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Release of nitric oxide from endothelial cells stimulated by YC-1, an activator of soluble guanylyl cyclase

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1 In this study we examined the endothelium-dependent effect of YC-1 – a benzyl indazole derivative which directly activates soluble guanylyl cyclase (sGC) – on vascular relaxation and nitric oxide (NO) and guanosine-3′,5′-cyclic monophosphate (cyclic GMP) in endothelial cells.

2 In preconstricted rat aortic rings with intact endothelium, YC-1 produced a concentration-dependent relaxation. However, the concentration response curve was shifted rightward to higher concentrations of YC-1, when (i) the aortas were pre-treated with L-N^G-nitroarginine methylester (L-NAME) or (ii) the endothelium was removed.

3 Incubation of bovine aortic endothelial cells (BAEC) with YC-1 produced a concentration-dependent NO synthesis and release as assessed using a porphyrinic microsensor. Pre-incubating cells with L-NAME or with 8-bromo-cyclic GMP decreased this effect indicating that the YC-1 stimulation of NO synthesis is due to an activation of nitric oxide synthase, but not to an elevation of cyclic GMP. No direct effect of YC-1 on recombinant endothelial constitutive NO synthase activity was observed.

4 The YC-1 stimulated NO release was reduced by 90%, when extracellular free calcium was diminished.

5 In human umbilical vein endothelial cells (HUVEC), YC-1 stimulated intracellular cyclic GMP production in a concentration- and time-dependent manner. Stimulation of cyclic GMP was greater with a maximum concentration of YC-1 compared to calcium ionophore A23187. Similar effects were observed in BAEC and rat microvascular coronary endothelial cells (RMCEC).

6 When HUVEC and RMCEC were pre-treated with L-N^G-nitroarginine (L-NOARG), the maximum YC-1 stimulated cyclic GMP increase was reduced by $\geqslant 50\%$.

7 These results indicate, that beside being a direct activator of sGC, YC-1 stimulates a NO-synthesis and release in endothelial cells which is independent of elevation of cyclic GMP but strictly dependent on extracellular calcium. The underlying mechanism needs to be determined further.

Keywords: Soluble guanylyl cyclase; guanosine-3′,5′-cyclic monophosphate (cyclic GMP); nitric oxide; YC-1; endothelial cells; relaxation; calcium

Abbreviations: BAEC, bovine aortic endothelial cells; cyclic GMP, guanosine-3′,5′- cyclic monophosphate; EGTA, ethyleneglycol-bis(β-aminoethylether)N,N,N′,N′-tetraacetic acid; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; IBMX, 3-isobutyl-1-methyl-xanthine; L-NAME, L-N^G-nitroarginine methyl ester; L-NOARG, L-N^G-nitroarginine; NO, nitric oxide; RMCEC, rat microvascular coronary endothelial cells; sGC, soluble guanylyl cyclase; YC-1, 3-(5′-hydroxymethyl-2′-furyl-benzylindazole).

Introduction

Guanylyl cyclases catalyse the conversion of guanosine triphosphate (GTP) to guanosine-3′,5′-cyclic monophosphate (cyclic GMP). This group of enzymes consists of two classes. The membrane-bound guanylyl cyclases are receptor-like enzymes which are activated upon extracellular binding of natriuretic peptides. In contrast, soluble guanylyl cyclases (sGC) act *via* their haem group as important intracellular receptors for nitric oxide (NO). Mammalian sGC's are heterodimers with two different α and β-subtypes. The $\alpha_1\beta_1$-heterodimer has been shown to be expressed ubiquitously. Recently, another heterodimer, $\alpha_2\beta_1$-sGC, was found in human placenta (Russwurm *et al*., 1998). Additionally, other α and β subtypes have been cloned from adult human brain (Giuili *et al*., 1992; 1993). However, re-sequencing of the human cDNA clones for the α_3 and β_3-subtype revealed several sequencing errors and indicate that these clones might represent the human homologues of the α_1 and β_1-subtype (Zabel *et al*., 1998).

Despite their widespread distribution, the role of soluble guanylyl cyclases has been clearly underestimated. One of the experimental problems so far has been the lack of selectively sGC-modulating compounds. However, two compounds have been recently described which modulate sGC activity *in vitro* and *in vivo* (for a review, see Koesling, 1998). ODQ (1*H*-[1,2,4]oxadiazolol-[4,3-*a*]quinoxalin-1-one) acts as an inhibitor of sGC-activity – presumably by oxidizing the sGC haem iron – while not affecting particulate GC, adenylate cyclase or NO synthase activity. In contrast to ODQ, YC-1 (3-(5′-hydroxymethyl-2′-furyl)1-benzylindazole) has been identified as a direct activator of sGC. Initially described as an inhibitor of platelet aggregation, YC-1 prolonged the tail bleeding time of conscious mice by 30 min after intraperitoneal administration (Ko *et al*., 1994). YC-1 elicited a direct, reversible activation of

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purified bovine lung $\alpha_1\beta_1$-subtype sGC by increasing the affinity for GTP and increasing the maximum enzyme activity. The YC-1 induced sGC activation could be inhibited by superoxide radicals and synergistically increased by NO (Mülsch et al., 1997). Friebe and coworkers demonstrated that NO-insensitive sGC mutants were not influenced either by SOD or by xanthine oxidase suggesting that the inhibition by superoxide might be due to its scavenging effect on atmospheric NO (Friebe *et al*., 1998b). Furthermore, it was reported that YC-1 turns carbon monoxide (CO) into a potent activator of sGC (Friebe *et al*., 1996).

YC-1 relaxed preconstricted, endothelium-denuded rabbit aortic rings in a concentration-dependent manner and markedly increased cyclic GMP levels. At a threshold concentration of YC-1, this vasodilation was potentiated by NO donating compounds (Mülsch *et al*., 1997). By measuring formation of nitrite and nitrate, these authors excluded the possibility that YC-1 leads to a release of NO. In this study, by using a more sensitive protocol for measuring NO, we identified a novel action for YC-1 in stimulating NO synthesis and release in endothelial cells, which contributes to YC-1 stimulated relaxation of endothelium-containing rat aortic rings and to YC-1 stimulated formation of cyclic GMP in cultured endothelial cells.

Methods

Vascular reactivity studies (organ bath)

Male Wistar rats (250–300 g) rats were decapitated and the chest and abdomen were opened through a medial sternotomy. The descending aorta was removed, cleaned of connective tissue and dissected into four rings (3 mm in length). Subsequently, aortic rings were mounted in a thermostatted (37°C) organ bath (Schuler-Organbad; Hugo Sachs Elektronik) for isometric measurement of contractile tone. The rings were equilibrated for 30 min under a resting tension of 1 g in carbogenated (95% O_2; 5% CO_2) Krebs-Henseleit solution, pH 7.4 (composition in mmol l^{-1}): Na^+ 144.0, K^+ 5.9, Cl^- 126.9, Ca^{2+} 1.6, Mg^{2+} 1.2; $H_2PO_4^-$ 1.2; SO_4^{2-} 1.2; HCO_3^- 25.0; D-glucose 11.1, in the presence of the cyclo-oxygenase inhibitor indomethacin (1 μmol l^{-1}). Then, rings were contracted with phenylephrine (1 μmol l^{-1}) and the functional integrity of the endothelium was tested by recording the relaxation stimulated by acetylcholine (1 μmol l^{-1}). Following a 30 min washout period, rings were contracted with phenylephrine (1 μmol l^{-1}) and the relaxant response to cumulative dose of YC-1 was assessed in the presence or absence of L-NAME (1 μmol l^{-1}). In some of the rings, the endothelium was removed by forcing a glass rod through the lumen prior to mounting into the organ bath. In order to test whether this denudation protocol resulted in an impaired reactivity of the vessel, intact and denuded rings were incubated in independent experiments with the NO donor SIN-1 (10 nmol l^{-1} to 1 μmol l^{-1}).

Isolation and cultivation of endothelial cells

Primary endothelial cell were isolated and cultured as described (Wohlfart *et al*., 1997). Briefly, HUVEC were seeded onto 12-well plates, whereas BAEC and RMCEC were seeded onto 6-well plates. Generally, plates were pre-coated with collagen I from rat tail (Becton Dickinson Labware, Heidelberg, Germany). Primary cells were used for the experiments 2 days after reaching confluence.

Measurement of NO

Nitric oxide was measured using a porphyrinic microsensor as previously described (Malinski & Taha, 1992; Wiemer *et al*., 1996). Briefly, the sensor was placed 10 ± 4 μm from the surface of the endothelial cells and the amperometric signal at constant potential of 0.67 V was measured using a Princeton Applied Research –PAR– model 273 voltametric analyser interfaced with an IBM 80486 computer with data acquisition and control software. Linear calibration curves were constructed for each sensor from 2 nmol l^{-1} to 2 μmol l^{-1} NO, before and after *in vitro* measurements, using aliquots of saturated NO prepared as described (Jia & Furchgott, 1993). In order to chelate extracellular calcium, the incubation buffer was supplemented in some experiments with ethyleneglycol-bis(β-aminoethylether)N,N,N′,N′-tetra-acetic acid (EGTA, 2 mmol l^{-1}) prior to the addition of YC-1.

Measurement of cyclic GMP

Intracellular cyclic GMP was measured as described (Wohlfart *et al*., 1997) with minor modifications given below. Briefly, after washing twice, endothelial cells were equilibrated for 15 min in warm HEPES/Tyrodes solution. Pre-incubation was started by addition of 3-isobutyl-1-methyl-xanthine (IBMX, 0.5 mmol l^{-1} final concentration) and superoxide dismutase (SOD, from bovine erythrocytes, 20 units ml^{-1}). Compounds were added after 15 min pre-incubation. YC-1 and calcium ionophore A23187 were prepared as stock solutions in dimethylsulphoxide (DMSO) and tested against a respective control (0.5% v v^{-1} final concentration of DMSO). Incubations were stopped at the times indicated in the result section by rapid aspiration of the supernatants and addition of an ice-cold mixture (15 : 85 v v^{-1}) of acetone and formic acid (1 mol l^{-1}). After at least 2 h extraction at -20°C the organic supernatants were lyophilized and cyclic GMP was determined using a commercially available radio immunoassay based upon the manufacturer's instructions (BIOTREND, Köln, Germany).

Materials

YC-1 (3-(5′-hydroxymethyl-2′-furyl-benzylindazole)) was synthesized in our medicinal chemistry laboratories as described by Yoshina & Kuo (1978) and used as a stock solution in DMSO. The highest DMSO concentration in the various test systems was 0.5% (v v^{-1}) and did not elicit effects *per se* on the parameters tested. Cell culture medium and additives were obtained from BioWhitaker (Verviers, Belgium) or GibcoBRL (Eggenstein, Germany). All other biochemicals were obtained in the highest purity available from Sigma (Deisenhofen, Germany) or E. Merck (Darmstadt, Germany).

Statistics

Unless otherwise indicate the results shown represent mean ± s.e.mean from at least three independent experiments. 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 ANOVA followed by the Bonferroni correction for comparison of multiple means. $P < 0.05$ was considered significant.

Results

Relaxation of rat aortic rings

YC-1 elicited a concentration-dependent relaxation in isolated intact and endothelium-denuded aortic rings of the rat, preconstricted with phenylephrine (1 μmol l^{-1}, Figure 1). The concentration-dependent YC-1 stimulated relaxation was shifted significantly to the right by about a factor of 10 in endothelium-denuded rings. A similar rightward shift was observed when intact rings were pre-incubated with the NO-synthase inhibitor, L-NAME (1 μmol l^{-1}). An endothelium-independent NO-donor (SIN-1, 10 nmol l^{-1} to 1 μmol l^{-1}) relaxed both intact and denuded vessels to a similar degree (data not shown).

NO release from endothelial cells

In characterizing the mechanism underlying this endothelium-dependent improvement of the YC-1 stimulated relaxation, we observed that incubation of BAEC with YC-1 led to a time-dependent NO release assessed by a porphyrinic microsensor placed near the surface of the cells (Figure 2). Maximum NO release was measured 2–3 s after addition of YC-1 and rapidly declined. YC-1 loses its ability to stimulate NO release in calcium-free medium. As estimated by the area under the curve, the YC-1 stimulated release decreased by about 90% in the presence of EGTA (2 mmol l^{-1}). In Figure 3 the concentration-response-curve for YC-1 stimulated NO release is shown. The NO release was significant at $\geqslant$20 μmol l^{-1}. The maximum response to YC-1 could not be assessed due to poor solubility of the compound at concentrations >100 μmol l^{-1}. Approximately 40% of the NO release could be blocked by pre-incubation with L-NAME (200 μmol l^{-1}). A similar degree of inhibition by L-NAME was observed in experiments in which NO release was stimulated by calcium ionophore A23187 (Figure 4b). Pre-incubation with 8-bromo-cyclic GMP (100 μmol l^{-1}), a membrane permeable stable cyclic GMP analogue, or L-NAME (200 μmol l^{-1}) resulted in a significant inhibition of the calcium ionophore or the YC-1 mediated NO (Figure 4).

Accumulation of cyclic GMP in endothelial cells

Figure 5 depicts a typical kinetic profile of intracellular cyclic GMP accumulation induced by YC-1 (30 μmol l^{-1}) in HUVEC. After a rapid onset, maximum cyclic GMP was reached after 5 min and remained constant for a further 15 min. The maximum YC-1 stimulated cyclic GMP increase was greater than that observed with maximum stimulation by calcium ionophore A23187 (1 μmol l^{-1}). The time course of effect of both compounds on cyclic GMP differed to that of bradykinin (100 nmol l^{-1}) which yielded a peak value at 3 min followed by a rapid decline. The concentration-response curve for YC-1 revealed a threshold value of 1 μmol l^{-1} (Figure 6). YC-1 (30 μmol l^{-1}) also increased cyclic GMP accumulation in macrovascular bovine aortic endothelial cells (BAEC) and in rat microvascular coronary endothelial cells (RMCEC, Figure 7).

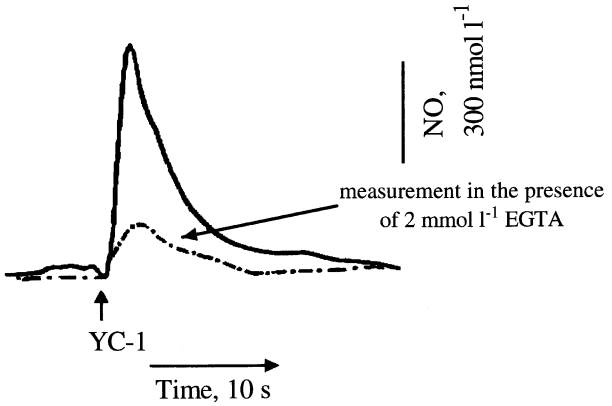

Figure 2 Effect of YC-1 on NO release from bovine aortic endothelial cells (BAEC) assessed by a porphyrinic microsensor as a function of time. In the presence of EGTA (2 mmol l^{-1}), the stimulation of NO release by YC-1 (80 μmol l−1) decreased by about 90%. Typical original tracings from a representative experiment are shown.

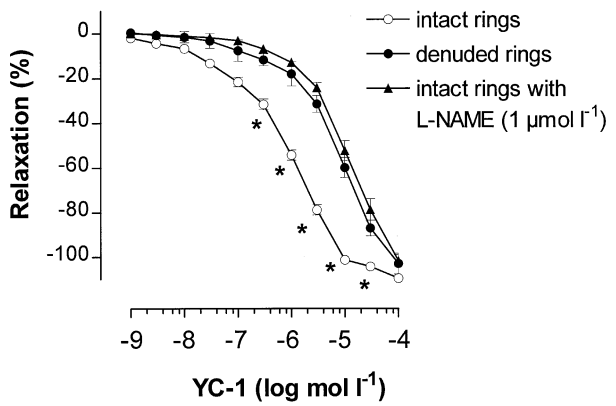

Figure 1 Effect of YC-1 on relaxation of rat aortic rings preconstricted with phenylephrine (1 μmol l^{-1}) as a function of concentration. The results are presented as means ± s.e.mean of four rings from a representative experiment. Three independent single experiments had similar results. * $P<0.05$ vs endothelium denuded rings and intact rings pre-incubated with L-NAME.

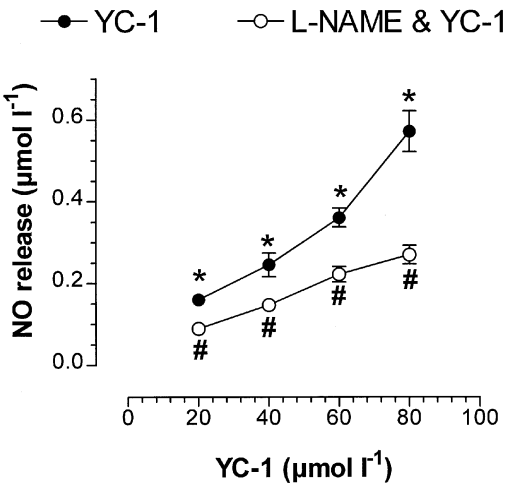

Figure 3 Effect of YC-1 on NO release from BAEC as a function of concentration. Data are shown for endothelial cells with and without pre-incubation for 15 min with L-NAME (200 μmol l^{-1}). The results are expressed as means ± s.e.mean from six independent experiments. *$P<0.05$ vs control (0.5% v v^{-1} DMSO, final concentration); #$P<0.05$ vs stimulation with YC-1 alone.

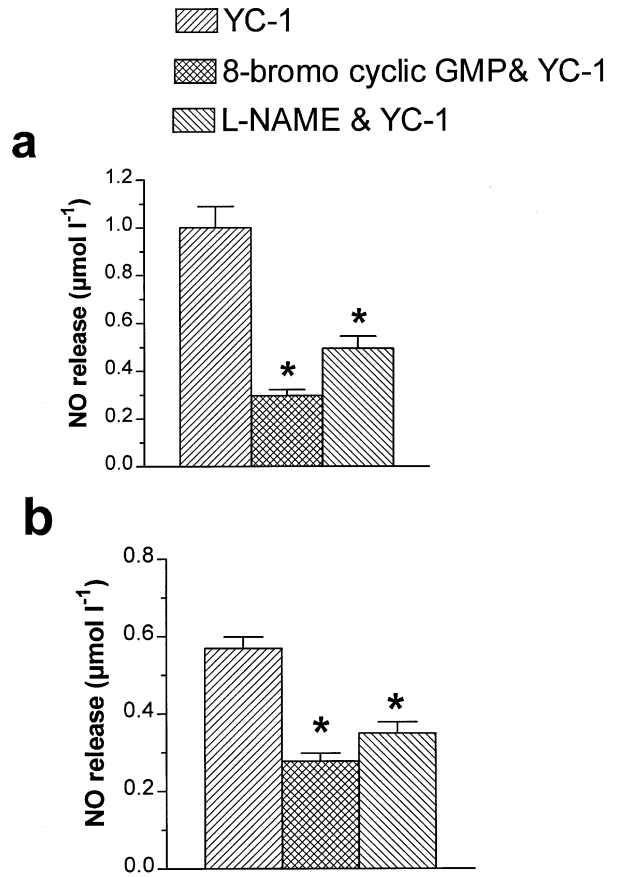

Figure 4 Effect of pre-incubation of BAEC for 15 min with L-NAME (200 μmol l^{-1}) on YC-1 (80 μmol l^{-1}) (a) or calcium ionophore A23187 (1 μmol l^{-1}) (b) stimulated NO release from BAEC. The results are expressed as mean ± s.e.mean from six independent experiments. $^*P<0.05$ vs stimulation with YC-1 alone.

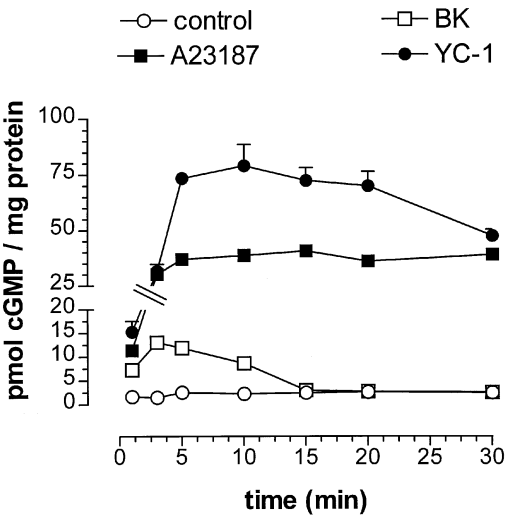

Figure 5 Time dependent increase in intracellular cyclic GMP accumulation in HUVEC induced by YC-1 (30 μmol l^{-1}), calcium ionophore A23187 (1 μmol l^{-1}) or bradykinin (BK, 100 nmol l^{-1}) compared to a solvent control (DMSO, 0.5% v v^{-1} final concentration). The results are presented as mean ± s.e.mean of four wells from a representative experiment. Two independent experiments on different cell batches showed similar results.

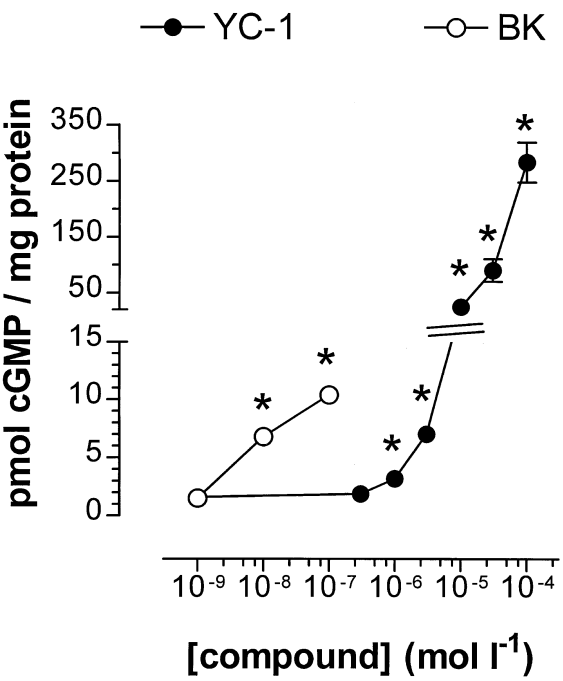

Figure 6 Concentration dependent effect of YC-1 (10 min incubation) on the intracellular accumulation of cyclic GMP in HUVEC compared to bradykinin (BK) stimulation (3 min incubation). The results are taken from a representative experiment and are expressed as the mean ± s.e.mean from four wells. Four independent experiments performed on different cell batches delivered similar results. $^*P<0.05$ vs control (0.5% v v^{-1} DMSO final concentration).

When HUVEC and RMCEC were pre-incubated with L-nitro-arginine (L-NOARG, 10 μmol l^{-1}) the YC-1 stimulated cyclic GMP synthesis was decreased by >50% (Figure 8). In independent experiments in both cell models, this concentration of L-NOARG totally inhibited the bradykinin or calcium ionophore induced cyclic GMP accumulation (data not shown).

Discussion

The role of soluble guanylyl cyclases has been underestimated in the past due to the lack of compounds which modulate enzyme activity specifically. This situation changed, once compounds with a higher specifity towards sGC could be identified. Besides NO itself, YC-1 represents the first activating pharmacophore of intracellular sGCs in a biological milieu. In initial experiments, YC-1 was investigated for its effect on platelet aggregation (Ko *et al*., 1994; Teng *et al*., 1997; Wu *et al*., 1995. Later detailed studies on isolated sGC followed. It was demonstrated that species independently, YC-1 activates isolated bovine, rat, and human $\alpha_1\beta_1$-subtype sGC (Friebe *et al*., 1996; Friebe & Koesling, 1998; Hoenicka *et al*., 1999; Mülsch *et al*., 1997; Zabel *et al*., 1998) as well as a recombinant form of the $\alpha_2\alpha_1$-subtype sGC (Russwurm *et al*., 1998).

Only a few studies have been performed so far to confirm a specific action of YC-1 on sGC expressed in vascular tissues and cell types. From relaxation studies with endothelium-denuded rat aortic rings, it was indirectly concluded that the YC-1 stimulated relaxation is mainly mediated by activation of sGC and subsequent increases in cyclic GMP (Wegener *et al*., 1997). However, later it was demonstrated in human platelets

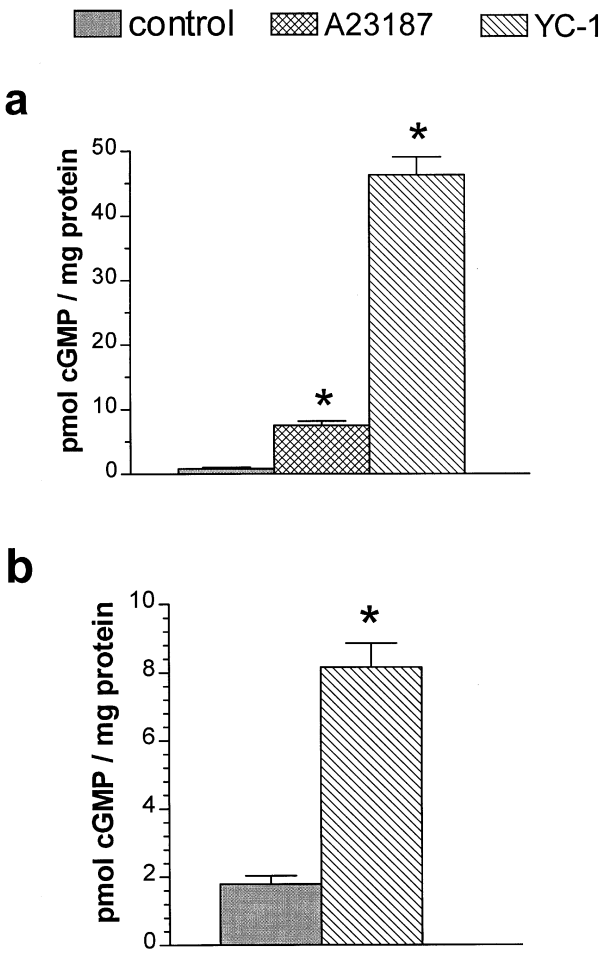

Figure 7 Intracellular cyclic GMP accumulation in bovine aortic endothelial cells (BAEC a) and in rat microvascular coronary endothelial cells (RMCEC b). Cells were incubated for 10 min with solvent (control, 0.5% final concentration of DMSO, calcium ionophore A23187 (1 μmol l^{-1}) or YC-1 (30 μmol l^{-1})). The results are presented as mean $\pm$ s.e.mean of three wells from representative experiments. Three independent experiments performed on different cell batches delivered similar results. *$P<0.05$ vs control (0.5% v v^{-1} DMSO, final concentration).

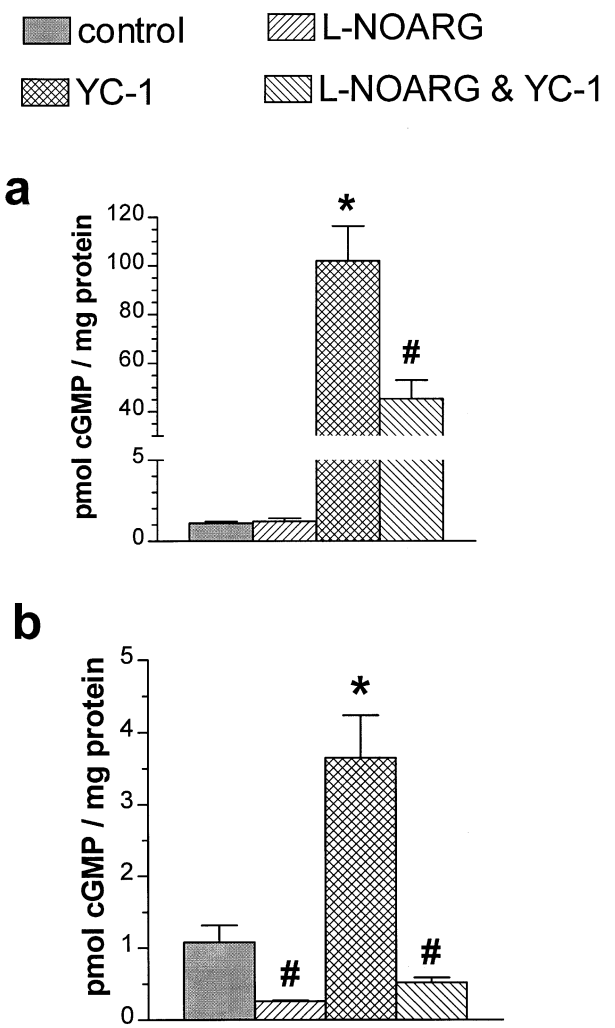

Figure 8 Inhibition of YC-1 stimulated cyclic GMP accumulation (30 μmol l^{-1} YC-1, 10 min) by pre-incubation for 15 min with L-NOARG (10 μmol l^{-1}) in HUVEC (a) and RMCEC (b). Results are given as mean $\pm$ s.e.mean of six wells from a representative experiment of each endothelial cell type. Three independent experiments performed on different cell batches showed similar results. *$P<0.05$ vs control (0.5% v v^{-1} DMSO, final concentration); #$P<0.05$ vs stimulation with YC-1 alone.

that beside stimulation of sGC, YC-1 also inhibited cyclic GMP-hydrolysing phosphodiesterases, thereby enhancing the overall effect on cyclic GMP (Friebe *et al*., 1998a). Inhibition of cyclic GMP-hydrolysing phosphodiesterases by higher concentrations of YC-1 (100 μmol l^{-1}) was also observed in extracts of rabbit aortae (H.H.H.W. Schmidt, personal communication 1998).

Our main finding of the present study is the identification of a novel action of YC-1 which seems to be mediated by a mechanism independent of activation of sGC. Using an established porphyrinic microsensor, we demonstrated that YC-1 concentration-dependently stimulated release of NO from BAEC. This release could be partially decreased by pre-incubation with L-NAME. The incomplete inhibitory action of L-NAME on YC-1 stimulated NO release even at higher concentrations could be attributed to the location where NO is detected. In contrast to any other protocol for detection of NO, the prophyrinic sensor measures NO on the surface of the endothelial cells closest to the localization of the endothelial NO synthase (eNOS) and hence delivers the highest concentrations of NO, which decay exponentially with the distance from the endothelial cell surface. Therefore, measurement with the porphyrinic sensor always indicates higher NO levels than those measured by different protocols. In our particular case, in the presence of L-NAME, a 40% decrease in NO on the surface of endothelial cells is translated to a near zero concentration at the site of measurement of cyclic GMP. By using calcium ionophore A23187, we confirmed that even for this control stimulus only a partial inhibition by L-NAME could be obtained with our experimental setup.

In order to provide further evidence for YC-1 stimulated NO release in endothelial cells, we performed measurements of intracellular cyclic GMP in cultured endothelial cells. YC-1 produced a strong intracellular cyclic GMP-synthesis which always exceeded cyclic GMP synthesis induced by calcium ionophore A23187. In previous studies, a synergistic activation of sGC by NO and YC-1 was demonstrated for purified bovine lung sGC (Friebe *et al*., 1996; Friebe & Koesling, 1998; Mülsch *et al*., 1997) and for intracellular sGC in cultured rat vascular smooth muscle cells, endothelium-denuded rabbit vessels and human platelets (Mülsch *et al*., 1997; Friebe *et al*., 1998a).

Hence, a synergistic activation of sGC by YC-1 and basal NO may provide an explanation for the greater effect of YC-1 compared to A23187. In our experiments, the YC-1 stimulated cyclic GMP formation was attenuated by pre-incubation with L-NOARG. It seems to be very unlikely, that this attenuation is only due to inhibition of baseline synthesis of NO. Despite the fact that in HUVEC pre-incubation with L-NOARG did not alter baseline cyclic GMP and hence baseline synthesis of NO, the large attenuation of YC-1 stimulated cyclic GMP formation by L-NOARG cleary suggests a YC-1 stimulated NO synthesis as a second mode of action.

In previous studies, the effect of YC-1 on vascular reactivity was investigated only on endothelium-denuded vascular rings (Mülsch *et al*., 1997; Wegener *et al*. 1997), most likely assuming that sGC in smooth muscle is the main target for YC-1 in the vasculature. In our experiments we noticed a marked difference between endothelium-denuded and intact rings with respect to reactivity towards YC-1. Approximately 10 fold higher concentrations of YC-1 were needed to relax preconstricted endothelium-denuded versus intact rings. In accordance with a previous study on isolated rabbit left carotid arteries (Hadoke *et al*., 1993), denudation of the vessel did not result in altered relaxation to an endothelium-independent control dilator (SIN-1). Therefore, it seems unlikely that denudation alone accounted for the apparent rightward shift of the response to YC-1. A similar rightward shift in the concentration-response curve of YC-1 stimulated relaxation could be observed, when intact rings were pre-treated with L-NAME. These findings are in accordance with our measurements of stimulation of NO synthesis and release in endothelial cells as a second mechanism of YC-1 but could also be explained by a synergistic action of basal endothelium-derived NO and YC-1 on sGC. Our vascular experiments with YC-1 indicate a possible disadvantage of this pharmacological agent compared to other dilators. The YC-1 stimulated vascular relaxation might be attenuated in situations associated with endothelial dysfunction, when the endothelium loses its integrity and/or ability to synthesise NO.

In our experiments in BAEC, we observed a dependency of NO release on the presence of extracellular calcium. In a buffer devoid of free calcium, YC-1 stimulated NO release was decreased by about 90%. A YC-1 mediated increase in intracellular calcium via modulation of calcium entry flux might be sufficient to explain the observed YC-1 stimulated NO synthesis and release. The diversity of calcium entry pathways and the precise mechanisms for their control in endothelial cells are still a matter of controversy. Because high-affinity antagonists clearly discriminating between these pathways are still lacking we could not investigate the dependency of YC-1 stimulated NO-release on extracellular calcium any further. In an attempt to identify additional second messengers involved in the YC-1 stimulated NO release, we observed that this activation is not due to a preceding increase in endothelial cyclic GMP. When BAEC were pre-incubated with 8-bromo-cyclic GMP, a membrane permeable stable cyclic GMP analogue, a decrease not an increase in the YC-1 stimulated NO-release was observed. Therefore, both processes, YC-1 stimulated NO and cyclic GMP synthesis, occur concomitantly in endothelial cells. We also investigated the effect of YC-1 on intracellular cyclic AMP but noticed no change in intracellular cyclic AMP synthesis in endothelial cells incubated under similar conditions with YC-1 (data not shown). These results confirm data from another study, in which YC-1, used in similar concentrations, did not increase cyclic AMP levels either in aortic rings or in ventricular cardiomyocytes (Wegener *et al*., 1997). Furthermore, a direct interaction of YC-1 with eNOS might also explain a stimulation of NO synthesis and release. However when human recombinant eNOS was incubated with YC-1, no significant difference in enzyme activity, assessed as conversion of radio-labelled L-arginine to L-citrulline, was observed (data not shown).

We demonstrate in this study that YC-1 is an activator of sGC in endothelial cells. However, stimulation of NO synthesis and release by YC-1 in endothelial cells is an additional mode of action of this compound which is independent of activation of sGC. Although the underlying calcium-dependent mechanism of this novel action of YC-1 needs to be determined further, future functional studies investigating the effects of YC-1 on intact cells and tissues should be interpreted very carefully with respect to these new observations.

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