AII-induced vasoconstriction in control-perfused kidneys and reduced the L-NAME-enhanced responses by 60 and 30%, respectively. In contrast to AII, treatment with CHAPS or F8Ab:Cpl augmented by 4.6 and 4.0 times, respectively, the vasoconstriction induced by NA in control-perfused kidneys. Moreover, treatment with CHAPS or F8Ab:Cpl was unable to

augment further the NA-induced vasoconstriction which had been enhanced by perfusing the kidneys in the presence of L-NAME. Consistent with our previous findings (Muller *et al*., 1997), the AT_1 antgonist L158809 (0.5 μM) virtually abolished the vasoconstriction induced by 0.1 nM AII in both intact and damaged kidneys. The AT_2 antagonist PD123319 (0.5 μM) further decreased the vasoconstriction induced by AII after partial endothelium damage, to a level which was not significantly different from that seen after AT_2 blockade in intact kidneys (Figure 5).

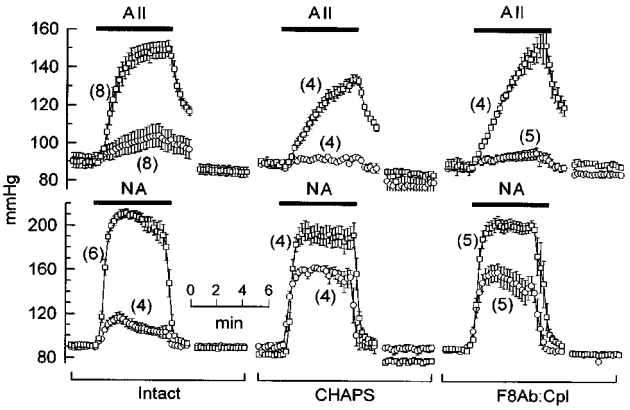

Figure 3 Changes in perfusion pressure depicting the vasoconstrictor effects of 0.1 nM AII and 3 μM NA in isolated kidneys perfused at constant flow with control perfusate (circles) or with perfusate containing 0.1 mM L-NAME (squares). To damage endothelium, kidneys were pretreated with either 0.2% CHAPS for 30 s or with F8Ab:Cpl (1/1000 : 2%) for 10 min, as indicated. Values are means and vertical lines show s.e.means, not corrected for overflow pressures (see Method section), for the number of kidney preparations indicated in parentheses.

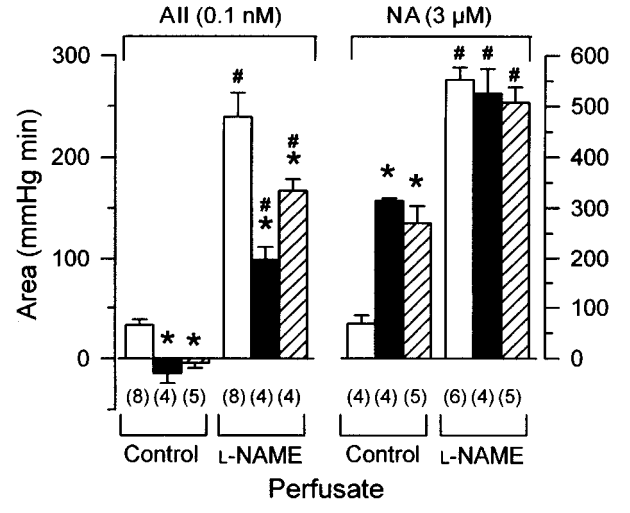

Figure 4 Effects of endothelium damage on the vasoconstrictions induced by 0.1 nM AII or 3 μM NA in isolated kidneys. Kidneys were perfused with either control perfusate or with control perfusate containing 0.1 mM L-NAME as indicated. The open columns represent the vasoconstrictions induced in kidneys with intact endothelium; the hatched and the solid columns represent the vasoconstrictions induced in endothelium-damaged kidneys pretreated with CHAPS or F8Ab:Cpl, respectively. Note different scales of axes for AII and NA. Values are means$\pm$s.e.means for the number of kidney preparations indicated in parentheses. #$P<0.05$ control *vs* L-NAME; *$P<0.05$ intact *vs* endothelium-damaged kidneys.

Effect of bosentan, ETYA and αNFV on NO-masked AII-induced vasoconstrictions

We next investigated whether endothelium-derived endothelin or arachidonic acid metabolites contribute to the potentiation of the effect of 0.1 nM AII by NO-synthesis inhibition. When added to the perfusate, 10 μM bosentan, a non-selective ET receptor antagonist, had no significant effect on the vasoconstriction induced by 0.1 nM AII (Figure 6). On the other hand, ETYA (2 μM), an analogue of arachidonic acid

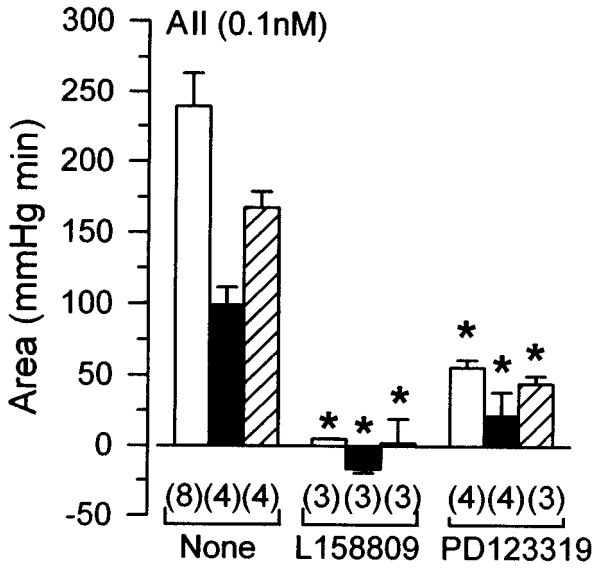

Figure 5 Effects of AT antagonists on the vasoconstriction induced by 0.1 nM AII in intact kidneys (open columns) or in kidneys in which endothelium had been damaged by treatment with either CHAPS (solid columns) or F8Ab:Cpl (hatched columns). Kidneys were perfused with 0.1 mM L-NAME alone (none) or with L-NAME and either 500 nM L158809 or 500 nM PD123319, as indicated. Values are means$\pm$s.e.means for the number of kidney preparations indicated in parentheses. *$P<0.05$: significant inhibitory effect of AT antagonist.

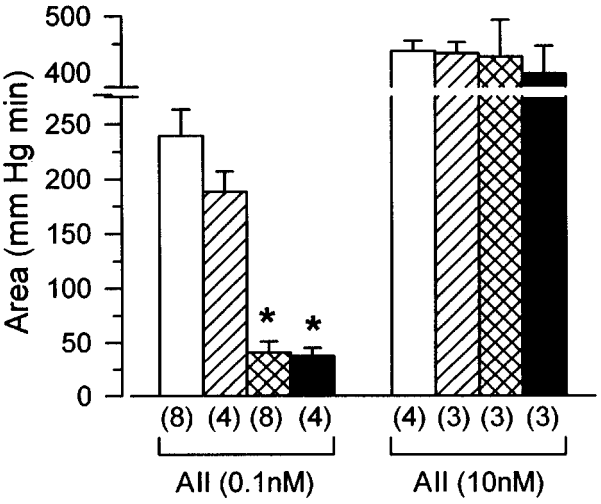

Figure 6 Vasoconstrictions induced by 0.1 nM or 10 nM AII in kidneys perfused with either L-NAME perfusate (open columns) or with L-NAME perfusate to which the following drugs had been added: 10 μM bosentan (hatched columns); 2 μM ETYA (cross-hatched columns); 10 μM αNFV (solid columns). Values are means $\pm$s.e.means for the number of kidney preparations indicated in parentheses. *$P<0.05$ *vs* L-NAME perfusate.

able to block all pathways of arachidonic acid metabolism, decreased by 83% the 0.1 nM AII-induced vasoconstriction. αNFV (10 μM), which has been shown to inhibit selectively several CytP450 isozymes, also blunted by 84% the 0.1 nM AII-induced vasoconstriction. By contrast, both ETYA and αNFV were unable to affect the vasoconstriction induced by 10 nM AII.

Discussion

We recently investigated, specifically, whether the AII-induced vasoconstriction enhanced by NO inhibition could occur via multiple AT receptors in the renal vasculature (Muller *et al*., 1997). We used the rat isolated kidney rendered non-filtering to minimize the involvement of tubule-mediated regulatory mechanisms on renovascular tonus. All kidney preparations were perfused in the presence of indomethacin in order to prevent the formation of endogenous prostaglandins. These studies revealed that the enhancement of the AII-induced vasoconstriction during NO-blockade comprises both a potentiated and an augmented component. Thus, the concentration of AII needed to induce half-maximal vasoconstriction (EC_{50}) was lowered by about one order of magnitude (from ~0.4 to ~0.04 nM) during L-NAME perfusion, and thereby unmasked strong vasoconstrictor responses to physiological concentrations of AII (0.01 – 1 nM). Moreover, the maximal effect (E_{max}) was augmented about 3 fold during NO synthesis inhibition. L158809, an AT_1 antagonist, abolished both the L-NAME-potentiated and the L-NAME-augmented components of the AII-induced vasoconstriction. Importantly, PD123319, a selective AT_2 antagonist, at concentrations which have been considered to be subtype selective (Brechler *et al*., 1993), reversed the leftwards-shift of the concentration-response curve and thereby increased the EC_{50} value back to the value seen in absence of L-NAME. The conclusion reached by these studies (Muller *et al*., 1997) is that endogenous NO inhibits AT_2-mediated potentiation of AT_1-mediated constrictions in the renal vasculature. Using exactly the same experimental conditions, we now show that the NO-masked AT_2-sensitive renal vasoconstriction induced by 0.1 nM AII is dependent upon both endothelium and the CytP450-derived arachidonic acid metabolites.

Endothelium damage and NO-blockade exert additive constrictor effects on basal renal vascular resistance

Because the endothelium cannot be readily removed from the vasculature of the intact kidney, we used two different approaches to damage endothelium. For the first approach, the kidneys were pretreated with the detergent CHAPS, which has previously been used under similar conditions by us and others, to remove endothelium in rat and rabbit isolated perfused kidneys (Bhardwaj & Moore, 1989; King & Brenner, 1991; Massfelder *et al*., 1996). In these studies, short exposure of the kidneys to CHAPS has been shown to decrease markedly acetylcholine or bradykinin-induced vasodilatation, without affecting the ability of the kidneys to vasoconstrict. The second approach is based on the fact that endothelial cells specifically express factor VIII-related antigens and that complement reacts with factor VIII-related antigens/antibody complexes, causing cell damage and lysis (Juncos *et al*., 1994). This method has been shown to eliminate selectively endothelium-dependent vasodilatation not only from micro-perfused afferent arteriole, but also from isolated kidneys without impairing smooth muscle responsiveness (Juncos *et al*., 1994; Massfelder *et al*., 1996). The actual degree of endothelium damage has been evaluated for both methods in the present study. Insofar as carbachol-induced vasodilatation can be taken as an index, CHAPS was three times more effective than F8Ab:Cpl in damaging endothelium. In agreement with this, CHAPS exhibited higher effects on both basal RVR and AII/NA-induced vasoconstrictions. Although the efficiency of the two methods used to damage endothelium differed, their effects were consistent.

It is widely accepted that NO is a major contributor to the basal relaxed state of the kidney (Stephan *et al*., 1990; Radermacher *et al*., 1990; Gardes *et al*., 1994; Bryand *et al*., 1995; Kaufmann *et al*., 1995; Navar *et al*., 1996; Ziyyat *et al*., 1996; Barthelmebs *et al*., 1996; Muller *et al*., 1997). We found that L-NAME, indeed, strongly increased RVR. On the other hand the effect of endothelium removal on basal vascular resistance of the isolated perfused kidney is not obvious from the literature (Navar *et al*., 1996). We found that both endothelium damaging methods increased RVR. This finding suggests that the various vasoactive compounds released by the endothelium, including NO, exert a net vasodilator effect on basal RVR. The fact that CHAPS and L-NAME had similar and additive effects on RVR suggests that endothelium-derived vasodilators different from NO – like the putative endothelium-derived hyperpolarizing factor (EDHF) – and that NO of extraendothelial origin contribute to the basal relaxed state. The involvement of vasodilator arachidonic acid metabolites has to be ruled out since the kidney preparations have been perfused in the presence of indomethacin and because neither ETYA nor αNFV affected RVR during NO blockade. Together, these results imply that during NO synthesis inhibition, the endothelium releases a dilator factor different from both NO and arachidonic acid metabolites in the isolated perfused kidney. Whatever it is, it was not the aim of the present study to clarify this casual finding. It should also be mentioned, that during NO blockade, neither AT nor ET antagonists were able to affect basal vascular resistance, indicating that endogenous AII or ET did not contribute to the basal tonus in our renal preparations.

Endothelium damage enhances the NA-induced vasoconstriction and blunts the AII-induced vasoconstriction

A major aim of the present investigation was to clarify the role played by the endothelium in the NO-masked AT_2-sensitive renal vasoconstriction induced by 0.1 nM AII. We hypothesized that an endothelium-derived vasoconstrictor system could be responsible for the NO-masked potentiation of the AII-induced vasoconstriction. If this hypothesis were true, then endothelium damage should inhibit and not mimic the L-NAME-dependent potentiation of the vasoconstriction induced by 0.1 nM AII. We found, indeed, that endothelium damage abolished this low vasoconstriction under basal conditions and markedly reduced its AT_2-sensitive potentiation by L-NAME. That endothelium damage inhibited only partially the L-NAME-induced potentiation is most likely a reflection of partial damage of the endothelium.

By comparing 0.1 nM AII to 3 μM NA we could determine whether the requirement of an endothelium-derived vasoconstrictor system was specific to AII. At the concentrations used, AII and NA induced low vasoconstrictions under basal conditions, while the relative enhancing effect of L-NAME on their constrictor effects were comparable. Contrary to AII, endothelium damage partially mimicked L-NAME in that it enhanced the NA-induced vasoconstriction in control-perfused

kidneys. That endothelium damage enhanced only partially the NA- induced vasoconstriction is again a reflection of a partial damage of endothelium. In support of this, treatment with CHAPS or F8Ab:Cpl was unable to augment further the NA-induced vasoconstriction which has been enhanced by perfusing the kidneys in the presence of L-NAME. It has been shown, that in the rat isolated perfused kidney, COX-derived vasoconstrictors are involved in the L-NAME-induced enhancement of the effect of NA on RVR (Ziyyat *et al*., 1996). In the present studies this has to be ruled out, as the kidneys were perfused in the presence of indomethacin.

Taken together, these results strongly suggest that endothelium-derived constrictors contribute critically to the AT_1/AT_2-mediated vasoconstriction induced by 0.1 nM AII during inhibition of NO synthesis. By contrast, the reduced availability of vasodilator endothelium-derived NO presumably plays a dominant role in the enhancing effect of L-NAME on the NA-induced vasoconstriction.

The NO-masked AT_1/AT_2-mediated renal vasoconstriction depends on CytP450-derived arachidonic acid metabolites

Bosentan, a non-selective ET receptor antagonist (Clozel *et al*., 1994) did not affect AII-induced vasoconstrictions, ruling out the possible participation of ET in the L-NAME-induced enhancement of AII-induced vasoconstriction. On the other hand, the quasi-abolition of the L-NAME-induced potentiation of the vasoconstriction induced by 0.1 nM AII after inhibition of all pathways of arachidonic acid metabolism with ETYA (McGiff, 1991), suggests that this constriction depends on the production and/or release of lipoxygenase or CytP450 derived eicosanoids. This hypothesis was strengthened and refined in experiments using αNFV, a widely recognized inhibitor of CytP450 enzymes (Chang *et al*., 1994; Oyekan, 1994) which, comparably to ETYA, inhibited the vasoconstriction induced by 0.1 nM AII during NO blockade. Since COX was blocked in our studies, it should be kept in mind that the availability of arachidonic acid for utilization by other pathways could have been artificially increased. However, it is of interest, that the vasoconstriction induced by a maximal effective concentration of AII (10 nM) during NO blockade, which has been shown to be insensitive to AT_2-receptor antagonists (Muller *et al*., 1997), was also insensitive to both ETYA and αNFV.

Previous investigations have already demonstrated that NO and NO-releasing compounds inhibit the synthesis of arachidonic acid-derived compounds, namely CytP450-derived eicosanoids both *in vivo* and *in vitro* (Wink *et al*., 1993; Oyekan, 1995; Ziyyat *et al*., 1996; Baylis *et al*., 1996; Bauersachs *et al*., 1996; Alonso-Galicia *et al*., 1997; Khatsenko & Kikkawa, 1997). Moreover, AII has been shown to trigger the release of metabolites of arachidonic acid from endothelium (Bottari *et al*., 1993; Douglas & Hopfer, 1994; Navar *et al*., 1996). In addition, the products of lipoxygenase and/or CytP450 enzyme systems are abundantly formed by the renovascular system, namely by the endothelium, and some of them are able to vasoconstrict the kidney (Oyekan, 1994; 1995; Navar *et al*., 1996; Imig *et al*., 1996). Also, AII has been shown to activate phospholipase A_2 leading to increased formation of arachidonic metabolites, including CytP450 metabolites (Bottari *et al*., 1993; Douglas & Hopfer, 1994; Navar *et al*., 1996).

The present study did not address any particular CytP450 vasoconstrictor product. There are two categories of vasoactive CytP450 metabolites, those catalyzed by epoxygenase enzymes which generate epoxyeicosatrienoic acids (EETs) and those enzymes which generate hydroxyeicosatetraenoic acids (HETEs). 5,6-, 8,9-, 11,12- and 14,15-EETs are generally potent vasodilators (Harder *et al*., 1995). On the other hand, in the *in vitro* blood-perfused juxtamedullary nephron preparation, 5,6-EET has been shown to constrict afferent and interlobular arterioles but these vasoconstrictions were COX-dependent (Imig *et al*., 1996a, b). HETEs, such as 20-HETE are potent constrictors (Harder *et al*., 1995; Imig *et al*., 1996a, b) and are produced in renal microvessels (Imig *et al*., 1996a, b). In addition, the vasoconstrictor response to exogenous 20-HETE is not altered by blockade of the COX, lipoxygenase and CytP450 pathways (Imig *et al*., 1996b). Finally, it has been shown more recently that inhibition of the formation of 20-HETE in renal arterioles contributes to the vasodilator response to NO (Alonso-Galicia *et al*., 1997). But, clearly, further studies are needed to identify the CytP450 metabolite involved in the potentiation of renal AII-induced vasoconstriction during NO blockade.

Our results demonstrate that, during NO inhibition, the classical AT_1-mediated constriction to a low concentration of AII becomes sensitive to AT_2 antagonists, depends on an intact endothelium and can be blocked by inhibition of eicosanoid synthesis. Therefore, we would suggest that the AII-mediated vasoconstriction, most likely through smooth muscle cell AT_1 receptors, is potentiated in the absence of NO by the release of eicosanoids from the endothelium through AT_2 receptors. According to this hypothesis, during NO blockade, eicosanoids are necessary for the potentiation of AT_1-mediated vasoconstriction but appear not to be sufficient to induce vasoconstriction in response to a low concentration of AII on their own.

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