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YC-1 potentiates the antiplatelet effect of hydrogen peroxide via sensitization of soluble guanylate cyclase

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Abstract

In the present study, we showed that 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1), a nitric oxide (NO)-independent activator of soluble guanylate cyclase, could potentiate H_2O_2-induced inhibition of platelet aggregation and increase of platelet cGMP levels. The synergistic effect of YC-1 and H_2O_2 on platelet aggregation and increases of cGMP were almost completely prevented by catalase and a selective soluble guanylate cyclase inhibitor (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), or partially attenuated by the hydroxyl radical scavenger mannitol. In contrast, superoxide dismutase failed to influence H_2O_2/YC-1-induced inhibition of aggregation. Furthermore, YC-1 could enhance the activation of soluble guanylate cyclase caused by $FeSO_4/H_2O_2$ and, this effect was prevented markedly by mannitol. These results suggest that YC-1 may enhance the antiaggregatory effect of H_2O_2 via the sensitization of platelet soluble guanylate cyclase. In addition, this phenomenon is, at least in part, dependent on H_2O_2-derived hydroxyl radical.

Keywords: YC-1; Hydrogen peroxide; Antiplatelet; Guanylate cyclase, soluble

1. Introduction

There is growing evidence that reactive oxygen species (ROS) play an important role in ischemia-reperfusion-induced injury and thrombotic reocclusion (Ambrosio et al., 1997; Forde and Fitzgerald, 1997). ROS may influence these pathologic process by interfering with various components in the vascular lumen, such as platelets, endothelial cells, leukocytes and coagulation factors. Hydrogen peroxide (H_2O_2) is one of most abundant ROS released in the vascular lumen following restoration of flow after a period of ischemia. It has been reported that H_2O_2 may promote thromboxane synthesis and thus enhances arachidonic acid- and collagen-induced platelet aggregation (Del Principe et al., 1985; Pratico et al., 1992). On the other hand, exposure to larger concentration of H_2O_2 may inhibit platelet aggregation caused by several, but not all, agonists via increases of cGMP formation (Canoso et al., 1974; Ambrosio et al., 1994), but the precise mechanism involved are not fully understood. Besides the conflicting effects of H_2O_2 on platelet aggregation by itself, H_2O_2 also interacts with other free radicals, including nitric oxide (NO). It has been reported that H_2O_2 may either enhance or reduce the antiplatelet activity of NO (Naseem and Bruckdorfer, 1995; Freedman et al., 1996; Naseem et al., 1996).

We previously reported that 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1), a derivative of benzylindazole, possesses antiplatelet activity in vitro and in vivo, and demonstrated that YC-1 inhibited platelet aggregation through NO-independent activation of soluble guanylate cyclase (Ko et al., 1994; Wu et al., 1995, 1997; Teng et al., 1997). In the present study, we investigated the interaction of YC-1 and H_2O_2 on platelet aggregation and cGMP formation. Our data suggested that YC-1 could enhance the antiaggregatory effect of H_2O_2 via the sensitization of platelet soluble guanylate cyclase.

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2. Materials and methods

2.1. Preparation of washed human platelets

Human platelet suspension was prepared according to the procedure previously described (Wu et al., 1994). Fresh whole blood anticoagulated with acid citrate dextrose (ACD) was obtained from healthy human volunteers who had not taken any drugs within the last 2 weeks. The platelets, after washing, were finally suspended in Tyrode's solution (mM): NaCl, 136.9; $CaCl_2$, 2; KCl, 2.7; $MgCl_2$, 2.1; $NaHCO_3$, 11.9; NaH_2PO_4 0.4; glucose, 5.6; pH 7.4 containing bovine serum albumin (3.5 mg/ml) at a concentration of 3×10^8 platelets/ml.

2.2. Measurement of platelet aggregation

Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log Co., USA) (Born, 1962). The platelet suspension was incubated with H_2O_2 and/or YC-1 at 37°C for 1 min under a stirring condition (1200 rpm) prior to the addition of the platelet inducer (thrombin). The extent of platelet aggregation was measured as the increase of light transmission at 5 min after the addition of thrombin.

2.3. Measurement of glutathione peroxidase and catalase activity

Glutathione peroxidase activity was determined by means of a glutathione reductase coupled reaction (Flohe and Gunzler, 1984). The assay mixture (1 ml) contained 1.5 mM H_2O_2, 1 U/ml glutathione peroxidase, 4 U/ml glutathione reductase, 0.12 mM NADPH, 1 mM glutathione, 0.1 mM EDTA and dimethyl sulfoxide (DMSO) or YC-1 in 50 mM phosphate buffer (pH 7.0) at 37°C. The reaction was started by addition of H_2O_2, then the decrease in absorbance at 340 nm was monitored with a recorder for 1 min.

Catalase activity was determined by monitoring the rate of decomposition of H_2O_2, as a function of the decrease in absorbance at 240 nm (Aebi, 1984). The assay mixture (1 ml) contained 10 mM H_2O_2, 20 U/ml catalase and DMSO or YC-1 in 50 mM phosphate buffer (pH 7.0) at 25°C. The reaction was started by addition of H_2O_2, then the decrease in absorbance was monitored with a recorder for 1 min.

2.4. Estimation of platelet cGMP contents

The method of Karniguian et al. (1982) was used. The platelet suspension was incubated with H_2O_2 and/or YC-1 at 37°C for 1 min under a stirring condition (1200 rpm) in a light-transmission aggregometer. The reaction was stopped by adding EDTA (10 mM) followed immediately by boiling for 2 min. Upon cooling to 4°C, cell debris was removed by centrifugation at $10,000 \times g$ for 5 min. The supernatant was then used to assay for cGMP using an enzyme immunoassay kit.

2.5. Determination of guanylate cyclase activity

Human washed platelets were prepared as described above but finally resuspended in Tris–HCl buffer (mM: Tris–HCl, 50; dithiothreitol, 1; EDTA, 0.2; pH 7.4). Platelets were disrupted by sonication at 4°C ($4–6 \times 10$ s; setting 5, Vibra cell, Sonics and Materials Inc.), and the homogenate was centrifuged at $30,000 \times g$ and 4°C for 15 min. The supernatant was used as a source of crude soluble guanylate cyclase. Protein content was determined with a protein assay kit (Bio-Rad). Guanylate cyclase activity was determined as previously described (Gerzer et al., 1983). The enzyme preparation was incubated in a final volume (200 μl) with other reactants as follows: GTP (0.2 mM containing 1×10^6 cpm [α-^{32}P]GTP), $MgCl_2$ (5 mM), cyclic GMP (2.5 mM), creatine phosphate (5 mM), creatine kinase (0.25 mg/ml) with H_2O_2, YC-1 or other test compounds in Tris–HCl buffer (50 mM, pH 7.4). The reaction was initiated by adding the enzyme preparation and, after incubation at 37°C for 10 min, was terminated by adding HCl (0.5 N, 200 μl). Next, the reaction mixture was heated to 100°C for 2 min and then cooled in an ice bath. Imidazole (1 mM, 200 μl) was added to each tube. GTP and cyclic GMP were separated on neutral alumina and the radioactivity of [^{32}P]cyclic GMP was determined in a liquid scintillation counter.

2.6. Statistics

Results are expressed as the mean ± standard error of the mean (S.E.M.) and comparisons were made using Student's *t*-test. A probability of 0.05 or less was considered significant.

2.7. Drugs

YC-1 was chemically synthesized as described previously (Yoshina and Kuo, 1978) and dissolved in DMSO for a stock solution. Before experiments, the stock solution of YC-1 was diluted with a mixture of water/isopropanol (2:1). The final concentration of DMSO and isopropanol in each experiment was less than 0.005% and 0.17% respectively, and did not significantly affect the parameters measured. H_2O_2 was purchased from Merck, Germany. Bovine thrombin was obtained from Parke Davis, USA. [α-^{32}P]GTP and cGMP enzyme immunoassay kit were purchased from Amersham, U.K. Protein assay kit from Bio-Rad. Catalase, superoxide dismutase (SOD), glutathione peroxidase, glucose oxidase, *N*-ethylmaleimide, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), mannitol, S-nitrosoglutathione (GSNO) and other chemicals were purchased from Sigma, USA.

3. Results

3.1. Effect of YC-1 and H_2O_2 on platelet aggregation of washed human platelets

In washed human platelets, H_2O_2 (10–100 μM) did not affect thrombin (0.05 U/ml)-induced platelet aggregation (Fig. 1). YC-1, an NO-independent soluble guanylate cyclase activator, at a subthreshold concentration (10 μM) which had no significant effect ($10.2 \pm 6.7\%$ inhibition, $n = 5$) on aggregation caused by thrombin, lead to a leftward shift of the concentration–response curve to H_2O_2 (Fig. 1). To eliminate H_2O_2 by addition of catalase (200 U/ml) or to block soluble guanylate cyclase with ODQ could completely abolish the synergistic antiaggregatory effect of H_2O_2/YC-1 (Fig. 2). In order to investigate whether other oxygen-derived free radicals were involved in the interaction of H_2O_2 and YC-1, the platelets were pretreated with SOD (200 U/ml) or mannitol (100 mM) for 1 min before the addition of H_2O_2/YC-1. The antiaggregatory effect of H_2O_2/YC-1 was partly attenuated by mannitol, but not by SOD (Fig. 2). Catalase, ODQ, SOD and mannitol alone did not affect thrombin-induced platelet aggregation at the concentration used (data not shown).

To investigate whether the potentiation of YC-1 on H_2O_2 is due to inhibition of glutathione peroxidase or catalase, which account for H_2O_2 breakdown in cells, we determined the effect of YC-1 on these two enzymes. At the concentration (10 μM) used in our experiments, YC-1 neither affected glutathione peroxidase activity nor catalase activity (data not shown).

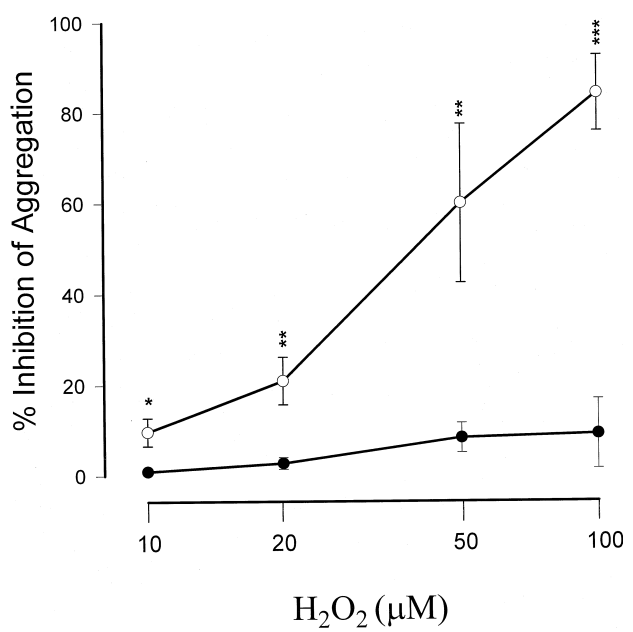

Fig. 1. Synergistic inhibition of H_2O_2 and YC-1 on thrombin-induced platelet aggregation. Washed platelets were preincubated with H_2O_2 (10–100 μM) before the addition of thrombin (0.05 U/ml) either in the presence (○) or absence (●) of YC-1 (10 μM), and aggregation was measured 5 min later. The H_2O_2 was added simultaneously with the YC-1. Percentage of inhibition is presented as mean ± S.E.M. ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with H_2O_2 alone.

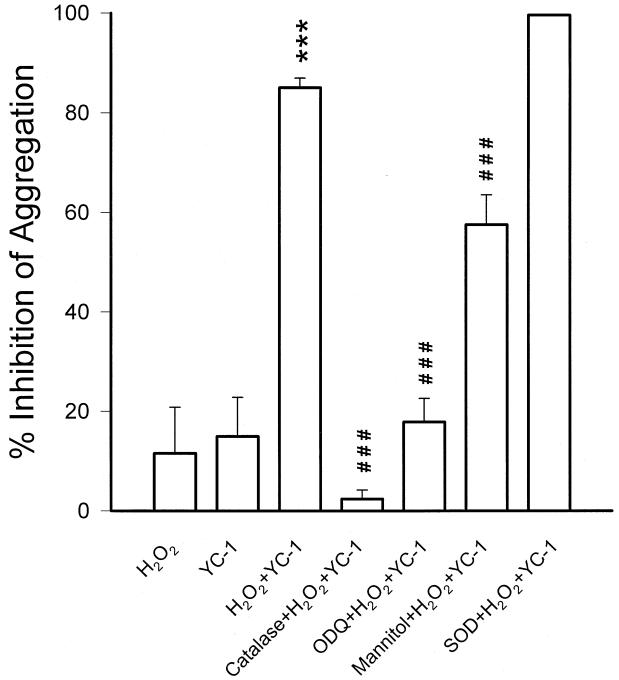

Fig. 2. Effect of reagents that suppress soluble guanylate cyclase or reactive oxygen species on the antiaggregatory activity of H_2O_2/YC-1. Washed platelets were incubated with H_2O_2 (100 μM) or/and YC-1 (10 μM) for 1 min before the addition of thrombin (0.05 U/ml), and aggregation was measured 5 min later. In some experiments, platelets were preincubated with catalase (200 U/ml), mannitol (100 mM) or SOD (200 U/ml) for 1 min, or with ODQ (2 μM) for 30 min before the addition of H_2O_2 and YC-1. Percentage of inhibition is presented as mean ± S.E.M. ($n = 4$). $^{***}P < 0.001$ as compared with H_2O_2 alone, $^{###}P < 0.001$ as compared with H_2O_2 + YC-1.

3.2. Effect of YC-1 and H_2O_2 on platelet cGMP levels

Although H_2O_2 (100 μM) or YC-1 (10 μM) applied alone elicited only a small increase in cGMP synthesis, simultaneous application of these two compounds synergistically elevated the cGMP levels of platelets (Fig. 3). In contrast, H_2O_2 (100 μM) and another soluble guanylate cyclase activator (GSNO, 10 μM) had a slight additive effect on platelet cGMP levels (Fig. 3). Additionally, the increase of platelet cGMP levels caused by H_2O_2/YC-1 was totally abolished by catalase or ODQ, while partly attenuated by the hydroxyl radical scavenger mannitol and DMSO (Fig. 3).

Similar to the synergism of YC-1 and H_2O_2, YC-1 also greatly enhanced the cGMP-elevating effect of GSNO. When YC-1 (10 μM) and GSNO (10 μM) applied alone, the cGMP concentration in platelets was 1.45 ± 0.09 and 6.29 ± 0.18 pmol/10^9 platelets respectively ($n = 4$; the

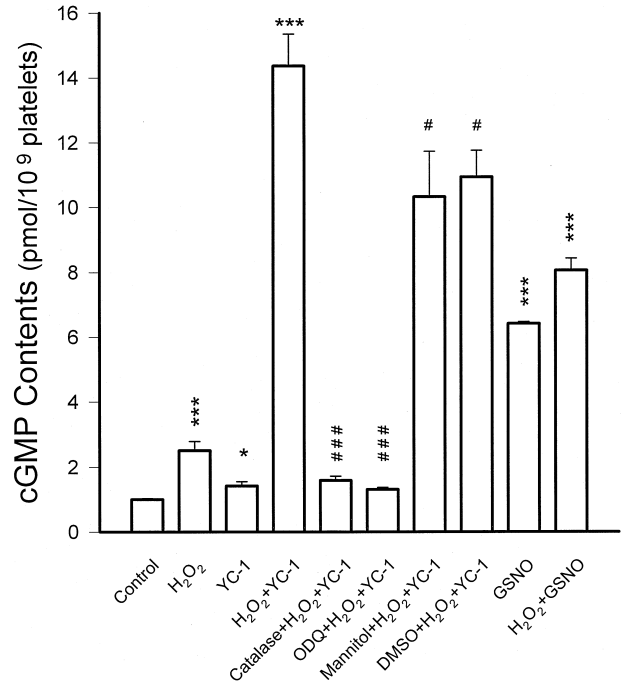

Fig. 3. Synergistic effect of H_2O_2 and YC-1 on the increases of platelet cGMP levels. Washed platelets were incubated with H_2O_2 (100 μM), YC-1 (10 μM) or GSNO (10 μM) for 1 min at 37°C. In some experiments, platelets were preincubated with catalase (200 U/ml), mannitol (100 mM) or DMSO (100 mM) for 1 min, or with ODQ (2 μM) for 30 min before the addition of H_2O_2 and YC-1. The cGMP contents were determined by enzyme immunoassay as described under Section 2. Values are presented as means ± S.E.M. ($n = 4$). $^{*}P < 0.05$, $^{***}P < 0.001$ as compared with control (vehicle); $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ as compared with H_2O_2 + YC-1.

basal value was 0.99 ± 0.03 pmol/10^9 platelets), however, simultaneous application of these two compounds synergistically elevated the cGMP levels of platelets (66.25 ± 19.92 pmol/10^9 platelets, $n = 4$).

3.3. Effect of YC-1 and glucose oxidase on platelet aggregation and cGMP levels

To further characterize the effect of H_2O_2, we tested platelet reactivity after 2 min of incubation with glucose oxidase (100 mU/ml), a H_2O_2 generator. At this condition, glucose oxidase generated a steady-state concentration (about 630 nM) of H_2O_2 (19). Glucose oxidase (100 mU/ml) alone did not affect thrombin (0.05 U/ml)-induced platelet aggregation (Fig. 4a), but elicited a small increase of cGMP levels in platelets (Fig. 4b). Combined application of YC-1 (10 μM) and glucose oxidase (100 mU/ml), however, synergistically inhibited platelet aggregation and elevated the cGMP levels (Fig. 4). Moreover, preincubation of platelets with catalase (200 U/ml) or ODQ (2 μM) almost completely abolished the synergistic antiaggregatory effect of glucose oxidase and YC-1 (Fig. 4a).

In addition, we found *N*-ethylmaleimide, an inhibitor of glutathione peroxidase, at a concentration (20 μM) which

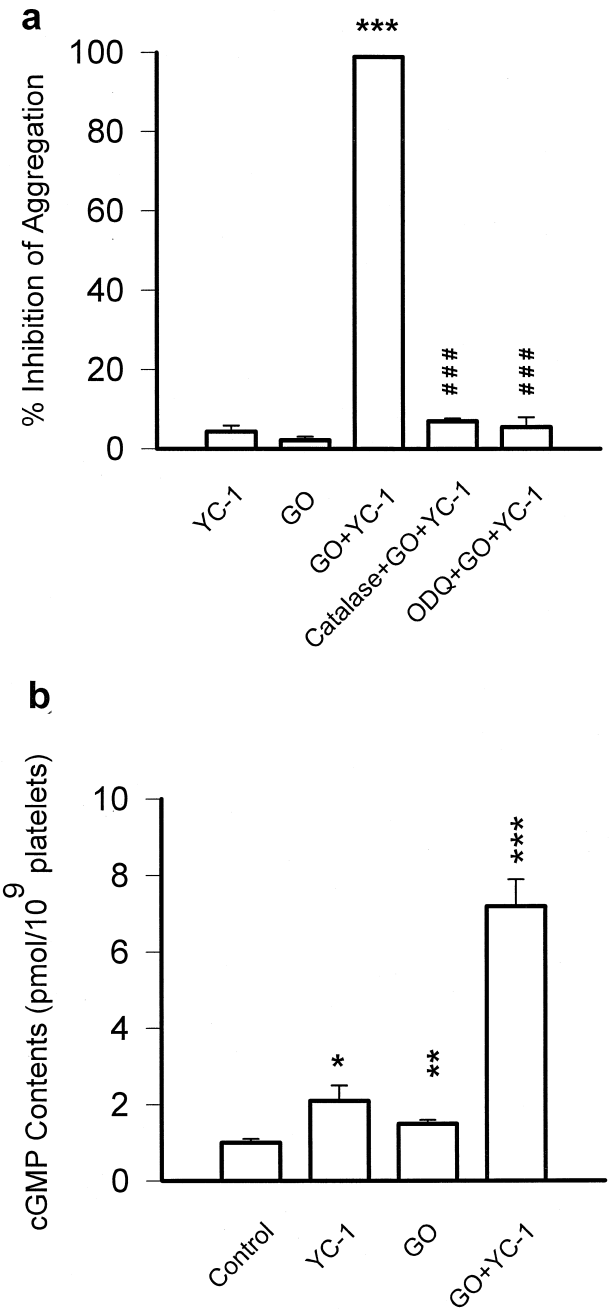

Fig. 4. Synergistic effect of glucose oxidase (GO) and YC-1 on the inhibition of platelet aggregation and the increase of platelet cGMP levels. (a) Platelets were incubated with GO (100 mU/ml) for 2 min or/and YC-1 (10 μM) for 1 min before the addition of thrombin (0.05 U/ml), and aggregation was measured 5 min later. In some experiments, platelets were preincubated with catalase (200 U/ml) for 1 min or with ODQ (2 μM) for 30 min before the addition of glucose oxidase and YC-1. (b) Platelets were incubated with glucose oxidase (100 mU/ml) for 2 min or/and YC-1 (10 μM) for 1 min at 37°C. The cGMP contents were determined by enzyme immunoassay. Values are presented as means ± S.E.M. ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with YC-1 (a) or vehicle (b); $^{\#\#\#}P < 0.001$ as compared with glucose oxidase + YC-1.

did not affect thrombin-induced platelet aggregation, also suppressed synergistically platelet aggregation with YC-1 (53.9 ± 5.7% inhibition, $n = 4$). Once again, this effect was prevented by catalase or ODQ (data not shown).

3.4. Effect of YC-1 and H_2O_2 on platelet soluble guanylate cyclase activity

Using the 30,000 × *g* supernatant from human platelet homogenate, we found H_2O_2 (100 μM) alone did not affect the activity of soluble guanylate cyclase. YC-1 (10 μM) elicited a small increase of soluble guanylate cyclase activity and, simultaneous application of H_2O_2 and YC-1 had some potentiatory effect on soluble guanylate cyclase activity (Fig. 5). To investigate whether transition metals were involved in regulation of the effect of H_2O_2 on soluble guanylate cyclase, $FeSO_4$ (30 μM) was added to the reaction mixture. In the presence of $FeSO_4$, H_2O_2 elicited a 2.3-fold increase in soluble guanylate cyclase activity. Additionally, YC-1 markedly potentiated the stimulatory effect of $H_2O_2/FeSO_4$ on soluble guanylate cyclase. Moreover, mannitol and DMSO largely prevented this potentiation (Fig. 5). While GSNO (10 μM) elicited a 6-fold increase in soluble guanylate cyclase activity, either H_2O_2 or $H_2O_2/FeSO_4$ attenuated rather than enhanced the stimulatory effect of GSNO (Fig. 5).

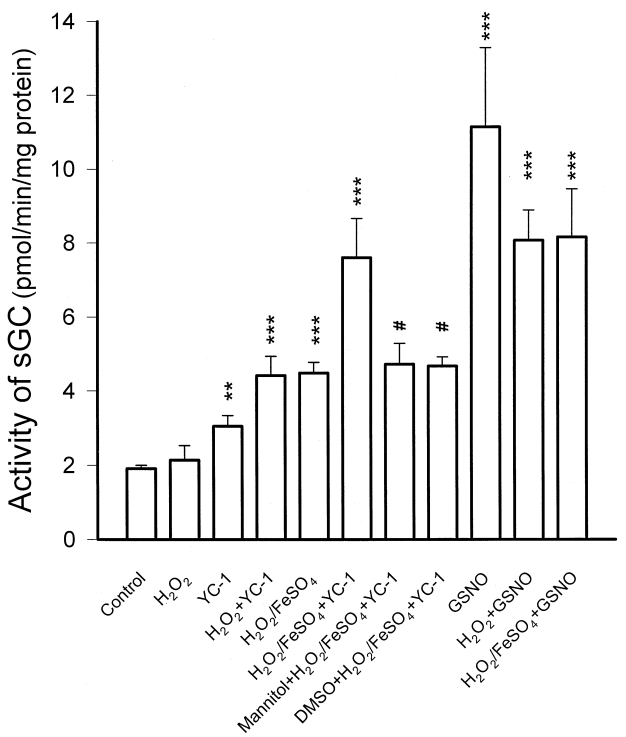

Fig. 5. Interaction of H_2O_2 and YC-1 on platelet soluble guanylate cyclase activity. The activity of platelet soluble guanylate cyclase in the 30,000 × *g* supernatant fraction was assayed in the presence of H_2O_2 (100 μM), YC-1 (10 μM), GSNO (10 μM) with or without $FeSO_4$ (30 μM) for 10 min at 37°C as described in Section 2. In some experiments, mannitol (100 mM) or DMSO (100 mM) was added to the reactants. Values are presented as means ± S.E.M. ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with control (vehicle); $^{\#}P < 0.05$ as compared with $H_2O_2/FeSO_4$ + YC-1.

4. Discussion

Previous studies had reported that H_2O_2, at submillimolar concentrations, could inhibit platelet activation caused by weak agonists, such as ADP, and this effect may be due to increase of platelet cGMP formation (Ambrosio et al., 1994). In the present work, H_2O_2 (10–100 μM) did not influence thrombin (0.05 U/ml)-induced platelet aggregation, although with a small raise of platelet cGMP levels, indicating the antiplatelet effect of H_2O_2 is limited. However, in the presence of YC-1, a novel activator of soluble guanylate cyclase, the antiaggregatory activity of H_2O_2 was dramatically potentiated, and accompanied by a marked increase of platelet cGMP levels. The elevation of cGMP contents was unlikely due to inhibition of cGMP breakdown by H_2O_2, since simultaneous application of H_2O_2 and another soluble guanylate cyclase activator GSNO had no synergistic effect on platelet cGMP levels. On the other hand, YC-1 has no effect on glutathione peroxidase and catalase, thus the potentiatory effect of YC-1 is not due to inhibition of H_2O_2 metabolism. Furthermore, ODQ, a selective inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995), completely blocked the capacity of YC-1/H_2O_2 to inhibit platelet aggregation and elevate platelet cGMP levels, suggesting their target of action is soluble guanylate cyclase.

In the present study, H_2O_2 at rather high concentration (10–100 μM) was required to inhibit platelet aggregation with YC-1. This may be due to the fact that the added H_2O_2 rapidly escaped or was rapidly metabolized in platelet suspensions, leading to fast decline of H_2O_2 concentrations (Burke and Wolin, 1987). To further characterize the effect of H_2O_2, glucose oxidase was used to generate a small (nanomolar) but constant flux of H_2O_2 (Burke and Wolin, 1987), and therefore more closely resemble the conditions found in vivo (Zweier et al., 1987; Bolli et al., 1988; Ambrosio et al., 1994). Similar to the interaction of H_2O_2 solution and YC-1, glucose oxidase acts synergistically with YC-1 both in the inhibition of platelet aggregation and the elevation of platelet cGMP levels. Moreover, the antiplatelet effect of glucose oxidase and YC-1 was also prevented by catalase or ODQ. *N*-Ethylmaleimide has been reported to inhibit glutathione peroxidase, a major enzyme which destroys H_2O_2 in platelets, and potentiates H_2O_2 formation induced by thrombin (Leoncini, 1994; Leoncini et al., 1991). We found *N*-ethylmaleimide also suppressed synergistically platelet aggregation with YC-1. Once again, this effect was prevented by catalase or ODQ. These results further support that YC-1 may act synergistically with exogeneous or endogeneous

H_2O_2 to inhibit platelet activation, and this effect is dependent on soluble guanylate cyclase/cGMP pathway.

Although H_2O_2 can increase cGMP formation in vascular smooth muscle cells and platelets (Burke and Wolin, 1987; Ambrosio et al., 1994), but it does not stimulate soluble guanylate cyclase in cell-free systems. One possibility is that H_2O_2 may indirectly regulate soluble guanylate cyclase through its metabolites in intact cells. In the presence of certain transition metals, such as Fe^{2+}, H_2O_2 can yield hydroxyl radical through Fenton reaction and, it is speculated that hydroxyl radical accounts for the stimulatory activity of H_2O_2 on soluble guanylate cyclase (Schmidt, 1992). In this study, we showed that H_2O_2 did not activate soluble guanylate cyclase unless in the presence of $FeSO_4$. In addition, the stimulatory effect of $H_2O_2/FeSO_4$ could be enhanced by YC-1, but the hydroxyl radical scavenger mannitol and DMSO largely abolished this enhancement. Furthermore, the synergism of H_2O_2 and YC-1 on inhibition of platelet aggregation and elevation of platelet cGMP levels were partly prevented by mannitol or DMSO. These results suggest that hydroxyl radical is involved in the interaction of H_2O_2 and YC-1. In contrast, since SOD failed to reverse H_2O_2/YC-1-induced inhibition of platelet aggregation, superoxide anion may not play a key role in this synergism.

Similar to the interaction of H_2O_2 and YC-1, it has been reported that H_2O_2 also acts synergistically with NO or GSNO on the inhibition of platelet aggregation (Naseem and Bruckdorfer, 1995; Naseem et al., 1996). However, the increase of cGMP levels of platelets caused by H_2O_2/NO is not correlated well with their antiaggregatory effect (Naseem and Bruckdorfer, 1995). According to these findings, we showed here that simultaneous application of H_2O_2 and GSNO had only a slight additive effect on platelet cGMP levels and, in a cell-free system, H_2O_2 even attenuated the soluble guanylate cyclase-stimulating activity of GSNO. Therefore, the potentiation of soluble guanylate cyclase activation may be not sufficient to account for the synergistic antiplatelet effect of H_2O_2/NO.

In contrast, (Friebe et al., 1996) have reported recently that YC-1, apart from activating soluble guanylate cyclase by itself, also greatly potentiating NO-, CO- and protoporphyrin IX-induced soluble guanylate cyclase stimulation. In the purified enzyme system, Friebe and his colleagues have showed that YC-1 increased the maximal soluble guanylate cyclase activity observed in the presence of NO by $>40\%$ and, shifted the concentration–response curve of NO stimulation by one order of magnitude to the left. Similar results also have been reported by Mülsch et al. (1997), Russwurm et al. (1998) and Zabel et al. (1998), whom demonstrated that YC-1 greatly enhanced the activity of NO to stimulate either purified or gene expressed soluble guanylate cyclase. In tissues or intact cells, YC-1 also potentiated the cGMP-elevating activity of NO or NO-donors, and had synergistic relaxing effect in vascular and tracheal smooth muscle (Mülsch et al., 1997; Hwang et al., 1999). Furthermore, since YC-1 potentiation is independent of the mode of soluble guanylate cyclase activation (NO, CO and protoporphyrin IX), it is suggested that YC-1 acts through stabilization of the activated configuration of soluble guanylate cyclase, thus sensitizing soluble guanylate cyclase towards its activators. Since YC-1 significantly potentiated $H_2O_2/FeSO_4$-induced soluble guanylate cyclase activation, and had synergistic effects with H_2O_2 on platelet aggregation and cGMP formation, we suggested that YC-1 potentiation results from sensitization of soluble guanylate cyclase towards H_2O_2 or H_2O_2-derived hydroxyl radical.

In conclusion, YC-1 may act synergistically with H_2O_2 to inhibit platelet aggregation via sensitization of soluble guanylate cyclase. Various pathological conditions such as ischemia-reperfusion (Forde and Fitzgerald, 1997), diabetes (Leoncini et al., 1997) and deficiency of plasma glutathione peroxidase (Freedman et al., 1996) were accompanied by increased H_2O_2 generation and hyperactivity of platelets. Therefore, the modulation of H_2O_2 on platelet aggregation by YC-1 might have potential pharmacotherapeutic implication in these diseases.

Acknowledgements

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