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Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme

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It took at least a decade to realize that the toxic gas NO is the physiological activator of soluble guanylyl cyclase (sGC), thereby acting as a signaling molecule in the nervous and cardiovascular systems. Despite its rather poor sGC-activating property, CO has also been implicated as a physiological stimulator of sGC in neurotransmission and vasorelaxation. Here, we establish YC-1 as a novel NO-independent sGC activator that potentiates both CO- and NO-induced sGC stimulation. As this potentiating effect is also observed with protoporphyrin IX which activates sGC independently of a gaseous ligand, we conclude that stabilization of the enzyme's active configuration is the underlying mechanism of YC-1's action. Moreover, the results obtained with YC-1 reveal that CO is capable of stimulating sGC to a degree similar to NO, and thus provide the molecular basis for CO functioning as a signaling molecule.

Keywords: carbon monoxide/cGMP/nitric oxide/sensitization/soluble guanylyl cyclase

Introduction

Nitric oxide (NO) has been implicated in a wide range of physiological functions including regulation of neuronal transmission (Garthwaite *et al.*, 1988; Snyder, 1992; Zhou *et al.*, 1993; Hawkins *et al.*, 1994) and smooth muscle tone (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Moncada and Higgs, 1995). Enzymatic formation of NO is catalyzed by the family of NO synthases (Marletta, 1994; Nathan and Xie, 1994). The constitutive neuronal and endothelial isoforms produce NO as a messenger molecule in a Ca^{2+}-dependent manner in response to various receptor agonists or physical stimuli.

Soluble guanylyl cyclase (sGC) is the major receptor for NO. By catalyzing the formation of cGMP from GTP (Arnold *et al.*, 1977; Waldman and Murad, 1987; Garbers and Lowe, 1994), sGC mediates many of the effects of NO as a signaling molecule in the nervous and cardiovascular systems. In contrast to the membrane-bound GC which has a homomeric structure, sGC is a heterodimeric enzyme ($\alpha_1\beta_1$) containing a non-covalently bound prosthetic heme group necessary for NO-induced stimulation (Ignarro *et al.*, 1982a). A recent report shows that the homologous C-terminal regions of the subunits, also conserved in the membrane-bound guanylyl cyclases and in the cytosolic domains of the adenylyl cyclases, are sufficient for the formation of cGMP, whereas the less conserved N-termini of the α and β subunits are required for NO responsiveness (Wedel *et al.*, 1995).

Spectral studies of sGC reveal a five-coordinated heme with a His residue as the axial ligand (Stone and Marletta, 1994). The His105 of the β_1 subunit is a possible candidate for the axial ligand of the heme group, as substitution with Phe resulted in an NO-insensitive, heme-deficient enzyme with basal activity remaining intact (Wedel *et al.*, 1994). Activation of sGC by NO is initiated by binding of NO to the heme iron and proceeds via breaking of the His–Fe bond and the formation of a five-coordinated nitrosyl–heme complex. The subsequent conformational change of the enzyme results in an up to 400-fold increase in cGMP production (Humbert *et al.*, 1990; Stone and Marletta, 1996). In accordance with the proposed mechanism of activation, protoporphyrin IX (PP-IX), the iron-free precursor of heme, stimulates sGC independently of NO (Ignarro *et al.*, 1982b) by mimicking the conformation of the NO–heme complex (Ignarro *et al.*, 1984) in the enzyme.

Another noxious gas, carbon monoxide (CO), has also been implicated as a physiological stimulator of sGC (Brüne and Ullrich, 1987; Furchgott and Jothianandan, 1991). CO is produced predominantly by the enzyme heme oxygenase. As the first step in heme degradation, heme is cleaved resulting in the formation of CO and biliverdin. Heme oxygenase exists as two isoforms of which type 1 is inducible and type 2 is constitutively expressed (Maines, 1988). Heme oxygenase 1 is highly expressed in liver and spleen, whereas heme oxygenase 2 is found in the brain. Interestingly, heme oxygenase 2 was shown by *in situ* hybridization to co-localize with sGC in brain (Verma *et al.*, 1993), suggesting a role for CO in neurotransmission (Verma *et al.*, 1993; Zhou *et al.*, 1993; Hawkins *et al.*, 1994; Ingi and Ronnett, 1995). The enzyme was also detected in endothelial cells (Christodoulides *et al.*, 1995; Zakhary *et al.*, 1996), thus possibly being involved in vasorelaxation induced by CO (Ramos *et al.*, 1988; Utz and Ullrich, 1991; Morita *et al.*, 1995; Zakhary *et al.*, 1996).

CO, similarly to NO, binds to sGC's prosthetic heme group with high affinity, but only leads to a 4- to 6-fold activation of the purified enzyme (Brüne and Ullrich, 1987; Stone and Marletta, 1994). In contrast to NO, CO forms a six-coordinated heme complex, with the His–Fe bond remaining intact. It has been suggested that dissociation of CO proceeds via a five-coordinated intermediate (Kharitonov *et al.*, 1995; Deinum *et al.*, 1996), which, by its structural similarity to the nitrosyl–heme complex, presumably is responsible for the observed stimulation of sGC. Comparing the respective fold stimulations induced by NO and CO, it still remains unclear how CO can exert the functions attributed to it as it is such a poor activator of sGC.

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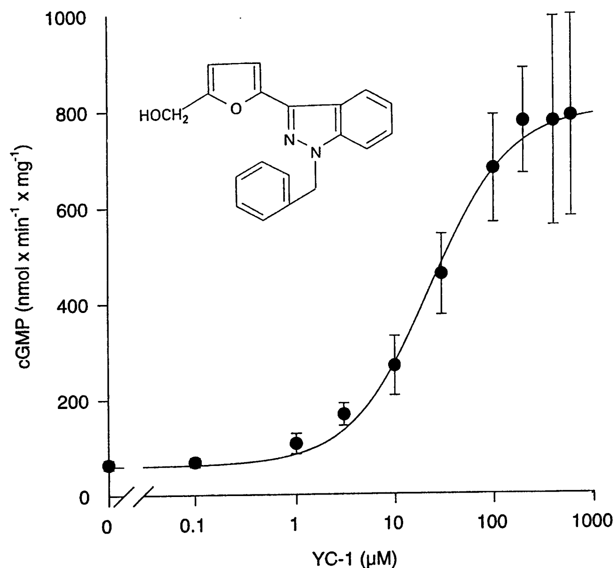

Fig. 1. Stimulation of purified soluble guanylyl cyclase by YC-1. Guanylyl cyclase activity was measured as described (Schultz and Böhme, 1984) with increasing concentrations of YC-1. Data are means ± SD from three independent experiments. The chemical structure of YC-1, a benzyl indazole derivative, is shown in the insert.

Little is known about the regulation of sGC by substances other than NO or CO. YC-1, a benzyl indazole derivative (Yoshina and Kuo, 1978), has been shown recently to inhibit platelet aggregation induced by various agents (Ko *et al.*, 1994; Wu *et al.*, 1995). The inhibitory effect was accompanied by a 6-fold increase in the intracellular cGMP concentration. YC-1 also stimulated cGMP formation in a cytosolic fraction of platelets, an effect which was not blocked by hemoglobin.

Here, we investigated the effect of YC-1 on purified sGC. Our data demonstrate that YC-1 is a non-NO-releasing, yet heme-dependent activator of sGC which, more importantly, potentiates NO- and CO-induced stimulation of the enzyme. As the stimulation induced by PP-IX was potentiated as well, we conclude that YC-1 acts by stabilizing the active conformation of the enzyme independently of the mechanism of activation. The modulation of the sensitivity of sGC towards different activating ligands repesents a novel physiological and pharmacological regulatory principle. In addition, our data show, for the first time, that in the presence of YC-1, CO is capable of stimulating sGC to an extent similar to NO.

Results

sGC stimulation by YC-1

In intact platelets and in a crude cytosolic fraction, YC-1 was shown to elevate cGMP in a concentration-dependent manner, with maximal effect at 100 μM (Wu *et al.*, 1995). Figure 1 shows that YC-1 is able to stimulate purified sGC. Maximal activation was observed with 200 μM YC-1, resulting in an up to 12-fold increase in cGMP formation (EC_{50} = 20 μM). Although the chemical structure of YC-1, a benzyl indazole derivative, is not indicative of NO-releasing properties (see Figure 1, insert), YC-1 might stimulate sGC by modulating the NO concentration in solution (Friebe *et al.*, 1996). In order to rule out the possibility that YC-1 stimulation of sGC is mediated by NO, sGC activity was measured in the presence of the NO scavenger oxyhemoglobin. In agreement with the data of Wu *et al.* (1995), we found that the stimulatory effect of YC-1 on sGC was independent of NO, since oxyhemoglobin did not prevent YC-1-induced stimulation (Table I).

To find out whether YC-1 exhibits a direct effect on cGMP catalysis, we tested YC-1 on an NO-insensitive sGC mutant. A point-mutated enzyme carrying a His→Phe substitution at position 105 on the β_1 subunit (β_1H105F) was shown previously in our laboratory (Wedel *et al.*, 1995) to exhibit intact basal activity but to be insensitve to NO. As shown in Table I, the NO-insensitive sGC mutant was not affected by YC-1.

The presence of the prosthetic heme group of sGC is necessary for the stimulatory effect of NO as heme-deficient sGC is insensitive to NO (Ignarro *et al.*, 1982a), reinforcing the notion that activation of the enzyme proceeds via the formation of a nitrosyl–heme complex (Ignarro *et al.*, 1986). As already implicated by the results with the β_1H105F mutant, we investigated whether YC-1 required the presence of the prosthetic heme group in order to stimulate sGC. Low concentrations (0.5%) of the non-ionic detergent Tween-20 lead to the removal of the prosthetic heme from the enzyme without destruction of basal enzyme activity (Foerster *et al.*, 1996). As shown in Table I, removal of heme by 0.5% Tween abolished the ability of YC-1 to stimulate sGC. These results demonstrate that the stimulatory effect of YC-1 on sGC is dependent on the presence of the prosthetic heme group and indicate that YC-1 does not alter the catalytic activity directly but is dependent on the prosthetic heme group.

Potentiation of NO- and CO-induced sGC stimulation

Since stimulation by YC-1 requires the presence of the regulatory heme group of sGC, we examined the influence of YC-1 on the stimulated enzyme. As shown in Figure 2A, YC-1 had a dramatic effect on NO activation. At a concentration of 200 μM, YC-1 increased the maximal sGC activity observed in the presence of NO by >40% (see also Table I). Moreover, YC-1 shifted the concentration–response curve of NO stimulation by one order of magnitude to the left. EC_{50} values for the NO donor diethylamine-NO (DEA-NO) obtained in the absence and presence of 200 μM YC-1 were 540 and 60 nM, respectively.

Under physiological conditions, NO as a signaling molecule is rather unlikely to occur at micromolar concentrations. Endogenously produced NO concentrations in the course of signal transduction processes are <100 nM (Varner and Beckman, 1995). Figure 2B illustrates the effects of YC-1 on sGC activity in the presence of physiological NO concentrations and shows that YC-1 caused a pronounced potentiation of NO stimulation. In the absence of YC-1, 30 nM DEA-NO caused a 6-fold increase in enzyme activity. Addition of 200 μM YC-1 potentiated this effect of NO, leading to a 75-fold increase in cGMP formation. Even a submaximal concentration of YC-1 (30 μM) caused a 40-fold sGC stimulation in the presence of 30 nM DEA-NO. These results indicate that YC-1 sensitized sGC towards NO.

Table I. Effect of YC-1 on purified soluble guanylyl cylase

Enzyme	Treatment	−YC-1		+YC-1	
		cGMP (nmol/min/mg)	Fold stimulation	cGMP (nmol/min/mg)	Fold stimulation
Purified sGC	control	64 ± 8	1	773 ± 80	12
	65 μM oxyHb	61 ± 1	1	792 ± 66	13
	0.5 % Tween-20	130 ± 89	2	139 ± 46	2
	10 μM DEA-NO	6340 ± 360	99	9030 ± 450	140
	10 μM PP-IX	1290 ± 350	20	3760 ± 800	58
	CO	218 ± 11	3	6840 ± 1400	106
Purified β_1H105F	control	73 ± 4	1	101 ± 5	1.4

Enzymatic activity of purified sGC was measured in the absence and presence of 200 μM YC-1 with the indicated substances (Schultz and Böhme, 1984). CO was administered as 100% in the vial's headspace. Values are means ± SD from three independent experiments.

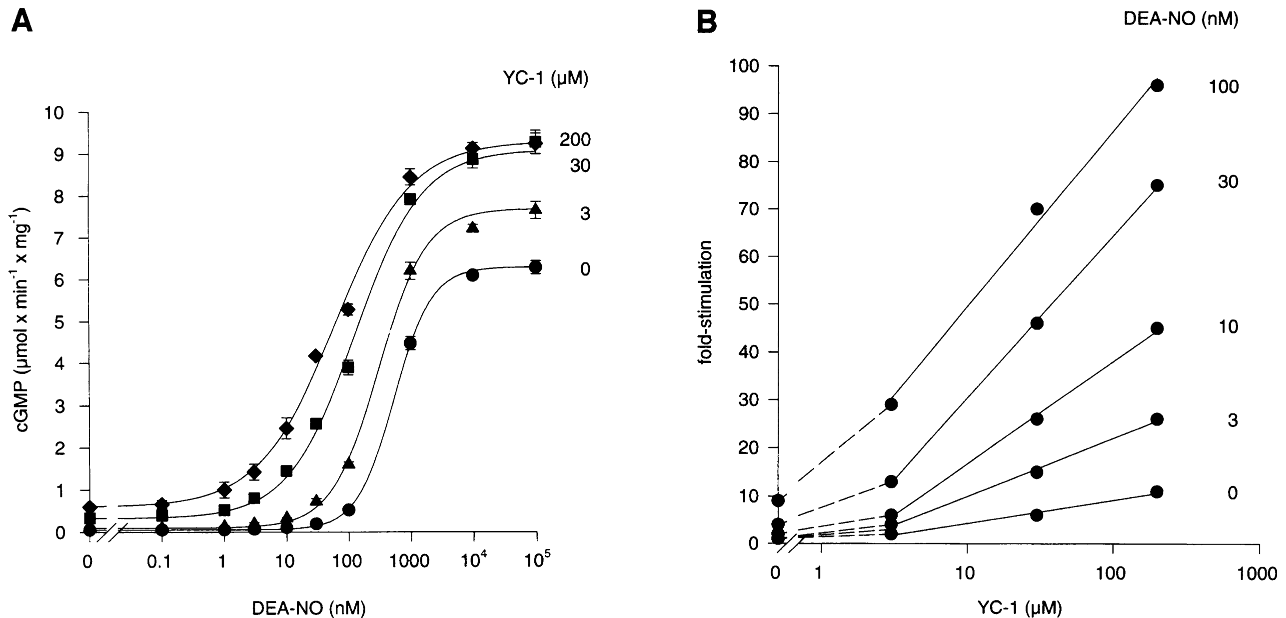

Fig. 2. Potentiation of NO-induced soluble guanylyl cyclase stimulation by YC-1. (**A**) Increasing concentrations of the NO donor diethylamine-NO (DEA-NO) were applied in the absence (●) or presence of 3 (▲), 30 (■) or 200 μM (◆) YC-1. Data are means ± SD from three independent experiments. (**B**) Plot of the data taken from (A) showing the effect of YC-1 on the DEA-NO-stimulated activity.

In order to investigate the underlying mechanism of the potentiating effect of YC-1, we used sGC activators other than NO. CO, having properties similar to NO with high affinity towards heme groups, has been implicated in various NO-mediated functions. Despite being a weak activator of sGC *in vitro*, CO is thought to function as a neurotransmitter and vasorelaxant, activating sGC by a mechanism similar to that of NO. As shown in Table I, CO stimulated purified sGC only 3-fold, which is in accordance with earlier results (Brüne and Ullrich, 1987; Stone and Marletta, 1994). In the presence of YC-1, the stimulatory effect of CO was potentiated tremendously, and CO was capable of stimulating sGC 106-fold, resulting in enzyme activation similar to that by NO.

To elucidate whether YC-1 acts by influencing binding of the gaseous ligand to the heme moiety, we tested YC-1 on the PP-IX-activated enzyme. PP-IX stimulates sGC by mimicking the heme conformation of the NO-activated enzyme, thus stimulating sGC independently of a gaseous ligand. Table I shows that stimulation by maximally effective concentrations of PP-IX was also potentiated by YC-1. These results suggest that activated enzyme molecules are affected by YC-1. Since YC-1 potentiation is independent of the mode of activation (NO, CO or PP-IX), we conclude that YC-1 acts through stabilization of the activated configuration of sGC.

In addition to the mechanistic implication of YC-1-induced potentiation, the high enzyme activities obtained with CO in the presence of YC-1 emphasize the possible role of CO as a physiological activator of sGC. The specific activities of the CO-stimulated enzyme (100% in the vial's headspace) were 220 and 6840 nmol/min/mg in the absence and presence of YC-1, respectively. Thus, maximal enzyme activity in the presence of YC-1 and CO was comparable with NO-stimulated sGC activity (see Table I). To elucidate whether YC-1 not only increased the V_{max} of the CO-stimulated enzyme but, in analogy to the results obtained with NO, also shifted the EC_{50} for CO to the left, we obtained the concentration–response curve of CO (administered from a saturated solution) in the absence and presence of YC-1. As shown in Figure 3, YC-1 caused a shift to the left of the concentration–response curve for CO in addition to the dramatic increase in V_{max}. This is the first demonstration that CO is able to stimulate sGC to a degree similar to NO, and the existence of an endogenous YC-1-like substance would provide the

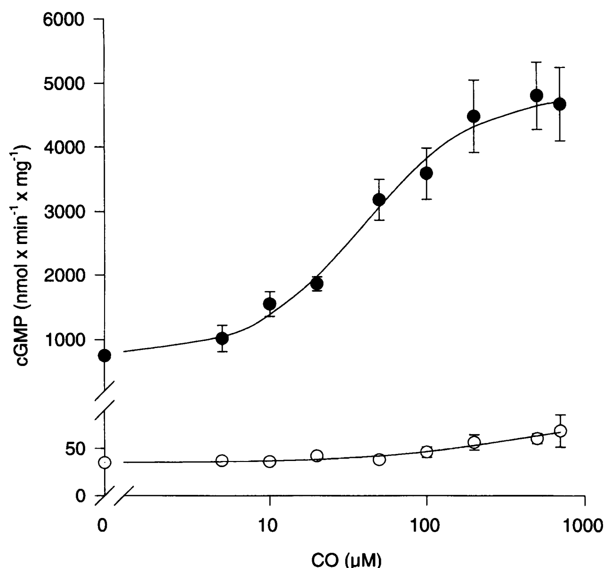

Fig. 3. Potentiation of CO-induced soluble guanylyl cyclase stimulation by YC-1. Increasing concentrations of CO were applied in the absence (○) or presence (●) of 200 μM YC-1. Data are the means ± SD from three independent experiments.

molecular basis for a physiological role for CO in the regulation of sGC.

Discussion

The identification of the regulation of soluble guanylyl cyclase by NO has been a milestone in the understanding of the physiology of neuronal and cardiovascular processes. So far, sGC is thought to be the main receptor for NO, thus representing a key component in NO-mediated signal transduction. Consequently, attention has been drawn to non-NO substances which may also activate sGC or modulate NO-stimulated sGC activity. In this study, we demonstrate that the novel sGC activator YC-1 affects sGC activity by a mechanism differing fundamentally from that exerted by the enzyme's so far only known physiological activator, NO, suggesting a novel pharmacological and physiological principle.

YC-1 led to an ~12-fold activation of purified sGC. This activation was NO- and CO-independent, as evidenced by the fact that oxyhemoglobin, which scavenges NO as well as CO, did not prevent stimulation. An NO-insensitive, yet catalytically active, sGC mutant and heme-depleted sGC were not stimulated by YC-1. These results show the heme requirement for YC-1 stimulation and demonstrate that YC-1 does not act on the catalytic domain of sGC directly but is dependent on the regulatory enzyme-bound heme group. Thus, YC-1 activates sGC by a novel mechanism.

In the presence of maximally effective NO concentrations, YC-1 was able even to enhance the rate of cGMP formation by >40%. The increase in NO-stimulated enzyme activity was surprising, as sGC activation achieved by NO had been considered as maximal. In addition to the increase in V_{max}, YC-1 shifted the EC_{50} for NO by one order of magnitude to the left, thereby sensitizing the enzyme. As depicted in Figure 2B, this potentiating YC-1 effect was prominent at physiological NO concentrations ranging between 1 and 100 nM (Varner and Beckman, 1995), thus pointing towards a possible therapeutic use.

Using CO, we showed that the potentiating effect of YC-1 is not restricted to NO. The poor (3-fold) activation of sGC by CO was increased dramatically by YC-1 (100-fold), yielding enzyme activities comparable with those of the NO-activated enzyme. Compared with the 40% increase in maximal activity obtained with NO, YC-1 raised CO-stimulated activity by >4000%.

Both NO and CO exert their stimulatory action as ligands of the prosthetic heme group. Therefore, we investigated whether YC-1 potentiation required the stimulation of sGC by a gasous heme binding ligand. PP-IX, the iron-free precursor of heme, stimulates sGC in a ligand-independent manner by mimicking the structure of the nitrosyl–heme complex in the activated enzyme. Similarly to the NO and CO stimulation, YC-1 potentiated the PP-IX-induced stimulation of sGC. We conclude that stabilization of the active conformation of sGC is the underlying mechanism of YC-1 potentiation and that induction of the active conformation, regardless of the nature of the activating species, is the prerequisite for the YC-1 effect. Thus, YC-1 appears to shift the equilibrium between inactive and active sGC molecules. In unstimulated conditions, the majority of sGC molecules will be in an inactive conformation. Yet, due to the exceptionally high stimulation factor of sGC, the potentiating effect of YC-1 on even a very small population of enzymes in the activated state would lead to a detectable increase in cGMP formation. Therefore, the 12-fold increase in unstimulated sGC activity by YC-1 may be explained by the stabilization of the activated configuration of a small enzyme population even in unstimulated conditions.

By increasing the responsiveness of sGC towards endogenously occurring NO, YC-1 may represent a novel class of drugs sensitizing the enzyme towards its physiological activator. Most NO donors commonly used in the treatment of coronary heart diseases have to undergo metabolic transformation to release NO; therefore, their action is limited to tissues expressing the metabolizing enzymes. In contrast, by reinforcing the effects of physiologically relevant NO concentrations, YC-1 will exert its effect directly at sites of endogenous NO production. Moreover, as YC-1 is independent of biotransformation, the nitrate tolerance ascribed to NO donor-metabolizing enzymes (Bennett *et al.*, 1994) should not occur.

Along with the pharmacological implications, the results with YC-1 suggest the existence of an endogenous modulator of sGC activity. In analogy to the possible role of endogenous benzodiazepine-like substances acting on the $GABA_A$ receptor (Macdonald and Olson, 1994), an endogenous YC-1 equivalent may alter the sensitivity of sGC towards NO and even more so towards CO.

Finally, our results reinforce the concept of a physiological role for CO as we provide evidence that CO in the presence of YC-1 is capable of stimulating sGC to an extent similar to NO. The proposal that CO is a physiological activator of sGC has been contrasted by the rather poor sGC-stimulatory properties of CO. An increasing number of reports on the physiological role of CO as a signal molecule in the nervous system are being published, along with publications repudiating CO's function in neuronal signal transduction.

In olfactory receptor neurons, the effect of CO on the intracellular cGMP concentrations (Verma *et al.*, 1993; Ingi and Ronnett, 1995; Leinders-Zufall *et al.*, 1995) strongly indicates the participation of CO in olfactory signal transduction, especially as these cells express high levels of heme oxygenase activity but no NO synthase activity (Ingi and Ronnett, 1995). In addition, CO was proposed as a candidate for the retrograde messenger for long-term potentiation (Stevens and Wang, 1993; Zhou *et al.*, 1993), although the results obtained remain controversial (Luo and Vincent, 1994; Meffert *et al.*, 1994; Poss *et al.*, 1995). A recent study proposes a model in which CO acts as modulator of the NO–cGMP signaling pathway in the brain (Ingi *et al.*, 1996).

The existence of an endogenous YC-1-like substance being expressed in a cell- or tissue-specific manner could explain the controversy concerning the role of CO in the above-mentioned systems. Depending on the presence of such an endogenous modulator, CO would display low or high sGC-activating properties. The existence of a YC-1-like modulator potentiating CO-induced stimulation of sGC would also account for the discrepancy between the cGMP-increasing effects of CO *in vivo* and the rather low stimulatory effects of CO on purified sGC *in vitro*. Identification and isolation of a possible YC-1-like endogenous activator would certainly help to understand the function of CO and sGC in the nervous and cardiovascular systems.

Materials and methods

Purification of soluble guanylyl cyclase and determination of guanylyl cyclase activity

sGC was purified from bovine lung to apparent homogeneity by an immunoaffinity purification procedure as described previously (Humbert *et al.*, 1990). The same method was applied to isolate the NO-insensitive β_1H105F mutant expressed in the baculovirus–Sf9 system (Wedel *et al.*, 1994).

Cyclase activity was measured by the conversion of [α-^{32}P]GTP to [^{32}P]cGMP at 37°C for 10 min. Reaction mixtures contained 3 mM Mg^{2+} as divalent metal ion, 3 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 1 mM cGMP, 300 μM GTP and 50 mM triethanolamine hydrochloride, pH 7.4, in a total volume of 0.1 ml. Reactions were stopped by $ZnCO_3$ precipitation, and [^{32}P]cGMP was isolated as described (Schultz and Böhme, 1984). All measurements were performed in duplicate and were repeated at least three times.

YC-1 was dissolved in dimethylsulfoxide (DMSO). The final DMSO concentration in all samples did not exceed 2% (v/v).

Preparation of CO solution

CO was administered as a saturated CO solution which was prepared by bubbling 100% CO through 50 mM triethanolamine buffer, pH 7.4 (10 min at room temperature). The saturated solution was diluted to the indicated final concentrations in the assay mixture and applied with a gas-tight syringe immediately before starting the incubation at 37°C.

Synthesis of oxyhemoglobin

Oxyhemoglobin was prepared in 50 mM triethanolamine-HCl, pH 7.0, by reducing bovine methemoglobin with sodium dithionite. Subsequently, reduced hemoglobin was desalted by passing over a Sephadex G-25 (PD-10) column. The concentration of oxyhemoglobin was determined photometrically.

Materials

YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole] was a generous gift from Bayer (Wuppertal, Germany). 2,2-Diethyl-1-nitroso-oxyhydrazine sodium salt (DEA-NO) was purchased from NCI Chemical Carcinogen Repository. Hemoglobin, PP-IX and hemin were obtained from Sigma, and Tween-20 was purchased from Boehringer Mannheim. [α-^{32}P]GTP (800 Ci/mmol) was from NEN-DuPont. CO (99.9%) was from Merck-Schuchhardt, Hohenbrunn, Germany.

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References

Arnold,W.P., Mittal,C.K., Katsuki,S. and Murad,F. (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3′,5′-cyclic monophosphate levels in various tissue preparations. *Proc. Natl Acad. Sci. USA*, **74**, 3203–3207.

Bennett,B.M., McDonald,B.J., Nigam,R. and Simon,W.C. (1994) Biotransformation of organic nitrates and vascular smooth muscle cell function. *Trends Pharmacol. Sci.*, **15**, 245–249.

Brüne,B. and Ullrich,V. (1987) Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol. Pharmacol.*, **32**, 497–504.

Christodoulides,N., Durante,W., Kroll,M.H. and Schafer,A.I. (1995) Vascular smooth muscle cell heme oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. *Circulation*, **91**, 2306–2309.

Deinum,G., Stone,J.R., Babcock,G.T. and Marletta,M.A. (1996) Binding of nitric oxide and carbon monoxide to soluble guanylate cyclase as observed with resonance Raman spectroscopy. *Biochemistry*, **35**, 1540–1547.

Foerster,J., Harteneck,C., Malkewitz,J., Schultz,G. and Koesling,D. (1996) A functional heme-binding site of soluble guanylyl cyclase requires intact N-termini of α_1 and β_1 subunits. *Eur. J. Biochem.*, **240**, 380–386.

Friebe,A., Malkewitz,J., Schultz,G. and Koesling,D. (1996) Positive effects of pollution? *Nature*, **382**, 120.

Furchgott,R.F. and Jothianandan,D. (1991) Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels*, **28**, 52–61.

Garbers,D.L. and Lowe,D.G. (1994) Guanylyl cyclase receptors. *J. Biol. Chem.*, **269**, 30741–30744.

Garthwaite,J., Charles,S.L. and Chess-Williams,R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as a intercellular messenger in the brain. *Nature*, **336**, 385–388.

Hawkins,R.D., Zhuo,M. and Arancio,O. (1994) Nitric oxide and carbon monoxide as possible retrograde messengers in hippocampal long-term potentiation. *J. Neurobiol.*, **25**, 652–665.

Humbert,P. *et al.* (1990) Purification of soluble guanylate cyclase from bovine lung by a new immunoaffinity chromatographic method. *Eur. J. Biochem.*, **190**, 273–278.

Ignarro,L.J., Dengan,J.N., Barricos,W.H., Kadowitz,P.J. and Wolin,M.S. (1982a) Activation of purified guanylate cyclase by nitric oxide requires heme: comparison of heme-deficient, heme-reconstituted and heme-containing forms of soluble enzyme from bovine lung. *Biochim. Biophys. Acta*, **718**, 49–59.

Ignarro,L.J., Wood,K.S. and Wolin,M.S. (1982b) Activation of purified soluble guanylate cyclase by protoporphyrin IX. *Proc. Natl Acad. Sci. USA*, **79**, 2870–2873.

Ignarro,L.J., Wood,K.S. and Wolin,M.S. (1984) Regulation of purified soluble guanylate cyclase by porphyrins and metalloporphyrins: a unifying concept. *Adv. Cyclic Nucleotide Res.*, **17**, 267–274.

Ignarro,L.J., Adams,J.B., Horwitz,P.M. and Wood,K.S. (1986) Activation of soluble guanylate cyclase by NO-hemoproteins involves NO–heme exchange: comparison of heme-containing and heme-deficient enzyme forms. *J. Biol. Chem.*, **261**, 4997–5002.

Ignarro,L.J., Buga,G.M., Wood,K.S., Byrns,R.E. and Chandhuri,G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl Acad. Sci. USA*, **84**, 9265–9269.

Ingi,T. and Ronnett,G.V. (1995) Direct demonstration of a physiological role for carbon monoxide in olfactory receptor neurons. *J. Neurosci.*, **15**, 8214–8222.

Ingi,T., Cheng,J. and Ronnett,G.V. (1996) Carbon monoxide: an endogenous modulator of the nitric oxide–cyclic GMP signaling system. *Neuron*, **16**, 835–842.

Kharitonov,V.G., Sharma,V.S., Pilz,R.B., Magde,D. and Koesling,D. (1995) Basis of guanylate cyclase activation by carbon monoxide. *Proc. Natl Acad. Sci. USA*, **92**, 2568–2571.

Ko,F.-N., Wu,C.-C., Kuo,S.-C., Lee,F.-Y. and Teng,C.-M. (1994) YC-1, a novel activator of platelet guanylate cyclase. *Blood*, **84**, 4226–4233.

Leinders-Zufall,T., Shepherd,G.M. and Zufall,F. (1995) Regulation of cyclic nucleotide-gated channels and membrane excitability in olfactory receptor cells by carbon monoxide. *J. Neurophysiol.*, **74**, 1498–1508.

Luo,D. and Vincent,S.R. (1994) Metalloporphyrins inhibit nitric oxide-dependent cGMP formation *in vivo*. *Eur. J. Pharmacol.*, **267**, 263–267.

Macdonald,R.L. and Olson,R.W. (1994) $GABA_A$ receptor channels. *Annu. Rev. Neurosci.*, **17**, 569–602.

Maines,M.D. (1988) Heme oxygenase: function, multiplicity, regulatory mechanism and clinical applications. *FASEB J.*, **2**, 2257–2568.

Marletta,M.A. (1994) Nitric oxide synthase: aspects concerning structure and catalysis. *Cell*, **78**, 927–930.

Meffert,M.K., Haley,J.E., Schuman,E.M., Schulman,H. and Madison,D.V. (1994) Inhibition of hippocampal heme oxygenase, nitric oxide synthase and long-term potentiation by metalloporphyrins. *Neuron*, **13**, 1225–1233.

Moncada,S. and Higgs,E.A. (1995) Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.*, **9**, 1319–1330.

Morita,T., Perrella,M.A., Lee,M.-E. and Kourembanas,S. (1995) Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc. Natl Acad. Sci. USA*, **92**, 1475–1479.

Nathan,C. and Xie,Q.-W. (1994) Nitric oxide synthases: roles, tolls and controls. *Cell*, **78**, 915–918.

Palmer,R.M.J., Ferrige,A.G. and Moncada,S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

Poss,K.D., Thomas,M.J., Ebralidze,A.K., O'Dell,T.J. and Tonegawa,S. (1995) Hippocampal long-term potentiation is normal in heme oxygenase-2 mutant mice. *Neuron*, **15**, 867–873.

Ramos,K.S., Lin,H. and McGrath,J.J. (1988) Modulation of cyclic guanosine monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide. *Biochem. Pharmacol.*, **38**, 1368–1370.

Schultz,G. and Böhme,E. (1984) Guanylate cyclase. GTP pyrophophate-lyase (cyclizing), E.C.4.6.1.2. In Bergmeyer,H.U. and Graβl,M. (eds), *Methods of Enzymatic Analysis*. Verlag Chemie, Weinheim, New York, Vol. IV, pp. 379–389.

Snyder,S.H. (1992) Nitric oxide: first in a class of neurotransmitters? *Science*, **257**, 494–496.

Stevens,C.F. and Wang,Y. (1993) Reversal of long-term potentiation by inhibitors of heme oxygenase. *Nature*, **364**, 147–149.

Stone,J.R. and Marletta,M.A. (1994) Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry*, **33**, 5636–5640.

Stone,J.R. and Marletta,M.A. (1996) Spectral and kinetic studies on the activation of soluble guanylyl cyclase by nitric oxide. *Biochemistry*, **35**, 1093–1099.

Utz,J. and Ullrich,V. (1991) Carbon monoxide relaxes ileum smooth muscle through activation of guanylate cyclase. *Biochem. Pharmacol.*, **41**, 1195–1201.

Varner,P.D. and Beckman,J.S. (1995) Nitric oxide toxicity in neuronal injury and degeneration. In Vincent,S.R. (ed.), *Nitric Oxide in the Nervous System*. Academic Press, London, UK, pp. 191–206.

Verma,A.D., Hirsch,J., Glatt,C.E., Ronnett,G.V. and Snyder,S.H. (1993) Carbon monoxide: a putative neuronal messenger. *Science*, **259**, 281–284.

Waldman,S.A. and Murad,F. (1987) Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.

Wedel,B., Humbert,P., Harteneck,C., Foerster,J., Malkewitz,J., Böhme,E., Schultz,G. and Koesling,D. (1994) Mutation of His-105 of the β_1-subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl Acad. Sci. USA*, **91**, 2592–2596.

Wedel,B., Harteneck,C., Foerster,J., Friebe,A., Schultz,G. and Koesling,D. (1995) Functional domains of soluble guanylyl cyclase. *J. Biol. Chem.*, **270**, 24871–24875.

Wu,C.-C., Ko,F.-N., Kuo,S.-C., Lee,F.-Y. and Teng,C.-M. (1995) YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. *Br. J. Pharmacol.*, **116**, 1973–1978.

Yoshina,S. and Kuo,S.C. (1978) Studies on heterocyclic compounds XXXV. Synthesis of furo[3,2-C]pyrazole derivatives. (2) electrophilic substitution of 1,3-diphenylfuro[3,2-C]pyrazole. *Yakugaku Zasshi*, **98**, 204.

Zakhary,R., Gaine,S.P., Dinerman,J.L., Ruat,M., Flavahan,N.A. and Snyder,S.H. (1996) Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation. *Proc. Natl Acad. Sci. USA*, **93**, 795–798.

Zhuo,M., Small,S.A., Kandel,E.R. and Hawkins,R.D. (1993) Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. *Science*, **260**, 1946–1950.

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