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Endothelium-dependent and NO-mediated desensitization to vasopressin in rat aorta

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1 The present study was performed to characterize the tachyphylaxis of rat aortae to vasopressin. Isometric tension generated by rat thoracic aorta sliced in 4 mm rings, was recorded.

2 Tension generated by intact rings increased with cumulative additions of vasopressin up to 10 nM (1.51±0.15 g). After this concentration, most rings lost their tension and relaxed to 1.09±0.17 g ($P<0.001$) despite further addition of vasopressin. This tachyphylaxis was not observed in endothelium-denuded rings (from 2.87±0.12 g to 2.68±0.17 g).

3 Repeated administrations of supramaximal concentration (100 nM) of vasopressin confirmed an enhanced desensitization in intact rings, compared to endothelium-denuded rings. No desensitization to phenylephrine was observed in intact or in endothelium-denuded rings.

4 Dose-response curves to a V_1 receptor agonist, [Phe^2,Ile^3,Orn^8]-vasopressin, and to a V_2 receptor agonist, [deamino-Cys^1,D-Arg^8]-vasopressin, were performed in intact preparations. An increase in tension, followed by a desensitization was observed with the V_1 receptor agonist. In contrast, the V_2 receptor agonist did not induce any response.

5 Pretreatment of intact aortic rings with the cyclo-oxygenase inhibitor, diclofenac (1 μM), did not prevent the desensitization to vasopressin. In contrast, NO synthase inhibition with N^G-nitro-L-arginine (30 μM) resulted in an attenuated desensitization to vasopressin in intact rings (from 2.46±0.17 to 2.25±0.22 g, NS).

6 To confirm the involvement of NO, endothelium-denuded rings were pretreated with sodium nitroprusside (SNP). At a concentration of 10 nM, SNP induced a desensitization to vasopressin comparable with that observed in intact rings.

7 Pretreatment of endothelium-denuded rings with 8-bromo-cyclic GMP (100 μM) reduced maximum contraction to vasopressin without producing any desensitization. In contrast, guanylate cyclase inhibition with either LY 83,583 (10 μM) or methylene blue (10 μM) blocked completely the desensitization of intact rings to vasopressin.

8 The results suggest that the endothelium-dependent tachyphylaxis to vasopressin is due to rapid desensitization and is mediated by NO. However, it is unclear whether this effect of NO involves cyclic GMP.

Keywords: Vasopressin; desensitization; endothelium; nitric oxide; aortic rings; cyclic GMP; V_{1a} receptors

Introduction

Vasopressin is a neurohypophysial hormone having multiple sites of action. It acts on V_2 receptors located in the renal tubule and collecting duct to mediate an antidiuretic effect (Manning *et al.*, 1993). Vasopressin is also a potent vasoconstrictor. The V_{1a} receptors found on smooth muscle cells are responsible for the vasopressor effect of this hormone. Recent studies have suggested an important role of vasopressin in the regulation of blood pressure. For example, vasopressin released during exercise contributes to the redistribution of cardiac output by increasing vascular resistance in abdominal viscera (Stebbins & Symons, 1993). Furthermore, blockade of V_1 receptors causes a lowering of blood pressure in animal models of hypertension (Yamada *et al.*, 1994; Burrell *et al.*, 1994).

Desensitization, defined as a decrease of cellular responsiveness to a hormone following a prolonged or repetitive exposure (Hausdorff *et al.*, 1990; Bouvier *et al.*, 1995), has been observed for vascular V_1 receptors (Caramelo *et al.*, 1991; Thibonnier, 1992). We have recently observed that, in rat aorta, the presence of an intact endothelium enhances the desensitization to vasopressin. The aim of this study was therefore to characterize the mechanisms by which the endothelium influences the desensitization of smooth muscle cells to vasopressin.

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Methods

Experimental model

All experiments were performed on male Sprague-Dawley rats (300-350 g, Charles River, St-Constant, Que., Canada). Each rat was anaesthetized with CO_2 and killed by decapitation. A medial laparotomy was then performed immediately to excise the thoracic aorta. The vessel was sectioned in four 4-mm rings after being gently dissected free of fat and connective tissue. In some rings, the endothelium was mechanically removed by inserting one arm of a fine forceps into the lumen and rolling each ring back and forth ten times on a gauze soaked with physiological solution (Krebs-Henseleit buffer). Rings were then mounted into 20-ml organ baths filled with oxygenated buffer (95% O_2, 5% CO_2) and warmed to 37°C. The preparations were connected to strain gauges (Grass FT03) and isometric tension was recorded on a Grass polygraph (model 79). After being mounted, the rings were allowed to stabilize for 20 min. The preparations were then stretched gradually to an optimal 4 g resting tension, and a standard constriction was induced with 40 mM KCl. The addition of acetylcholine (1 μM) to KCl preconstricted rings confirmed either the absence (no relaxation) or presence (relaxation) of endothelium. The preparations were then allowed to stabilize for an additional 30 min before experimentation. All subsequent constrictions were expressed as a percentage of the standard constriction (40 mM KCl).

Experimental protocols

Two different experimental protocols were studied: firstly, dose-response curves to vasopressin and phenylephrine in intact (E+) and endothelium-denuded (E−) preparations, were compared in the absence or presence of a given pretreatment. The pretreating drugs were N^G-nitro-L-arginine (30 μM) and 8-bromo-cyclic GMP (100 μM) administered 30 min before the first dose of the dose-response curves, diclofenac (1 μM), LY 83,583 (10 μM), and methylene blue (10 μM) administered 15 min before, and sodium nitroprusside (SNP, 10 nM) administered only 5 min before the first dose. Secondly, repeated administrations of supramaximal concentration (10 fold) of vasopressin (100 nM) or phenylephrine (100 μM), in intact and endothelium-denuded preparations were performed. For this latter protocol, each preparation was exposed for 20 min to the administered vasoconstrictor, followed by intermittent washes performed within a 45 min stabilization period.

Drugs

Fresh solutions of acetylcholine (Sigma, St-Louis, MO, U.S.A.) phenylephrine (ICN Pharmaceuticals, Montreal, Canada), N^G-nitro-L-arginine (ICN Pharmaceuticals), and methylene blue (Fisher Scientific Limited, Montreal, Canada) were prepared in distilled water. Diclofenac (Sigma) was dissolved in NaCl solution (0.9%). SNP (ICN Pharmaceuticals) was dissolved in sodium acetate (1 mM). Stock solutions (2.3×10^{-4} M) of [Arg^8]-vasopressin (Sigma), the V_2 receptor agonist, [deamino-Cys^1,D-Arg^8]-vasopressin (Sigma), and the V_1 receptor agonist, [Phe^2,Ile^3,Orn^8]-vasopressin (Peninsula Laboratories Inc, Belmont, CA, U.S.A.), were prepared in acetic acid (0.2 N) containing 0.1% bovine serum albumen (BSA). A stock solution of 8-bromo-cyclic GMP (10 mM) was prepared in water. LY 83,583 (6-(phenylamino)-5,8-quinolinedione, RBI, Natick, MA, U.S.A.) was dissolved in dimethylsulphoxide (DMSO) to obtain a 100 mM stock solution. All subsequent dilutions of drugs were made in distilled water.

Statistical analysis

Values represent the mean ± s.e.mean. Dose-response curves were analysed by a curve-fitting analysis programme (AllFit for Windows version 2.1). Some dose response curves could not be fitted as a sigmoid, due to the loss of tension observed at higher concentrations. For these curves, Student's paired t test was used to compare maximum tension with the tension observed at the end of the curve, and Student's unpaired t test was used to compare dose-response curves to vasopressin with and without pretreatment. Probability less than 0.05 ($P<0.05$) was considered statistically significant (two-tail tests).

Results

Cumulative additions of vasopressin to endothelium-denuded rings induced increases in tension that remained sustained at high concentrations in most preparations (Figure 1). In contrast, cumulative additions of vasopressin to intact rings caused only transient increases in tension, followed by a loss of responsiveness when higher concentrations were added (Figure 1). This altered response of intact rings to vasopressin is also reflected in dose-response curves, with a right-shift and a marked reduction in maximum response, compared to endothelium-denuded rings (Figure 2). In addition, in intact preparations, tension induced by vasopressin reached a maximum value (1.51 ± 0.15 g) at 10 nM and fell thereafter despite further addition of vasopressin (1.09 ± 0.17 g, at 100 nM, $P<0.001$). In contrast, no significant loss of tension was observed in endothelium-denuded preparations (from 2.87 ± 0.12 g at 10 nM to 2.68 ± 0.17 g at 100 nM). The presence of an intact endothelium decreased maximal constriction

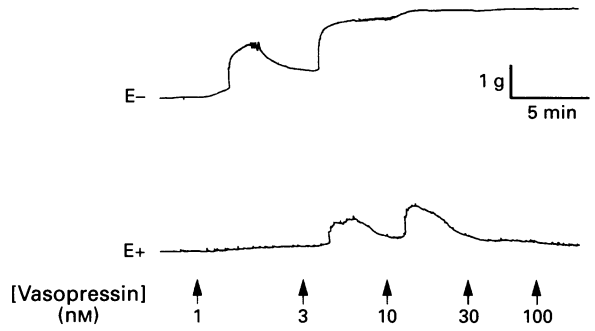

Figure 1 Original tracings of a dose-response curve to vasopressin in an intact preparation (lower panel) and an endothelium-denuded preparation (upper panel) obtained from the same aorta. The final concentration of vasopressin after each cumulative addition is marked by an arrow. Time and tension calibrations are illustrated by the horizontal and vertical bars, respectively.

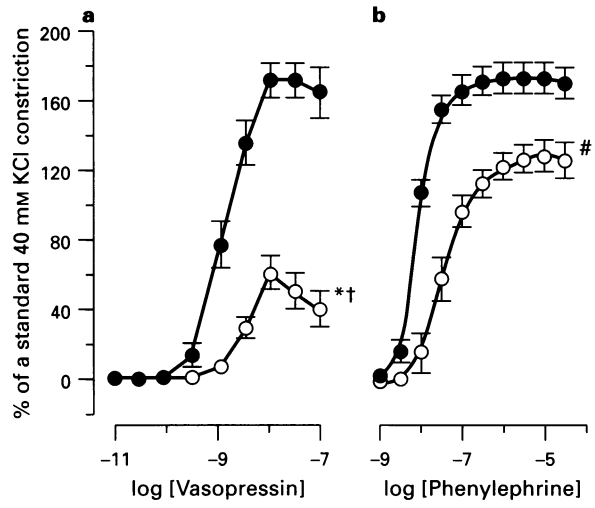

Figure 2 Dose-response curves to vasopressin (a, $n=16$), and to phenylephrine (b, $n=6-12$), in intact (○) and endothelium-denuded preparations (●). *$P<0.05$ compared with the maximal response observed at 10 nM; †$P<0.05$ compared with the corresponding response in endothelium-denuded preparations; #indicates a different ($P<0.05$) maximal response estimated by curve fitting.

and reduced sensitivity of aortic rings to phenylephrine (Figure 2). However, in contrast to the observation made with vasopressin, no loss of tension was observed with phenylephrine.

To confirm that the loss of tension to vasopressin was due to desensitization, repeated administrations of a supramaximal concentration (10 fold) of vasopressin was used. The contractile response of endothelium-denuded rings to vasopressin was significantly attenuated after a third administration, when compared with the first one (Table 1). However, a greater loss of responsiveness was observed in the presence of an intact endothelium: the contractile response to the third administration of vasopressin was 72 ± 4% of the first one in endothelium-denuded rings, compared with 16 ± 4% ($P<0.05$) in intact rings (Table 1). No reduction in the contractile response to phenylephrine was observed, in either endothelium-denuded or intact preparations (Table 1).

In intact preparations, the V_2 receptor agonist [deamino-Cys^1,D-Arg^8]-vasopressin, did not induce any contractile response (Figure 3). The dose-response curve to the V_1 receptor agonist, [Phe^2,Ile^3,Orn^8]-vasopressin, was shifted to the right compared to vasopressin, but reached a comparable maximal contraction. Moreover, cumulative addition of the V_1 receptor agonist to a concentration of 3 μM, was accompanied by a loss of responsiveness, similar to the one observed with vasopressin, with the maximal tension (1.08 ± 0.39 g) falling to 0.21 ± 0.14 g (Figure 3).

To test whether the loss of tension observed with vasopressin was caused by a prostaglandin release, dose-response curves to vasopressin were performed in the presence of an inhibitor of cyclo-oxygenase, diclofenac (1 μM). Dose-response curves to vasopressin in endothelium-denuded rings under control conditions and after pretreatment with diclofenac were superimposed (data not shown). In intact preparations, diclofenac did not prevent the loss of tension observed at the highest concentration of vasopressin (Figure 4). The contribution of NO to the endothelium-mediated loss of responsiveness to vasopressin was assessed by use of the NO synthase inhibitor, N^G-nitro-L-arginine (30 μM). In endothelium-denuded rings, dose-response curves to vasopressin under control conditions and after N^G-nitro-L-arginine pretreatment were comparable (data not shown). However, in intact preparations, the loss of tension was prevented by N^G-nitro-L-arginine, compared with untreated preparations (Figure 4). A left-shift of the dose-response curve (from an EC_{50} of 3.29 ± 0.25 to 2.22 ± 0.15 nM, $P<0.01$) and an increased maximal constriction were also observed with this pretreatment (Figure 4).

To confirm the involvement of NO, sodium nitroprusside was administered as a pretreatment in endothelium-denuded preparations in order to perform dose-response curves to vasopressin during constant formation of NO. Three concentrations of SNP (1, 10 and 100 nM) were used in this study. At a concentration of 1 nM, SNP had no effect on the contractile response to vasopressin (data not shown). In contrast, vasopressin was unable to overcome the relaxant effect of 100 nM SNP (data not shown). Pretreatment with 10 nM SNP in endothelium-denuded preparations reduced maximal constriction, and caused a rightward shift of the dose-response curve to vasopressin and phenylephrine (Figure 5) compared with control rings. In addition, pretreatment with SNP mimicked the loss of tension (from 1.02 ± 0.21 g to 0.38 ± 0.15 g, $P<0.05$) to vasopressin normally observed in intact preparations (Figure 5). No loss of tension to phenylephrine was observed with SNP (Figure 5).

To evaluate whether cyclic GMP was involved in the NO-mediated loss of responsiveness to vasopressin, pretreatment with 8-bromo-cyclic GMP (1 μM, 10 μM, 100 μM) was performed in endothelium-denuded preparations. With lower concentrations of 8-bromo-cyclic GMP (1 μM and 10 μM), dose-response curves to vasopressin were similar to those in control endothelium-denuded rings (data not shown). A reduction in maximal constriction by 100 μM 8-bromo-cyclic GMP was observed with vasopressin and a rightward

Table 1 Effect of the endothelium on the contractile responsiveness[1] to repeated administrations of vasopressin and phenylephrine

	Repeated administrations		
	first	*second*	*third*
Vasopressin (100 nM)			
E+ ($n=6$)	66.7 ± 7.7	17.5 ± 1.9*	6.8 ± 0.6*
E- ($n=6$)	138.1 ± 7.3	116.8 ± 9.4*	75.9 ± 15.9*
Phenylephrine (100 μM)			
E+ ($n=5$)	133.5 ± 14.8	139.8 ± 11.6	137.5 ± 13.3
E- ($n=6$)	157.2 ± 10.7	168.2 ± 20.2	166.7 ± 17.8

[1]Expressed as % of a standard 40 mM KCl constriction.
*$P<0.05$, compared with the first administration.

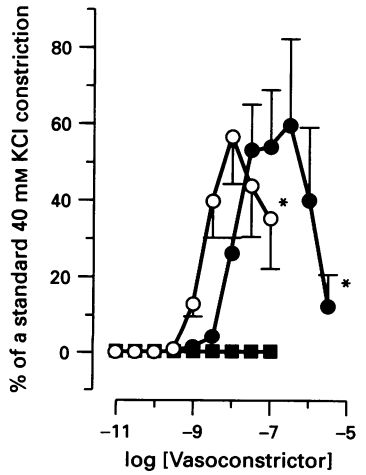

Figure 3 Dose-response curves to vasopressin (○, $n=13$), to a V_1 receptor agonist, [Phe^2,Ile^3,Orn^8]-vasopressin (●, $n=14$), and to a V_2 receptor agonist, [deamino-Cys^1,D-Arg^8]-vasopressin (■, $n=6$), in intact preparations. *$P<0.05$ compared with the maximal response (10 nM for vasopressin and 300 nM for the V_1 receptor agonist).

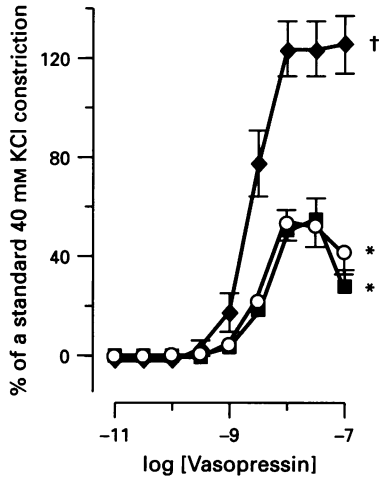

Figure 4 Dose-response curves to vasopressin in intact preparations under control conditions (○, $n=27$), and after a pretreatment with 1 μM diclofenac (■, $n=19$), or 30 μM N^G-nitro-L-arginine (◆, $n=15$). *$P<0.05$ compared with the corresponding maximal response. †$P<0.05$ compared with the corresponding response in control preparations.

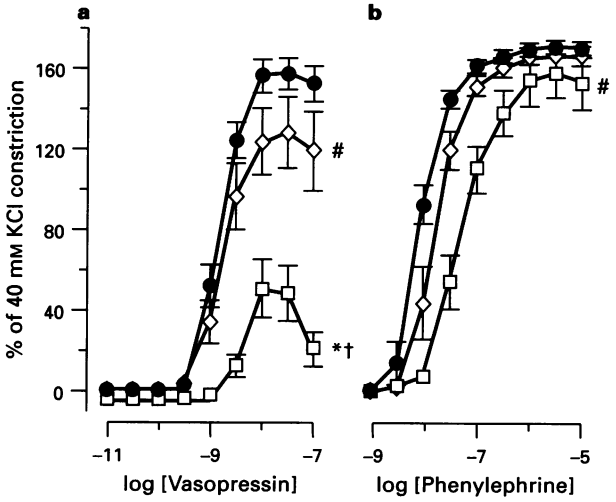

Figure 5 Dose-response curves to vasopressin (a) and to phenylephrine (b) in endothelium-denuded preparations under control conditions (●, $n=6$), and after a pretreatment with either 10 nM sodium nitroprusside (□, $n=6$) or 100 μM 8-bromo-cyclic GMP (◇, $n=12$). *$P<0.05$ compared with the maximal response observed at 10 nM; †$P<0.05$ compared with the corresponding response in control preparations; #indicates a different ($P<0.05$) maximal response estimated by curve fitting.

shift of the dose-response curve to phenylephrine was observed in the same conditions (Figure 5). However, no loss of tension was observed with 8-bromo-cyclic GMP (100 μM), either with vasopressin or with phenylephrine (Figure 5).

The involvement of cyclic GMP was assessed by use of a different approach. Pretreatment of intact rings with either LY 83,583 (10 μM) or methylene blue (10 μM) was used to inhibit soluble guanylate cyclase. Intact rings treated with the LY 83,583 vehicle (0.01% DMSO) were also studied. Intact rings treated with methylene blue vehicle (20 μl water) were used as controls. In control rings, a significant loss of tension to vasopressin was observed (Figure 6). In contrast, the dose-response curve to vasopressin in DMSO-treated rings was blunted, with no significant desensitization (Figure 6). The maximal contractile response to vasopressin was significantly reduced in LY 83,583-treated rings, compared with methylene blue-treated rings, which might be attributed to the depressant effect of DMSO (Figure 6). Inhibition of cyclic GMP formation with either LY 83,583 or methylene blue prevented the loss of tension to vasopressin (Figure 6).

Discussion

In the present study, we have demonstrated that the presence of an intact endothelium enhances the desensitization of rat aorta to vasopressin. This effect of endothelium appears to be specific, inasmuch as it was not observed with phenylephrine. Desensitization of intact rings to vasopressin was prevented in the presence of an NO synthase inhibitor. Furthermore, an exogenous supply of NO with SNP in endothelium-denuded rings mimicked the desensitization to vasopressin normally observed in intact rings. These data strongly suggest a major role of NO in the endothelium-mediated enhancement of the desensitization to vasopressin. Although we could not mimic the desensitization with 8-bromo-cyclic GMP, the data obtained with two inhibitors of guanylate cyclase would suggest a contribution of cyclic GMP in this effect of NO.

The role of the endothelium in regulation of vascular tone is well known. Dose-response curves to several vasoconstrictors in arterial rings with an intact endothelium are frequently shifted towards higher concentrations, compared with those in endothelium-denuded rings. This has been observed in the present study with both vasopressin and phenylephrine. A shift to the right of dose-response curves in intact preparations can also be observed with receptor-independent vasoconstrictors like KCl (E. Millette and D. Lamontagne, unpublished observation). Besides this physiological antagonistic effect, the endothelium can modulate the response to vasoconstrictors in a totally different way: intact preparations lost their responsiveness at the highest concentrations of vasopressin. Experiments with repetitive exposure to supramaximal concentrations of vasopressin confirmed an enhancement of vasopressin desensitization by the endothelium.

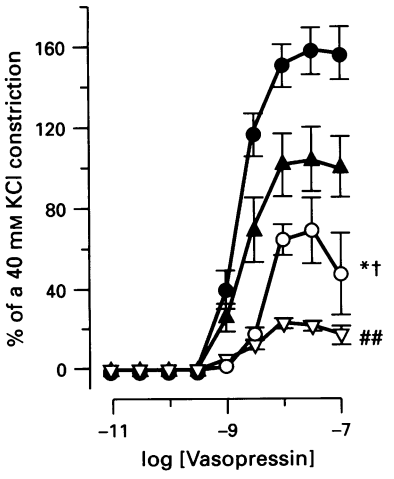

Figure 6 Dose-response curves to vasopressin in endothelium-denuded preparations after pretreatment with either LY 83,583 (10 μM, ▲, $n=6$) or methylene blue (10 μM, ●, $n=11$) and their respective vehicle-treated controls (0.01% DMSO, △, $n=6$, and water ○, $n=7$). *$P<0.05$ compared with the corresponding maximal response; †$P<0.05$ compared with the corresponding response in rings treated with methylene blue; ##indicates a different ($P<0.01$) maximal response estimated by curve fitting.

A few reports of receptor modulation by NO can be found in the literature. For example, it has been recently demonstrated that long exposure of cultured vascular smooth muscle cells to NO, either with NO-generating drugs or lipopolysaccharide treatment, decreased binding sites to labelled angiotensin II (Cahill *et al.*, 1995). Other examples of modulation of receptor-mediated responses can be found in the nervous system. Sodium nitroprusside induces a reduction in bradykinin responsiveness of peripheral sensory fibres in rats (Rueff *et al.*, 1994). NO can also decrease L-glutamate binding (Fujimori & Pan-Hou, 1991) and NMDA receptor-mediated calcium responses (Manzoni & Bockaert, 1993) in neuronal cells. To our knowledge, this is the first report of the effect of NO on desensitization to vasopressin. Furthermore, our results have important physiological significance, since they demonstrate that endogenous NO, released from a normal endothelium can, in addition to vasorelaxation, modulate the responsiveness of adjacent smooth muscle cells to circulating vasoconstrictors.

In our experiments, enhancement of desensitization to vasopressin by the endothelium could be the result of a passive basal release of NO, or an active vasopressin-induced release of NO. Vasopressin is known to produce endothelium-dependent, NO-mediated relaxation of cerebral (Cosentino *et al.*, 1993; Toda *et al.*, 1993; Suzuki *et al.*, 1993) and pulmonary arteries (Evora *et al.*, 1993), as well as in arteries of the human forearm (Tagawa *et al.*, 1993). However, using a bioassay based on the model described by Rubanyi *et al.* (1985), we were unable to demonstrate any vasopressin-induced NO release from rat thoracic aortae (E. Dumont and D. Lamontagne, unpublished observation). Furthermore, intact rat aortic rings preconstricted with 0.3 μM phenylephrine did not relax to either vasopressin (10 nM to 100 nM) or the V_2 receptor agonist, [deamino-Cys^1,D-Arg^8]-vasopressin (E. Millette and D. Lamontagne, unpublished observation). Therefore, it is likely that the endothelium-mediated enhancement of vasopressin desensitization is the result of a passive and constant basal release of NO. This is supported by our observation that a constant supply of exogenous NO with SNP in endothelium-denuded rings restored the desensitization to vasopressin. Although no attempt was made to measure the actual concentration of NO in our experiments with SNP, this agent induced a shift to the right of the dose-response curve to phenylephrine, similar to the one observed when intact rings were compared with endothelium-denuded rings. This suggests that the concentration of NO produced by SNP in our conditions is within the same order of magnitude as that passively released in an intact arterial ring.

The V_2 receptor agonist, [deamino-Cys^1,D-Arg^8]-vasopressin, did not induce any measurable constriction in rat thoracic aortic rings. Furthermore, the dose-response to the V_1 receptor agonist, [Phe^2,Ile^3,Orn^8]-vasopressin, displayed a bell shape characteristic of desensitization at higher concentrations. Therefore, desensitization of rat aorta to vasopressin may reflect desensitization of V_1 receptors. The mechanism of desensitization of V_1 receptors has been the subject of several studies. V_1 receptor desensitization results from rapid uncoupling from phospholipase C (Cantau *et al.*, 1988) followed by a decrease in V_1 binding sites (Cantau *et al.*, 1988; Grier, III *et al.*, 1989; Lutz *et al.*, 1990). The decrease in cell-surface vasopressin binding sites results most probably from receptor-mediated endocytosis and internalization of vasopressin receptors (Lutz *et al.*, 1991). Little is known about the in-

tracellular signalling pathways involved in vasopressin desensitization. It has been reported in cultured vascular smooth muscle cells that protein kinase C downregulation inhibits vasopressin desensitization (Caramelo *et al.*, 1991). In contrast, the mechanism of desensitization of cloned V_{1a} receptors expressed in *Xenopus* oocytes does not involve either protein kinase C or Ca^{2+} and cyclic AMP (Nathanson *et al.*, 1994).

Several biological responses to NO are mediated through activation of soluble guanylate cyclase and formation of cyclic GMP. The effects of cyclic GMP are numerous, including decrease in intracellular calcium (Collins *et al.*, 1986), inhibition of phosphatidylinositol hydrolysis (Rapoport, 1986) and IP_3 formation (Lang & Lewis, 1991a), and inhibition of PKC activation (Lang & Lewis, 1991b). We investigated whether the effect of NO on vasopressin desensitization involved cyclic GMP. When endothelium-denuded rings were incubated with 8-bromo-cyclic GMP, vasopressin desensitization was not restored. This would argue against a contribution of cyclic GMP. On the other hand, inhibition of soluble guanylate cyclase with either LY 83,583 or methylene blue prevented vasopressin desensitization in intact rings. The latter suggests a role for cyclic GMP. However, these inhibitors can affect the NO-cyclic GMP pathway at several levels. Besides inhibition of guanylate cyclase, both LY 83,583 (Kontos & Wei, 1993; Lee & Wurster, 1995) and methylene blue (Marczin *et al.*, 1992) can generate oxygen free radicals which could reduce the half life of NO. In addition, LY 83,583 has been reported to interfere with the release of NO (Mülsch *et al.*, 1988). Therefore, the results with guanylate cyclase inhibitors must be interpreted with caution.

The molecular mechanisms involved in vasopressin V_1 receptor desensitization are unknown. The cloned human V_{1a} receptor contains several consensus sequences for phosphorylation by protein kinases (Thibonnier *et al.*, 1994). Thus, phosphorylation of the receptor by kinases, including the cyclic GMP-activated PKG, may participate in V_1 receptor desensitization. Although highly speculative, inhibition of receptor palmitoylation by NO may provide an alternative cyclic GMP-independent mechanism. It has been reported that NO inhibits palmitoylation in cultured PC-12 cells (Hess *et al.*, 1993). Three potential sites for palmitoylation were identified in the C-terminal region of the cloned human V_{1a} receptor (Thibonnier *et al.*, 1994). It has been suggested that cyclic palmitoylation-depalmitoylation of the β_2-adrenoceptor regulates receptor coupling (Bouvier *et al.*, 1995). Therefore, inhibition of vasopressin V_{1a} receptor palmitoylation by NO could favour uncoupling of the receptor and desensitization. Clearly, additional work is required to elucidate the mechanisms involved in the NO-mediated desensitization to vasopressin.

In conclusion, the endothelium enhances the desensitization of rat thoracic aortae to vasopressin. This effect of the endothelium is mediated by NO. Conflicting results obtained with 8-bromo-cyclic GMP and the guanylate cyclase inhibitors preclude a clear conclusion on the involvement of cyclic GMP in this NO-mediated effect.

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