British Journal of Pharmacology (1995) 115, 186–190

Inhibition by zinc protoporphyrin-IX of receptor-mediated relaxation of the rat aorta in a manner distinct from inhibition of haem oxygenase

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1 Carbon monoxide (CO), produced by haem oxygenase through degradation of haem, has been claimed to be a neuromessenger and a possible regulator of vascular tone. We examined whether the haem oxygenase inhibitor, zinc protoporphyrin-IX (ZnPP) and other porphyrins affect the relaxation evoked by various agents in the rat isolated aorta.

2 Pretreatment with ZnPP (0.1 mM) virtually abolished the relaxation evoked by vasoactive intestinal peptide (VIP) and atrial natriuretic peptide (ANP). ZnPP also evoked a rightward shift of the concentration-response curve for the relaxation induced by acetylcholine.

3 In contrast, ZnPP did not affect the relaxation evoked by forskolin and 3-morpholino-sydnonimine, agents which directly activate adenylate and guanylate cyclase, respectively.

4 Although, less effective than ZnPP, tin protoporphyrin-IX (SnPP; 0.1 mM) and protoporphyrin-IX (PP; 0.1 mM) also attenuated the VIP-evoked relaxation.

5 The elevation of cyclic AMP and cyclic GMP levels evoked by VIP and ANP, respectively, were abolished by pretreatment with ZnPP (0.1 mM).

6 ZnPP, SnPP and PP did not affect the contraction evoked by phenylephrine.

7 The results show that ZnPP inhibits relaxation induced by VIP, ANP and acetylcholine, probably by interfering with membrane receptor-coupled signal transduction pathways. This inhibition does not seem to be dependent upon inhibition of haem oxygenase. The lack of specificity of the haem oxygenase inhibiting metalloporphyrins makes them less suitable as pharmacological tools in the investigation of a messenger role for CO.

Keywords: Metalloporphyrins; carbon monoxide; blood vessel; relaxation; cyclic nucleotides; second messenger; vasoactive intestinal peptide; atrial natriuretic peptide; acetylcholine

Introduction

Substantial evidence has indicated that nitric oxide (NO) is an endogenous mediator of various physiological processes in the brain and periphery (e.g. Moncada *et al.*, 1991). It was recently suggested that another gas, carbon monoxide (CO), which is produced by microsomal haem oxygenase, also may be a neuronal messenger in the brain (Maines *et al.*, 1993; Verma *et al.*, 1993). Both NO and CO are known to increase guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in various tissues (Brüne & Ullrich, 1987; Furchgott & Jothianandan, 1991). Haem oxygenase is the rate limiting step in the degradation of haem-containing compounds, resulting in the formation of CO and biliverdin (e.g. Maines *et al.*, 1993). Haem oxygenase exists in two isoforms haem oxygenase-1 and haem oxygenase-2 (Maines *et al.*, 1993). High levels of haem oxygenase or haem oxygenase-2 mRNA have been found in the rat liver, spleen and brain (Vreman & Stevenson, 1988; Verma *et al.*, 1993).

It has been speculated that CO, which shares many properties with NO, may be a regulator of vascular tone (Marks *et al.*, 1991; Schmidt, 1992). The evidence for such a view is, however, circumstantial. Like NO, exogenously applied CO has been shown to relax various isolated blood vessels and to increase cyclic GMP levels (Furchgott & Jothianandan, 1991; Moncada *et al.*, 1991; Lefer *et al.*, 1993; Zygmunt *et al.*, 1994). We have recently demonstrated haem oxygenase activity in various blood vessel homogenates, including rat aorta, by measurement of CO production using a gas chromatographic method (Grundemar *et al.*, 1995). Certain metalloporphyrins, like zinc protoporphyrin-IX (ZnPP) and tin protoporphyrin-IX (SnPP), are haem oxygenase inhibitors, of which primarily ZnPP has been used in studies suggesting a messenger role for CO in the brain and periphery (e.g. Verma *et al.*, 1993; Rattan & Chakder, 1993).

However, little is known about the specificity of these metalloporphyrins. The aim of the study was to examine whether ZnPP and other porphyrins affect relaxation evoked by agents with different modes of action in the rat isolated aorta.

Methods

Mechanical activity

Female Sprague-Dawley rats (250–300 g) were killed by CO_2 asphyxia and the thoracic aorta was dissected out. The aorta was placed in an ice-cold Krebs solution and cut into 2 mm long ring segments. The preparations were transferred to thermostatically controlled (37°C) 5 ml tissue baths containing Krebs solution bubbled with 5% CO_2 and 95% O_2, resulting in a pH of 7.4, and mounted between two L-formed hooks, one of which was attached to a force transducer (Grass FT03) for measurement of mechanical activity, and the other was connected to a sledge, which allowed adjustment of the passive tension of the vessel. The recordings were made on a Grass polygraph, 7D or 7E. The vessels were repeatedly stretched for 1 h until a stable resting tension of 8 mN was obtained. The contractile capacity was examined by adding an isotonic 60 mM potassium Krebs solution (for composition see below). In order to construct concentration-

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response curves, drugs were added in a cumulative manner. Relaxation was studied in preparations precontracted by phenylephrine (0.1–3 μM), corresponding to 50-70% of the contraction obtained by potassium Krebs solution. The potassium-induced contraction amounted to 10.6 ± 0.2 mN ($n = 86$).

Measurement of cyclic GMP and adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentrations

Cyclic nucleotide levels were analyzed in aortic segments after recording of mechanical activity. The preparations were separated into two groups, either incubated with ZnPP 0.1 mM or with vehicle only (control). The content of cyclic nucleotides in the aortic segments was measured (1) at the tension level obtained after phenylephrine-contraction, (2) after exposure to VIP 1 μM, and (3) after exposure to ANP 30 nM. When the vessels had reached a stable tension level, they were rapidly removed from the tissue bath and frozen in liquid nitrogen. The tissue was homogenized in 2 ml 10% trichloroacetic acid (TCA), with a glass-glass homogenizer, and centrifuged at 1500 *g* (4°C) for 10 min. The protein content in the pellets was determined by the method described by Bradford (1976), with bovine serum albumin used as standard. The supernatants were extracted 5 times with 5 ml of water-saturated diethyl ether. The aqueous phase was evaporated and the residue stored at −20°C. Residues were dissolved in 0.05 M sodium acetate, and the amounts of cyclic GMP and cyclic AMP were quantitated by using [^{125}I]-cyclic GMP and [^{125}I]-cyclic AMP RIA kits (RIANEN, Du Pont Company, Boston, MA, U.S.A.). [^{3}H]-cyclic AMP was added to the TCA tissue homogenate in order to determine the recovery of cyclic GMP and cyclic AMP during the ether extraction. The mean recovery was 80%.

Solutions

The normal Krebs solution used had the following composition (in mM): NaCl 119, KCl 4.6, $NaHCO_3$ 15, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11. High K^+-Krebs solution contained: KCl 60, NaCl 60, $NaHCO_3$ 15, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11.

Drugs

The chemicals were obtained from the following sources: acetylcholine (Aldrich, Steinheim, Germany), forskolin, protoporphyrin-IX, vasoactive intestinal peptide (VIP; Sigma Chemical Company, St Louis, MO, U.S.A.), atrial natriuretic peptide (ANP; rat; Peninsula Laboratories Inc, Belmont, CA, U.S.A.), 3-morpholino-sydnonimine, (SIN-1; Casella AG, Germany), ZnPP and SnPP (Porphyrin Products Inc, Logan, UT, U.S.A.). ZnPP and SnPP were dissolved in 0.2 M NaOH and PP in alcohol and 0.2 M HCl. All other drugs were dissolved in and diluted with saline. The preincubation time with ZnPP, SnPP and PP was 60 min. All experiments were performed in darkness by covering the tissue bath with a black plastic film.

Calculations and statistics

Results are expressed as mean ± s.e. mean. When the statistical difference between two means was determined, Student's unpaired two-tailed *t* test was used. $P < 0.05$ was regarded as significant. Outliers were checked for by Dixon's gap test. (*n*) refers to the number of vessels examined, each from a different animal. pIC_{50}, the negative logarithm of the concentration that evokes a 50% relaxation of the precontracted vessel, was determined by regression analysis using the values immediately above and below half maximum response.

Results

Effects of porphyrins on vascular tone

Pretreatment with ZnPP (0.1 mM) did not affect the basal tone of the rat isolated aorta. However, in phenylephrine-contracted vessels ZnPP (0.1 mM) virtually abolished the relaxation evoked by VIP and ANP (Table 1, Figure 1 a and b). In addition, ZnPP evoked a rightward shift of the concentration-response curve for the relaxation induced by acetylcholine (Table 1). In contrast, ZnPP did not affect the relaxation evoked by forskolin and SIN-1, agents which directly activate adenylate and guanylate cyclase, respectively (Table 1, Figure 2 a and b). Although, less effective than ZnPP, SnPP and PP (each 0.1 mM) also attenuated the VIP-evoked relaxation (Table 1). ZnPP, SnPP and PP had no effect on the initial contraction evoked by phenylephrine. This contraction amounted to 56 ± 2%, 54 ± 6%, 52 ± 3% and 58 ± 2% of the K^+-response in vessels treated with ZnPP, SnPP, PP, and in untreated vessels, respectively. The corresponding phenylephrine concentrations were 0.7 ± 0.2 μM, 0.7 ± 0.5 μM, 0.5 ± 0.1 μM and 0.6 ± 1.0 μM in ZnPP, SnPP, PP, and untreated vessels, respectively.

Effects of zinc protoporphyrin on cyclic AMP and cyclic GMP levels

The effects of ZnPP on cyclic nucleotide levels are shown in Figures 3 and 4. VIP, but not ANP increased the cyclic AMP level in the rat isolated aorta. Pretreatment with ZnPP (0.1 mM) abolished the VIP-evoked increase of cyclic AMP. ZnPP *per se* did not affect the basal cyclic AMP level. ANP, but not VIP increased the cyclic GMP level in the aorta. Pretreatment with ZnPP abolished the ANP-evoked increase of cyclic GMP. ZnPP *per se* did not appear to affect the basal cyclic GMP level.

Discussion

The original suggestion that endogenous CO may be a neuronal messenger was based upon the observation that haem oxygenase-2 or its mRNA was detected in certain neuronal structures in the brain and that it was associated with guanylate cyclase mRNA (e.g. Maines *et al.*, 1993;

Table 1 Inhibitory effects of porphyrins on relaxation evoked by various vasodilator agents in the rat isolated aorta

	pIC_{50} (M)	P	n
VIP	6.90 ± 0.31		
VIP + ZnPP (0.1 mM)	–	NA	6
VIP	*7.64 ± 0.22		
VIP + SnPP (0.1 mM)	*6.62 ± 0.23	<0.01	6
VIP	6.37 ± 0.16		
VIP + PP (0.1 mM)	7.10 ± 0.23	<0.05	6
ANP	8.95 ± 0.08		
ANP + ZnPP (0.1 mM)	–	NA	6
Acetylcholine	7.79 ± 0.20		
Acetylcholine + ZnPP (0.1 mM)	6.98 ± 0.21	<0.05	6
Forskolin	7.07 ± 0.11		
Forskolin + ZnPP (0.1 mM)	6.89 ± 0.12	NS	6
SIN-1	6.62 ± 0.13		
SIN-1 + ZnPP (0.1 mM)	6.61 ± 0.15	NS	7

For abbreviations, see text
*pIC_{25} value since no pIC_{50} value could be determined after SnPP pretreatment. NS not statistically significant. NA, not applicable. –, the maximum relaxation was less than 25%.

Verma *et al.*, 1993). A series of metalloporphyrins, such as ZnPP and SnPP have been used clinically for inhibition of haem oxygenase activity in efforts to prevent jaundice (Maines, 1988). ZnPP has also been used as a pharmacological tool in order to establish a messenger role for CO both in the central and peripheral nervous systems. ZnPP has for instance been shown to inhibit long-term potentiation in the rat hippocampus (Zhuo *et al.*, 1993; Stevens & Wang, 1993), to lower cyclic GMP activity in olfactory neurones (Verma *et al.*, 1993), to block glutamate-evoked effects in the rat nucleus tractus solitarii (Glaum & Miller, 1993), and to reduce depolarization-induced glutamate release in cortical synaptoneurosomes (Shinomura *et al.*, 1994). In the periphery, ZnPP has been shown to inhibit neurally mediated relaxation of the opossum anal sphincter (Rattan & Chakder, 1993), and potassium currents in human jejunal smooth muscle cells (Farrugia *et al.*, 1993). These results have been interpreted to mean that endogenous CO may be a messenger in the brain and gut.

However, concern has been raised about the specificity of metalloporphyrins (Morris & Collinridge, 1993). Some metalloporphyrins are light-sensitive and in the presence of light they are inactive on haem oxygenase (Greenbaum & Kappas, 1991). It has, however, been suggested that ZnPP is less light-sensitive than other metalloporphyrins (Vreman *et al.*, 1990). Nonetheless, we have recently shown that ZnPP (at a concentration 10 times lower than that used in the present study), when exposed to ordinary laboratory light conditions, but not in darkness, abolished acetylcholine-induced vasodilatation in a manner probably distinct from inhibition of haem oxygenase (Zygmunt *et al.*, 1994). In order to avoid unspecific effects of light-exposed ZnPP, the experiments in the present study were carried out in darkness.

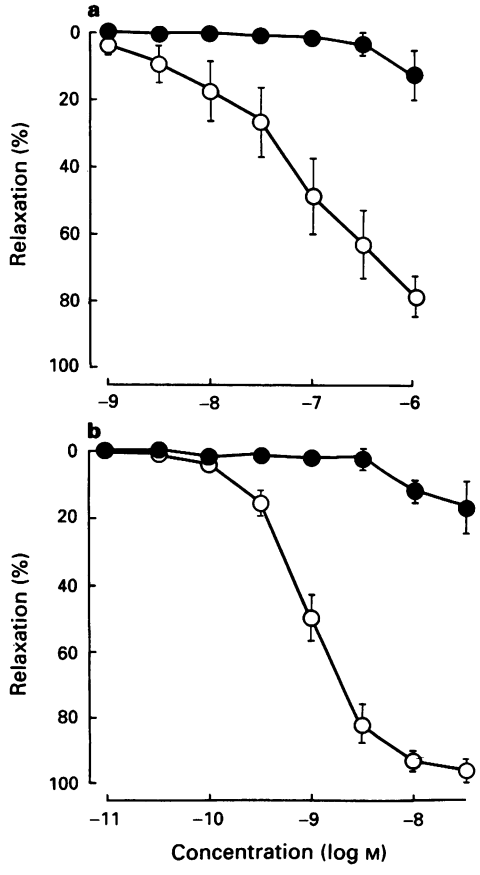

Figure 1 Effect of increasing concentrations of vasoactive intestinal peptide (VIP) (a) or atrial natriuretic peptide (ANP) (b) without pretreatment (○) and pretreated with ZnPP (0.1 mM) (●) on the rat isolated aorta precontracted with phenylephrine.

Recently, haem oxygenase activity in homogenates from the rat aorta has been demonstrated (Grundemar *et al.*, 1995). The results from the present study have shown that ZnPP virtually abolished VIP- and ANP-evoked relaxation in the rat isolated aorta. Also the acetylcholine-induced relaxation was attenuated by ZnPP. These compounds relax vessels

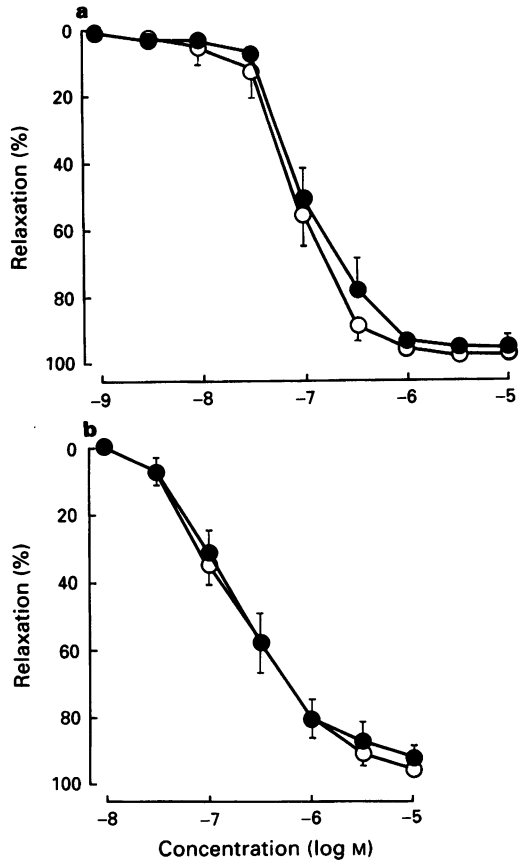

Figure 2 Effect of increasing concentrations of forskolin (a) or morpholino sydnonimine (SIN-1) (b) without pretreatment (○) and pretreated with ZnPP (0.1 mM) (●) on the rat isolated aorta precontracted with phenylephrine.

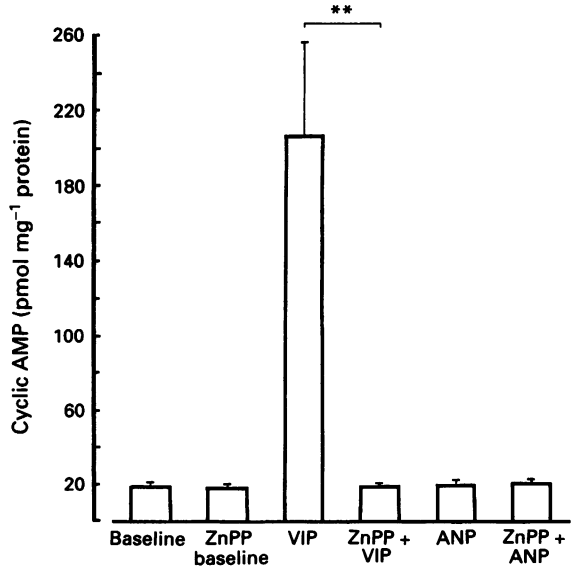

Figure 3 Concentrations of cyclic AMP (pmol mg^{-1} protein) in aortic segments after exposure to vasoactive intestinal peptide (VIP, 1 μM) or atrial natriuretic peptide (ANP, 30 nM), with or without pretreatment with ZnPP 0.1 mM. $^{**}P<0.01$, $n=6$.

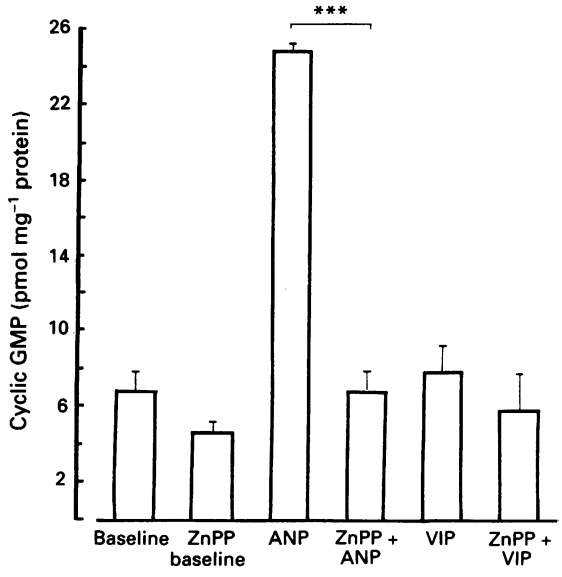

Figure 4 Concentrations of cyclic GMP (pmol mg^{-1} protein) in aortic segments after exposure to vasoactive intestinal peptide (VIP, 1 μM) or atrial natriuretic peptide (ANP, 30 nM), with or without pretreatment of ZnPP 0.1 mM. ***$P<0.001$, $n=6$.

via activation of different intracellular pathways. The VIP-induced relaxation in rat aorta is associated with activation of adenylate cyclase (Schoeffter & Stocklet, 1985), and ANP activates particulate guanylate cyclase in vascular smooth muscle cells (e.g. Winquist & Hintze, 1990). Acetylcholine releases NO from the endothelium. NO, in turn, activates soluble guanylate cyclase (Caulfield, 1993). Furthermore, we found that the VIP- and ANP-induced elevations of cyclic AMP and cyclic GMP levels, respectively, were inhibited by ZnPP. By contrast, the relaxation evoked by the stimulator of adenylate cyclase, forskolin, or the NO donator, SIN-1, was unaffected by ZnPP.

Thus, ZnPP was shown to inhibit relaxation and increases in cyclic AMP and cyclic GMP levels evoked by mediators, which act through activation of membrane-associated receptors, but not relaxation evoked by agents, which directly stimulate adenylate cyclase or guanylate cyclase. The effect was not specific for ZnPP, since SnPP and PP (the latter does not inhibit haem oxygenase) also attenuated the VIP-induced relaxation. Moreover, the phenylephrine-evoked contraction was unaffected by ZnPP, and the other porphyrins, suggesting that ZnPP did not affect the contractile capacity of the blood vessels. It seems that ZnPP interfered with relaxant but not contractile signal transduction pathways within the plasma membrane.

The concentration of ZnPP used in the present series of experiments (0.1 mM) was within the concentration-range used in studies suggesting a messenger role for CO (10 μM–2 mM ; Farrugia *et al.*, 1993; Rattan & Chakder, 1993; Verma *et al.*, 1993; Zhuo *et al.*, 1993). ZnPP and other synthetic metalloporphyrins have been shown to inhibit soluble guanylate cyclase in tissue homogenates (Ignarro *et al.*, 1984). Since ZnPP was unable to inhibit the relaxant effect of SIN-1, which activates soluble guanylate cyclase it is possible that ZnPP has a poor ability to reach this cytosol-located enzyme in the intact tissue. As there is no obvious common denominator between the receptors of VIP and ANP and the respective target protein (adenylate cyclase and particulate guanylate cyclase, respectively) and the mechanism by which acetylcholine releases NO from the endothelium, the effect of ZnPP seems to be unspecific.

Taken together, the present results suggest that ZnPP inhibits relaxation induced by VIP, ANP and acetylcholine, at least partly by interfering with receptor-coupled dilator signal transduction pathways in the plasma membrane. These effects of ZnPP seem to be distinct from inhibition of microsomal haem oxygenase. It appears that the lack of specificity of the haem oxygenase inhibiting metalloporphyrins makes them unsuitable as pharmacological tools in the investigation of a messenger role for CO.

This study was supported by the Swedish Medical Research Council (grant no 6837), the Medical Faculty, University of Lund, the Crafoord Foundation, the Royal Physiographic Society, Lund, and the Swedish Society for Medical Research, Sweden.

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(Received September 5, 1994
Revised December 23, 1994
Accepted February 3, 1995)