

YC-1 Potentiates Nitric Oxide- and Carbon Monoxide-Induced Cyclic GMP Effects in Human Platelets

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ABSTRACT

Nitric oxide (NO), the physiological activator of soluble guanylyl cyclase (sGC), induces inhibitory effects on platelet activation via elevation of cGMP levels and stimulation of the cGMP-dependent protein kinase. YC-1, a benzylindazole derivative, was shown to activate sGC in intact platelets, resulting in inhibition of platelet aggregation. In a previous study, we demonstrated that YC-1 not only stimulates purified sGC but also potentiates the stimulatory action of submaximally effective NO and carbon monoxide (CO) concentrations. Here, we investigated the potentiating effect of YC-1 in intact platelets. YC-1 together with NO or CO led to complete inhibition of platelet aggregation at concentrations that were ineffective by themselves. Maximally effective 2,2-diethyl-1-nitroso-oxyhydrazine (3 μM) and YC-1 (100 μM) concentrations each elevated the cGMP levels in intact platelets approximately 13-fold, and ad-

ministration of the two drugs together resulted in enormous potentiation of cGMP formation, which greatly exceeded the effect on the purified enzyme and yielded a >1300-fold increase in cGMP levels. Similar results were obtained using CO instead of NO. Furthermore, YC-1 not only stimulated sGC but also inhibited cGMP-hydrolyzing phosphodiesterases in platelets. The enormous elevation of cGMP levels led to enhanced phosphorylation of the cGMP-dependent protein kinase substrate vasodilator-stimulated phosphoprotein. Thus, by the combination of two effects (i.e., potentiation of NO-induced sGC stimulation and phosphodiesterase inhibition), YC-1-like substances are potent activators of the sGC/cGMP pathways and are therefore interesting candidates to act as modulators of cGMP-mediated effects, especially within the cardiovascular system.

The stimulation of sGC by the signaling molecule NO induces important functional changes within the cardiovascular system, i.e., vasorelaxation and inhibition of platelet activation/aggregation (Ignarro *et al.*, 1987; Waldman and Murad, 1987; Moncada and Higgs, 1995). sGC, a heterodimeric hemoprotein that is stimulated up to 400-fold by NO, catalyzes the conversion of GTP to cGMP (Humbert *et al.*, 1990; Stone and Marletta, 1996). In addition to NO, CO has been suggested to be an activator of sGC, although its role as a signaling molecule has been controversial because of its rather poor sGC-stimulating properties (Brüne and Ullrich, 1987; Stone and Marletta, 1994). CO was reported to mediate vasorelaxation (Utz and Ullrich, 1991; Morita *et al.*, 1995; Zakhary *et al.*, 1996), as well as inhibition of platelet aggregation (Brüne and Ullrich, 1987).

Recently, the new substance YC-1, which is a benzylindazole derivative, has been identified as an inhibitor of platelet

aggregation (Ko *et al.*, 1994; Wu *et al.*, 1995). Inhibition was accompanied by a 10-fold increase in the intracellular cGMP concentration. In addition to platelets, cGMP-increasing effects of YC-1 have been reported for smooth muscle cells, resulting in relaxation (Mülsch *et al.*, 1997; Wegener and Nawrath, 1997). We showed that YC-1 stimulated purified sGC 10-fold, potentiated the effect of submaximally effective NO concentrations, and increased the V_{max} of the NO-stimulated enzyme by >40% (Friebe *et al.*, 1996). Treatment with CO alone resulted in only 3-fold activation of sGC. However, in the presence of YC-1, CO produced an enormous (up to 100-fold) stimulation of the purified enzyme. Analysis of the mechanism of action suggested a reduction of the dissociation rate of either gaseous activator produced by binding to an allosteric site (Friebe and Koesling, 1998), although in a very recent report no change in the CO dissociation rate was observed (Stone and Marletta, 1998).

In platelets, stimulation of sGC with subsequent elevation of the cyclic nucleotide cGMP leads to inhibition of the acti-

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ABBREVIATIONS: sGC, soluble guanylyl cyclase; NO, nitric oxide; CO, carbon monoxide; DEA-NO, 2,2-diethyl-1-nitroso-oxyhydrazine; TEA, triethanolamine; IBMX, 3-isobutyl-1-methylxanthine; RIA, radioimmunoassay; VASP, vasodilator-stimulated phosphoprotein; wP, washed platelets; cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; PDE, phosphodiesterase; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

vation (adhesion, secretion, and aggregation) induced by various stimuli, such as ADP, thrombin, thromboxane A_2 , or collagen. These inhibitory effects of cGMP on platelet activation are primarily mediated by the activation of cGK, although the cGMP-inhibited cAMP PDE is an additional cGMP target (Eigenthaler *et al.*, 1993; Butt and Walter, 1997b). Among the many postulated target proteins, VASP is an established cGK and cAK substrate *in vitro* and in intact cells (Lohmann *et al.*, 1997; Smolenski *et al.*, 1998).

This study was designed to investigate the synergistic effects of YC-1 and NO/CO in intact platelets. YC-1 together with NO or CO, at concentrations that were by themselves ineffective, led to complete inhibition of platelet aggregation, which was associated with a tremendous (>1000-fold) increase in intraplatelet cGMP levels. Much to our surprise, YC-1 inhibited IBMX-sensitive cGMP PDEs, in addition to stimulating sGC. The enormous elevation of cGMP also caused enhanced phosphorylation of the cGK target VASP in intact human platelets. Thus, YC-1-like substances are intriguing candidates to act as potent modulators of cGMP-mediated effects within the cardiovascular system.

Materials and Methods

Preparation of human wP. Venous blood from healthy volunteers was drawn into solution containing 2.5 mM trisodium citrate, 1.37 mM citric acid, and 2.0 mM glucose, using 19-gauge needles. Blood was centrifuged for 10 min at 18° and $350 \times g$. Platelet-rich plasma was collected and recentrifuged for 12 min at 18° and $700 \times g$. The platelet pellet was resuspended in platelet buffer (5 mM HEPES, pH 7.4, 150 mM NaCl, 0.55 mM NaH_2PO_4 , 7 mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose) and adjusted to $2-3 \times 10^8$ platelets/ml.

Aggregation of human wP. wP ($200 \mu l$) were preincubated at 37° , with constant stirring (1000 rpm), in a Daiichi four-channel aggregometer (KDK Corp., Kyoto, Japan). Platelet aggregation was measured as the increase in light transmission in 5 min, starting immediately after the addition of thrombin (0.05 unit/ml) or the thromboxane A_2 receptor agonist U46619 (1 μM). DEA-NO and/or YC-1 were administered 3 min before addition of the aggregating agonist. When CO was used, wP were bubbled for 10 sec with 100% CO gas and preincubated for 3 min before addition of agonist.

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

RIA of intracellular cGMP and cAMP. After preincubation of wP (100 μl) for 5 min at 37° , either DEA-NO, YC-1, or the combination of the two was added and wP were incubated for an additional 5 min. When CO was used, wP were bubbled with 100% CO gas instead of being treated with DEA-NO. The incubation was stopped by the addition of ice-cold ethanol (final concentration, 66%), and vials were placed on ice for 30 min. Samples were then centrifuged for 15 min at 4° at $14,000 \times g$, and supernatants were dried in a Speed-Vac centrifuge. Preparation of tracer, acetylation of samples and standards, and incubation with antibody were performed as described by Brooker *et al.* (1979) and Rosenthal (1983). The assay was carried out in duplicate with different dilutions of samples.

Phosphorylation of VASP. Platelets ($8-10 \times 10^6$) were treated as described under RIA of intracellular cGMP and cAMP. After a 5-min incubation, SDS-containing Laemmli buffer was added, and samples were heated to 95° for 5 min and separated on 9% SDS-polyacrylamide gels. After electrophoresis, proteins were blotted onto nitrocellulose filters. Phosphorylation of VASP was detected using a monoclonal antibody (0.5 $\mu g/ml$) specific for VASP phosphorylated at the cGK-preferred site Ser239 (Smolenski *et al.*, 1998), followed by a peroxidase-labeled anti-mouse antibody (Sigma), with

subsequent detection with an enhanced chemiluminescence kit (Amersham).

Measurement of PDE activity. Cyclic nucleotide hydrolysis was measured in human platelet cytosol prepared from platelet concentrates. For the preparation of cytosol, the concentrate was handled as described above to obtain wP. wP were then recentrifuged, and the platelet pellet was washed twice with TEA buffer containing 50 mM TEA hydrochloride, pH 7.4, 75 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, and 0.2 mM benzamidine and was resuspended in the same buffer. Platelets were then lysed by sonication, and a cytosolic fraction was obtained by ultracentrifugation at 4° for 20 min at $200,000 \times g$.

PDE activity was measured by the conversion of [^{32}P]cGMP (prepared by conversion from [α - ^{32}P]GTP using purified sGC) to guanosine and [^{32}P]phosphate at 37° for 10 min. Reaction mixtures contained 0.6–4 μg of cytosolic protein, [^{32}P]cGMP (30,000–60,000 cpm) plus nonradioactive cGMP (10 nM, 1 μM , or 10 μM), 3 mM Mg^{2+} , 3 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 1 unit of alkaline phosphatase, and 50 mM TEA hydrochloride, pH 7.4, in a total volume of 0.2 ml. Reactions were stopped by the addition of ice-cold charcoal suspension (20% activated charcoal in 50 mM KH_2PO_4 , pH 2.3). After pelleting of the charcoal by centrifugation, [^{32}P]phosphate was measured in the supernatant. All measurements represent the mean \pm standard error of at least three independent experiments performed in duplicate.

Materials. YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] was obtained from Cayman Chemicals (Grünberg, Germany). DEA-NO (sodium salt) was purchased from Research Biochemicals International (Natick, MA). Thrombin, U46619, and monosuccinyl-tyrosyl-cGMP were obtained from Sigma, and alkaline phosphatase was from Boehringer Mannheim. Activated charcoal was from Riedel-de Haën. [α - ^{32}P]GTP (800 Ci/mmol) was from NEN-DuPont, and $Na^{125}I$ was purchased from Amersham. CO was from AGA Gas (Berlin, Germany).

Results

To study the possible synergistic effects of YC-1 and NO on platelet aggregation, we stimulated wP with thrombin and U46619 in the absence and presence of DEA-NO and YC-1. Fig. 1A shows that platelet aggregation induced by the stable thromboxane A_2 receptor agonist U46619 (1 μM) was not influenced by preincubation with either 1 nM DEA-NO or 1 μM YC-1. However, the combination of the two drugs led to full inhibition of aggregation. Similar results were obtained with thrombin (0.05 unit/ml) (Fig. 1B). Here, neither 10 nM DEA-NO nor 3 μM YC-1 alone influenced the aggregatory response, whereas the two substances together completely blocked aggregation.

Fig. 2 shows the concentration dependence of this potentiating effect. YC-1 at concentrations between 1 and 5 μM did not have an antiaggregatory effect on thrombin (0.05 unit/ml)-induced platelet aggregation in the absence of NO (Fig. 2A). In combination with 10 nM DEA-NO, YC-1 led to a concentration-dependent inhibition of aggregation. Similar results were obtained using YC-1, at concentrations between 0.5 and 1.5 μM , upon stimulation of wP with U46619 (1 μM) (Fig. 2B). YC-1 was more potent in inhibiting aggregation induced by U46619 than that induced by thrombin, which is in agreement with the findings of Wu *et al.* (1995).

CO, which itself barely activates purified sGC (maximally 6-fold), becomes an effective activator of the enzyme in the presence of YC-1 (100-fold) (Friebe *et al.*, 1996). We wanted to investigate whether this effect has its functional correlate in intact platelets. Whereas CO (100%) alone had no effect on

thrombin (0.05 unit/ml)-induced platelet aggregation, the combination with YC-1 (1, 3, or 5 μM) led to a concentration-dependent inhibition of aggregation (Fig. 2A). Similar results were obtained using U46619 (1 μM) (Fig. 2B). Therefore, we

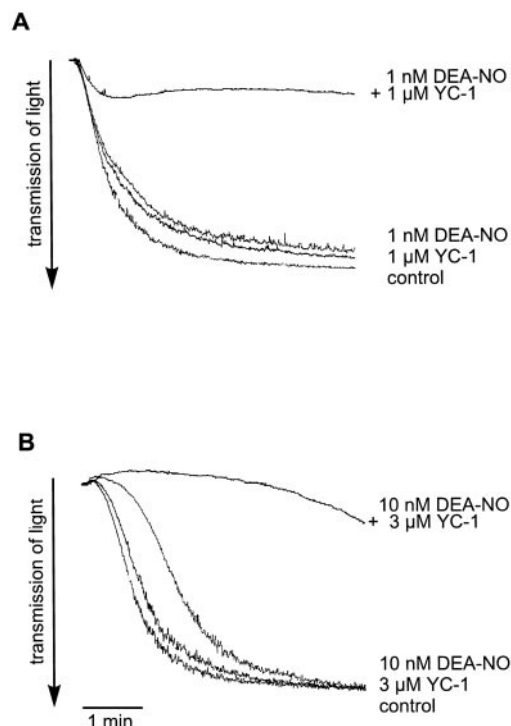


Fig. 1. Synergistic inhibition of platelet aggregation by YC-1 and NO. wP were preincubated with the indicated concentrations of YC-1 and/or DEA-NO for 5 min at 37°. The subsequent response to stimulation with U46619 (1 μM) (A) or thrombin (0.05 unit/ml) (B) was recorded as the increase in light transmission.

conclude that the enormous sGC-activating property of CO in the presence of YC-1 is not only an *in vitro* effect on the purified enzyme but also occurs in intact cells.

Next, we asked whether these potentiating effects on aggregation are indeed mediated by cGMP. Fig. 3 shows the intracellular cGMP levels measured by RIA. Basal cGMP concentrations were 1.5 ± 0.5 pmol/ 10^9 platelets. NO alone led to a concentration-dependent increase in the intracellular cGMP content; maximal stimulation of sGC (10 μM DEA-NO) resulted in an approximately 13-fold increase (20.0 ± 0.6 pmol/ 10^9 platelets) (Fig. 3A), as seen by others (Eigenthaler *et al.*, 1992). YC-1 at 100 μM caused a similar, approximately 13-fold increase in intracellular cGMP (20.0 ± 3.4 pmol/ 10^9 platelets) in the absence of NO. Addition of increasing concentrations of DEA-NO led to an enormous rise in the cGMP level (2070 ± 190 pmol/ 10^9 platelets). This corresponds to a >1300-fold increase in cGMP, an elevation that to our knowledge had not been previously found. As seen with purified sGC, YC-1 evoked a leftward shift of the EC_{50} value for NO of 1 order of magnitude (Fig. 3A, *inset*).

Fig. 3B shows the potentiation of CO-stimulated cGMP production by YC-1 in intact platelets. CO by itself did not increase cGMP synthesis. In the presence of increasing concentrations of YC-1, which by themselves resulted in up to 13-fold elevation of cGMP levels, CO (100% gas) led to a >1000-fold (1430 ± 130 pmol/ 10^9 platelets) enhancement of intraplatelet cGMP levels.

Next, we studied the effect of YC-1 on cGMP degradation. As shown in Fig. 4A, YC-1 at 100 μM reduced PDE activity in platelet cytosol by approximately 50%; addition of NO had no further influence. IBMX (1 mM), a nonselective PDE inhibitor, decreased PDE activity by 80%. The presence of YC-1 did not further inhibit cGMP breakdown. Fig. 4B shows the concentration dependence of PDE inhibition by YC-1 in hu-

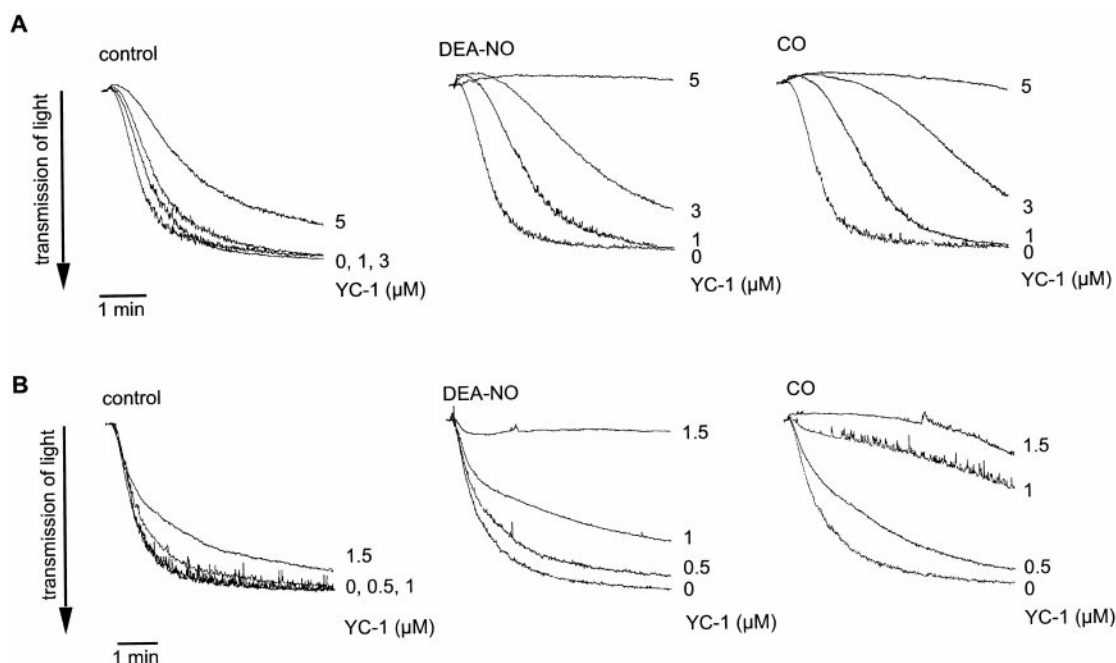


Fig. 2. Potentiating effect of YC-1 on NO- and CO-induced inhibition of platelet aggregation. A, wP were preincubated with 0, 1, 3, or 5 μM YC-1, in the presence of buffer, DEA-NO (10 nM), or CO (100%), for 5 min at 37° and were then stimulated with thrombin (0.05 unit/ml). B, Similarly, wP were preincubated with 0, 0.5, 1, or 1.5 μM YC-1, in the presence of buffer, DEA-NO (1 nM), or CO (100%), for 5 min at 37° and were then stimulated with U46619 (1 μM). Platelet aggregation was recorded as the increase in light transmission. Shown are representative curves from at least four experiments.

man platelet cytosol. At 10 nM and 1 μ M cGMP, half-maximal inhibition was seen with approximately 20 μ M YC-1 (data not shown), a concentration similar to the EC_{50} value for the activation of purified sGC (Friebe *et al.*, 1996). We conclude that YC-1, in addition to activating sGC in human platelets, inhibits cGMP breakdown by IBMX-sensitive PDEs. In the presence of IBMX (1 mM), the YC-1-induced PDE inhibition is abrogated; therefore, the YC-1-induced increase in intraplatelet cGMP levels shown in Fig. 4C is solely the result of sGC activation. NO-stimulated cGMP levels were approximately 3-fold higher than those evoked by YC-1, showing that the sGC-stimulating property of YC-1 is less than that of NO. Nevertheless, YC-1 still leads to a 10-fold increase in the maximally NO-stimulated catalytic rate, showing that the potentiating action of YC-1 is not based solely on PDE inhibition. This 10-fold increase in cGMP formation cannot be

observed with the purified enzyme, for which the maximal catalytic rate is raised only 1.4-fold.

Next, we determined the cAMP levels in intact platelets. Fig. 4D shows that YC-1 increased the cAMP concentration in wP approximately 1.3-fold, from 13 to 19 pmol/ 10^9 plate-

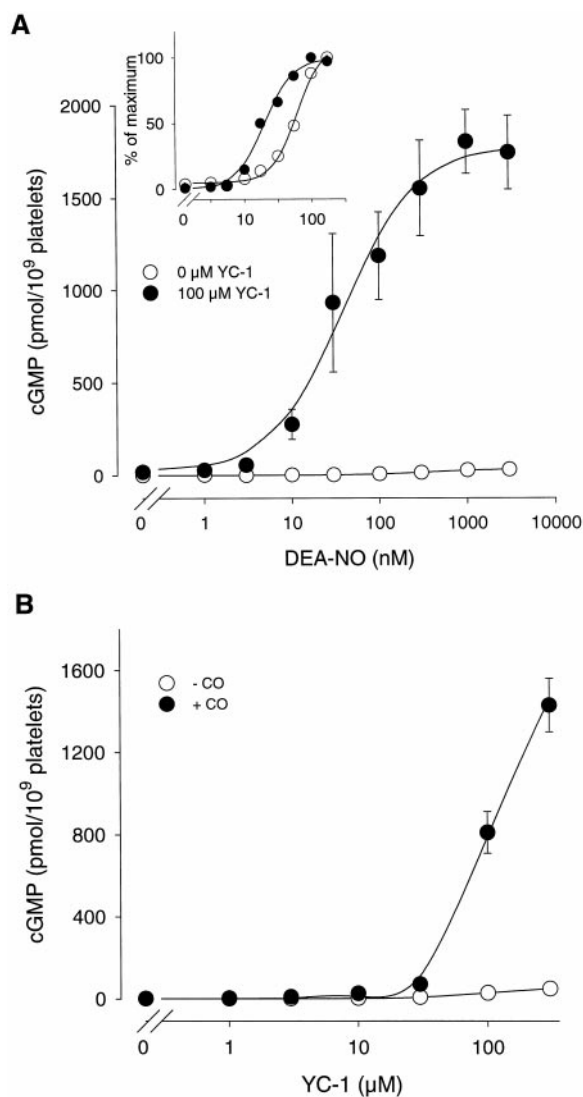


Fig. 3. Potentiation of NO- and CO-stimulated cGMP levels in intact human platelets by YC-1. cGMP concentrations in intact human platelets were measured by RIA after incubation of wP for 5 min at 37°. A, DEA-NO-induced cGMP production was determined in the absence or presence of 100 μ M YC-1. For better illustration of the leftward shift of the EC_{50} value, the percentage of maximal stimulation is also shown (inset). B, The concentration-response relationship for YC-1 in the absence or presence of CO (100%) is shown. Data are mean \pm standard error from six (DEA-NO) or three (CO) independent experiments.

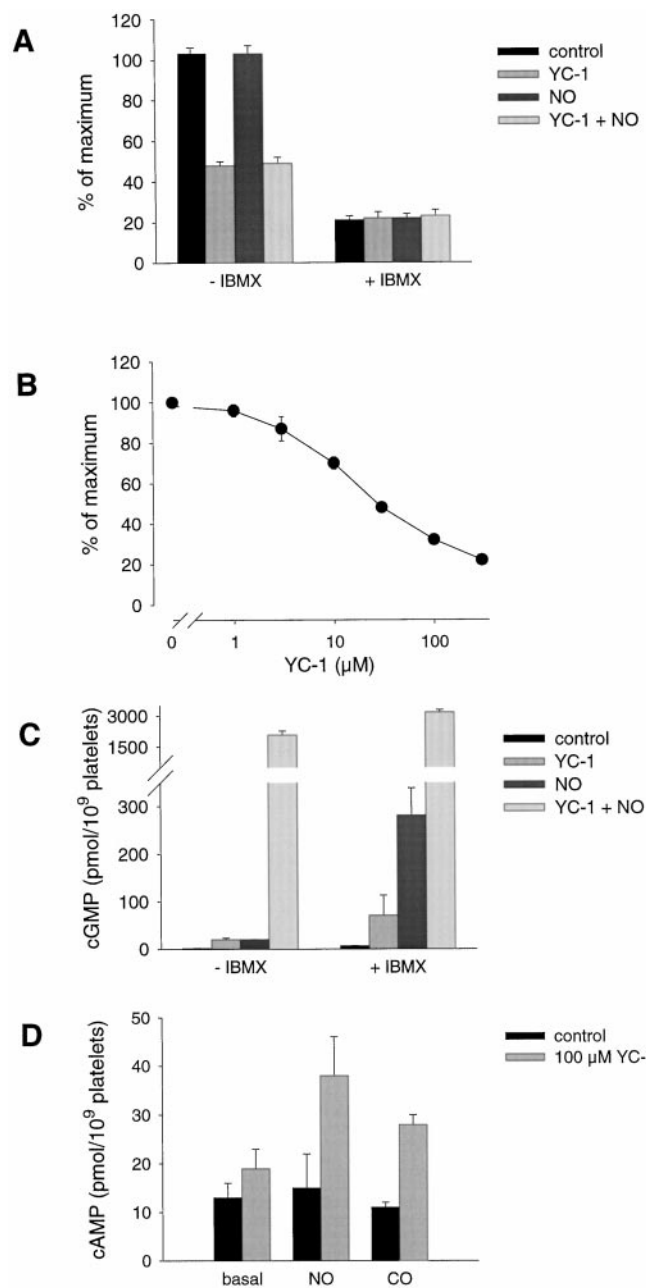


Fig. 4. Effect of YC-1 on cGMP hydrolysis in human platelet cytosol and cGMP and cAMP production in intact human platelets. A, cGMP hydrolysis (10 μ M cGMP) in the cytosol of human platelets, in the absence or presence of 10 μ M DEA-NO, 100 μ M YC-1, or the combination of the two, was measured with and without 1 mM IBMX. The amount of [32 P]phosphate released in the absence of IBMX under control conditions was taken as 100%. B, Concentration-dependent inhibition by YC-1 of cGMP-degrading PDEs in the cytosol of human platelets was measured in the presence of 1 μ M cGMP. C, wP were preincubated with either buffer, 100 μ M YC-1, 1 μ M DEA-NO, or the combination of the two, in the absence or presence of 1 mM IBMX, for 5 min at 37°. cGMP accumulation was measured by RIA. D, cAMP levels in intact platelets were measured by RIA. wP were preincubated with buffer, 10 μ M DEA-NO, or CO (100%), in the absence or presence of 100 μ M YC-1. Data in A–D are mean \pm standard error from three or more independent experiments.

lets; this effect is similar to the increase seen by Wu *et al.* (1995). Neither NO nor CO alone had an effect on the cAMP content of intact platelets. In combination with YC-1, NO and CO led to 2.9- and 2.5-fold increases in cAMP levels, respectively.

In a last approach, we studied whether the enormous rise in intracellular cGMP induced by YC-1 and NO also results in enhanced cGMP-induced and cGK-mediated protein phosphorylation. Although both cGK and cAK phosphorylate VASP at two major sites (Ser239 and Ser157), cGK and cAK prefer Ser239 and Ser157, respectively (Halbrügge *et al.*, 1990; Butt and Walter, 1997a). Phosphorylation of Ser157 is associated with a shift in the apparent molecular mass of VASP in SDS-polyacrylamide gel electrophoresis, from 46 to 50 kDa. VASP Ser239 phosphorylation can be detected by a new monoclonal antibody (Smolenski *et al.*, 1998). Fig. 5 shows the concentration-dependent increase in VASP Ser239 phosphorylation induced by YC-1 under nonstimulated, NO-stimulated, and CO-stimulated conditions, as detected using this antibody. YC-1 alone produced VASP Ser239 phosphorylation, associated with a partial VASP shift (Ser157 phosphorylation), only at high concentrations (10–300 μ M). In the presence of CO (100%), which alone did not evoke detectable VASP phosphorylation, the signal was enhanced and the shift to the larger phosphorylated form was seen with lower YC-1 concentrations. The effect of DEA-NO, which at 1 μ M alone caused considerable phosphorylation of VASP at Ser239 and Ser157, was further increased by YC-1, leading to total conversion of VASP to the 50-kDa form. This shows that the potentiation of NO- and CO-stimulated cGMP production in intact human platelets by YC-1 can be detected at the level of VASP phosphorylation.

Discussion

In a previous report (Friebe *et al.*, 1996), we demonstrated the NO-independent stimulatory effect of YC-1 and its synergism with NO and CO on purified sGC. Here, we investigated whether these effects of YC-1 also occur in intact cells. This is indeed the case, inasmuch as YC-1 potentiated the antiaggregatory platelet response of ineffective concentrations of NO and CO, leading to complete inhibition of aggre-

gation induced by two different agonists (thrombin and U46619).

Using a RIA, we showed that the synergism of YC-1 and NO leading to inhibition of aggregation was associated with an enormous increase in intraplatelet cGMP levels. Maximal increases in cGMP levels induced by NO or YC-1 alone were approximately 13-fold each. The two substances applied together resulted in a tremendous (>1000-fold) increase in the intracellular cGMP concentration. This huge increase in cGMP is only partly explained by the unexpected finding that YC-1 inhibits PDE activity in human platelets (see below). Despite this PDE inhibition, YC-1 still exerts a direct potentiating effect on maximally NO-stimulated sGC. However, the effect of YC-1 on maximally NO-activated cGMP production in intact platelets differs from that on purified sGC; whereas YC-1 led to an only 1.4-fold increase in the NO-stimulated catalytic rate (Friebe *et al.*, 1996), the potentiation observed in intact platelets was 10-fold. In cytosol obtained from human platelets, the potentiation of NO-stimulated cGMP formation by YC-1 was similar to that of the purified enzyme (data not shown). The differing behavior of sGC toward YC-1 and NO in cytosolic fractions and intact cells cannot be explained so far.

As seen with NO, the platelet response to CO was potentiated by YC-1. CO alone did not elicit an antiaggregatory response, whereas in combination with YC-1, at concentrations that were ineffective by themselves, CO blocked the aggregation of wP. cGMP levels evoked by CO in the presence of high micromolar YC-1 concentrations were similar to those elicited by NO plus YC-1. These data show that the potentiation of CO-induced sGC stimulation by YC-1 is not an *in vitro* artifact with the purified enzyme but may play a role in intact cells.

Despite the enormous increase in cGMP, YC-1 plus NO augmented cAMP only approximately 3-fold. We conclude that the synergistic action of YC-1 and NO/CO does not directly (via stimulation of adenylyl cyclase) or indirectly (via inhibition of cAMP degradation) lead to a pronounced increase in cAMP levels.

A previously unknown property of YC-1 described here is its ability to inhibit cGMP-hydrolyzing PDEs. This finding is in contrast to the data of Ko *et al.* (1994), who did not show an effect of YC-1 on cyclic nucleotide hydrolysis. This discrepancy may be explained by species differences, because their experiments were carried out with rat platelets. In our hands, YC-1 at 100 μ M decreased cGMP PDE activity in the cytosol of human platelets by 50%. IBMX, a nonspecific PDE inhibitor, caused 80% inhibition of cGMP hydrolysis; the activity was not further influenced by YC-1 (Fig. 4A). Therefore, we conclude that YC-1 inhibits IBMX-sensitive PDEs in human platelets. The experiments performed in intact platelets in the presence of IBMX allow differentiation between the sGC-stimulating and PDE-inhibiting effects of YC-1 (Fig. 4C). Without IBMX, the effect of YC-1 is the result of sGC stimulation and inhibition of cGMP degradation; the cGMP elevation observed in the presence of IBMX reflects the sGC-activating property of YC-1, which is less than that of NO. In human platelets, there are three distinct forms of PDEs (types II, III, and V), which differ in their cyclic nucleotide specificity and kinetic properties (Butt and Walter, 1997b). Because the cGMP-binding PDE (type V) is the major cGMP PDE in human platelets, it is likely that the inhibitory effect of YC-1 on cGMP hydrolysis is the result of inhibition of this

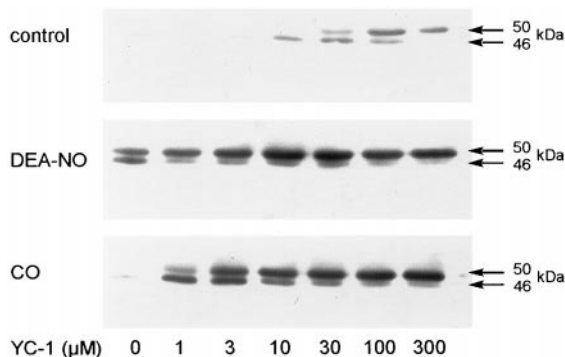


Fig. 5. Effect of NO and CO on the phosphorylation of VASP in the presence of increasing concentrations of YC-1. wP were preincubated with buffer, DEA-NO (1 μ M), or CO (100%), in the presence of increasing concentrations of YC-1, for 5 min at 37°. Proteins were then separated on a 9% SDS-polyacrylamide gel, and phosphorylated VASP was detected using a monoclonal anti-phospho-VASP antibody. The two proteins represent Ser239-phosphorylated VASP and the shifted form (shifted from 46 to 50 kDa), indicating additional phosphorylation of VASP at Ser157.

PDE. The small increase in the platelet cAMP levels observed in response to YC-1 may be the result of some inhibition of the cGMP-inhibited cAMP PDE (type III).

In addition to the PDE-inhibiting and sGC-stimulating actions of YC-1, the potentiating effect of YC-1 with respect to NO and CO stimulation is of great importance. The resulting tremendous increase in platelet cGMP levels induces additional intracellular signaling, as demonstrated by increased phosphorylation of the cyclic nucleotide-dependent protein kinase target VASP. It will be interesting to determine whether this phosphorylation is the result of activation of only cGK or also cAK (through the moderately increased cAMP levels and/or the enormously elevated cGMP levels). Additional experiments should show whether the enormous cGMP-elevating effect of YC-1 represents a general feature of cGMP modulation.

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