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Carbon monoxide-induced vasorelaxation and the underlying mechanisms

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1 Carbon monoxide (CO) induced a concentration-dependent relaxation of isolated rat tail artery tissues which were precontracted with phenylephrine or U-46619. This vasorelaxing effect of CO was independent of the presence of the intact endothelium.

2 The CO-induced vasorelaxation was partially inhibited by the blockade of either the cyclicGMP pathway or big-conductance calcium-activated K (K_{Ca}) channels. When both the cyclicGMP pathway and K_{Ca} channels were blocked, the CO-induced vasorelaxation was completely abolished.

3 Incubation of vascular tissues with hemin, in order to enhance the endogenous production of CO, suppressed the phenylephrine-induced vasocontraction in a time- and concentration-dependent manner. The hemin-induced suppression of the vascular contractile response to phenylephrine was abolished after the vascular tissues were co-incubated with either oxyhaemoglobin or zinc protoporphyrin-IX, suggesting an induced endogenous generation of CO from vascular tissues.

4 The effect of hemin incubation on vascular contractility did not involve the endogenous generation of nitric oxide.

5 Our results suggest that CO may activate both a cyclicGMP signalling pathway and K_{Ca} channels in the same vascular tissues, and that the endogenously generated CO may significantly affect the vascular contractile responses.

Keywords: Carbon monoxide; vascular smooth muscle; contraction; relaxation; cyclicGMP; K channels; haeme oxygenase

Introduction

Carbon monoxide (CO), commonly found in exhaust fumes and cigarette smoke, has been traditionally viewed as a life-threatening toxic gas (Barinaga, 1993). It inhibits intracellular oxidoreductases as well as O_2 transport from blood to tissue as it binds to haemoglobin, competing with O_2 (Coburn & Forman, 1987). Interestingly, CO also induces relaxations of vascular tissues (Coburn, 1979; Lin & McGrath, 1988; Graser *et al*., 1990) and cultured vascular smooth muscle cells (Ramos *et al*., 1989). The vascular effects of CO are not due to hypoxia or functional hypoxia (McFaul & McGrath, 1987), not mediated by prostaglandins and not related to the stimulation of adrenoceptors or adenosine (Martin *et al*., 1985; Lin & McGrath, 1989). Despite many efforts and years of investigation, the effects of exogenous or endogenous CO on different vascular tissues or vascular smooth muscle cells have not been systemically characterized and the mechanisms underlying CO-mediated vasorelaxation remain largely elusive.

Recently, haeme oxygenase, which cleaves the haeme ring to form biliverdin and CO (Marks *et al*., 1991), has been located in vascular smooth muscle cells (Christodoulides *et al*., 1995; Morita *et al*., 1995). The production of CO from rat aortic tissues has been directly measured (Cook *et al*., 1995). These studies emphasize the resemblance of CO to nitric oxide (NO), another endogenous gaseous vasorelaxant factor. Considering that the physiological vascular effect of NO has been firmly established, CO is proposed to perform both physiologically (Furchgott & Jothianandan, 1991) and pathophysiologically (Levere *et al*., 1990; Thunedborg *et al*., 1995) vasoactive functions.

In the present study, we examined the vasorelaxing effects of both exogenously applied and endogenously generated CO. Two possible mechanisms mediating the CO effects, namely, the guanosine 3′:5′-cyclic monophosphate (cyclic-GMP) signalling pathway and calcium-activated K (K_{Ca}) channels, were respectively investigated. Our results may help to establish the role of CO in a new class of endogenous gaseous vasoactive factors which can 'glide right through the membranes in their path' and transmit messages 'through membranes that have no vesicle-releasing machinery' (Barinaga, 1993).

Methods

Measurement of tension development in rat tail artery tissues

The method used was that described by Wang *et al*. (1996). Tail arteries from male Sprague-Dawley rats (150–200 g) (6–8 weeks old) were isolated. Segments of tail arteries (approximately 15 mm in length) were cut into helical strips and mounted in a 10 ml organ bath chamber filled with a Krebs bicarbonate saline (bubbled with 95% O_2/5% CO_2). These tissue strips were always stretched to a basal force of 0.7 g and equilibrated for 1 h before the experiments were begun. Indomethacin (1 μM) was routinely added to the Krebs saline which was composed of (in mM): NaCl 115, KCl 5.4, $MgSO_4$, 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 1.8. The endothelium was removed from the vascular strips with a rubbing procedure and the lack of endothelium was confirmed by the failure of acetylcholine (1 μM) to relax the tissue. In some experiments, tail artery rings of approximately 3 mm in width were prepared with intact endothelium. Those rings were mounted on two tungsten wires in a 10 ml organ bath. The tension development of vascular strips or rings was measured at 37°C with FT 03 force displacement transducers (Grass Ins. Co., Quincy). Data acquisition and analysis were accomplished with a Biopac system (Biopac Systems, Inc., Golata) including the MP100 WS acquisition units, TCI 100 amplifiers, an AcKnowledge software (3.01), universal modules, and a Macintosh computer.

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Chemicals and data process

To prepare CO solution, 20 ml of stock solution in a sealed glass tube was bubbled with a stream of CO (purity, 99.9999%; Canadian Liquid Air Ltd.) for 20 min under the pressure of 100 kPa at 37°C. One microlitre of this CO-saturated solution contains 30 ng of the gas (Vedernikov *et al.*, 1989). The stock solution of CO was freshly prepared before each experiment and then immediately diluted to the desired concentration with the bath solution. The estimated CO concentration was based on the solubility of CO at 37°C, the extent of dilution of the CO-saturated solution, and the assumption that the loss of the added CO from the bath solution at the time of our experiments was negligible. Because the assumption was not strictly correct, actual concentration of CO might be somewhat lower than the estimated concentration (Graser *et al.*, 1990). As tissue responses to CO varied, the modulation of CO effects by different agents was consistently analysed by comparing the effects of CO on the same tissue before and after different treatments. Vascular tissues were always exposed to CO in a static bath solution (not superfused). CO was removed from the bath by completely displacing the CO-containing solution with a fresh CO-free bath solution at least three times.

Zinc protoporphyrin-IX was purchased from Aldrich (Milwaukee, U.S.A.). Hemin, charybdotoxin (ChTX), apamin, methylene blue (MB), phenylephrine (PE), U-46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$), indomethacin, N^{ω}-nitro-L-arginine (L-NOARG), and acetylcholine were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Rp-8-Br-cyclicGMPS (Rp-8-bromoguanosine-3′,5′-cyclic monophosphorothioate) was from Biolog Life Science Institute (La Jolla, U.S.A.). Osmolalities and pH of all recording solutions were adjusted to 290 mOsm and 7.4, respectively.

The vasorelaxing effects of CO were evaluated by comparing the changes in the PE-induced tonic contraction force, i.e. the amplitudes of plateau phase contraction, before and after the application of CO. The data are expressed as means ± s.e.mean and analysed by using Student's *t* test or analysis of variance in conjunction with the Newman-Keuls test where applicable. The curve-fitting was performed by use of software of Microcal Origin (version 4.1, Microcal Software Inc., Northampton, U.S.A.). Group differences were considered statistically significant at the level of $P<0.05$.

Results

CO had no effect on the basal force of the vascular strips. For example, the basal forces of tail artery strips were 0.64 ± 0.03 g and 0.63 ± 0.03 g before and after CO (100 μM), respectively ($n=8$, $P>0.05$). After CO was applied to rat tail artery strips, which were precontracted with phenylephrine (PE, 1 μM), a sustained vasorelaxation occurred immediately (Figure 1a). Upon removal of CO from the bath solution, the isometric tension level of the vascular tissues before the application of CO was regained. The vasorelaxant effect of CO depended on the concentrations of CO in the bath solution with a detectable threshold concentration of 1 μM. At 1 mM, which is the saturated concentration of CO in saline, CO caused a 80.2 ± 3.4% relaxation of precontracted tissues ($n=24$). To ascertain that the CO-induced vasorelaxation was not due to antagonism of α-adrenoceptors, another vasoconstricting agent, U-46619, was used to raise the tonic tension of rat tail artery tissues. The U-46619-induced vasocontraction results mainly from the release of intracellular calcium and is independent of the adrenergic tone (Cocks *et al.*, 1993). Figure 1b shows that CO suppressed significantly the U-46619-induced vasocontraction. In the presence of CO (300 μM), the U-46619-induced tissue contraction was inhibited by 91 ± 6% ($n=4$, $P<0.01$). The vasorelaxant effect of CO was also not mediated by endothelium since an intact endothelium had been removed from the vascular strips (see Methods) before the tension measurement. Whether the presence of endothelium would modify the effect

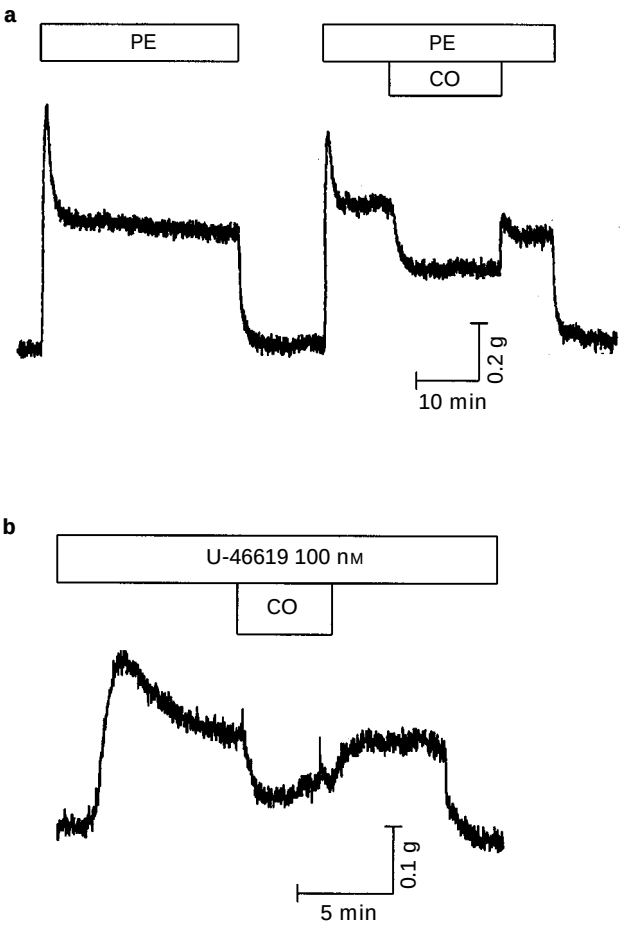

Figure 1 The effect of carbon monoxide (CO) on the isometric tension development of rat tail artery strips. (a) Actual isometric contraction record showing that CO at 300 μM reversibly relaxed tail artery strip precontracted with phenylephrine (PE, 1 μM). (b) Actual isometric contraction record showing that CO at 300 μM reversibly relaxed tail artery strip precontracted with U-46619.

of CO on smooth muscles was further studied in rat tail artery rings with intact endothelium. The presence of a functionally intact endothelium in tail artery rings was indicated by the acetylcholine (1 μM)-induced relaxation (Figure 2a). In these ring preparations ($n=4$), CO (300 μM) induced a 59 ± 10% relaxation, which was similar to a 60 ± 5.2% relaxation induced by CO at the same concentration in endothelium-free tail artery strips ($n=20$). That the CO-induced vasorelaxation may be mediated by the activation of a cyclicGMP signal transduction pathway in vascular smooth muscles was investigated by preincubating the tissues with Rp-8-Br-cyclicGMPS. The PE-induced tonic contraction forces were 0.39 ± 0.03 g and 0.42 ± 0.05 g above the resting tension level before and after pretreatment with 30 μM Rp-8-Br-cyclicGMPS ($n=8$, $P>0.05$). This Rp-8-Br-cyclicGMPS pretreatment significantly suppressed, although not completely eliminated, the CO-induced vasorelaxation (Figure 3). Figure 3b showed that Rp-8-Br-cyclicGMPS (30 μM) partially inhibited the CO-induced concentration-dependent vasorelaxation. The inhibitory effect of Rp-8-Br-cyclicGMPS was significant only when the concentrations of CO were greater than 30 μM. These data indicate that CO may relax vascular tissues via a cyclicGMP-independent pathway at lower concentrations, but may activate both cyclicGMP-dependent and cyclicGMP-independent pathways at higher concentrations.

To examine whether the partial inhibitory effect of Rp-8-Br-cyclicGMPS (30 μM) on the CO-induced concentration-dependent vasorelaxation was due to the incomplete blockade of the cyclicGMP-dependent pathway, the effect of Rp-8-Br-cyclicGMPS at 100 μM as well as the interaction of Rp-8-Br-

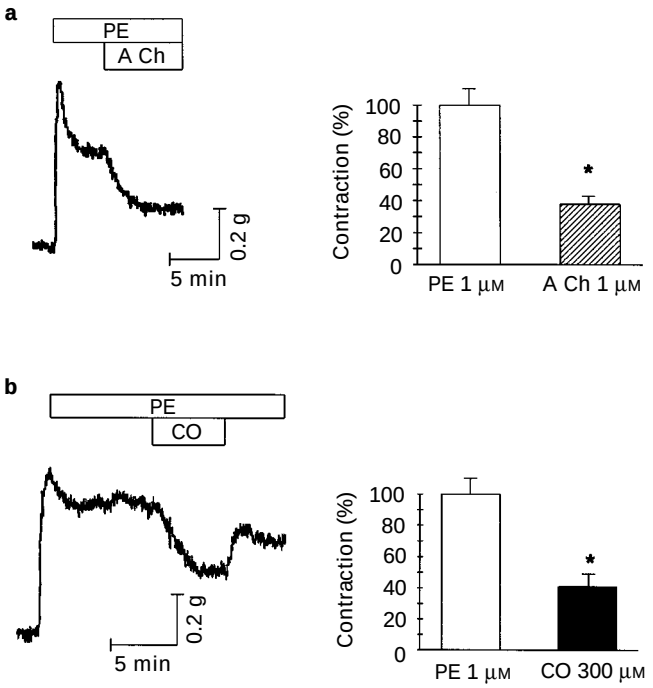

Figure 2 The relaxation of rat tail artery rings with an intact endothelium precontracted with phenylephrine (PE). (a) The acetylcholine (ACh)-induced relaxation of vascular rings (left, one actual record; right, the summary of 4 rings, $*P<0.01$). (b) The CO-induced relaxation of vascular rings (left, one actual record; right, the summary of 4 rings, $*P<0.01$).

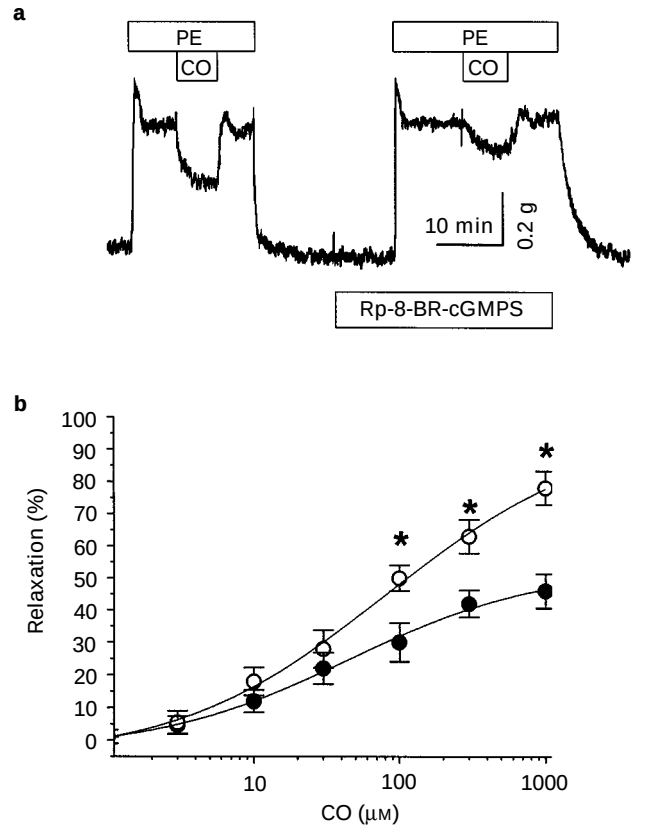

Figure 3 The inhibitory effect of Rp-8-Br-cyclicGMPS (30 μM) on the CO-induced vasorelaxation. (a) Actual isometric contraction record showing that Rp-8-Br-cyclicGMPS suppressed the CO (100 μM)-induced relaxation of a tail artery strip precontracted with phenylephrine (PE,1 μM). (b) The concentration-dependent vasorelaxation induced by CO in the absence (○) and presence (●) of Rp-8-Br-cyclicGMPS (30 μM). Vertical lines show s.e.mean; $n=8-10$ per data point. $*P<0.05$.

cyclicGMPS with methylene blue (MB), an inhibitor of soluble cytosolic guanylyl cyclase, were further studied. Figure 4 shows the results from three groups of experiments. In each set, the CO-induced relaxation of vascular strips precontracted with PE (1 μM) was first recorded. After a 30 min recovery period the tissue strips were incubated with Rp-8-Br-cyclicGMPS alone or together with MB for 1 h. Subsequently, PE (1 μM) was introduced to induce vascular contraction, and the relaxant effect of CO was tested again. The inhibitory effects of Rp-8-Br-cyclicGMPS on the CO-induced vasorelaxation were not different between the two concentration levels ($P>0.05$). This lends support to the notion that the maximum inhibition of cyclicGMP-dependent protein kinase (PKG) might have been achieved with 30 μM Rp-8-Br-cyclicGMPS (Romey *et al.*, 1984; Thunedborg *et al.*, 1995). The CO-induced relaxations of the vascular tissues pretreated with Rp-8-Br-cyclicGMPS plus MB and the tissues pretreated with Rp-8-Br-cyclicGMPS alone were also not significantly different (Figure 4), further supporting the contention that maximal inhibition of the cyclicGMP pathway was produced by Rp-8-Br-cyclicGMPS at 30 μM.

Calcium-activated K channels play an important role in the regulation of vascular tone. To examine whether the CO-induced vasorelaxation was due to the opening of K_{Ca} channels in vascular smooth muscles, the effects of charybdotoxin (ChTX) or apamin were examined. The vascular tissues were pretreated with ChTX (300 nM) or apamin (100 nM) for 60 min. Subsequently, the CO (300 μM)-induced relaxation of the vascular strips precontracted with PE (1 μM) was examined. ChTX had no effect on either the resting tension level or the PE-induced tonic contraction (Figure 5a). In the presence of ChTX, the CO-induced vasorelaxation was reduced significantly from $46\pm6\%$ to $22\pm8\%$ ($P<0.05$) (Figure 5b). Co-application of ChTX with Rp-8-Br-cyclicGMPS (30 μM) to pretreat the tissues completely inhibited the relaxant effect of CO (Figure 6). In contrast, apamin had no effect on the CO-induced vasorelaxation. Also, the inhibitory effect of Rp-8-Br-cyclicGMPS (30 μM) on the CO-induced vasorelaxation was not potentiated by apamin (Figure 6).

In the next series of experiments, we examined whether endogenously generated CO affected vascular tone. Tail artery strips were pre-incubated in the dark for 2 h with hemin (4 μM). It was expected that hemin would induce the inducible

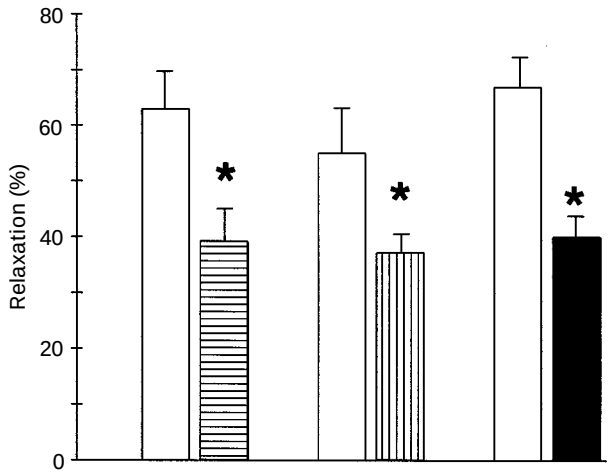

Figure 4 The inhibitory effect of Rp-8-Br-cyclicGMPS on the CO (300 μM)-induced vasorelaxation. The vascular strips were pretreated with Rp-8-Br-cyclicGMPS alone or together with methylene blue (MB). The CO-induced relaxations of PE (1 μM)-precontracted strips were compared before (open columns) and after different pretreatments. Horizontal hatched column, vertical hatched column, and solid column represent pretreatments of vascular tissues with Rp-8-Br-cyclicGMPS (30 μM), Rp-8-Br-cyclicGMPS (100 μM), and Rp-8-Br-cyclicGMPS (30 μM) plus MB (3 μM), respectively. $*P<0.05$; $n=8$ for each group.

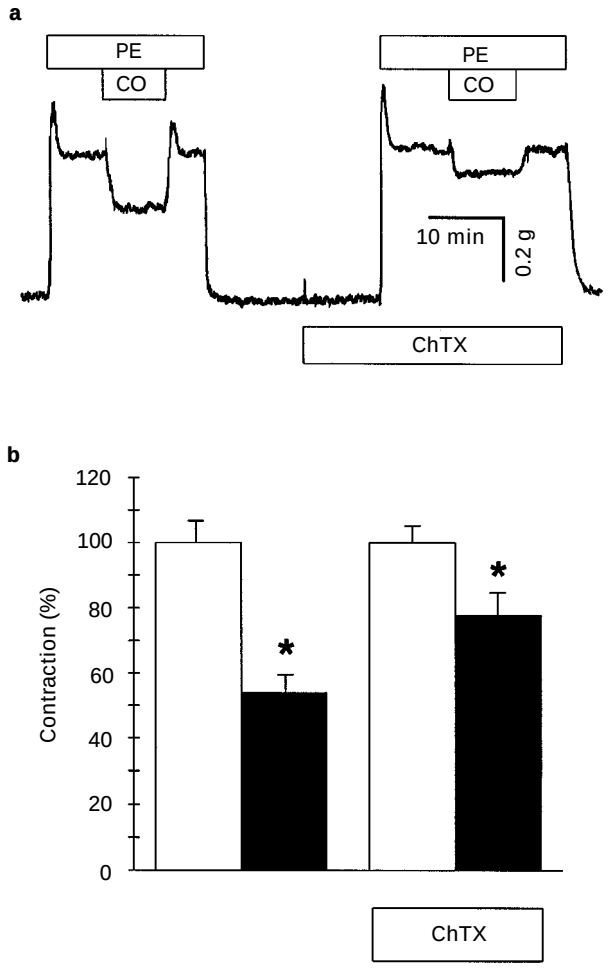

Figure 5 The inhibitory effect of charybdotoxin (ChTX, 300 nM) on the CO-induced vasorelaxation. (a) Actual isometric contraction record showing that ChTX suppressed the CO (100 μM)-induced relaxation of the vascular strip precontracted with PE (1 μM). (b) Summary of the effect of ChTX on the vasorelaxation induced by CO (100 μM, $n=8$). The tonic contractions of vascular tissues induced by PE before and after the application of CO were represented by open and solid columns, respectively. $^*P<0.05$.

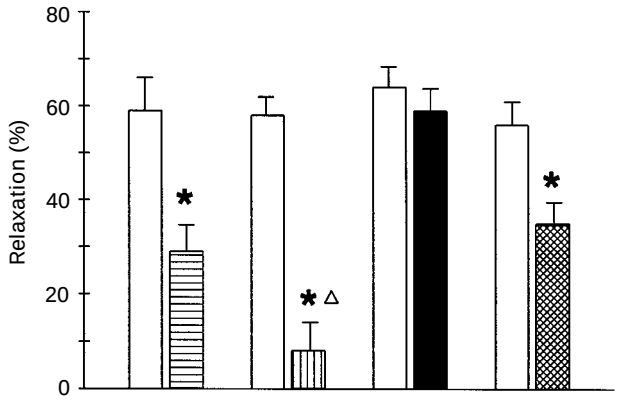

Figure 6 The modulation of the CO-induced vasorelaxation by different agents. The CO-induced relaxations of PE (1 μM)-precontracted strips were compared before (open columns) and after different pretreatments. Horizontal hatched column, vertical hatched column, solid column, and cross-hatched column represent pretreatments of vascular tissues with ChTX (300 nM), ChTX plus 8-Br-cyclicGMPS (30 μM), apamin (100 nM), and apamin plus Rp-8-Br-cyclicGMPS (30 μM), respectively. $^*P<0.05$ (comparison between open columns and hatched columns). $^{\triangle}P>0.05$ (comparison between the PE-induced tonic contraction levels in the absence and presence of CO). $n=8$ for each group.

form of haeme oxygenase, thus promoting the endogenous generation of CO from vascular wall. However, this short period of incubation did not alter the PE-induced vascular contraction (not shown). Subsequently, we prolonged the incubation period to 6 h. Control experiments showed that a 6 h incubation of vascular tissues in normal bath solution did not change the resting tension level or the PE-induced concentration-dependent vasocontraction (Figure 7a). Interestingly, the concentration-dependent vasocontraction induced by PE was significantly suppressed by incubating tail artery tissues with hemin (4 μM) for 6 h (Figure 7b). Different effects of hemin at different incubation periods suggest that hemin may not have a direct effect on vascular contractility but may induce the endogenous production of CO in a time-dependent manner. At a higher concentration (20 μM), the inhibitory effect of hemin on the PE-induced concentration-dependent vasocontraction was further potentiated (Figure 7c).

To elucidate the involvement of endogenously produced CO in the effect of hemin incubation, vascular tissues were incubated with both hemin and oxyhaemoglobin. This co-incubation abolished the shift in the PE dose-response curve induced by hemin incubation alone (Figure 8a). As oxyhaemoglobin can scavenge both CO and NO, we tested whether the effect of hemin incubation could be related to the induced release of NO from vascular smooth muscles treated with L-NOARG to inhibit NO synthesis. Incubation with L-NOARG alone had no effect on the basal contractile force of the endothelium-free vascular tissues (not shown). Figure 8b shows that the vascular contractile response to PE after incubation with both hemin and L-NOARG (30 μM) was still suppressed significantly, thus ruling out the contribution of endogenous NO to the vascular effect of hemin. If hemin acts specifically on haeme oxygenase, the blockade of this enzyme should abolish the effect of hemin. This hypothesis was confirmed in our study, as shown in Figure 8c. The PE-induced concentration-dependent vascular contraction was not changed by co-incubation of vascular tissues with hemin (20 μM) and zinc protoporphyrin-IX (10 μM). It should be pointed out that pre-incubating vascular tissues in the dark with zinc protoporphyrin-IX (10 μM) for a period of 2 to 6 h did not change the PE-induced vasocontraction (not shown).

Discussion

CO-induced vasodilatation has been observed for decades (Coburn, 1979; Duke & Killick, 1952), far preceding the discovery of NO-induced vasodilatation. However, it is only since the vascular effects of NO have become increasingly clear that the vascular effect of CO has come back into light. This was inspired partially by the concept of a new class of gaseous vasoactive factors, to which NO and CO may belong; and partially by the discovery of a wide distribution of haeme oxygenase subtypes in various cell types and by the direct measurement of CO production from vascular smooth muscle cells (Christodoulides *et al*., 1995; Cook *et al*., 1995; Morita *et al*., 1995). From our present study and other documented studies, the vascular effect of CO appears to be similar to that of NO. Both gases can be generated from the vascular wall via the constitutive and inducible forms of haeme oxygenase or NO synthases, respectively. Both relax vascular tissues and activate the cyclicGMP pathway. Therefore, whether the vascular effects of exogenously applied CO were due to contamination by trace NO of the commercial CO gas used in our experiments should be addressed. The possibility is unlikely if not impossible. During the preparation of CO-saturated solution, the air-tight container was not purged of O_2. Therefore, that NO may remain in such a preparation of CO stock solution is unrealistic, even if there were trace amounts of NO in commercial CO gas. Furthermore, in our experiments CO-saturated solution was directly added in a bolus into the organ bath, not continuously superfused. The vasorelaxation was sustained as long as CO was present. This observation can be

properly explained by the chemical stability of CO, but cannot be due to contamination by NO since NO only exerts transient effects due to its short life span (seconds).

Remarkable differences between the vascular effects of CO and NO do exist. While the generation of NO is endothelium-dependent, that of CO is not dependent on the presence of an intact endothelium. Both NO and CO participate in the regulation of vascular tone, but with different approaches. It is possible that endothelium-derived NO relaxes smooth muscles in a transient and fast reacting manner, whereas smooth

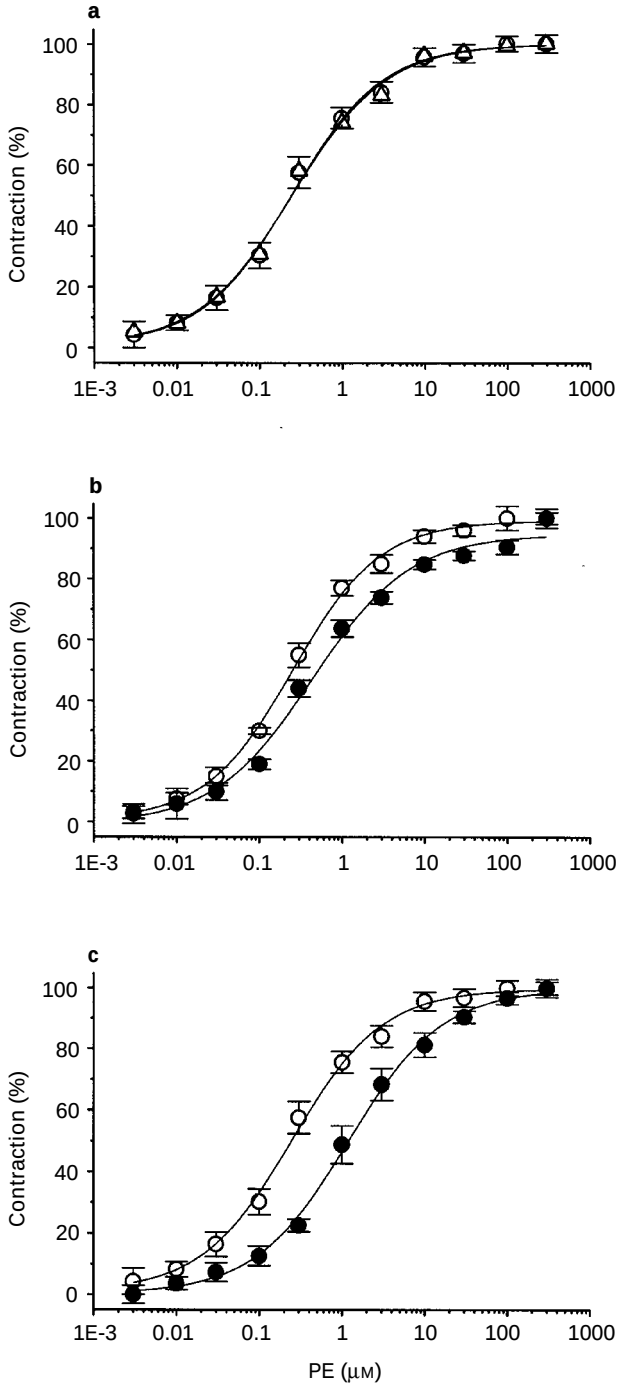

Figure 7 The concentration-dependent vasocontraction induced by phenylephrine (PE) after a 6 h incubation. (a) The contractile responses of vascular tissues to PE before (○) and after (△) incubation in the normal bath solution were not different. (b) Hemin (4 μM) incubation (●) of vascular tissues significantly inhibited the PE-induced vasocontraction. (c) Hemin (20 μM) incubation (●) of vascular tissues significantly inhibited the PE-induced vasocontraction. $n=8$ per data point.

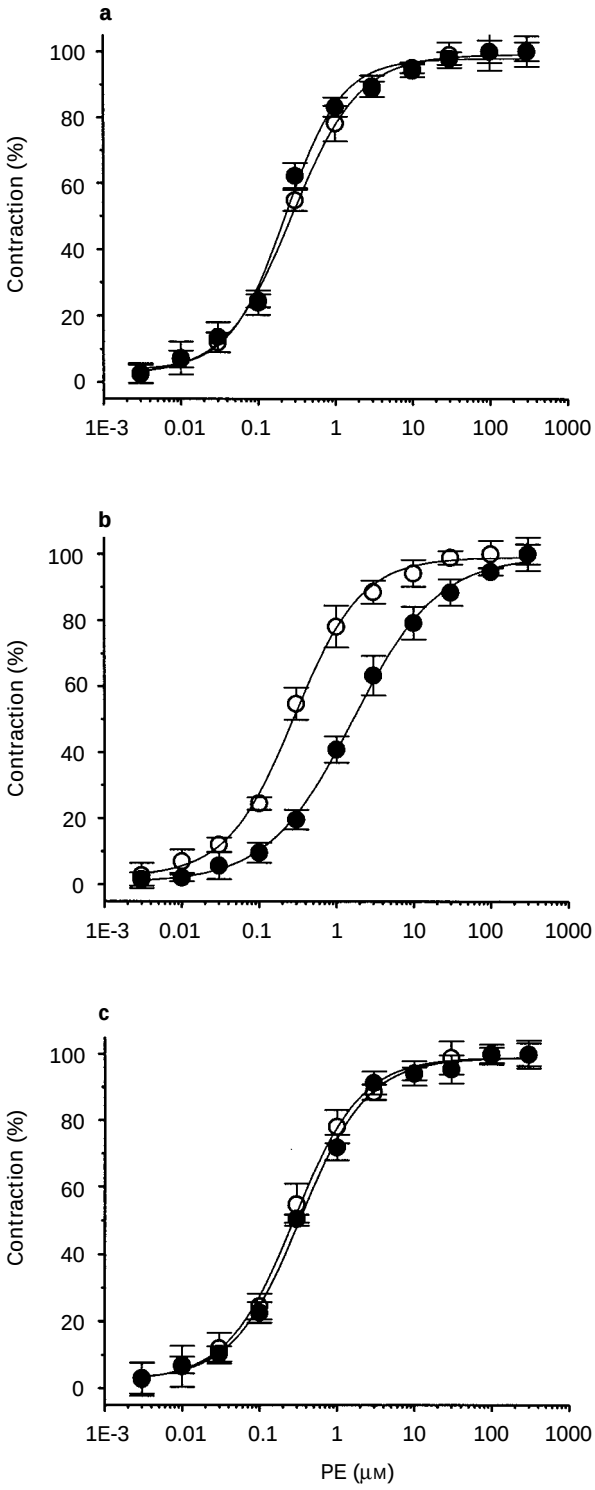

Figure 8 The effects on the phenylephrine (PE)-induced vasocontraction of hemin (20 μM) incubation 6 h after different treatments. (a) The inhibition of the PE-induced vasocontraction by hemin incubation was abolished by co-incubation with oxyhaemoglobin (1 μM), which was added to the bath solution during the last hour of hemin incubation. (b) The inhibition of the PE-induced vasocontraction by hemin incubation in the presence of L-NOARG (30 μM). (c) The inhibition of the PE-induced vasocontraction by hemin incubation was abolished by co-incubation with zinc protoporphyrin-IX (10 μM). The tonic contractions of vascular tissues induced by PE before and after incubation with hemin plus oxyhaemoglobin (a), hemin plus L-NOARG (b), or hemin plus zinc protoporphyrin-IX (c), are represented by (○) and (●), respectively. $n=8-10$ per data point.

muscle-derived CO exerts an autocrine and/or paracrine vascular effect to provide a long-lasting and slow reacting mechanism. In certain physiological or pathophysiological situations, NO and CO may work together to coordinate the fine tuning of vascular tone. Under other conditions, the functional role of one gas may prevail over the other. For instance, the increased accumulation of cyclicGMP in vascular smooth muscle cells in hypoxia was mainly due to the increased production of CO, not NO, in smooth muscle cells (Morita *et al*., 1995). In the case of CO-induced hypoxia, the cellular production of NO may be increased to dilate blood vessels to optimize the cellular oxygen supply.

Our study demonstrated that CO effectively relaxed precontracted rat tail artery. This effect of CO was consistently observed irrespective of whether the tissue was precontracted with PE to raise the adrenergic tone, or with U-46619 to promote intracellular calcium release. Furthermore, the effect of CO was not mediated by endothelium as in endothelium-free vascular strips and in endothelium-intact vascular rings CO induced a reduction in the isometric contraction force to a similar extent. During the course of our experiments, we experienced a variance in the tissue responses to CO. However, in the same tissue, CO always produced concentration-dependent relaxations with a threshold concentration as low as 1 μM. As indicated previously, the concentration of CO was calculated based on ideal conditions. The actual concentrations of CO applied to the vascular tissues in our experiments might be lower than expected. Although the generation of CO from rat (Cook *et al*., 1995) and human (Grundemar *et al*., 1995) vascular smooth muscles *in vitro* has been determined, the physiological concentration of CO in the adjacent neighbourhood of vascular smooth muscle cells *in vivo* is still unknown, as with NO. Nevertheless, studies in the brain tissues (Nathanson *et al*., 1995) have indicated that the concentrations of CO produced *in vivo* are higher than that of NO and are at least as great as those used *in vitro* to stimulate guanylyl cyclase (1–200 μM). Whether the effective concentration (equal or higher than 1 μM) of CO to induce vasorelaxation is within the physiological range or not needs to be investigated further.

Both inducible (HO-1) and constitutive (HO-2) forms of haeme oxygenase have been identified in aortic smooth muscles (Christodoulides *et al*., 1995). While hemin can induce HO-1, zinc protoporphyrin-IX can inhibit both HO-1 and HO-2. In our study, endogenous production of CO was promoted experimentally by incubating vascular tissues with hemin. Due to the technical limitation, we were not able to measure directly and acutely the basal production of CO from vascular tissues, while maintaining their functional integrity. Therefore, the reduced vascular contractile responses to PE stimulation after hemin treatment have to be interpreted as indirect evidence of endogenous production of CO. It is known that hemin at micromolar concentration range would easily permeate cell membrane bilayer (Kirschner-Zilber *et al*., 1982). Our data show that a 2 h incubation of vascular tissues with hemin 4 μM did not affect the vascular contractile response to PE. This observation not only indicates that the induction of HO-1 in rat tail artery needs a longer incubation period but also rules out a direct toxic effect of hemin on vascular contractility. It has been shown that the cellular effects of hemin are both time- and concentration-dependent (Kirschner-Zilber *et al*., 1982). For example, incubating rat aortic smooth muscle cells for 15 min with hemin (20 μM) failed to induce a significant increase in the cellular cyclicGMP content, whereas a 24 h incubation did. In our experiments, when the incubation time with hemin (4 μM) was prolonged to 6 h (Figure 7b), a significant inhibition of the vascular contractile responses to PE was recorded. An even stronger inhibition was observed with a 6 h incubation of vascular tissues with hemin at 20 μM. These results suggest that the endogenous production of CO was induced by hemin, which may partially antagonize the contractile effect of PE. Moreover, the reduced efficiency of PE after hemin incubation to contract vascular tissues could be reversed by co-incubation of vascular tissues with oxyhaemoglobin. This provides further evidence supporting the endogenous production of CO since our observations can be interpreted by assuming the scavenge of endogenously generated CO by oxyhaemoglobin. We also examined the inhibitory effect of zinc protoporphyrin-IX on HO-1 and HO-2 (Zhuo *et al*., 1993) by use of our bioassay method. Incubation of rat tail artery tissues *in vitro* with zinc protoporphyrin-IX alone did not elicit significant changes in the PE-induced vasocontraction. This indicates that the basal production of CO or the expression of HO-2 in rat tail artery tissues under our experimental conditions may not be significant enough to exert a visible vascular effect. That the co-incubation of vascular tissues with hemin and zinc protoporphyrin-IX abolished the inhibitory effect of hemin incubation alone on the PE-induced vasocontraction, on the other hand, supports the possibility of specific effects of both hemin and zinc protoporphyrin-IX on HO-1. The involvement of endogenous NO production from vascular tissues after hemin incubation can be excluded, since the presence of L-NOARG in the incubation solution did not inhibit the hemin-induced reduction of the effect of PE (Figure 8b). Together with previous results on the endogenous vascular production of CO (Cook *et al*., 1995; Christodoulides *et al*., 1995), our data provide functional evidence, albeit indirect, for the vascular effect of endogenously produced CO.

In the present study, we examined two possible mechanisms for the vascular effects of CO, i.e. the increase in cyclicGMP levels in smooth muscle cells and the enhancement of K channel activities.

The CO-induced increase in cyclicGMP content of smooth muscle cells has been well documented (Graser *et al*., 1990; Furchgott & Jothianandan, 1991; Trischmann *et al*., 1991). CO activates soluble guanylyl cyclase via a stimulating interaction with the haeme in the regulatory subunit of guanylyl cyclase (Trischmann *et al*., 1991). The increased cyclicGMP would consequently decrease the intracellular free calcium concentration of smooth muscle cells (Lincoln *et al*., 1994) through the inhibition of inositol triphosphate (IP_3) formation, the activation of Ca^{2+}-ATPase, and the inhibition of voltage-dependent calcium channels. In rat tail artery tissues, CO also acts on the cyclicGMP signal transduction pathway. Rp-8-Br-cyclicGMPS is a membrane permeable and specific inhibitor of cyclicGMP-dependent protein kinase (Nakazawa & Imai, 1994). In our experiment, Rp-8-Br-cyclicGMPS at a maximal dose, or RP-8-Br-cyclicGMPS plus MB inhibited partially the CO-induced vasorelaxation. These results not only indicate that the vascular effect of CO is mediated by cyclicGMP-dependent protein kinase, but also suggest that other cyclicGMP-independent mechanisms may be involved since a portion of the effects of CO was resistant to the modulation of the cyclicGMP pathway.

To address the nature of the cyclicGMP-independent vascular effects of CO, we set out to explore the role of K channels. Since smooth muscle cells have a high input resistance, K^+ efflux resulting from the activation of even a small number of K channels will hyperpolarize cell membranes. The membrane hyperpolarization will not only inactivate voltage-dependent calcium channels, but also inhibit agonist-induced increase in IP_3 and reduce Ca^{2+} sensitivity, leading to vasorelaxation. Apamin is a specific blocker for small conductance K_{Ca} channels (Romey *et al*., 1984). The inability of apamin to affect the CO-induced vasorelaxation in our study indicates the lack of involvement of small conductance K_{Ca} channels in the vascular effect of CO. ChTX has been widely used as a selective inhibitor of big conductance K_{Ca} channels, and it inhibited the cyclicGMP-independent vasorelaxing effect of CO in our study. Taken together, our results strongly suggest that the activation of big conductance K_{Ca} channels constitutes an important mechanism for the CO-induced vasorelaxation. It is known that a hydrogen bond can be formed between CO and imidazole group of histidine residues, hence affecting the protein function (Yang & Phillips, 1996). If a similar mechanism is applied to K_{Ca} channel protein, a chemical inter-

action between CO and certain amino acid residues may be responsible for the reversible activation of K_{Ca} channels by CO. Interestingly, it has been shown that CO decreases K_{Ca} channel currents in smooth muscle cells isolated from urinary bladder of the guinea-pig (Trischman *et al*., 1991). The effect of CO has been hypothesized as a consequence of the CO-induced decrease in intracellular free calcium concentration. However, a decrease in K_{Ca} channel current would lead to membrane depolarization and muscle contraction, at least to abort muscle relaxation. It seems difficult to correlate the inhibitory effect of CO on K_{Ca} channels with the CO-induced muscle relaxation. The discrepancy between our results and the study by Trischman *et al*. (1991) may be due partially to the tissue difference, since the functional regulation and ion channel distribution may be quite different between visceral smooth muscles and vascular smooth muscles.

In conclusion, the present study shows, for the first time, the vasorelaxant effect of CO on rat tail artery smooth muscle, which is often used as a model of a peripheral resistance vessel. Many studies have demonstrated various effects of CO on the cyclicGMP pathway in different tissue preparations, but whether the vascular effect of CO is solely dependent on cyclicGMP pathway is not clear. Our study demonstrated the co-existence of a cyclicGMP-dependent pathway and a cyclicGMP-independent mechanism in the same vascular tissue, both of which are responsible for the CO-induced vasorelaxation. An increase in cellular cyclicGMP levels under conditions of enhanced endogenous CO formation has been shown previously. However, the functional significance of the endogenous CO has not been established to date. We established a bioassay, in the present study, to monitor indirectly the vascular effect of endogenously generated CO. Our results represent the first observation that endogenously generated CO plays a significant modulatory role in the regulation of vascular contractile responses. Since significant changes in the generation of endogenous CO have been observed in different pathophysiological situations, such as hypoxia (Morita *et al*., 1995), subarachnoid haemorrhage (Matz *et al*., 1996), hypertension (Levere *et al*., 1990) or chronic uraemia (Thunedborg *et al*., 1995), our results may also help to understand better these CO-related vascular abnormalities.

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