Nitric Oxide/cAMP Interactions in the Control of Rat Renal Vascular Resistance

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Abstract—This study aimed to characterize the interaction between nitric oxide (NO)- and cAMP-related pathways in the control of renal blood flow. Using the isolated perfused rat kidney model, we determined the effects of inhibition of NO formation by $N^\omega$-nitro-L-arginine methyl ester (L-NAME; 1 mmol/L) and of NO administration by sodium nitroprusside (SNP, 10 μmol/L) on renal vascular resistance under conditions of elevated vascular cAMP levels. cAMP levels were increased either by adenylyl cyclase activation via isoproterenol or by inhibition of cAMP phosphodiesterases (PDEs) 1, 3, and 4. We found that L-NAME markedly increased vascular resistance and that this effect was completely reversed by SNP. Both isoproterenol and inhibitors of the cAMP PDEs lowered basal vascular resistance. In the presence of isoproterenol (3 nmol/L) and inhibitors of PDE-1 [8-methoxymethyl-1-methyl-3-(2-methylpropyl)-xanthine; 8-MM-IBMX, 20 μmol/L] and PDE-4 (rolipram, 20 μmol/L), L-NAME again substantially increased vascular resistance, and this effect of L-NAME was completely reversed by SNP. In the presence of the PDE-3 inhibitors milrinone (20 μmol/L) and trequinsin (200 nmol/L), however, both L-NAME and SNP failed to exert any additional effects. Because PDE-3 is a cGMP-inhibited cAMP PDE and because the vasodilatory effect of SNP was abrogated by the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) (20 μmol/L), our findings are compatible with the idea that an action of NO on PDE-3 could account for the vasodilatory properties of NO on the renal vasculature. Moreover, our findings suggest that PDE-3 activity is an important determinant of renal vascular resistance. (Circ Res. 1999;84:186-192.)

Key Words: cGMP ▲ phosphodiesterase ▲ renal blood flow

During the last few years, a body of evidence has been established indicating that endothelial-derived nitric oxide (NO) is a physiologically relevant determinant of renal blood flow.1–4 Although NO does not interfere with the steady-state myogenic autoregulation of renal blood flow, it appears more as a direct vasodilator acting independently of the renal perfusion pressure. The cellular mechanisms by which NO lowers renal vascular resistance has not yet been characterized. In general, several pathways have been suggested by which NO could relax vascular smooth muscle cells, all of them involving an enhanced formation of cGMP initiated by an activation of soluble guanylate cyclase through NO.5,6 Thus, cGMP-dependent protein kinases (G kinases) may phosphorylate myosin light chain kinase and thereby attenuate the activity of this particular enzyme essential for smooth muscle cell contraction.7 G kinases may also enhance calcium extrusion from the cytosol and thus lower the concentration of the critical activator of contraction.8–11 G kinase may also activate potassium channels to hyperpolarize vascular smooth muscle and thus prevent the activation of voltage-gated calcium channels triggering transmembrane calcium influx.12,13

The relative contribution of these processes to the vasodilatory action of NO in the kidney is not known. In experiments with isolated perfused rat kidneys, it has been found that membrane-permeable G kinase activators decrease and G kinase inhibitors increase vascular resistance,14 which would be compatible with the idea that G kinase could be relevantly involved in the vasodilatory action of NO. In the same study, however, it was also noted that the NO donor sodium nitroprusside is still able to lower renal vascular resistance in the presence of G kinase inhibitors, suggesting that NO may also act via G kinase independent actions.14

Given that the vasodilatory action requires cGMP, but not necessarily G kinase activity, attention is directed to cGMP-regulated cAMP phosphodiesterases (PDEs). cAMP is also a well-characterized second messenger, mediating relaxation of vascular smooth muscle cells and increasing renal blood flow.15 It is well known that both cGMP-activated and cGMP-inhibited cAMP phosphodiesterases exist, classified as the PDE-2 and PDE-3 family, respectively.16 In view of the vasodilatory properties of NO and with regard to the vasodilatory action of elevated intracellular cAMP levels, it is conceivable that the PDE-3 family, rather than the PDE-2
family, may be involved in the signaling pathway stimulated by NO. For PDE-3, 2 subforms exist that have been cloned from rat and human libraries and were recently named as PDE-3A and PDE-3B, respectively. PDE-3A is most abundant in adipose tissues, and PDE-3B in heart and vascular smooth muscle cells. In fact, a recent in situ hybridization study has provided solid evidence for the expression of PDE-3B in the vasculature of rat kidneys. There are hints from previous studies using PDE-3 inhibitors suggesting that PDEs can influence myocardial contractility and smooth muscle relaxation via regulating cAMP pools. However, the functional relevance of this enzyme in the renal vasculature has not yet been examined.

It was our interest, therefore, to determine the relevance of cAMP PDEs for renal vascular resistance in comparison with the effect of NO. For our experiments, we chose the isolated rat kidney perfused at constant pressure, which has been demonstrated to be a valuable tool for the study of renal blood flow regulation. In this model, we examined the effects of inhibition and stimulation of NO formation alone and in combination with pharmacological modulations of cAMP levels, such as during stimulation of adenylate cyclase or specific inhibition of PDE subtypes. Moreover, we compared the mRNA abundance of different PDEs (PDE-1, PDE-2, PDE-3, and PDE-4) in preparations of afferent arterioles using RNase protection assays. Our findings suggest that among all cAMP PDEs, PDE-3B is most abundant in renal afferent vessels and that pharmacological inhibition of PDE-3 mimics the effects of NO on renal vascular resistance.

Materials and Methods

Isolated Perfused Rat Kidney

Male Sprague-Dawley rats (250 to 300 g body weight) having free access to commercial pellet chow and tap water were obtained from Charles River Germany and used for the present study. All animal experiments were conducted according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the German Law on the Protection of Animals. Kidney perfusion was performed in a recycling system. In brief, the animals were anesthetized with 150 mg/kg of 5-ethyl-(1'-methyl-propyl)-2-thiobarbituric acid (Inactin, Byk Gulden). Volume loss during the preparation was substituted by intermittent injections of physiological saline via a catheter inserted into the jugular vein. After opening of the abdominal cavity by a mid-line incision, the right kidney was exposed and placed in a thermoregulated metal chamber. The right ureter was cannulated with a small polypropylene tube (PP-10), which was connected to a larger polyethylene catheter (PE-50). After intravenous heparin injection (2 U/g), the aorta was clamped distal to the right renal artery, and the large vessels branching off the abdominal aorta were ligated. A double-barreled cannula was inserted into the abdominal aorta and placed close to the origin of the right renal artery. After ligation of the aorta proximal to the right renal artery, the aortic clamp was quickly removed, and perfusion was started in situ with an initial flow rate of 8 mL/min. The right kidney was excised, and perfusion at constant pressure (100 mm Hg) was established. To this end, the renal artery pressure was monitored through the inner part of the perfusion cannula (Statham transducer P10 EZ), and the pressure signal was used for feedback control of a peristaltic pump. The perfusion circuit was closed by draining the venous effluent via a metal cannula back into a reservoir (200 to 220 mL). The basic perfusion medium, which was taken from a thermostated (37°C) reservoir, consisted of a modified Krebs-Henseleit solution containing (in mmol/L) the following: glucose 8.7, pyruvate 0.3, l-lactate 2.0, α-ketoglutarate 1.0, l-malate 1.0, urea 6.0, and all physiological amino acids in concentrations between 0.2 and 2.0 mmol/L. The perfusate was supplemented with 6 g/100 mL BSA, 1 mg/100 mL vasopressin 8-lysine, and freshly washed human red blood cells (10% hematocrit). Ampicillin 3 mg/100 mL and floxacillin 3 mg/100 mL were added to inhibit possible bacterial growth in the medium. To improve the functional preservation of the preparation, the perfusate was continuously dialyzed against a 25-fold volume of the same composition but lacking erythrocytes and albumin. For oxygenation of the perfusion medium, the dialysate was gassed with a 95% O2/5% CO2 mixture. Under these conditions, both glomerular filtration and filtration fraction remain stable for at least 90 minutes at values of about 1 mL/min×g and 7%, respectively.24 Perfuse flow rates were obtained from the revolutions of the peristaltic pump, which was calibrated before and after each experiment. Renal flow rate and perfusion pressure were continuously monitored by a potentiometric recorder. After establishing the reperfusion loop, perfusate flow rates usually stabilized within 15 minutes. Stock solutions of the drugs to be tested were dissolved in freshly prepared perfusate and infused into the arterial limb of the perfusion circuit directly before the kidneys at 3% of the rate of perfusate flow. Renal vascular resistance was calculated as the ratio of perfusion pressure over flow rate.

Preparation of Isolated Afferent Arterioles and Cortex

Afferent arterioles from rat kidneys were isolated by a method of Chatziantoniou and Arendshorst,25 as described previously. In brief, kidneys were infused with a magnetized iron oxide suspension (1% Fe3O4 in saline) for 0.5 to 1 minute at the constant pressure of 120 mm Hg, then excised and placed in a phosphate buffer solution. Cortical tissue was homogenized and renal preglomerular vessels, glomeruli, and surrounding connective tissue were removed from the homogenate using a magnet. The vascular tissue was then passed through needles of decreasing size and sieved through a 125-μm sieve mesh screen, detaching connective tissue, removing iron oxide from the large vessels, and separating afferent arterioles from glomeruli. The vascular tissue consists primarily of afferent arterioles (<50 μm in diameter). The remaining tissue from the top of the sieve was used for subsequent RNA isolation.

For preparation of cortical tissues, kidneys were placed in a physiological salt solution on ice, and the cortex was carefully dissected from the outer medulla with a scalpel blade. Cortical tissue samples were frozen in liquid nitrogen and kept at −80°C until RNA isolation.

Extraction of RNA

Total RNA from afferent arterioles and cortex was isolated from frozen samples according to the standard method of Chomczynski and Sacchi.
RNase Protection Assays for Renin, β-Actin, and PDEs

RNase Protection Assays for renin and β-actin were done as described previously. 27,28

RNase Protection Assay for PDE-1, PDE-2, PDE-3, and PDE-4

To generate antisense RNA fragments suitable to detect the different rat PDE mRNAs, we constructed a transcription vector for each PDE. After reverse transcription (RT) of 500 ng total kidney RNA, the obtained cDNA was amplified by polymerase chain reaction (PCR). For amplification, specific primers for each PDE were used, as presented in the Table.

RT-PCR was performed as described previously 32 using standard protocols, and amplified fragments were cloned in the polylinker site of the pSP73 transcription vector (Promega) after BamHI/EcoRI digestion. Subcloning procedures of the cDNA fragments and transformation into competent EcoliDH5α cells were also performed according to standard protocols. Sequencing of the inserts was done by SEQUISERVE (Dr Metzger, Vaterstetten, Germany), and this confirmed the identity of the inserts with the published sequences. For PDE-3B, the human sequence had already been published, and sequence comparison with the GCG (Genetic Computer Group) program confirmed the sequence homology between our cloned rat PDE-3B fragment and human PDE-3B. After linearization with HindIII for PDE-1C, PDE-2A, PDE-3A, and PDE-4C and PVUII for PDE-3B, 32P-labeled antisense probes were obtained by in vitro transcription with SP6 polymerase. In vitro transcription, hybridization, and RNase protection assay were performed as described previously. 32 Protected mRNA fragments were separated on an 8% denaturing polyacrylamide gel. Radioactivity was detected and counted with an Instant Imager 2024 electronic autoradiograph (Packard). Data in counts per minute (cpm) obtained for the different PDE mRNAs depend on the length of protected mRNA fragments; therefore, mRNA data of the PDEs were normalized to the length of the protected renin fragment.

Statistics

For evaluating the significance of changes of renal vascular resistance induced by a certain experimental maneuver, all resistance values calculated within this experimental period (normally 4 values) were averaged and compared with the average values of vascular resistance of the preceding period. Student paired t test and ANOVA were used to calculate levels of significance. A value of P<0.05 was considered to be significant. For mRNA measurements, the levels of significance were calculated by ANOVA followed by Student unpaired t test. A value of P<0.05 was considered to be significant.
Results

Expression of cAMP PDE mRNA in Renal Afferent Arterioles

To obtain information about the distribution of cAMP PDEs in renal afferent resistance vessels, we compared the abundance of cAMP PDE mRNAs between preparations of pooled kidney cortex and pooled afferent arteriole preparation from Sprague-Dawley rats. As shown in Figure 1, transcripts for all PDEs examined in the present study, namely PDE-1, PDE-2, PDE-3A, PDE-3B, and PDE-4, were detected in both cortex and vessel preparation. However, only PDE-3B displayed an enrichment in the afferent vessel versus the total kidney cortex. For comparison, renin mRNA was assayed, and a 4-fold accumulation was found in the vessel preparation versus the total kidney cortex. Moreover, PDE-3B mRNA was the most abundant PDE mRNA in afferent arterioles in comparison with the other PDE subfamilies, and PDE-3B mRNA expression was ≈2-fold higher than the other cGMP-inhibited PDE-3A mRNA.

Influence of NO on Vascular Resistance in Isolated Perfused Kidneys

To characterize the role of NO for renal vascular resistance, the effects of inhibition of endogenous NO formation by Nω-nitro-L-arginine methyl ester (L-NAME; 1 mmol/L) and of exogenous administration of NO by the NO donor sodium nitroprusside (SNP; 10 μmol/L) were examined. As shown in Figure 2A, L-NAME increased basal vascular resistance from ≈8.5 to 16 mm Hg · min⁻¹ · g⁻¹ · mL⁻¹, whereas the NO donor SNP completely reversed the L-NAME effect, returning resistance to basal values in the presence of L-NAME.

Influence of NO on Vascular Resistance During Adenylate Cyclase Stimulation

To study the interference of NO-mediated vasodilatation with cAMP-induced vasodilatation, the effects of L-NAME and SNP were examined during adenylate cyclase stimulation. Stimulation of adenylate cyclase activity was achieved with the β-adrenoreceptor agonist isoproterenol. With 3 nmol/L isoproterenol, basal renal vascular resistance tended to decrease (P>0.05) (Figure 2B). L-NAME again increased vascular resistance close to 16 mm Hg · min⁻¹ · g⁻¹ · mL⁻¹, and SNP completely reversed the constrictor effect of L-NAME in the presence of isoproterenol (3 nmol/L) (Figure 2B).

Effect of NO on Vascular Resistance During cAMP PDE Inhibition

As an alternative maneuver to increase vascular cAMP levels, we used established cAMP PDE inhibitors. Rolipram
(20 μmol/L) was used to inhibit PDE-4, and as shown in Figure 3A, rolipram significantly lowered vascular resistance. In the presence of rolipram, L-NAME significantly increased vascular resistance, and this effect of L-NAME was abrogated by SNP (Figure 3A). Similar results were obtained with the PDE-1 inhibitor 8-methoxymethyl-l-methyl-3-(2-methylpropyl)-xanthine (8-MM-IBMX) (20 μmol/L), which also significantly reduced resistance but did not change the vasoconstrictor action of L-NAME nor the vasodilator action of SNP (Figure 3C).

Different results were obtained when PDE-3 activity was inhibited. The PDE-3 inhibitor trequinsin (200 nmol/L) lowered vascular resistance, as did the other PDE inhibitors. However, in marked contrast to the previously mentioned experiments, trequinsin virtually abolished any vasoconstrictor effect of L-NAME (Figure 3B). Also, the addition of SNP failed to exert any effect on vascular resistance in the presence of trequinsin (Figure 3B). Milrinone is another well-established inhibitor of PDE-3. As shown in Figure 4A, milrinone (20 μmol/L) produced the same effects as trequinsin (Figure 3B). In particular, any changes of vascular resistance by L-NAME or SNP were abolished in the presence of milrinone (Figure 4A).

Because PDE-3 cleaves not only cAMP but also cGMP, although at a slower rate, we compared the role of cGMP formation for the vasodilatation produced by SNP and milrinone. For inhibition of cGMP formation, we used the established guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ). As shown in Figure 4B, the vasodilatory effect of SNP was blunted by the guanylate cyclase inhibitor ODQ (20 μmol/L). The vasodilatory effect of milrinone was not changed by ODQ (Figure 4C).

**Effect of Angiotensin II and 8-Bromo-cGMP on Vascular Resistance During PDE Inhibition**

Because neither L-NAME nor SNP caused significant changes in renal vascular resistance after PDE-3 inhibition by trequinsin (Figure 3B) or milrinone (Figure 4A), it seemed reasonable to propose that PDE inhibitors might have impaired the regulation of vascular tone. To test the general vasoactivity after addition of PDE-3 inhibitors, we used the vasoconstrictor angiotensin II and the vasodilator 8-bromo-cGMP (8-Br-cGMP). As shown in Figure 5A, angiotensin II (1 nmol/L) significantly increased renal vascular resistance.
whereas 8-Br-cGMP (30 μmol/L) significantly lowered renal vascular resistance (Figure 5B), both during milrinone (20 μmol/L) treatment.

Discussion

The present study characterized the interference of NO with the cAMP pathway in the control of renal vascular resistance. In accordance with previous studies,1–4 our results indicate that inhibition of endogenous NO formation substantially increases renal vascular resistance, emphasizing the relevance of NO for the control of renal blood flow during basal conditions. The fact that the effects of NO synthase inhibition could be neutralized by exogenous NO donation supports the assumption that the effects of the arginine-derivative L-NAME were related to the inhibition of endogenous NO formation rather than to nonspecific effects. Our findings now show that this characteristic effect of NO on renal vascular resistance is still apparent if vascular cAMP levels are increased by adenylyl cyclase activation through isoproterenol. Similarly, in the presence of putative inhibitors of cAMP PDE-1 and cAMP PDE-4, which per se lowered basal resistance, blockade of NO formation markedly increased vascular resistance, and NO donation neutralized the vasoconstrictor effect of NO synthase inhibition.

In striking contrast, all effects of NO synthase inhibition and of NO donation on renal vascular resistance were virtually abolished by putative inhibitors of cAMP PDE-3, which themselves appeared as vasodilators. Certainly, the conclusions drawn from our experiments critically depend on the specificity of the PDE inhibitor drugs used, the modes of action presented in previous reports, and the unimpaired vascular responsiveness of the isolated perfused kidney after PDE inhibition. The latter is suggested by the findings that angiotensin II increased renal vascular resistance, and 8-Br-cGMP decreased renal vascular resistance after application of milrinone (Figure 5). An effect of NO mediated by increased intracellular cAMP would be supported if it were sensitive to blockade of adenylyl cyclase. However, despite experimental attempts, we could not test for this assumption, because we found no potent inhibitor of adenylyl cyclase for the isolated perfused kidney.

Nonetheless, we think that the comprehensive set of evidence derived from our findings converge to indicate that PDE-3 plays an important role in the regulation of renal vascular resistance, at least in rats. Thus, a comparison of mRNA expression of different cAMP PDEs in a preglomerular arteriolar preparation revealed new information about the predominance of PDE-3 over other cAMP PDEs (Figure 1). Such a significant expression of PDE-3B in rat renal vessels has previously been demonstrated by in situ hybridization experiments.19 Moreover, similar results were obtained when 2 structurally different drugs were used to inhibit PDE-3 (Figures 3B and 4A). Our results agree with the commonly held notion that NO lowers renal vascular resistance via cGMP formation (Figure 4B). Because PDE-3 is a cGMP-inhibited cAMP PDE,16 it is conceivable that NO leads to inhibition of PDE-3. In this case, pharmacological inhibition of PDE-3 would be expected to mimic the effects of NO on PDE-3, an intriguing prediction that is supported by our experiments. Taking all these arguments together, PDE-3 activity is an important determinant of renal vascular resistance that could mediate the vasodilatory action of cGMP induced by NO.

The expression of PDE-3 has been demonstrated for a variety of organs other than the kidney.19,35 In the circulatory system, including heart and blood vessels, PDE-3B appears to predominate over PDE-3A,16–18 which apparently also holds for renal afferent arterioles. Recently, it has been found that PDE-3B is more sensitive than PDE-3A toward inhibition by cGMP by about one order of magnitude,35,36 suggesting that PDE-3B is preferentially regulated by cGMP. There is accumulating evidence that in myocardium and smooth muscle cells, PDE-3B is critically involved in the vasodilatatory actions of NO.16,37–39 Our findings therefore agree with a more general concept about the interaction of the NO and cAMP pathway in the control of vascular resistance. In this view, the expression of PDE-3B would reportedly determine the efficacy of NO to induce vasodilatation. Because an impaired activity of NO in the wall of blood vessels leads to hypertension,40–42 it is conceivable that an altered expression of PDE-3B could lead to an increased tone of arterioles. For the kidney, this could be of particular relevance, because an increased renovascular resistance is considered an important reason for systemic hypertension.3,16,43 Given that the regulation of PDE-3B expression in the walls of blood vessels, including renal arterioles, is not yet known, future studies are required to investigate the regulation of the expression of this special enzyme in the context of hypertension.

Note Added in Proof

While our manuscript was in press, we realized that some uncertainty exists with regard to the nomenclature of PDE-3. To avoid misunderstanding of our data, we wish to emphasize that we termed the myocardial form of PDE-3 as PDE-3B and the adipose site form as PDE-3A.

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