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# Role of endothelium-derived relaxing factors in adrenomedullin-induced vasodilation in the rat kidney

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**Abstract**

The present study aimed to evaluate the contributions of endothelium-derived hyperpolarizing factor (EDHF), the nitric oxide (NO)-cGMP pathway, and prostaglandins to adrenomedullin-induced vasodilation in isolated rat kidney. Inhibition of the NO-cGMP pathway with $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) or 1*H*-[1,2,4]oxadiazolo-[4,3*a*]quinoxalin-1-one (ODQ) reduced the maximal vasodilator response to adrenomedullin by approximately 50%. Pretreatment of the vessels with the potassium channel inhibitor, tetraethylammonium or increased extracellular $K^+$, also decreased the maximal response to adrenomedullin by approximately 50%. The simultaneous administration of blockers of both endothelium-derived relaxing factors had a combined effect that almost suppressed adrenomedullin-induced vasodilation. The administration of indomethacin did not modify the renal response to adrenomedullin. Our results suggest that the vasodilator response to adrenomedullin in the isolated perfused kidney of rats is mediated by EDHF and NO to a similar extent. Our data also provide evidence that prostaglandins play no role in the vasodilator response to adrenomedullin in the renal vasculature. 

*Keywords:* Kidney, rat; Adrenomedullin; Vasodilation; Nitric oxide (NO); EDHF (endothelium-derived hyperpolarizing factor)

## 1. Introduction

Adrenomedullin is a widely distributed multifunctional regulatory peptide isolated from human pheochromocytoma cells (Kitamura et al., 1993), which is activated under physiological and pathophysiological conditions (Hinson et al., 2000; Cases et al., 2000). Adrenomedullin has potent cardiovascular and renal actions (Hinson et al., 2000; Cases et al., 2000; Schell et al., 1996), and exhibits potent vasodilator activity in the renal vasculature (Hirata et al., 1995; Hayakawa et al., 1999).

Adrenomedullin stimulates cyclic AMP (cAMP) formation in vascular smooth muscle cells (Ishizaka et al., 1994) and vascular endothelial cells (Shimekake et al., 1995). It is well established that the increase in intracellular cAMP of vascular smooth muscle cells is associated with endothelium-independent vasodilation. In aortic endothelial cells, however, adrenomedullin stimulates two signal transduction pathways, adenylate cyclase and intracellular calcium (Hirata et al., 1995; Shimekake et al., 1995). The latter mechanism can trigger nitric oxide synthase (NOS) activity.

Several studies have been conducted to explore the mechanisms of adrenomedullin-induced vasodilation. The contribution of NO seems to depend on the vascular bed studied. Miura et al. (1995) reported that renal vasodilation caused by intra-arterial administration of adrenomedullin in dogs was suppressed by $N^{\omega}$-nitro-L-arginine and that a large dose of L-arginine remedied this suppression. Gardiner et al. (1995) found that adrenomedullin-induced vasodilation in rat hindquarter was only slightly inhibited by $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), and Heaton et al. (1995) also observed that L-NAME did not antagonize the vasodilator effect of adrenomedullin in pulmonary vessels of rat. In the isolated perfused kidney, adrenomedullin increased NO release (Hirata et al., 1995), and the same group recently reported that the NO-cGMP pathway is involved in the mechanism of adrenomedullin-induced renal vasodilation (Hayakawa et al., 1999).

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Other putative endothelium-derived mediators include the endothelium-derived hyperpolarizing factor (EDHF), which is an unidentified diffusible substance that relaxes vascular smooth muscle through hyperpolarization by the opening of $K^+$ channels (Feletou and Vanhoutte, 1999; Garland et al., 1995; Mombouli and Vanhoutte, 1997; Quilley et al., 1997), and prostaglandins (Garland et al., 1995; Mombouli and Vanhoutte, 1997; Quilley et al., 1997; Koller et al., 1989). The contribution of these possible mediators of adrenomedullin-induced vasodilation in the isolated perfused kidney has not been fully established, although EDHF-dependent mechanisms have been identified as an important component of acetylcholine-induced endothelium-dependent vasodilation (Vargas et al., 1994) and $P_{2Y}$ receptor agonist stimulation (Vargas et al., 1996) in isolated perfused rat kidney.

The present study was undertaken to determine the contribution of EDHF, NO and prostaglandins to the vasodilator effects of adrenomedullin on the entire renal vasculature. To explore the participation of these mediators, we used: increased extracellular $K^+$ and tetraethylammonium as unspecific $K^+$ channel blockers (Adeagbo and Triggle., 1993) to examine the possible involvement of EDHF; L-NAME and 1*H*-[1,2,4]oxadiazolo-[4,3*a*]quinoxalin-1-one (ODQ), inhibitors of NO synthase (Ishii et al., 1990) and guanylyl cyclase (Schrammel et al., 1996), respectively, to further analyze the participation of the NO-cGMP pathway; and indomethacin, to determine the possible role of prostaglandins (Bhardwaj and Moore, 1988).

## 2. Materials and methods

### 2.1. Animals and isolated perfused kidney preparation

This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Male Wistar rats ($300 \pm 10$ g, $n = 54$) were maintained on standard chow and tap water ad libitum. The animals were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). The abdomen was opened through a midline incision and the left renal and superior mesenteric arteries were exposed. In order to maintain continuous renal perfusion, the superior mesenteric artery was cannulated with a beveled 18-gauge needle advanced into the left renal artery and secured with ligatures. The kidney was perfused at a constant flow rate (5 ml/g of kidney weight per minute) by means of a roller pump (IPS-4, Ismatec, Zurich) with Tyrode solution (37 °C) of the following composition (mM): NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.1; $NaHCO_3$, 12.0; $NaH_2PO_4$, 0.42; D(+)-glucose 5.6, aerated with 5% $CO_2$ in $O_2$. The kidney was then dissected clear from its surrounding tissues and placed in a chamber containing the Tyrode solution at 37 °C. Renal vascular responses were recorded (TRA-021 transducer connected to a two-channel Letigraph 2000 recorder, Letica, Barcelona) as changes in the renal perfusion pressure downstream from the pump.

### 2.2. Experimental protocol

The experiments evaluated the effects on the renal response to adrenomedullin under the following conditions ($n = 6$, each group): (1) no treatment; (2) after pretreatment with L-NAME ($10^{-4}$ mol/l) to inhibit NO synthesis; (3) ODQ ($10^{-5}$ mol/l, dissolved in ethanol 0.01%) to inhibit guanylyl cyclase; (4) after pretreatment with increased extracellular $K^+$ (80 mmol/l) or (5) tetraethylammonium ($3 \times 10^{-3}$ mol/l), as non-specific $K^+$ channel inhibitors; (6) indomethacin ($10^{-5}$ mol/l, dissolved in $Na_2CO_3$ 0.5 mol/l), as inhibitor of prostaglandins; (7) after combined pretreatment with L-NAME and tetraethylammonium at the doses mentioned above; (8) after combined pretreatment with ODQ and tetraethylammonium at the doses mentioned above; (9) after combined pretreatment with increased $K^+$ and L-NAME at the doses mentioned above. All these inhibitors were added to the perfusate after the stabilization period, and 30 min was allowed before the start of the dose–response curves. The inhibitors were present throughout the experiment. The renal vasculature was preconstricted with phenylephrine infused at a rate to produce a final concentration of $10^{-6}$ mol/l in the perfusate. After a 20-min period of stabilization with raised tone, the dose–response curve to adrenomedullin ($10^{-7}$ to $10^{-5}$ g) and the response to a single dose of acetylcholine ($10^{-5}$ g), which was used as reference compound for endothelium-dependent vasodilation, were obtained under normal conditions or after the administration of the inhibitors, using separate preparations as controls and for each inhibitor. When the inhibitors were administered, the dose of phenylephrine was adjusted to achieve an increase in renal perfusion pressure (level of preconstriction) similar to that observed in untreated preparations ($126 \pm 3$ mm Hg). Dose–response curves were constructed by injecting boluses of 50 μl/g kidney of the agonist. Injection of these volumes caused a small and transient increase in renal perfusion pressure that preceded the agonist-evoked response. A control injection of vehicle was given to each preparation, in order to verify that the

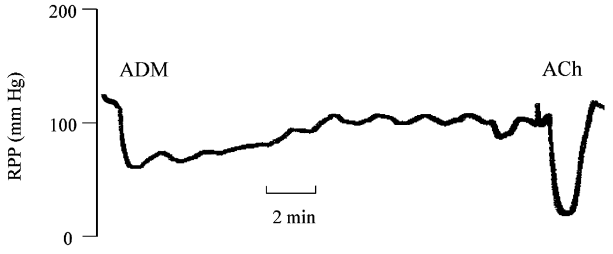


Fig. 1. Representative tracing of the vasodilator response to the maximal dose of adrenomedullin (ADM) and acetylcholine (ACh) expressed as renal perfusion pressure (RPP) in the isolated perfused rat kidney. Depressor response to adrenomedullin lasted approximately 16 min.

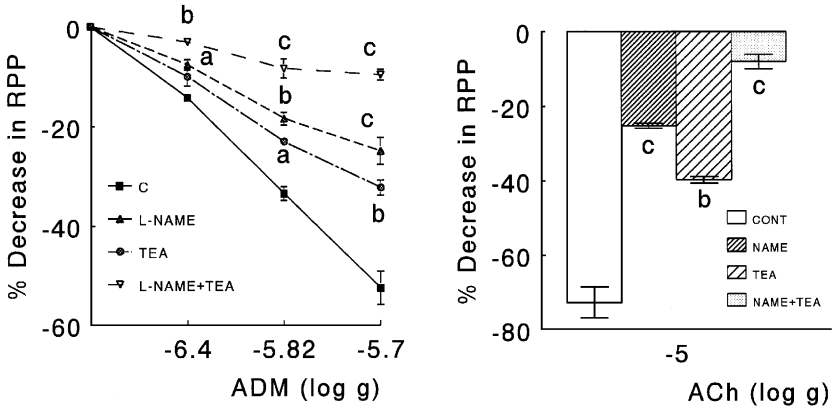


Fig. 2. Effects of L-NAME ($10^{-4}$ mol/l), TEA ($3 \times 10^{-3}$ mol/l), or L-NAME plus TEA on decreases in renal perfusion pressure (RPP) in response to ADM and ACh. a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$ compared with normal conditions. Data are expressed as means ± S.E.M. ($n = 6$ in each group). Doses are expressed per gram of kidney.

responses to the drug injections were not artifacts. The minimum time interval between successive doses of an agonist was 15 min. When necessary, these periods were extended until the previous response had disappeared. The minimum time interval between the administration of different agonists was 20 min. The dose–response curves in the presence of the inhibitors were compared with the respective dose–response curves under normal conditions. To obtain solutions containing 80 mmol/l of $K^+$, equimolar concentrations of NaCl were replaced by KCl in the Tyrode solution. The changes in renal perfusion pressure in response to the vasodilators were expressed as percentages of the decrease in renal perfusion pressure of the vasoconstriction obtained with phenylephrine or KCl. Dose–response curves were made using the peak response.

### 2.3. Drugs

The following drugs were used: pentobarbital sodium (Nembutal, Serva, Heidelberg, Germany), adrenomedullin, acetylcholine chloride, L-phenylephrine hydrochloride, $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), tetraethylammonium bromide, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ), and indomethacin (all Sigma).

### 2.4. Statistical analysis

The dose–response curves in the presence or absence of the inhibitors (L-NAME, ODQ, tetraethylammonium, increased $K^+$, or indomethacin) were compared using a three-factor random block design (rat, dose, and treatment). When the results of the analysis of variance were significant, Tukey's *t* and Neumann–Keuls tests were applied (Snedecor and Cochran, 1980).

## 3. Results

Fig. 1 illustrates the depressant dose–response curve for adrenomedullin in the isolated perfused rat kidney. Adreno-

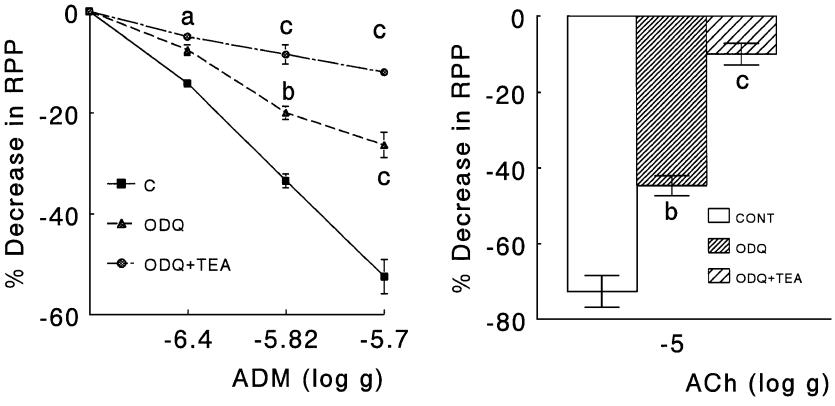


Fig. 3. Effects of ODQ ($10^{-5}$ mol/l), TEA ($3 \times 10^{-3}$ mol/l), or ODQ plus TEA on decreases in renal perfusion pressure (RPP) in response to ADM and Ach. a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$ compared with normal conditions. Data are expressed as means ± S.E.M. ($n = 6$ in each group). Doses are expressed per gram of kidney.

medullin showed a potent dose-dependent depressor action. The maximal vasodilator response was less (52.5 ± 3.4%) than that observed with acetylcholine (72.7 ± 4.2). However, adrenomedullin showed a far longer-lasting effect. In the graphic integration (area: mmHg·min), the depressor response induced by adrenomedullin was dramatically greater (320 ± 20 $mm^2$) than that induced by acetylcholine (76 ± 10 $mm^2$).

Fig. 2 depicts the effects of L-NAME, tetraethylammonium, and L-NAME plus tetraethylammonium on the adrenomedullin dose–response curve and on the response to the single dose of acetylcholine. L-NAME significantly decreased the responsiveness to adrenomedullin and acetylcholine. The administration of tetraethylammonium also shifted to the right the dose–response curve to both adrenomedullin, especially at the highest dose, and acetylcholine. The simultaneous administration of both L-NAME and tetraethylammonium further attenuated the vasodilator responses to adrenomedullin and acetylcholine produced by the administration of L-NAME and tetraethylammonium separately.

Pretreatment of the preparations with the cGMP inhibitor, ODQ, significantly reduced the response to adrenomedullin and the response to acetylcholine. When the renal vascular bed was pretreated with ODQ and tetraethylammonium, the vasodilator response to both agonists was again further reduced as compared with that under untreated conditions or with pretreatment by each inhibitor alone (Fig. 3).

Similarly, the increased $K^+$ concentration reduced the depressor response to adrenomedullin and acetylcholine and the simultaneous administration of both increased $K^+$ and L-NAME suppressed the dose-related decrease in renal perfusion pressure produced by both vasodilators (Fig. 4).

The administration of the prostaglandin inhibitor, indomethacin, did not modify the dose–response curves to adrenomedullin or acetylcholine (data not shown).

## 4. Discussion

The main finding we now report is that EDHF seems to play an important role in the vasodilator effect of adrenomedullin in the renal vascular bed. Our results indicate that the vasodilator response to adrenomedullin involves EDHF and NO but not the cyclooxygenase-derived mediators of vasodilation. Our results also confirm that adrenomedullin is a potent vasodilator in the isolated perfused renal vascular bed (Hirata et al., 1995; Hayakawa et al., 1999). Adrenomedullin produced a long-lasting vasodilator response, but the maximal adrenomedullin-induced depressor response was lower than that induced by acetylcholine.

The administration of L-NAME or ODQ markedly inhibited adrenomedullin-induced renal vasodilation (Figs. 2 and 3). However, a large part of the vasodilator effect of adrenomedullin was not prevented by these inhibitors. This incomplete inhibition of adrenomedullin-induced vasodilation is consistent with previously reported data (Hayakawa et al., 1999) for rat aorta and for the same preparation. The participation of the NO pathway in renal adrenomedullin-induced vasodilation seems to depend on the species studied. Thus, NO also mediated responses to adrenomedullin in the renal vascular bed of the dog (Miura et al., 1995) and the rabbit (Hjelmqvist et al., 1997), whereas NOS inhibitors were inactive in the cat kidney (Champion et al., 1997).

The depressor response to adrenomedullin in the presence of the inhibitor of guanylyl cyclase (26.4 ± 2.5%) was similar to that obtained with the NO synthesis inhibitor (24.9 ± 2.7%). However, the response to acetylcholine was significantly greater (44.7 ± 2.7%) than that obtained with the NO synthesis inhibitor (25.2 ± 0.7%), suggesting that the NO released in renal vessels in response to acetylcholine was not acting exclusively through the cGMP pathway. It is known that NO directly activates $K^+$ channels directly, both ATP-sensitive (Murphy and Brayden, 1995) and calcium-

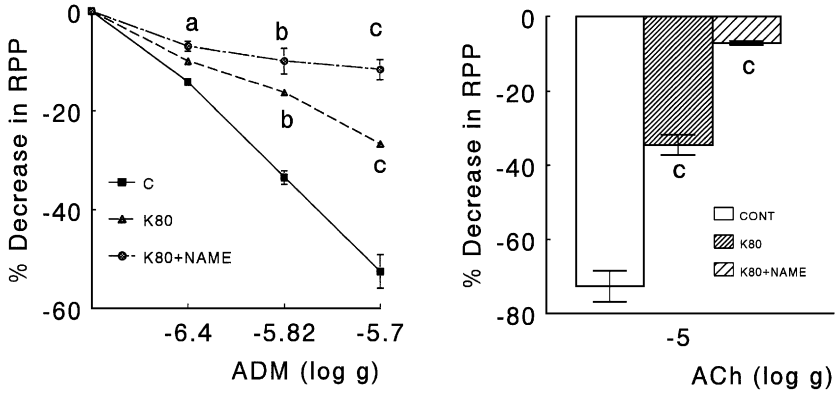


Fig. 4. Effects of $K^+$ (80 mmol/l), L-NAME ($10^{-4}$ mol/l), or L-NAME plus $K^+$ on decreases in renal perfusion pressure (RPP) in response to ADM and Ach. a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$ compared with normal conditions. Data are expressed as means ± S.E.M. ($n = 6$ in each group). Doses are expressed per gram of kidney.

dependent (Bolotina et al., 1994). Thus, it is likely that the differences observed between the L-NAME-treated and ODQ-treated preparations regarding their response to acetylcholine may be caused by the blockade of some $K^+$ channels directly activated by the release of NO.

Various authors have pointed out the importance of EDHF in the vasodilator effect of endothelium-dependent agonists (Feletou and Vanhoutte, 1999; Garland et al., 1995; Mombouli and Vanhoutte, 1997; Quilley et al., 1997). EDHF acts on vascular smooth muscle by opening $K^+$ channels, but the exact type of $K^+$ channel involved is still unclear and may vary between species (Feletou and Vanhoutte, 1999). Hence, to evaluate the activity of this factor we used increased extracellular $K^+$ and tetraethylammonium as non-specific blockers of $K^+$ channels, because tetraethylammonium inhibits voltage-dependent, $Ca^{2+}$-activated (Nagao and Vanhoutte., 1993) and ATP-dependent $K^+$ channels (Edwards and Weston., 1993). Increased extracellular $K^+$ and tetraethylammonium have been reported to inhibit strongly endothelium-dependent vasodilation in the renal vasculature (Vargas et al., 1994, 1996; Adeagbo and Triggle, 1993). The present study showed that blockade of $K^+$ channels influences adrenomedullin-induced vasodilation markedly and that the simultaneous inhibition of NO-cGMP and $K^+$ channels almost completely inhibits the depressor response to adrenomedullin in the renal vasculature. This indicates that $K^+$ channels (EDHF) are an important mechanism contributing to the vasodilator action of adrenomedullin in the renal vascular bed. This conclusion is based on the results obtained in groups treated with tetraethylammonium or high $K^+$ alone or in combination with NO-cGMP inhibitors (ODQ + tetraethylammonium and L-NAME + tetraethylammonium). First, tetraethylammonium and high $K^+$ produced marked inhibition of the adrenomedullin response. Second, in the presence of NO-cGMP inhibition, tetraethylammonium inhibited the vasodilator response to adrenomedullin, suggesting that the vasodilator response is mediated by both NO and $K^+$ channels independently of NO. Furthermore, our data indicate that the vasodilator effects of adrenomedullin may be almost fully explained by the simultaneous activity of NO and EDHF, both of which contribute equally. Because of the reported lack of specificity of tetraethylammonium and 80 mM $K^+$, the type of $K^+$ channels involved could not be identified in the present study. The results presented here show that further studies are warranted, using more specific drugs that inhibit selectively the various $K^+$ channels.

It has been reported that vasodilator prostaglandins might also contribute to adrenomedullin-induced vasodilation in rat pulmonary artery rings (Yang et al., 1996) and that prostaglandins may participate in the renal actions of adrenomedullin (Jougasaki et al., 1997). We therefore analyzed the dose–response curve to adrenomedullin in the isolated kidney after pretreatment with indomethacin. Our experiment showed that indomethacin at a dose ($10^{-5}$ M) that produces 90% inhibition of the response to these substances in rat isolated kidney (Bhardwaj and Moore., 1988) did not shift the dose–response curves to adrenomedullin (data not shown). The result did not provide evidence for a possible contribution of prostaglandins as endothelium-derived mediators of adrenomedullin-induced vasodilation in the isolated kidney. This finding is consistent with other reports that the vasodilator effect of other endothelium-dependent (Vargas et al., 1994, 1996) agonists does not differ whether the prostaglandin synthesis inhibitor, indomethacin, is present or absent.

In conclusion, our results suggest that the vasodilator response to adrenomedullin in the rat renal vasculature comprises at least two components, each responsible for approximately half the response: (1) NO-mediated vasodilation through the cGMP pathway, and (2) a remaining vasodilator effect due to EDHF activity. Our data also indicate that prostaglandins do not play a role in the vasodilator response to adrenomedullin in the rat renal vascular bed.

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