British Journal of Pharmacology (1997) 120, 681–689

Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators

[1]Alexander Mülsch, Johann Bauersachs, Andreas Schäfer, *Johannes-Peter Stasch, *Raimund Kast & Rudi Busse

Zentrum der Physiologie, Klinikum der Johann-Wolfgang-Goethe Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt, and *Institut für Herz- and Kreislaufforschung, Bayer AG, D-42096 Wuppertal, Germany

1 We studied the effects of 3-(5′-hydroxymethyl-2′furyl)-1-benzyl indazole (YC-1) on the activity of purified soluble guanylyl cyclase (sGC), the formation of guanosine-3′:5′ cyclic monophosphate (cyclic GMP) in vascular smooth muscle cells (VSMC), and on the tone of rabbit isolated aortic rings preconstricted by phenylephrine (PE). In addition, we assessed the combined effect of YC-1, and either NO donors, or superoxide anions on these parameters.

2 YC-1 elicited a direct concentration-dependent activation of sGC (EC_{50} 18.6±2.0 μM), which was rapid in onset and quickly reversible upon dilution. YC-1 altered the enzyme kinetics with respect to GTP by decreasing K_M and increasing V_{max}. Activation of sGC by a combination of sodium nitroprusside (SNP) and YC-1 was superadditive at low and less than additive at high concentrations, indicating a synergistic activation of the enzyme by both agents. A specific inhibitor of sGC, 1H-(1,2,4)-oxdiazolo-(4,3-a)-6-bromo-quinoxazin-1-one (NS 2028), abolished activation of the enzyme by either compound.

3 YC-1 induced a concentration-dependent increase in intracellular cyclic GMP levels in rat cultured aortic VSMC, which was completely inhibited by NS 2028. YC-1 applied at the same concentration as SNP elicited 2.5 fold higher cyclic GMP formation. Cyclic GMP-increases in response to SNP and YC-1 were additive.

4 YC-1 relaxed preconstricted endothelium-denuded rabbit aortic rings in a concentration-dependent manner (50% at 20 μM) and markedly increased cyclic GMP levels. Relaxations were inhibited by NS 2028. A concentration of YC-1 (3 μM), which elicited only minor effects on relaxation and cyclic GMP, increased the vasodilator potency of SNP and nitroglycerin (NTG) by 10 fold and markedly enhanced SNP- and NTG-induced cyclic GMP formation.

5 Basal and YC-1-stimulated sGC activity was sensitive to inhibition by superoxide (O_2^-) generated by xanthine/xanthine oxidase, and was protected from this inhibition by superoxide dismutase (SOD). YC-1-stimulated sGC was also sensitive to inhibition by endogenously generated (O_2^- in rat preconstricted endothelium-denuded aortic rings. Relaxation to YC-1 was significantly attenuated in aortae from spontaneously hypertensive rats (SHR), which generated O_2^- at a higher rate than aortae from normotensive Wistar Kyoto rats (WKY). SOD restored the vasodilator responsiveness of SHR rings to YC-1.

6 In conclusion, these results indicate that YC-1 is an NO-independent, O_2^--sensitive, direct activator of sGC in VSMC and exerts vasorelaxation by increasing intracellular cyclic GMP levels. The additive or even synergistic responses to NO-donors and YC-1 in cultured VSMC and isolated aortic rings apparently reflect the direct synergistic action of YC-1 and NO on the sGC. The synergism revealed in this *in vitro* study suggests that low doses of YC-1 may be of therapeutic value by permitting the reduction of nitrovasodilator dosage.

Keywords: YC-1 (3-(5′-hydroxymethyl-2′furyl)-1-benzyl indazole); nitric oxide; oxygen radicals; soluble guanylyl cyclase; vascular smooth muscle; relaxation; NS 2028 (1H-(1,2,4)-oxdiazolo-(4,3-a)-6-bromo-quinoxazin-1-one)

Introduction

It is generally accepted that nitrovasodilators like sodium nitroprusside (SNP) and nitroglycerin (NTG) inhibit vascular constriction, platelet activation and smooth muscle cell proliferation by releasing nitric oxide (NO) and activating guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent and, in some vascular beds, also cyclic GMP-independent effector pathways (Ignarro, 1990; Lincoln & Cornwell, 1993). However, besides these therapeutically beneficial effects, nitrovasodilator therapy carries the risk of generating adverse effects of NO. Nitrate tolerance is one such adverse phenomenon observed in patients after prolonged exposure to repetitive nitrate administration and may result from enhanced superoxide anion (O_2^-) generation within the vascular wall (Münzel *et al.*, 1995). Furthermore, the formation of NO from nitrovasodilators occurs at much higher rates in liver and kidney than in vascular tissues (Mülsch *et al.*, 1995a,b; Laursen *et al.*, 1996), creating a potential hazard to the detoxifying function of these organs. For example, enhanced NO formation in the liver has been shown to inhibit cytochrome P450 activity (Stadler *et al.*, 1994). In general, high levels of NO exert cytotoxic actions by diverse molecular mechanisms (Esumi & Tannenbaum, 1994; Billiar, 1995), which may be related to the generation of peroxynitrite and hydroxyl radicals. Such adverse effects of NO donors could be avoided by

[1] Author for correspondence.

using NO-independent activators of the soluble guanylyl cyclase (sGC). However, the toxicity and/or chemical instability of currently available NO-independent activators of sGC, such as protoporphyrin IX (Ignarro *et al.*, 1982), polyunsaturated fatty acids (Gerzer *et al.*, 1983), fatty acid endoperoxides (Graff *et al.*, 1978), diamide, 4,4′-dithiopyridine (Wu *et al.*, 1992) and carbon monoxide (Brüne *et al.*, 1990), precludes a clinical application of these compounds.

Recently, the benzylindazole-derivative YC-1 has been described as an inhibitor of platelet aggregation (Ko *et al.*, 1994; Wu *et al.*, 1995) and smooth muscle cell proliferation (Yu *et al.*, 1995), presumably acting via direct, NO-independent activation of sGC without involvement of cyclic GMP phosphodiesterases and the cyclic AMP pathway. However, no characterization of the vasodilator activity of YC-1 or its interaction with the purified sGC was performed.

The present study focusses on the action of YC-1 on sGC purified to homogeneity from bovine lung, on its vasodilator activity in rabbit and rat endothelium-denuded aorta, as well as on the interaction between YC-1 and either NO-donors or O_2^- at the enzymatic and cellular levels.

Methods

Determination of sGC activity

Enzyme activity was assessed by formation of cyclic [^{32}P]-GMP from [α^{32}P]-guanosine 5′-triphosphate (GTP). sGC (8 μg protein ml^{-1}) was incubated at 37°C for 10 min, or as indicated otherwise, in a Tris-HCl-buffered solution (30 mM, pH 7.4), containing 200 μM [α^{32}P]-GTP (0.2 μCi), 200 μM unlabelled cyclic GMP, 3 mM $MgCl_2$, 1 mg ml^{-1} bovine serum albumin, 10 mM creatine phosphate, 100 μg ml^{-1} creatine phosphokinase (1 unit ml^{-1}), 0.5 mM diethylenetriamine-pentaacetic acid (DETAPAC) and 3 mM glutathione (GSH) (buffer I). YC-1, SNP and the sGC inhibitor NS 2028 were added as indicated to make-up a final volume of 100 μl per sample. Some experiments were carried out in the presence of the O_2^--generating system xanthine (0.2 mM)/xanthine oxidase (0.1–3 u ml^{-1}), in the absence and presence of superoxide dismutase (SOD, 1 μM).

In dilution experiments sGC (80 μg protein ml^{-1}) was preincubated in the absence and presence of 30 μM YC-1 in buffer I, but without substrate (GTP), for 5 min at 37°C. Then the incubates were diluted 10 fold, and GC activity was assessed by addition of [α^{32}P]-GTP and a further 10 min incubation in the presence of YC-1 (3 or 30 μM).

Incubations were performed in duplicate and were stopped by co-precipitation of 5′-nucleotides with zinc carbonate. Following centrifugation (10 min, 10,000 *g*) cyclic [^{32}P]-GMP was isolated from the supernatant by chromatography on acid alumina. The amount of cyclic [^{32}P]-GMP formed was determined by liquid scintillation counting. The specific activity of sGC (nmol cyclic GMP formed mg^{-1} GC min^{-1} incubation time) was calculated as described previously (Mülsch *et al.*, 1988).

Smooth muscle cell culture

Smooth muscle cells were isolated by elastase/collagenase digestions of thoracic aortae from male Wistar-Kyoto rats as described previously (Schini *et al.*, 1991). The cells were seeded on 24-well multiwell plates and cultured in Waymouth medium supplemented by non-essential amino acids, penicillin (50 u ml^{-1}), streptomycin (50 μg ml^{-1}), and foetal bovine serum (7.5% v/v). Confluent cultures were serially passaged by trypsinization (0.05% trypsin, 0.02% EDTA). Passages 10 to 17 were used for the assessment of intracellular cyclic GMP responses. Confluent cultures were washed three times in HEPES-modified Tyrode solution of the following composition (mM): $CaCl_2$ 1.8, KCl 2.7, $MgCl_2$ 0.5, NaCl 137, HEPES 10, NaH_2PO_4 0.36 and glucose 5, pH adjusted to 7.35 at 37°C by NaOH. After 10 min the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; 0.5 mM) was added and 20 min later SNP, YC-1, or both, in the concentrations indicated in the results section. In some experiments NS 2028 was also present in the incubation buffer. Following 10 min exposure to SNP and/or YC-1 (at 37°C), the medium was removed and the reaction stopped by the addition of ice-cold trichloroacetic acid (TCA; 6% w/v). Cyclic GMP was thereafter determined by use of a commercially available radioimmunoassay. Results are expressed as pmol cyclic GMP per well, which contained approximately 10^5 cells (30 μg protein).

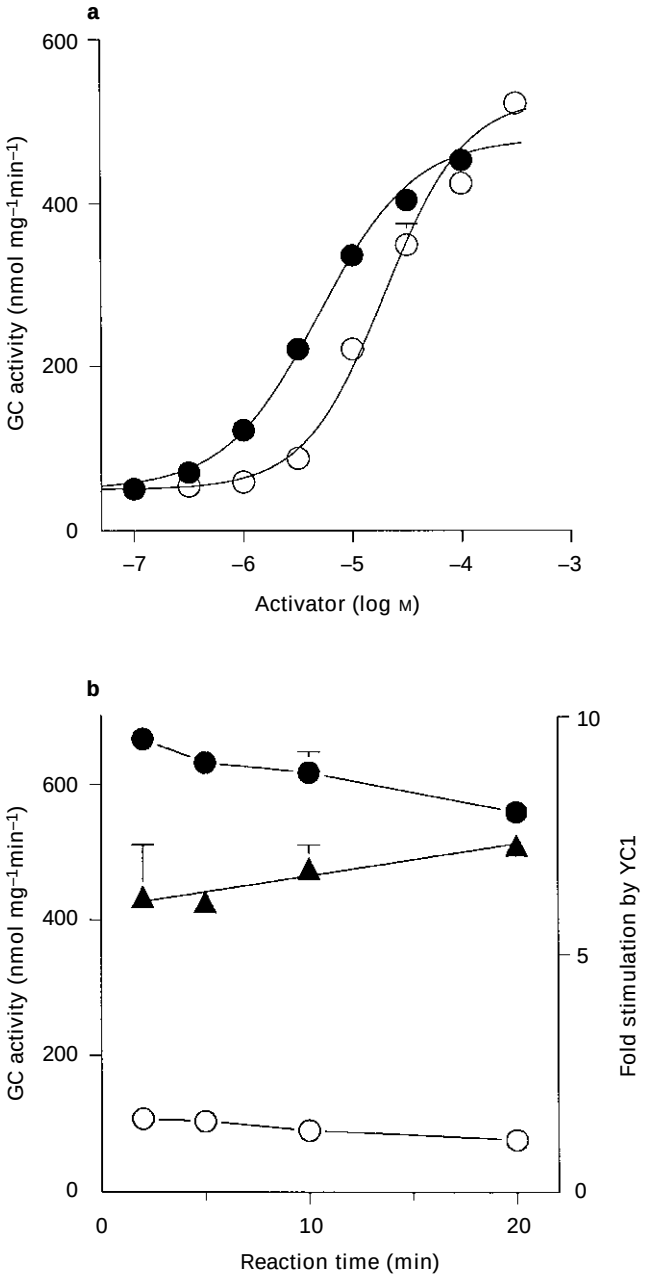

Figure 1 Activation of purified soluble guanylyl cyclase (sGC) by YC-1. (a) Soluble GC was incubated for 10 min in the presence of either sodium nitroprusside (0.1–100 μM; ●) or YC-1 (0.3–300 μM; ○). For calculation of the EC_{50} values the data were fitted to a logistic equation represented by the line graphs. (b) Time course of the activation of sGC by YC-1. Activity of sGC (left axis) was determined in the presence of either solvent (○) or YC-1 (100 μM; ●) for the reaction time indicated. The ratio of YC-1-enhanced vs basal sGC activity (▲) is displayed on the right axis. The data represent the mean from 3 independent determinations; vertical lines show s.e.mean.

Assessment of vasodilator responses

The descending thoracic aorta was removed from anaesthetized (60 mg kg^{-1} sodium pentobarbitone, i.v.) and exsanguinated male New Zealand white rabbits (1.5–2.5 kg body weight; Charles River, Sulzfeld, Germany), cleaned of connective tissue, and cut into rings 4 mm in length. The endothelium was removed mechanically and rings were mounted in thermostated (37°C) organ baths (Schuler-Organbad: Hugo Sachs Elektronik, March, Germany) connected to a force transducer (K30, Hugo Sachs Elektronik) for isometric measurement of contractile tone. The rings were equilibrated for 30 min under a resting tension of 2 g in carbogenated (95% O_2, 5% CO_2) Krebs-Henseleit solution, pH 7.4, of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 12 and EDTA-Ca-salt 25 μM, in the presence of the cyclo-oxygenase inhibitor diclofenac (1 μM). Rings were subsequently contracted by 1 μM phenylephrine (PE) and removal of the endothelium was functionally tested by the absence of the relaxant response to acetylcholine (1 μM). Following a 30 min washout period the rings were contracted to approximately 5 g by PE (1 μM), and the relaxant responses to cumulative doses of YC-1, NTG or SNP, were assessed, in the case of NTG and SNP, also in the presence of YC-1 (3 μM).

In order to assess the influence of endogenously generated O_2^- radicals on vascular responses to YC-1 additional experiments were performed with endothelium-denuded aortic rings from normotensive Wistar Kyoto rats (WKY) and

Table 1 Reversibility of YC-1-induced activation of sGC by dilution

Concentration of YC-1 (μM) during		*sGC-activity*
Preincubation	*Incubation*	(nmol mg^{-1} min^{-1})
0	0	58.1 ± 1.3
0	3	153.3 ± 0.5
0	30	372.6 ± 10.3
30	3	159.4 ± 11.1
30	30	453.5 ± 21.5

The sGC was preincubated for 5 min at 37°C with or without YC-1 (30 μM). Then formation of cyclic [^{32}P]-GMP was determined for a further 10 min after 10 fold dilution of the mixture and adjustment of the final concentration of YC-1 as listed under 'incubation'. Data are mean ± s.e.mean of 3 experiments performed in duplicate. GC activation elicited by 3 μM YC-1 was not significantly affected by preincubation with 30 μM YC-1 ($P>0.05$, ANOVA).

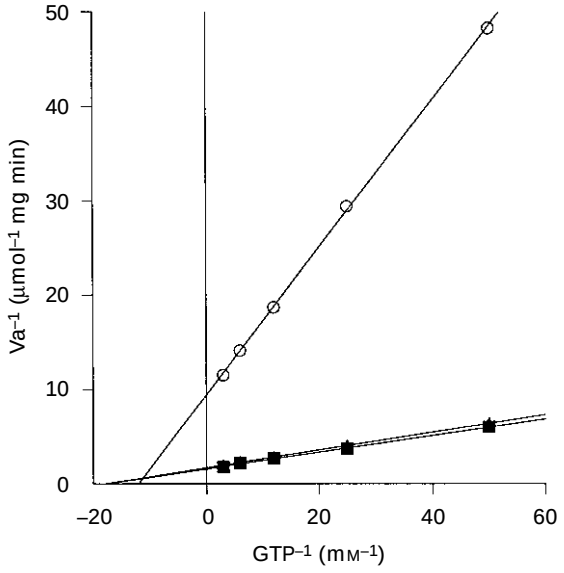

Figure 2 Lineweaver-Burk analysis of YC-1-dependent sGC activation kinetics. The specific activity of sGC was determined in the absence (○) and presence of YC-1 (▲ 30 μM; ■ 100 μM), and with different concentrations of GTP (20–320 μM). Double-reciprocal diagram of basal and YC-1-stimulated sGC activity (V_a) vs. substrate concentration (GTP). The extrapolated linear graphs intersect above the x-axis, indicating mixed-type activation kinetics (change in V_{max} and K_M). The data represent means of 3 independent determinations; the s.e.mean fell within symbol size.

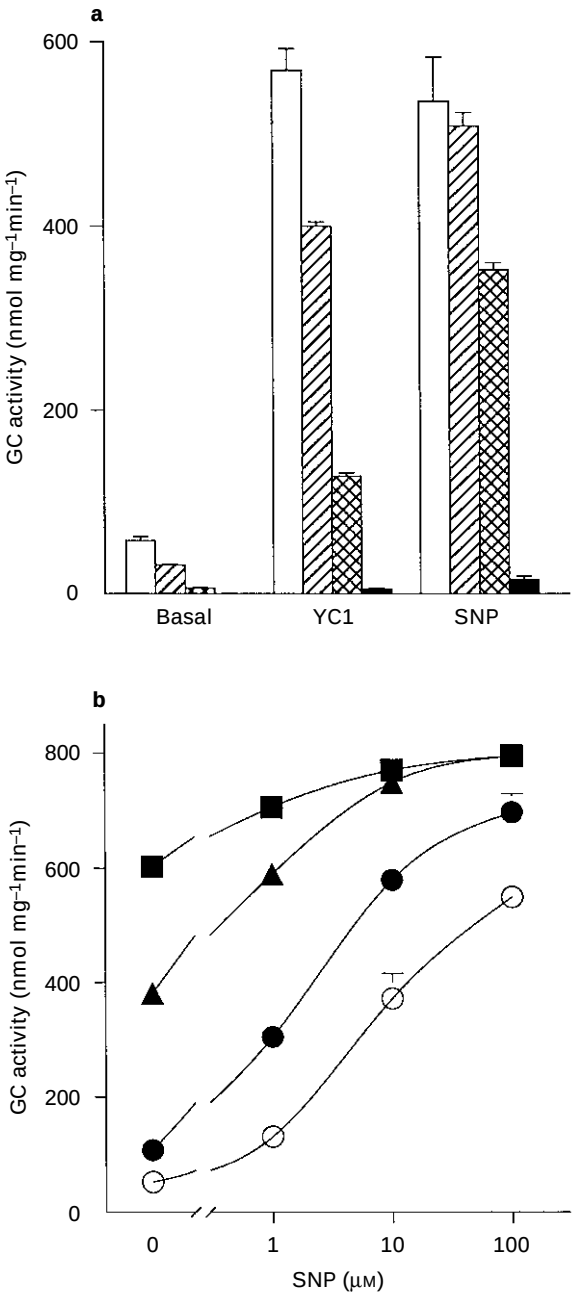

Figure 3 Effect of YC-1 and SNP on sGC activity and its sensitivity to NS 2028. (a) The activity of sGC was determined in the presence of either solvent (basal), YC-1 (100 μM) or SNP (100 μM) and in the absence (open columns) and presence of NS 2028 (hatched columns 0.1 μM; cross-hatched columns 1 μM; solid columns 10 μM). The data represent the mean ± s.e.mean from 3 independent experiments. (b) Activation of sGC by YC-1 and SNP. The concentration-dependent activation of sGC by SNP (0–100 μM) was assessed in the absence (○) and presence of YC-1 (● 3 μM; ▲ 30 μM; ■ 300 μM). Results are expressed as the mean from 3 independent experiments; vertical lines show s.e.mean. Activation by 100 μM YC-1 and SNP was significantly higher than activation by each agent alone ($P<0.05$; ANOVA).

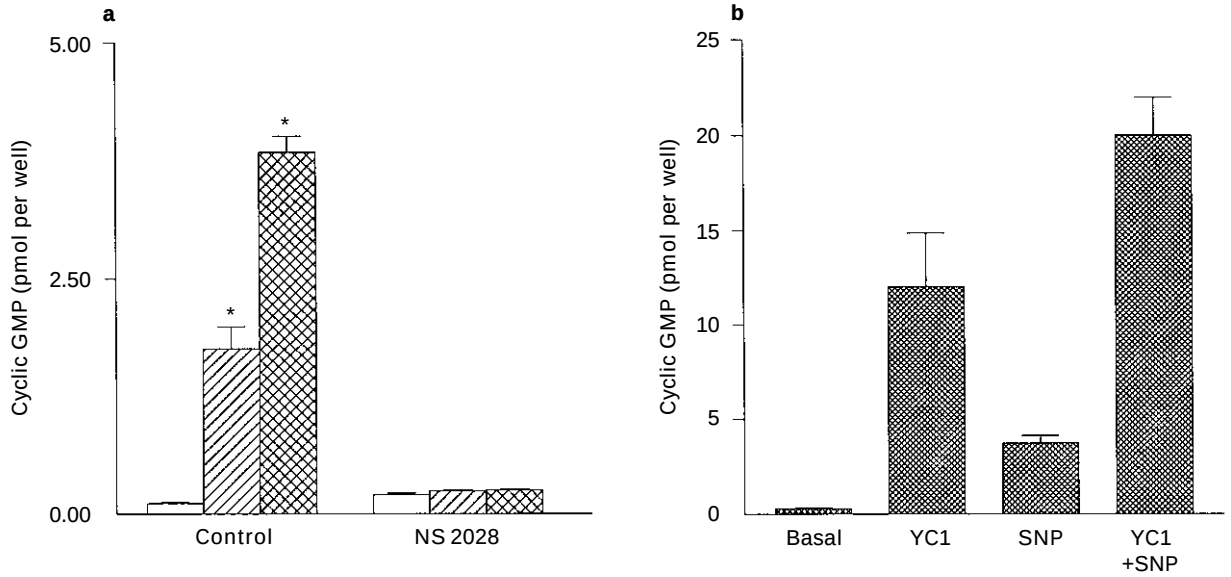

Figure 4 Effect of YC-1 on cyclic GMP levels in cultured vascular smooth muscle cells. (a) Cyclic GMP was determined in rat cultured aortic smooth muscle cells incubated for 10 min in the absence (open columns) and presence of YC-1 (hatched columns 10 μM; cross-hatched columns 100 μM) and in the absence (Control) and presence of NS 2028 (1 μM). YC-1-elicited cyclic GMP formation was significantly inhibited by NS 2028 (*$P<0.05$; ANOVA). (b) Cyclic GMP was determined in rat cultured aortic smooth muscle cells incubated for 10 min in the presence of either solvent (basal), YC-1 (100 μM), SNP (100 μM), or both SNP and YC-1 (100 μM each). Results are expressed as the mean$\pm$s.e.mean from 3 independent experiments. All values were significantly different from each other ($P<0.05$; ANOVA).

spontaneously hypertensive rats (SHR). These rings were mounted in organ baths as described for the rabbit rings, except that passive tension was adjusted to 2 g. The O_2^--generation of the rings was assessed by lucigenin-enhanced chemiluminescence as described previously (Hecker *et al.*, 1996).

Determination of cyclic GMP

To determine the content of cyclic GMP of the rabbit rings during relaxation, the rings were removed from the organ baths, blotted on filter paper, and each ring was homogenized in 600 μl ice-cold TCA (6%) by means of a glass-type potter-Elvejehm. Subsequently TCA homogenates were further processed and cyclic GMP and protein contents determined as described for cultured cells. Results are expressed as pmol cyclic GMP mg^{-1} protein.

Determination of nitrate and nitrite

YC-1 (500 μM) was incubated for up to 1 h at 37°C in the sGC incubation buffer in a final volume of 100 μl. Thereafter 100 μl aqueous buffered solution consisting of KH_2PO_4 (100 mM, pH 7.4) and EDTA (0.1 mM), 25 μl NADPH (5 mM), 12.5 μl nitrate reductase (0.05 units) and 12.5 μl FAD (100 μM) were added and the mixture was incubated for 30 min at 37°C to facilitate enzymatic conversion of nitrate to nitrite. Subsequently 20 μl lactate dehydrogenase (5 units; rabbit muscle) and 30 μl pyruvate (100 mM) were added and NADPH was consumed during 10 min at 37°C. The mixture was placed on ice for 5 min. Protein was precipitated by addition of ice-cold acetonitril (125 μl) and the mixture was cleared by centrifugation (15 min, 10,000 r.p.m.) at 4°C. A sample (200 μl) of the clear supernatant was pipetted on microtitreplates and nitrite was determined as a red azodye by addition of 45 μl sulphanilamide (1.8% in 1 N HCl) and 35 μl naphtylethylene diamine (0.3% in 1 N HCl) according to Griess (Green *et al.*, 1982). The absorbance at 570 nm was read after 20 min incubation at 20°C by a microplate reader. Calibration was performed by adding known amounts of nitrate to the sGC incubation buffer. The detection limit was 1 μM nitrate/nitrite, the recovery of nitrate was >90%.

Materials

Soluble GC was purified to apparent homogeneity from bovine lung (Mülsch & Gerzer, 1991). This preparation consists of subunits α_1 and β_1, as previously determined by sequence analysis (Kössling *et al.*, 1988; 1990) and confirmed in the presently used preparations by Western blot analysis (data not shown). YC-1 ((3-(5′-hydroxymethyl-2′furyl)-1-benzyl indazole) was synthetized as described by Yoshina & Kuo (1978) and was used as a 10 mM stock solution in DMSO. The highest DMSO concentration in the various test systems was 3% (v/v), and did not elicit any effects *per se* on the parameters tested. NS 2028 (1*H*-(1,2,4)-oxdiazolo-(4,3-a)-6-bromo-quinoxazin-1-one) was a generous gift from Dr S. Olesen (Neurosearch, Copenhagen, Denmark), stock solutions (10 mM) were prepared in DMSO. NTG was provided as a trituration in lactose (10% NTG) by Pohl-Boskamp (Hohenlockstedt, Germany). [α^{32}P]-GTP was from NEN-Dupont (Dreieich, Germany). Waymouth medium was obtained from PAN Systems Chemische Produkte GmbH (Aidenbach, Germany). Foetal bovine serum and antibiotics were obtained from Boehringer Mannheim (Mannheim, Germany). All other biochemicals were obtained in the highest purity available from Sigma (Deisenhofen, Germany), or Merck (Darmstadt, Germany).

Statistics

Unless otherwise indicated the results shown represent means$\pm$s.e.mean from at least 3 independent experiments performed in duplicate. In line graphs s.e.mean is indicated by error bars. In some figures the error bars fell within the symbol size. Statistical analysis was performed by Student's paired *t* test (two-tailed) or by ANOVA followed by the Bonferroni correction for comparison of multiple means. $P<0.05$ was considered significant.

Results

Activation of purified soluble guanylyl cyclase (sGC) by YC-1

YC-1 elicited a direct concentration-dependent stimulation of basal sGC activity (up to 10 fold at 300 μM; Figure 1a). YC-1 and SNP displayed a similar efficacy (V_{max}), but YC-1 was significantly less potent than the NO donor (EC_{50}: SNP 4.7 ± 0.5 μM vs. YC-1 18.6 ± 2.0 μM; $P<0.05$). Activation of sGC by YC-1 was rapid in onset and stable for at least 20 min at 37°C (Figure 1b). It was also rapidly reversible upon dilution according to the law of mass action (Table 1). Activation kinetics with respect to GTP were assessed by Lineweaver-Burk analysis (Figure 2). YC-1 stimulated GC activity by mixed-type activation kinetics, i.e., YC-1 (at 100 μM) increased V_{max} (650 vs. 107 nmol mg^{-1} min^{-1}) and decreased the K_M (54 ± 3 vs. 84 ± 3 μM). NS 2028, a specific inhibitor of sGC (Olesen *et al.*, 1996) and chemically related to 1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Garthwaite *et al.*, 1995), abrogated activation of sGC by YC-1 and SNP (Figure 3a).

YC-1 markedly enhanced activation of sGC in response to SNP in a concentration-dependent manner increasing both, the potency and efficacy of SNP for sGC activation (Figure 3b). The interaction between YC-1 and SNP was additive in that at maximally efficacious concentrations of SNP YC-1 further increased the enzyme activity (Figure 3b). A similar relationship was evident following the application of SNP to sGC maximally stimulated by YC-1. Other NO-releasing activators of sGC, such as 3-morpholino-sydnonimine and dinitrosyl-iron-di-L-cysteine complex elicited comparable effects (data not shown).

Cyclic GMP responses in cultured vascular smooth muscle cells

YC-1 elicited a concentration-dependent increase in intracellular cyclic GMP in rat cultured aortic vascular smooth muscle

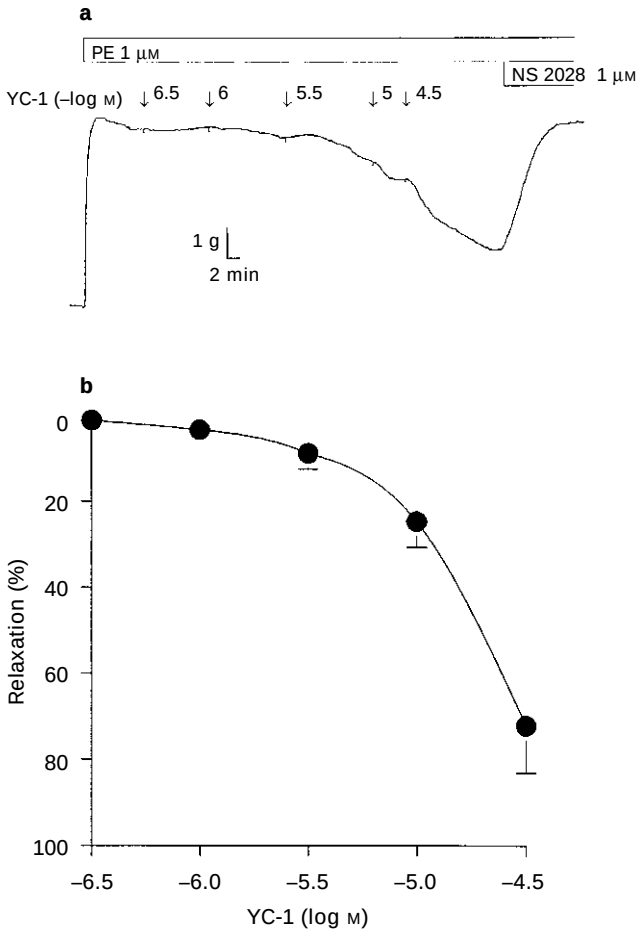

Figure 5 Concentration-response curve illustrating the effect of YC-1 on the vascular tone of rabbit preconstricted, endothelium-denuded aortic rings. (a) Original tracing and (b) statistical summary representing the relationship between YC-1 and vascular relaxation in rabbit aortic rings preconstricted with phenylephrine (PE; 1 μM). In some experiments NS 2028 (1 μM) was added to rings following the establishment of a stable maximal response to YC-1. Results in (b) are expressed as the mean from 6 independent experiments; vertical lines show s.e.mean. Since solubility limitations prevented the application of maximal relaxing concentrations of YC-1 the concentration-response curve could not be completed.

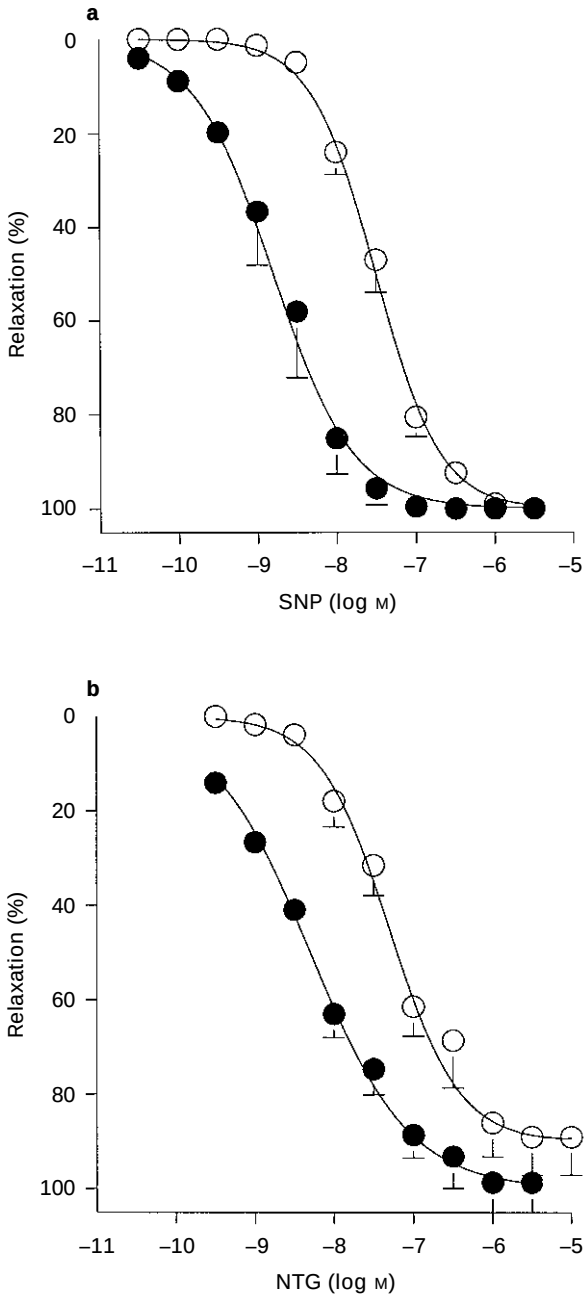

Figure 6 Effect of YC-1 on the relaxation to nitrovasodilators in rabbit endothelium-denuded aortic rings. Aortic rings preconstricted with phenylephrine (1 μM) were exposed to increasing concentrations of (a) SNP and (b) NTG. Experiments were performed in the absence (○) and presence of YC-1 (●; 3 μM). Results are expressed as the mean from 4 independent experiments; vertical lines show s.e.mean. For calculation of the EC_{50} values the data were fitted to a logistic equation represented by the line graphs. YC-1 significantly increased the vasodilator potency (EC_{50}) of SNP and NTG ($P<0.05$; t test for area under curve).

cells, which was abolished by preincubation with NS 2028 (Figure 4a). The increase in cyclic GMP elicited by 100 μM YC-1 was about 3 fold higher than that elicited by 100 μM SNP (Figure 4b). Cyclic GMP responses to 100 μM SNP and YC-1 were additive (Figure 4b).

Relaxation of rabbit endothelium-denuded aortic rings

In rabbit endothelium-denuded aortic rings, preconstricted with phenylephrine (1 μM), YC-1 (0.3–30 μM) elicited a concentration-dependent relaxation (Figure 5a,b). In contrast to the rapid onset of the relaxant response to SNP or NTG (data not shown), the YC-1-induced relaxation was slow to develop (Figure 5a). Addition of NS 2028 (1 μM) to aortic rings which had attained maximum relaxation to YC-1 completely reversed the relaxation (Figure 5a). To assess the interaction of YC-1 and NO donors at the vascular level the effect of YC-1-pretreatment on SNP- and NTG-induced relaxations was investigated. YC-1 at a concentration (3 μM), which on its own caused only a slight relaxation (8.9±3%), led to a leftward shift of the concentration response-curve to SNP and NTG with an approximately 10 fold increase in vasodilator potency (Figure 6a,b). Half-maximal relaxation was observed with

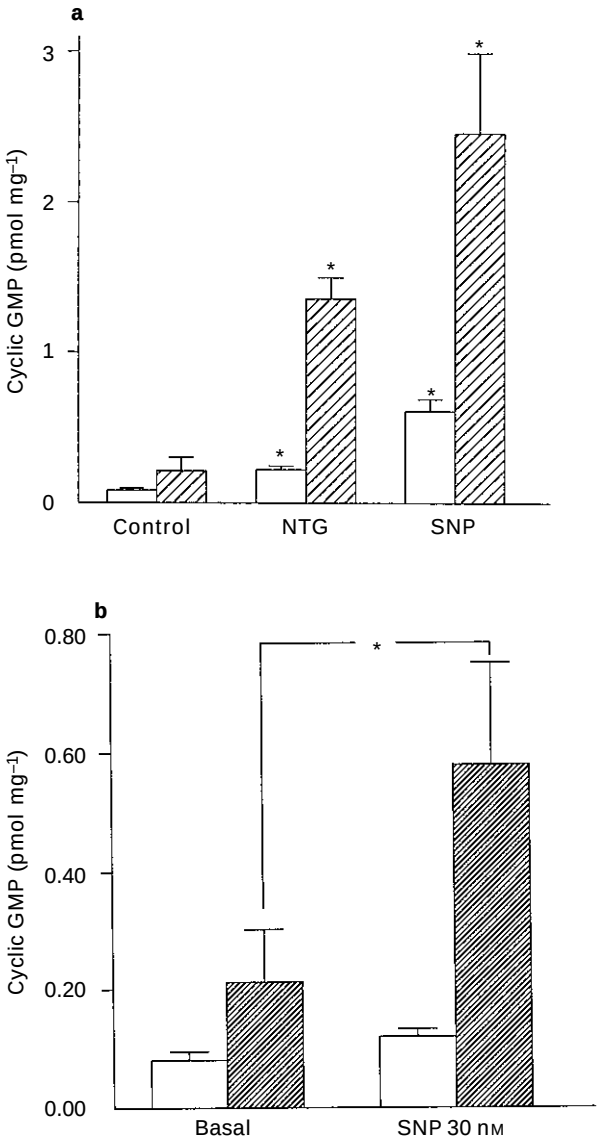

Figure 7 Potentiating effect of YC-1 on the cyclic GMP increase elicited by nitrovasodilators in rabbit endothelium-denuded aortic rings. (a) Cyclic GMP was assessed in PE-constricted rings following the addition of either solvent (control), or after attainment of maximal relaxation by NTG (10 μM) or SNP (3 μM). Experiments were performed in the absence (open columns) or presence of YC-1 (3 μM; hatched columns). *Indicates $P<0.05$ for NTG and SNP vs. control (ANOVA). (b) Cyclic GMP was assessed in PE-constricted rings following the addition of either solvent (basal), or SNP (30 nM), in the absence (open columns) and presence of YC-1 (3 μM; hatched columns). Results are expressed as the mean±s.e.mean from 7 independent experiments. *$P<0.05$ (ANOVA).

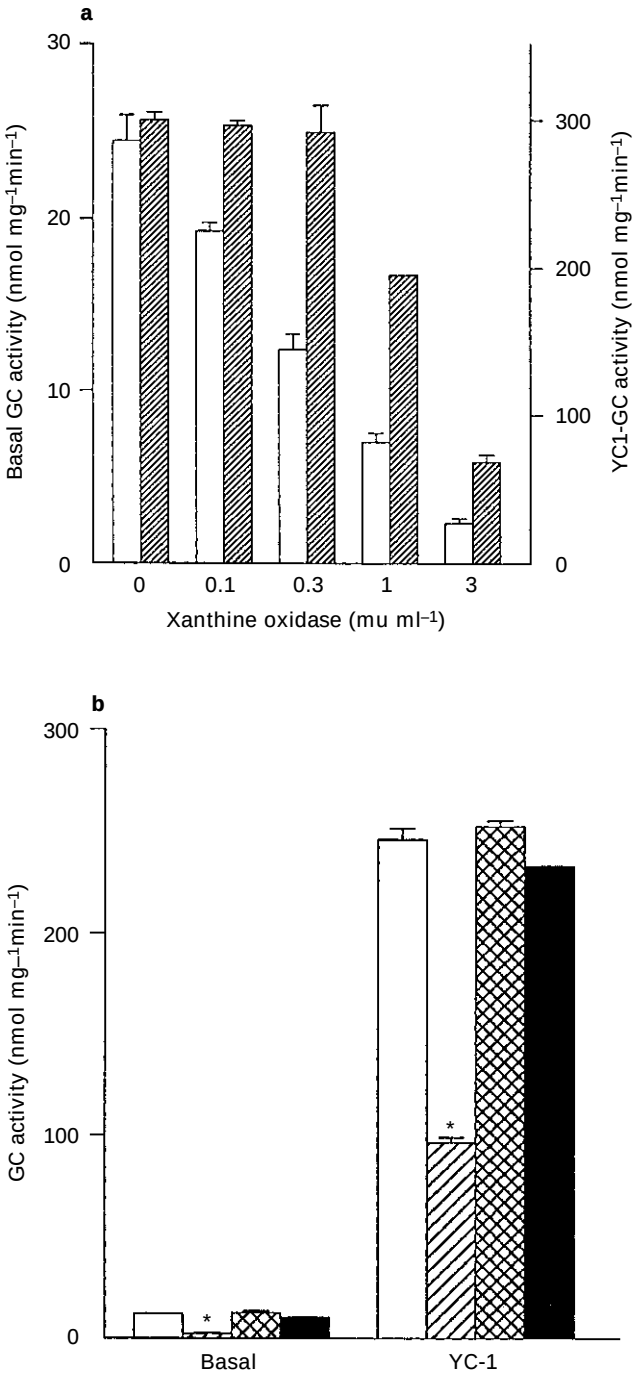

Figure 8 Effect of O_2^- radicals on basal and YC-1-stimulated sGC activity. (a) The basal (open columns, left axis) and YC-1-stimulated activity of sGC (hatched columns; right axis) were determined in the presence of xanthine (0.2 mM) and xanthine oxidase (0.1–3 mu ml^{-1}). XO significantly inhibited basal sGC activity at all concentrations tested and YC-1-enhanced sGC-activity at 1 and 3 mu ml^{-1} ($P<0.05$; ANOVA). (b) The basal and YC-1-stimulated activity of sGC (YC-1; 100 μM) were determined in the absence (open columns) and presence of xanthine oxidase (XO, 1 mu ml^{-1}; hatched columns), superoxide dismutase (SOD, 1 μM; cross-hatched columns), or XO and SOD (solid columns). Significant inhibition of sGC-activity by XO (*$P<0.05$) was prevented by SOD. Data represent mean±s.e.mean from 3 independent experiments in (a) and (b).

38±9 nM SNP and 57±23 nM NTG in the absence of YC-1 and with 3.2±1.5 nM SNP and 5.7±1.8 nM NTG in the presence of YC-1.

Cyclic GMP responses in rabbit aortic rings

The potentiation of SNP- and NTG-induced relaxations by YC-1 was mirrored by an increase in cyclic GMP concentration in the aortic rings (Figure 7a,b). While YC-1 (3 μM) and SNP (30 nM) applied alone elicited only slight increases in cyclic GMP, cyclic GMP levels were markedly elevated when both compounds were applied simultaneously (Figure 7b). Moreover, the cyclic GMP increase induced by high concentrations of SNP (3 μM) and NTG (10 μM), which induced complete relaxations of preconstricted aortic rings, was enhanced several fold in the presence of YC-1 (3 μM) (Figure 7a).

Effect of superoxide anion radicals (O_2^-) on basal and YC-1-stimulated activity of sGC and YC-1-elicited relaxation of rat aorta

Since O_2^- inhibits NO-induced vascular relaxation (Cherry *et al.*, 1990) and has been described as an inhibitor of sGC (Mülsch *et al.*, 1989; Brüne *et al.*, 1990) the effect of the O_2^--generating system xanthine/xanthine oxidase (XO) on basal and YC-1-enhanced sGC-activity was investigated. In the presence of xanthine (0.2 mM) XO induced a concentration-dependent decrease in basal sGC activity (Figure 8a). This inhibitory effect was half-maximal at 0.3 mu ml^{-1} XO and was prevented by SOD (1 μM) (Figure 8b). Xanthine/XO also decreased YC-1 (100 μM)-stimulated sGC-activity, although a higher concentration of XO was required to achieve a significant inhibition of YC-1-enhanced as compared to basal sGC-activity (Figure 8a). This inhibitory effect of the O_2^--generating system was also prevented by SOD (Figure 8b). To assess the influence of endogenously generated O_2^- on YC-1-induced relaxations the vascular responses to the sGC activator were compared in endothelium-denuded rings from Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR), which generated O_2^- in significantly different amounts. By using lucigenin-dependent chemiluminescence endogenous O_2^--generation was determined as 3.26±0.48 pmol mg^{-1} min^{-1} in aortic rings from WKY compared with 4.83±0.18 pmol mg^{-1} min^{-1} in aortic rings from SHR ($n=4$; $P<0.05$). In aortic rings from WKY, preconstricted with PE (1 μM), YC-1 induced a concentration-dependent relaxation (Figure 9a), which was slightly enhanced by SOD (0.1 μM) (Figure 9a,b). There was a clear attenuation of YC-1-induced relaxations of aortic rings from SHR compared to WKY (Figure 9a,b), which was restored following the application of SOD (0.1 μM). Subsequent administration of NS 2028 (3 μM) completely reversed YC-1- and SOD-elicited relaxations.

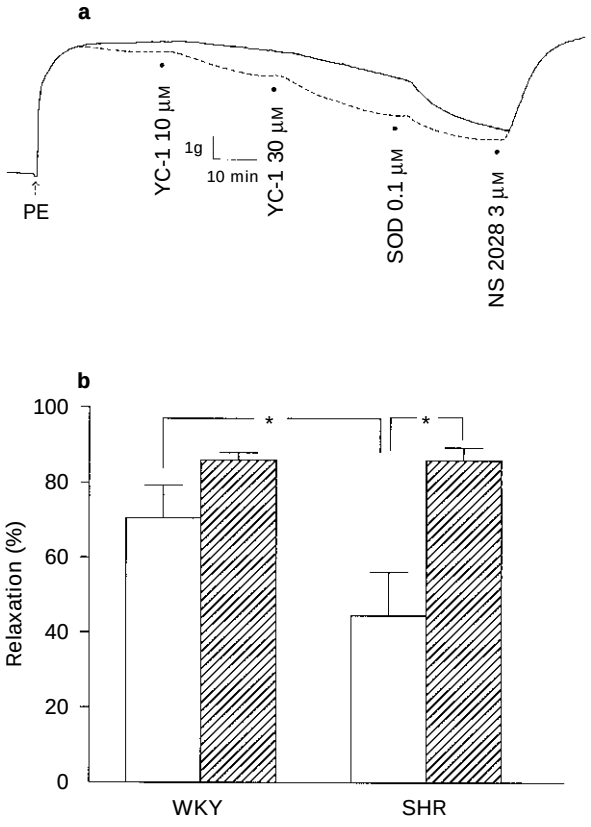

Figure 9 Effect of superoxide dismutase (SOD) on the vasodilator activity of YC-1 in preconstricted, endothelium-denuded rat aortic rings from normotensive (WKY) and spontaneously hypertensive rats (SHR). (a) Original tracings demonstrating the effect of SOD (0.1 μM) on YC-1 (30 μM)-elicited relaxations of PE (1 μM)-constricted aortic rings from SHR (upper tracing) and WKY (lower dotted tracing). NS 2028 (3 μM) completely reversed relaxations to YC-1 and SOD. (b) Relaxations (% reversal of PE-elicited contractions) elicited by YC-1 (30 μM) in the absence (open columns) and presence of SOD (hatched columns). Data represent mean± s.e.mean from 4 independent experiments. *Indicates significant difference ($P<0.05$; ANOVA).

Assessment of NO release from YC-1

To exclude the (minor) possibility that YC-1 activated sGC by NO generated spontaneously by oxygenation of one of its two nitrogen atoms, YC-1 (500 μM) was incubated for up to one hour in the sGC assay buffer. Then the mixture was assessed for the stable NO metabolites nitrate/nitrite. Neither NO metabolite could be detected.

Discussion

The present study demonstrates that YC-1 is a direct and reversible activator of haem-containing sGC purified from bovine lung. Moreover, our data revealed that the molecular mechanism of sGC activation was apparently different from that of NO donors and was sensitive to O_2^- radicals. YC-1 did not activate sGC by release of NO, as verified by measuring formation of nitrite and nitrate, stable metabolites of NO, by the Griess reaction. At the highest concentration of YC-1 achieved in the sGC test solution (0.5 mM) no nitrite/nitrate could be detected even after 1 h of incubation with YC-1 at 37°C, confirming the hypothesis that YC-1 is an NO-independent direct activator of sGC.

This NO-independent action may account for the observed additive or even synergistic activation of sGC by YC-1 and NO donors. This synergistic effect is best explained by assuming an interaction of YC-1 with the enzyme at a side distal to the heme, which functions as the primary NO receptor and triggers enzyme activation (DeRubertis *et al.*, 1978; Gerzer *et al.*, 1981; Ignarro, 1990). Combined evidence derived from UV/VIS, Raman and electron spin resonance spectra of NO-exposed purified sGC from bovine lung indicates that the heme-iron is penta-coordinated in the nitrosyl complex (Gerzer *et al.*, 1981; Mülsch & Böhme, 1984; Mülsch, 1986; Yu *et al.*, 1994; Stone *et al.*, 1995). Deletion mutant analysis of the $\alpha_1\beta_1$ heterodimer provided evidence that histidine $105\beta_1$ functions as an axial 5th or 6th ligand in the native resting enzyme (Wedel *et al.*, 1994). This and another histidine residue are presumably displaced upon NO binding (Yu *et al.*, 1994), thereby relieving strain from the tertiary protein structure. A subsequent conformational change of the protein backbone increases the catalytic activity of the enzyme. According to the reaction scheme set forth previously (intramolecular attack of the 3′-OH group on the α-phosphorous of GTP and proton transfer to a basic acceptor side; Senter *et al.*, 1983) and the changes in the reaction kinetics effected by binding of NO (decrease in K_M and increase in V_{max}) a conformational change

may stimulate catalysis in several ways. It may increase the binding affinity for the GTP-Mg^{2+} substrate, accelerate proton transfer from the 3′-hydroxyl-group of GTP to the basic acceptor side, and promote dissociation of the enzyme-product complex. In preliminary experiments YC-1 induced only minor changes in the soret absorption (430 nm) of the native enzyme (A. Mülsch, unpublished results). Therefore, it is conceivable that YC-1 activates sGC by inducing the aforementioned conformational change, without direct binding to the heme-iron. Future studies will help to elucidate whether YC-1 acts as an allosteric regulator and displaces the axial ligands of the enzyme from the heme iron, as occurs during the conversion of haemoglobin from the R- to T-state by inositolhexaphosphate. Alternatively, the enzyme's catalytic centre may be switched to a more active conformation by YC-1 without affecting heme-iron ligation.

Similar to the findings obtained with the purified enzyme a synergism between YC-1 and SNP was observed with regard to cyclic GMP increases in cultured vascular smooth muscle cells and in endothelium-denuded aortic rings of the rabbit. These results showed that responses of sGC to YC-1 in native and cultured vascular smooth muscle cells were qualitatively similar to that of the purified enzyme from bovine lung. However, in rat cultured aortic cells YC-1 was more efficacious than SNP in eliciting cyclic GMP formation. This contrasted with the apparently equal efficacy of both compounds on the purified enzyme. The reason for this discrepancy is unknown. However, cyclic GMP increases observed in response to YC-1 and SNP were completely inhibited by the specific inhibitor of sGC NS 2028, indicating that YC-1 elicited cyclic GMP formation in native and cultured vascular smooth muscle cells by activating the soluble isoform of GC. The synergistic effect of YC-1 on NO donor-elicited cyclic GMP formation accounted for the synergistic vasodilator response to YC-1 and NO donors, as assessed in rabbit aortic rings. Both compounds applied together in concentrations which, given alone, elicited only small increases in cyclic GMP and partial relaxation (3 μM YC-1 and 30 nM SNP), induced a pronounced increase in cyclic GMP and a complete relaxation of the aortic rings. Furthermore, YC-1 (3 μM) induced a ten fold decrease in the EC_{50} for SNP- and NTG-elicited relaxations. It is tempting to extrapolate these data to the *in vivo* situation, in which low plasma levels of YC-1 may potentiate the vasodilator activity of NO donors. Thus, it may be possible to decrease the dose of nitrovasodilators necessary to achieve a hypotensive response in patients, reducing the risk of adverse effects stemming from nitrovasodilator-derived NO, which is generated at a high rate in some organs (Mülsch *et al*., 1995a,b; Laursen *et al*., 1996). However this hypothesis on the potential therapeutic benefits of YC-1 remains to be tested in *in vivo* experiments.

A point to consider in the clinical setting is that activation of sGC by nitrovasodilators (Mülsch *et al*., 1989; Cherry *et al*., 1990) is sensitive to inhibition by O_2^- radicals. A somewhat unexpected finding was that both the basal and the YC-1-stimulated activity of sGC were inhibited by the O_2^--generating system xanthine/XO. In control experiments it was ascertained that the more stable O_2^--metabolite hydrogen peroxide in a concentration up to 20 μM did not affect sGC activity *in vitro* (data not shown). Since SOD prevented inhibition of sGC by xanthine/XO, O_2^- almost certainly is the inhibitory agent. The sites of interaction of O_2^- with sGC are unknown. Likely acceptor sites are the heme-iron, or some other O_2^--reactive moiety, such as a cysteine-thiol or enzyme-bound copper (Gerzer *et al*., 1981). The inhibition of sGC-activity by O_2^-, independent of the previously recognized scavenging of NO, indicates that sGC may be directly regulated by O_2^--levels *in vivo*. This hypothesis was supported by our observation that the vasodilator activity of YC-1 was decreased in endothelium-denuded aortic rings from SHR as compared to normotensive WKY rats. Two pieces of evidence suggested that endogenous O_2^- formation accounted for this difference in vasodilator sensitivity: firstly, the aortic rings from SHR generated significantly more O_2^- than the rings from WKY, and secondly, addition of SOD abolished the difference in vasodilator responsiveness to YC-1. Therefore, it remains to be investigated whether YC-1 is still able to potentiate the vasodilator activity of NO donors under pathophysiological conditions associated with increased vascular O_2^- generation, such as in hypercholesterolaemia (Ohara *et al*., 1993) and nitrate tolerance (Münzel *et al*., 1995).

In conclusion, YC-1 is a direct, NO-independent and O_2^--sensitive activator of sGC. The isolated enzyme and the enzyme present in its native cellular environment exhibit similar synergistic responses to the combined action of YC-1 and NO-releasing compounds. This synergistic action is functionally reflected by a pronounced increase in the vasodilator potency of SNP and NTG in the presence of non-relaxing concentrations of YC-1. The therapeutic benefit of this synergism remains to be exploited.

We would like to thank Dr Ingrid Fleming for valuable suggestions during the preparation of this manuscript and Michaela Stächele for skillful technical assistance. The antibodies to α_1 and β_1 subunits of sGC were kindly provided by Prof. Peter Yuen, University of Tennessee, Memphis, U.S.A.

References

BILLIAR, T.R. (1995). Nitric oxide. Novel biology with clinical relevance. *Annal. Surg*., **4,** 339–349.

BRÜNE, B., SCHMIDT, K.U. & ULLRICH, V. (1990). Activation of soluble guanylate cyclase by carbon monoxide and inhibition by superoxide anion. *Eur. J. Biochem*., **192,** 683–688.

CHERRY, P.D., OMAR, H.A., FARRELL, K.A., STUART, J.S. & WOLIN, M.S. (1990). Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. *Am. J. Physiol*., **259,** H1056–H1062.

DERUBERTIS, F.R., CRAVEN, P.A. & PRATT, D.W. (1978). Electron spin resonance study of the role of nitrosyl-heme in the activation of guanylate cyclase by nitrosoguanidine and related agonists. *Biochem. Biophys. Res. Commun*., **83,** 158–167.

ESUMI, H. & TANNENBAUM, S.R. (1994). U.S.-Japan cooperative cancer research progam: Seminar on nitric oxide synthase and carcinogenesis. *Cancer Res*., **54,** 297–301.

GARTHWAITE, J., SOUTHAM, E., BOULTON, C.L., NIELSEN, E.B., SCHMIDT, K. & MAYER, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one. *Mol. Pharmacol*., **48,** 184–188.

GERZER, R., BÖHME, E., HOFMANN, F. & SCHULTZ, G. (1981). Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett*., **132,** 71–74.

GERZER, R., HAMET, P., ROSS, A.H., LAWSON, J.A. & HARDMAN, J.G. (1983). Calcium-induced release from platelet membranes of fatty acids that modulate soluble guanylate cyclase. *J. Pharmacol. Exp. Ther*., **226,** 180–186.

GRAFF, G., STEPHENSON, J.H., GLASS, D.B., HADDOX, M.K. & GOLDBERG, N.D. (1978). Activation of soluble splenic cell guanylate cyclase by prostaglandin endoperoxides and fatty acid hydroperoxides. *J. Biol. Chem*., **253,** 7662–7676.

GREEN, L., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P., WISHNOOK, T. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem*., **126,** 131–138.

HECKER, M., PREIß, C., SCHINI-KERTH, V.B. & BUSSE, R. (1996). Antioxidants differentially affect nuclear factor κB-mediated nitric oxide synthase expression in vascular smooth muscle cells. *FEBS Lett*., **380,** 224–228.

IGNARRO, L.J. (1990). Haem-dependent activation of guanylate cyclase and cyclic GMP formation by endogenous nitric oxide: A unique transduction mechanism for transcellular signalling. *Pharmacol. Toxicol.*, **67,** 1–7.

IGNARRO, L.J., WOOD, K.S. & WOLIN, M.S. (1982). Activation of purified soluble guanylate cyclase by protoporphyrin IX. *Proc. Natl. Acad. Sci. U.S.A.*, **79,** 2870–2873.

KO, F.N., WU, C.C., LEE, F.Y. & TENG, C.M. (1994). YC-1, a novel activator of platelet guanylate cyclase. *Blood*, **84,** 4226–4233.

KÖSSLING, D., HERZ, J., GAUSEPOHL, H., NIROOMAND, F., HINSCH, K.D., MÜLSCH, A., BÖHME, E., SCHULTZ, G. & FRANK, R. (1988). The primary structure of the 70 kDa subunit of bovine soluble guanylate cyclase. *FEBS Lett.*, **239,** 29–34.

KÖSSLING, D., HARTENECK, C., HUMBERT, P., BOSSERHOFF, A., FRANK, R., SCHULTZ, G. & BÖHME, E. (1990). The primary structure of the larger subunit of soluble guanylyl cyclase from bovine lung. *FEBS Lett.*, **266,** 128–132.

LAURSEN, J.B., MÜLSCH, A., BOESGARD, S., MORDVINTCEV, P.I., TRAUTNER, S., GRUHN, N., NIELSEN-KUDSK, J.E., BUSSE, R. & ALDERSHVILE, J. (1996). Nitrate tolerance in vivo is not associated with reduced bioconversion of nitroglycerin to nitric oxide. *Circulation*, **94,** 2241–2247.

LINCOLN, T.M. & CORNWELL, T.L. (1993). Intracellular cyclic GMP receptor proteins. *FASEB J.*, **7,** 328–338.

MÜLSCH, A. (1986). *Untersuchungen zur Regulation der löslichen Guanylat-Cyclase*. Doctoral thesis, University of Heidelberg, Germany.

MÜLSCH, A., BARA, A., MORDVINTCEV, P., VANIN, A. & BUSSE, R. (1995a). Specificity of different organic nitrates to elicit NO formation in vascular tissues and organs of living rabbits. *Br. J. Pharmacol.*, **116,** 2743–2749.

MÜLSCH, A. & BÖHME, E. (1984). UV/VIS- and EPR-spectroscopic investigation of soluble guanylate cyclase. Activation by NO and sydnonimines. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **325,** R31.

MÜLSCH, A., BUSSE, R., LIEBAU, S. & FÖRSTERMANN, U. (1988). LY 83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.*, **247,** 283–288.

MÜLSCH, A. & GERZER, R. (1991). Purification of heme-containing soluble guanylyl cyclase. In *Methods Enzymol.*, Vol. 195, *Adenyl Cyclase, G Proteins, and Guanylyl Cyclase*. ed. Johnson, J.A. & Corbin, J.D. pp. 377–383. San Diego: Academic Press.

MÜLSCH, A., LÜCKHOFF, A., POHL, U., BUSSE, R. & BASSENGE, E. (1989). LY 83583 (6-anilino-5,8-quinolinedione) blocks nitrovasodilator-induced cyclic GMP increases and inhibition of platelet activation. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **340,** 119–125.

MÜLSCH, A., MORDVINTCEV, P., BARA, A., JUNG, F., CLEMENT, B. & BUSSE, R. (1995b). In vivo spin trapping of glycerol trinitrate-derived nitric oxide in blood vessels and organs. *Circulation*, **92,** 1876–1882.

MÜNZEL, T., SAYEGH, H., FREEMAN, B.A., TARPEY, M. & HARRISON, D.G. (1995). Evidence for enhanced vascular superoxide anion production in nitrate tolerance. *J. Clin. Invest.*, **95,** 187–194.

OHARA, Y., PETERSON, T.E. & HARRISON, D.G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.*, **91,** 2546–2551.

OLESEN, S.P., DREJER, J., AXELSSON, O., MOLDT, P., BANG, L., MÜLSCH, A., NIELSEN-KUDSK, J.E. & BUSSE, R. (1996). NS 2028, a novel inhibitor of the NO sensitive guanylyl cyclase. *2nd Intern. Conf. Biochemistry & Molecular Biology of Nitric Oxide*, Los Angeles.

SCHINI, J.B., JUNQUERO, D.C., SCOTT-BURDEN, T. & VANHOUTTE, P.M. (1991). Interleukin-1β induces the production of an L-arginine-derived relaxing factor from cultured smooth muscle cells from rat aorta. *Biochem. Biophys. Res. Commun.*, **176,** 114–121.

SENTER, P.D., ECKSTEIN, F.E., MÜLSCH, A. & BÖHME, E. (1983). The stereochemical course of the reaction catalyzed by soluble bovine lung guanylate cyclase. *J. Biol. Chem.*, **258,** 6741–6745.

STADLER, J., TROCKFELD, J., SCHMALIX, W.A., BRILL, T., SIEWERT, J.R., GREIM, H. & DOEHMER, J. (1994). Inhibition of cytochromes P4501A by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 3559–3563.

STONE, J.R., SANDS, R.H., DUNHAM, W.R. & MARLETTA, M.A. (1995). Electron paramagnetic resonance spectral evidence for the formation of a pentacoordinate nitrosyl-heme complex on soluble guanylate cyclase. *Biochem. Biophys. Res. Commun.*, **207,** 572–577.

WEDEL, B., HUMBERT, P., HARTENECK, C., FOERSTER, J., MALKEWITZ, J., BÖHME, E., SCHULTZ, G. & KOESLING, D. (1994). Mutation of His-105 in the β_1 subunit yields a nitric-oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 2592–2596.

WU, C.C., KO, F.N., KUO, S.C., LEE, F.Y. & TENG, C.M. (1995). YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. *Br. J. Pharmacol.*, **116,** 1973–1978.

WU, X.B., BRÜNE, B., VON APPEN, F. & ULLRICH, V. (1992). Reversible activation of soluble guanylate cyclase by oxidizing agents. *Arch. Biochem. Biophys.*, **294,** 75–82.

YOSHINA, S. & 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,** 204–208.

YU, A.E., HU, S., SPIRO, T.G. & BURSTYN, J.N. (1994). Resonance Raman spectroscopy of soluble guanylyl cyclase reveals displacement of distal and proximal heme ligands by NO. *J. Am. Chem. Soc.*, **116,** 4117–4118.

YU, S.M., CHENG, Z.J., GUH, J.H., LEE, F.Y. & KUO, S.C. (1995). Mechanism of anti-proliferation caused by YC-1, an indazole derivative, in cultured rat A10 vascular smooth muscle cells. *Biochem. J.*, **306,** 787–792.

(Received August 27, 1996
Revised November 12, 1996
Accepted November 21, 1996)