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Inhibitory effect of YC-1 on the hypoxic induction of erythropoietin and vascular endothelial growth factor in Hep3B cells

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Abstract

YC-1 is a newly developed agent that inhibits platelet aggregation and vascular contraction. Although its effects are independent of nitric oxide (NO), it mimics some of the biological actions of NO. For example, it stimulates soluble guanylate cyclase (sGC) and increases intracellular cGMP concentration. Here, we tested the possibility that YC-1 inhibits hypoxia-inducible factor (HIF)-1-mediated hypoxic responses, as does NO. Hep3B cells were used during the course of this work to observe hypoxic induction of erythropoietin (EPO) and vascular endothelial growth factor (VEGF), and the effects of YC-1 were compared with those of a NO donor, sodium nitropurruside (SNP). In hypoxic cells, YC-1 blocked the induction of EPO and VEGF mRNAs, and inhibited the DNA-binding activity of HIF-1. It suppressed the hypoxic accumulation of HIF-1α, but not its mRNA level. It also reduced HIF-1α accumulation induced by cobalt and desferrioxamine. Treatment with antioxidants did not recover the HIF-1α suppressed by YC-1. We examined whether these effects of YC-1 are related to the sGC/cGMP signal transduction system. Two sGC inhibitors examined failed to block the effects of YC-1, and 8-bromo-cGMP did not mimic actions of YC-1. The effects of YC-1 on the hypoxic responses were comparable with those of SNP. These results suggest that YC-1 and SNP suppressed the hypoxic responses by post-translationally inhibiting HIF-1α accumulation. The YC-1 effect may be linked with the metal-related oxygen sensing pathway, and is not due to the stimulation of sGC. This observation implies that the inhibitory effects of YC-1 on hypoxic responses can be developed to suppress EPO-overproduction by tumor cells and tumor angiogenesis.

Keywords: YC-1; Hypoxia; Hypoxia-inducible factor 1

1. Introduction

The reduction in oxygen tension stimulates the induction of hypoxia-inducible genes such as EPO, VEGF, phosphoglycerate kinase 1, enolase 1, aldolase A, and lactic dehydrogenase A [1]. The expression of these genes in hypoxic cells is up-regulated by a basic helix-loop-helix transcription factor, HIF-1 [2]. HIF-1 is composed of HIF-1α and HIF-1β, both of which belong to the PAS family of basic helix-loop-helix transcription factors [3]. HIF-1α and HIF-1β mRNAs are constitutively expressed in a number of mammalian cell lines under normoxic and hypoxic conditions, suggesting that HIF-1 is regulated by some post-translational mechanism. At the protein level, HIF-1α protein is markedly increased by hypoxia, whereas HIF-1β protein is constitutively present regardless of oxygen tension [4]. Under normoxia, HIF-1α is remarkably unstable and degraded through the ubiquitin-proteasome pathway [5]. Under hypoxia, it becomes stable and accumulates to be dimerized with HIF-1β, resulting in DNA binding and transactivation. In addition, transition metal ions (cobalt and nickel), iron chelating agent (desferrioxamine), and antioxi-

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Abbreviations: EPO, erythropoietin; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; NO, nitric oxide; NOS, nitric oxide synthase; CO, carbon monoxide; sGC, soluble guanylate cyclase; cGMP, cyclic GMP; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; SNP, sodium nitroprusside; ODQ, 1*H*-(1,2,4)oxadiazole (4,3a)quinoxatin-1-one; MB, methylene blue; NAME, N(G)-nitro-L-arginine methyl ester; RT-PCR, reverse transcription polymerase chain reaction; and EMSA, electrophoretic mobility gel shift.

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dants (thioredoxin) stabilize HIF-1α under normoxia, which strongly suggests that the stability of HIF-1α is related to the metal- and the redox state-dependent processes [6,7].

NO mediates a variety of biological effects such as vasodilation, cytotoxicity, and the regulation of the neuroendocrine function [8]. Its main target site is iron, bound within certain proteins as heme or iron-sulfur complexes. For instance, it binds to the heme component of sGC and then stimulates the enzymatic conversion of GTP to cGMP [9], which is responsible for the overwhelming majority of biological effects mediated by NO. Nevertheless, owing to its radical and lipophilic properties, NO can diffuse randomly away from its point of synthesis to interact with various intracellular molecules. The reaction of NO with metabolic enzymes or viral DNA, or its reaction with superoxide anion to yield peroxynitrite, may be important in the cytotoxic effects of inflammatory cells in removing pathogens. This biological reactivity of NO is also responsible for tissue injury in ischemia or inflammatory diseases [10].

Hypoxia appears to stimulate NO production through the induction of inducible NOS [11]. Conversely, NO inhibits hypoxic responses, such as the hypoxic induction of EPO [12,13] or VEGF [14], through the suppression of HIF-1α accumulation and the inactivation of HIF-1 transactivating activity. This NO-mediated inhibition of hypoxic responses is possibly involved in the exaggeration of tissue injury by NO in the hypoxic region. However, the mechanism of the inhibitory effect of NO on the hypoxic responses is not well understood. Three possible explanations for the NO effect can be suggested: binding to sGC and the stimulation of cGMP production, interaction with intracellular molecules regulating HIF-1α stability, and binding to the heme structure of a putative oxygen sensor, thereby locking the sensor in an oxygenated form.

The newly developed substance YC-1, which inhibits platelet aggregation [15,16] and vascular contraction [17], has been proven to be a new pharmacological principle that may lead to a potential therapeutic advantage in cardiovascular diseases. In platelet and vascular smooth muscle, the effect of YC-1 is accompanied by an increase in intracellular cGMP concentration via the stimulation of sGC activity. Although these effects of YC-1 are very similar to those of NO, the YC-1 stimulation of sGC is independent of NO [18]. However, how YC-1 stimulates the enzyme remains unclear. Likewise, since it mimics the biological actions of NO, we tested the possibility that YC-1 inhibits the HIF-1-mediated hypoxic response, as does NO. Our experiments show that both YC-1 and a NO donor suppressed the hypoxic induction of EPO and VEGF mRNAs in Hep3B cells, by inhibiting HIF-1α accumulation at the post-translational step. The YC-1 effect may be linked with the metal-related pathway of oxygen sensing, but is not due to the stimulation of sGC. This observation suggests that YC-1 can be a reasonable compound for the development of novel pharmacological approaches to the inhibition of hypoxic responses such as EPO-overproduction by tumor cells and tumor angiogenesis. Moreover, it can be developed as a new pharmacological tool for the study of the oxygen sensing mechanism.

2. Materials and methods

2.1. Materials

YC-1 was kindly supplied by Yung-Shin Pharmaceutical Industry (Taiwan). SNP, ODQ, and MB were purchased from Calbiochem. Other materials were obtained from Sigma or companies indicated in the text. Horseradish peroxidase-conjugated sheep anti-rat antiserum and the enhanced chemiluminiscence plus kit were purchased from Amersham, and [α-^{32}P]CTP (500 Ci/mmol) was from NEN-DuPont.

2.2. Cell culture and hypoxic induction

Hep3B cells were cultured in α-modified Eagle's medium (GIBCO/BRL), supplemented with 10% heat-inactivated fetal calf serum (GIBCO/BRL), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°. Oxygen tensions in the incubator (Vision Sci Co., model 9108MS2, Seoul, Korea) were either 140 mm Hg (20% O_2, v/v, normoxia) or 7 mm Hg (1% O_2, v/v, hypoxia). Cells were subjected to hypoxic induction at a cell density of 1×10^5 cells/cm^2.

2.3. Nuclear extract preparation

Cells were quickly cooled by placing the plates on ice and were then washed twice in ice-cold PBS. Scraped cells were centrifuged at 1,000 *g* for 5 min at 4° and then washed twice with PBS. They were resuspended in a lysis buffer containing 0.2% Nonidet P-40 and the nuclear proteins were extracted as previously described [19].

2.4. Electrophoretic mobility gel shift (EMSA) assay

The oligonucleotide probe used in the gel shift assay for HIF-1 consisted of the sequence 5′-ACCGGCCCTACGT-GCTGTCTCAC-3′. The probe was labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, and purified as previously described [19]. DNA-protein binding reactions were carried out for 20 min at 4° in a total volume of 20 μL, containing 5 μg of nuclear extract, 0.4 μg of sonicated, denatured calf thymus DNA, and 1×10^4 cpm of oligonucleotide probe in 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol and run on 5% non-denaturing polyacrylamide gels. Electrophoresis was performed at 200 V in a 0.3 × TBE buffer (15 mM Tris, 30 mM boric acid, and 0.06 mM EDTA, at pH 7.5) at 4° and dried gels were

autoradiographed. For supershift analysis, 1 μL of rat HIF-1α antiserum to was added to the completed EMSA reaction mixture and incubated for 2 hr at 4° prior to loading.

2.5. Western blot assay

For Western blot analysis, 20 μg of cell extract was separated on a 4% SDS/polyacrylamide gel and transferred to an Immobilon-P membrane (Milipore). Immobilized proteins were incubated overnight 4° with rat anti-HIF-1α, diluted 1:5000 in 5% nonfat milk in TBS/0.1% Tween-20 (TTBS). Horseradish peroxidase-conjugated sheep anti-rat antiserum (Amersham) was used as a secondary antibody (1:5000 dilution in 5% nonfat milk in TTBS). After extensive washing with TTBS, the complexes were visualized using enhanced chemiluminiscence plus (Amersham) according to the manufacturer's instructions. HIF-1 antiserum was generated in rats against a bacterially expressed fragment encompassing amino acids 418-698 of human HIF-1α as previously described [19].

2.6. Semiquantitative RT-PCR

Total RNA was isolated from cells by TRIZOL (GIBCO/BRL) according to the manufacturer's instructions, resuspended in nuclease-free water (Promega), and its absorbance read at A260 and A280. The quality of the RNA was checked on a 1% denaturing agarose gel to ensure the presence of the 28 S and 18 S ribosomal bands. mRNA expressions were measured using a semi-quantitative RT-PCR. One μg of total RNA was added to a 50-μl RT-PCR reaction (PCR-Access, Promega). The reaction master mix was prepared according to the manufacturer's instructions to give final concentrations of 1 × avian myeloblastosis virus/Tfl reaction buffer, 0.2 mM dNTPs, 5 μCi [α-^{32}P]CTP, 1.5 mM $MgSO_4$, 0.1 unit/ml avian myeloblastosis virus reverse transcriptase, 0.1 unit/μl Tfl DNA polymerase, and 250 nM concentrations of the primers. For quantitation of mRNA, primers were used in a reaction involving one cycle of reverse transcription at 48° for 1 hr and 20 cycles of denaturation at 94° for 30 sec, annealing at 56° for 30 sec, and elongation at 70° for 1 min. The resulting PCR fragments (5 μL) were electrophoresed on 4% polyacrylamide gels at 100 V in a 0.3 × TBE buffer (15 mM Tris, 30 mM boric acid, 0.06 mM EDTA, pH 7.5) at 4°, and dried gels were autoradiographed. The nucleotide sequences of the primers were 5′-CTGGAGAGGTACCTCTTGGA-3′ and 5′-CCTGTGTACAGCTTCAGCTT-3′ for EPO; 5′-AACTTTCTGCTGTCTTGG-3′ and 5′-TTTGGTCTGCATTCACAT-3′ for VEGF; 5′-CCCCAGATTCAGGATCAGACA-3′ and 5′-CCATCATGTTCCATTTTTCGC-3′for HIF-1α; 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-ATCTCTTGCTCGAAGTCCAG-3′ for β-actin.

3. Results

3.1. Effect of YC-1 on HIF-1-mediated hypoxic response

Hep3B cells, a hepatoma cell line, were used to examine the cellular response to hypoxia. Hep3B cells were exposed to hypoxia or normoxia for 16 hr, and total cellular RNA was isolated for semi-quantitative RT-PCR. Both EPO and VEGF mRNAs were markedly increased in cells exposed to hypoxia, compared with the normoxic controls (Fig. 1A). To examine the effect of YC-1 on the hypoxic induction of these mRNAs, the cells were treated with YC-1 for 16 hr under the hypoxic condition. YC-1 (100 and 200 μM) reduced the mRNA level of EPO and VEGF under hypoxia, whereas it was found to have no effect on the beta-actin mRNA level. NO-releasing SNP (5 and 10 μM), which was used to compare YC-1 and NO effects on the hypoxic response, also reduced these mRNAs in hypoxic cells. Under this condition, neither YC-1 nor SNP treatment caused a release of lactate dehydrogenase into the culture medium (data not shown). This result indicates that the hypoxic induction of EPO and VEGF mRNAs is suppressed by YC-1 in Hep3B cells.

HIF-1 DNA binding activity to the hypoxia response element of the EPO gene was examined to determine whether YC-1 affects the HIF-1 binding. HIF-1/DNA binding was greatly increased by hypoxia, and both YC-1 (100 μM) and SNP (5 μM) under hypoxic conditions inhibited HIF-1/DNA binding (Fig. 1B). The super-shift by the anti-HIF-1α antibody of the HIF-1 band showed that the upper band represented the HIF-1/DNA complex. Taken together, these results suggest that YC-1 and SNP inhibit the hypoxic activation of HIF-1, resulting in the suppression of EPO and VEGF in hypoxic Hep3B cells.

3.2. Effect of YC-1 on the hypoxic accumulation of HIF-1α protein

Several cellular steps are required to activate HIF-1: accumulation of HIF-1α protein, nuclear translocation, formation of HIF-1 complex with HIF-1β protein, and binding to DNA. Among these steps, the HIF-1α accumulation in hypoxia is considered to be a key step in the hypoxic EPO induction. Thus, we examined whether YC-1 affects the hypoxic accumulation of HIF-1α protein. The protein level of HIF-1α was greatly increased by hypoxia, while that in normoxic cells was negligible. 100 μM or more concentrations of YC-1 inhibited the hypoxic accumulation of HIF-1α, but at 25 μM or less concentrations the HIF-1α level was unaffected (Fig. 2A). In contrast, the density of a non-specific protein band (NS) was unaltered by YC-1 treatment, which suggests that the YC-1 effect is specific to HIF-1α. The effect of YC-1 on HIF-1α level was also examined in normoxic cells to examine the possibility that YC-1 affects HIF-1α protein under normoxic conditions. The result showed that YC-1 had no effect on HIF-1α

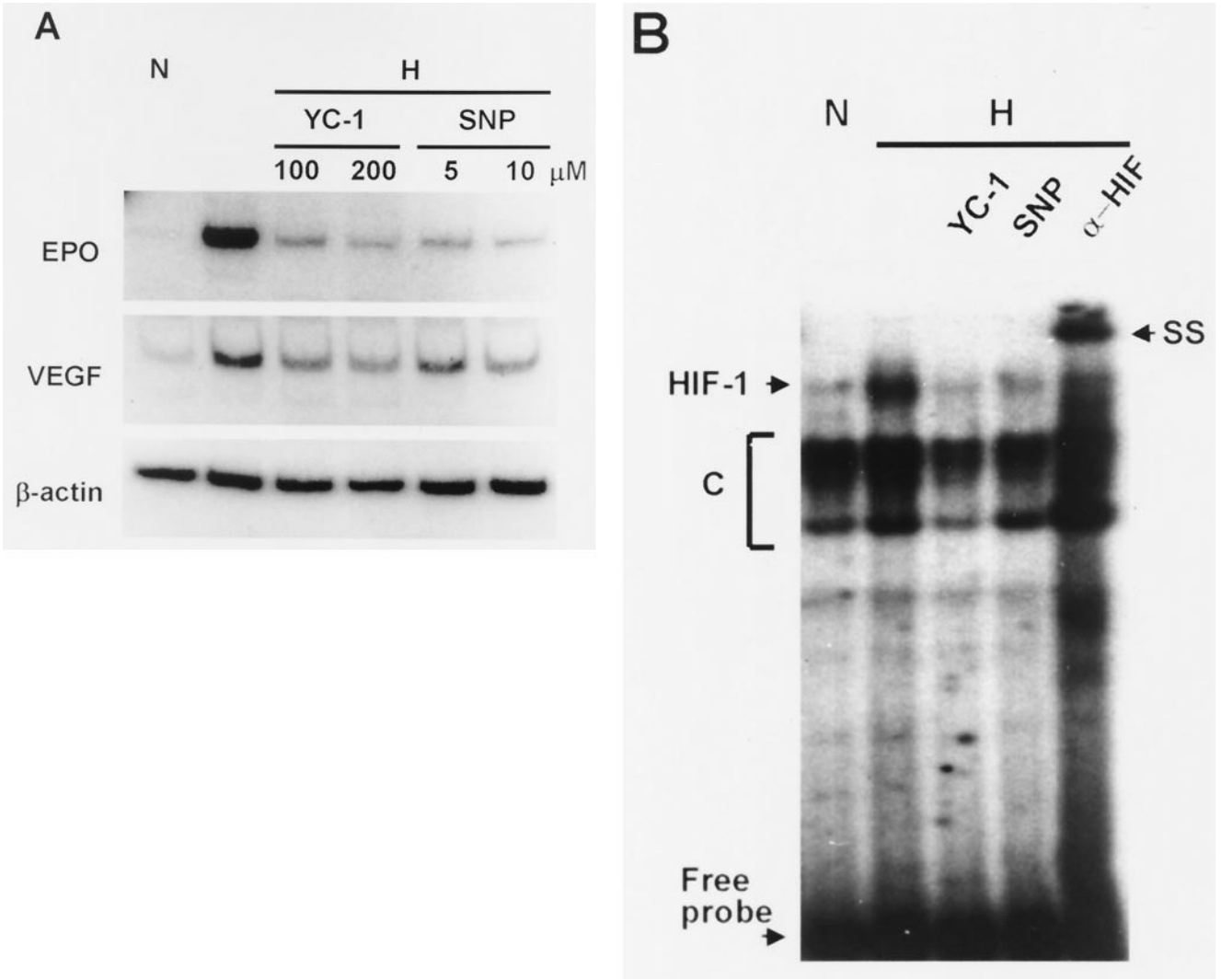

Fig. 1. Effects of YC-1 and SNP on HIF-1-mediated induction of EPO and VEGF. (**A**) Hypoxic induction of EPO and VEGF mRNAs. EPO, VEGF and β-actin mRNAs were isolated from Hep3B cells subjected to normoxia (N) or hypoxia (H) for 16 h, and analyzed by semi-quantitative RT-PCR. YC-1 or SNP was added to the culture media 5 min prior to hypoxic incubation. (**B**) DNA binding of HIF-1. HIF-1 was extracted from the nuclei of Hep3B cells subjected to normoxia (N) or 4 h-hypoxia (H) in the absence and presence of 100 μM YC-1 or 5 μM SNP, and analyzed by EMSA. For supershift analysis, 1 μl of HIF-1α anti-serum was added to the completed EMSA reaction mixture. HIF-1 binding (HIF-1), constitutive binding (C), and supershifted band (SS) are indicated. The data presented in each panel are representative of three separate experiments.

protein level in normoxic cells (Fig. 2B). In addition, we examined the effect of SNP on HIF-1α protein in hypoxic and normoxic cells. As expected, SNP suppressed the hypoxic accumulation of HIF-1α protein (Fig. 2C). 2.5 μM SNP reduced the HIF-1α level of hypoxic cells to the normoxic control level, but higher concentrations had no further effect, which was comparable with the inhibitory effect of SNP on the activity of HIF-1 by EMSA, as shown in Fig. 1B. In normoxic cells, SNP failed to induce HIF-1α accumulation over the range of concentrations examined, as shown in Fig. 2D. To rule out the possibility of the suppression of HIF-1α at the transcriptional level, HIF-1α mRNA levels were examined using semi-quantitative RT-PCR with [α-^{32}P]CTP. The HIF-1α mRNA levels were unaffected by hypoxia and treatment with YC-1 or SNP (Fig. 2E). These findings suggest that YC-1 and SNP inhibit the expression of EPO and VEGF genes by blocking the hypoxic accumulation of HIF-1α at the post-translational step.

3.3. Effect of YC-1 on the oxygen sensing pathway

Transition metals such as iron and cobalt and the intracellular redox state are considered to regulate the stability of HIF-1α at downstream of the oxygen sensor [4,20]. To examine whether YC-1 affects these processes that regulate the stability of HIF-1α, Hep3B cells were treated with cobalt ion, an iron chelator (desferrioxamine), and antioxidants (Trolox and N-acetyl cysteine). Both cobalt and desferrioxamine induced HIF-1α accumulation in normoxic cells as shown in Fig. 3A (lane 2 and 6). YC-1 blocked HIF-1α accumulation induced by cobalt or desferrioxamine

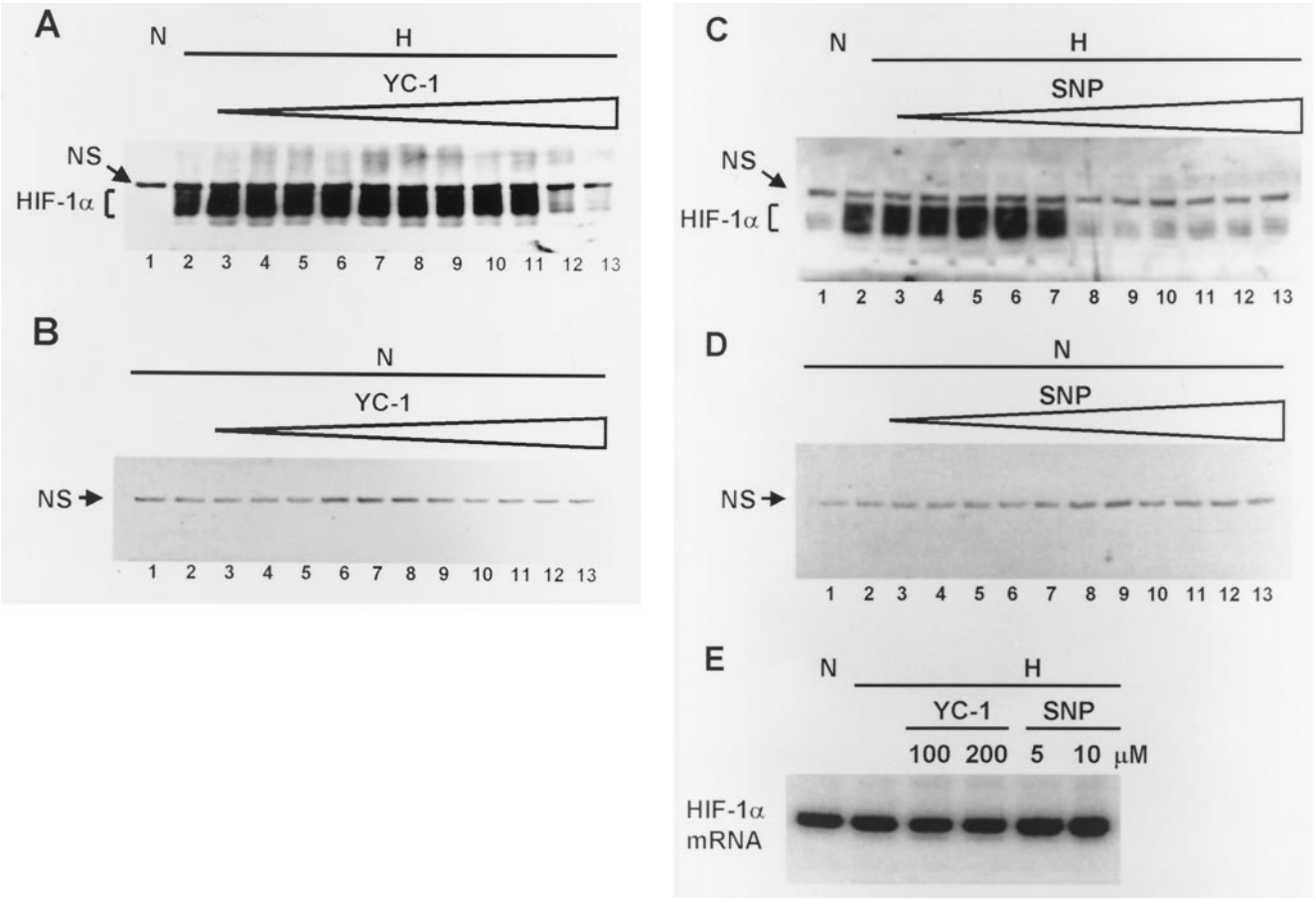

Fig. 2. Effects of YC-1 and SNP on the hypoxic accumulation of HIF-1α protein. After 4-h hypoxic incubation, HIF-1α protein was isolated and analyzed by Western blot. YC-1 or SNP was added to the culture media 5 min prior to 4-hr incubation of hypoxia (**A, C**) or normoxia (**B, D**). The HIF-1α protein band (HIF-1α) and the non-specific protein band (NS) are indicated. The concentrations of agents at lanes 3 to 13 were 0.002, 0.01, 0.02, 0.05, 0.25, 1, 2.5, 5, 25, 100, 200 μM in order. **E**, HIF-1α mRNAs were isolated from Hep3B cells subjected to normoxia (N) or hypoxia (H) for 4 hr, and analyzed by semi-quantitative RT-PCR. YC-1 or SNP was added to the culture medium 5 min prior to hypoxic incubation.

treatment (lane 3 and 7). However, SNP did not block desferrioxamine-induced accumulation of HIF-1α (lane 8), whereas YC-1 blocked the cobalt-induced HIF-1α (lane 4). On the other hand, the antioxidants, trolox and N-acetyl cysteine, failed to recover HIF-1α levels suppressed by YC-1 and SNP, as shown in Fig. 3B (lanes 4, 5, 7, and 8). These results suggest that the suppression mechanism of YC-1 on hypoxic responses is likely to be linked with the metal ion-related pathway of oxygen sensing but not with the cellular redox state. In addition, the mechanism of the YC-1 effect seems to be in part different from that of SNP in terms of the response to desferrioxamine-induced HIF-1α.

3.4. cGMP-independent action of YC-1

Both YC-1 and NO have been known to increase the intracellular cGMP levels by stimulating sGC, in platelet and smooth muscle cells, but their binding sites in sGC seem to differ. Furthermore, YC-1 can inhibit the activity of cGMP-hydrolyzing phosphodiesterase, which may be another mechanism that promotes an increase in cGMP [15]. Recently, it has been reported that a combination of YC-1 and NO donor results in an enormous increase in cGMP production. The co-administration of these two drugs increased the cGMP level 1000-fold or more, in human platelets [15], guinea-pig trachea [21], and in an *in-vitro* reaction system containing purified sGC [22]. This combination shows promise as a means of maximizing the intracellular cGMP content. Therefore, to test whether sGC or cGMP are involved in HIF-1α suppression by YC-1 and SNP, we treated hypoxic cells with two inhibitors of sGC, ODQ and MB. Neither inhibitor suppressed the action of either YC-1 or SNP on the hypoxic HIF-1α accumulation, as shown in Fig. 4. These results suggest that the effects of YC-1 and SNP are not related to the stimulation of sGC. To further check the possibility that cGMP is elevated by a mechanism which does not involve the stimulation of sGC, we tested the effect of combined treatment with 50 μM YC-1 and 1 μM SNP because the combination results in an enormous increase in cGMP production. At these concentrations, both YC-1 and SNP when used alone, showed a borderline effect

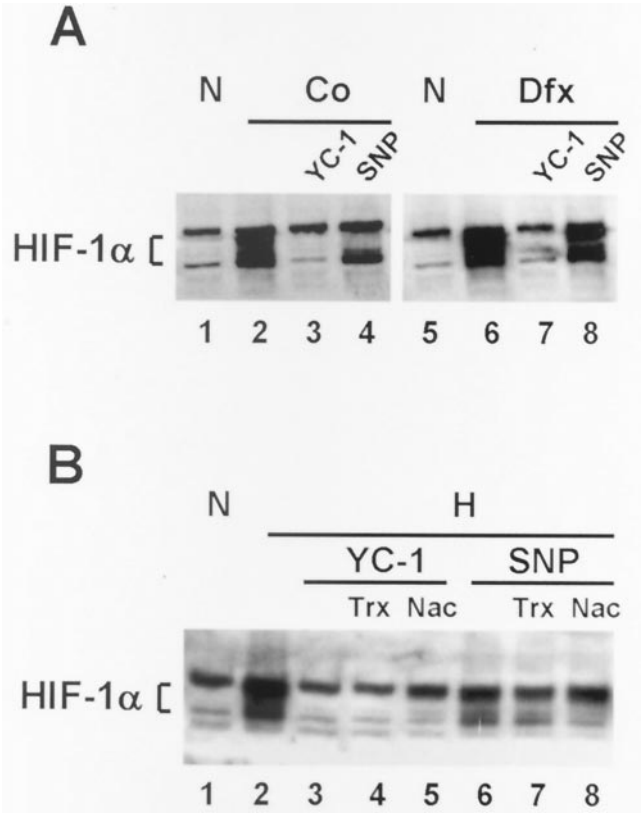

Fig. 3. Effects of YC-1 and SNP on the oxygen sensing pathway. (**A**) After a 4-hr treatment of Hep3B cells with 75 μM $CoCl_2$ (Co) and 130 μM desferrioxamine (Dfx) in the absence and presence of 100 μM YC-1 or 5 μM SNP, HIF-1α protein is analyzed by Western blot. (**B**) After a 4-hr treatment with 5 μM Trolox (Trx) and 20 mM N-acetyl cystein (Nac) in the presence of 100 μM YC-1 or 5 μM SNP, HIF-1α protein is analyzed by Western blot.

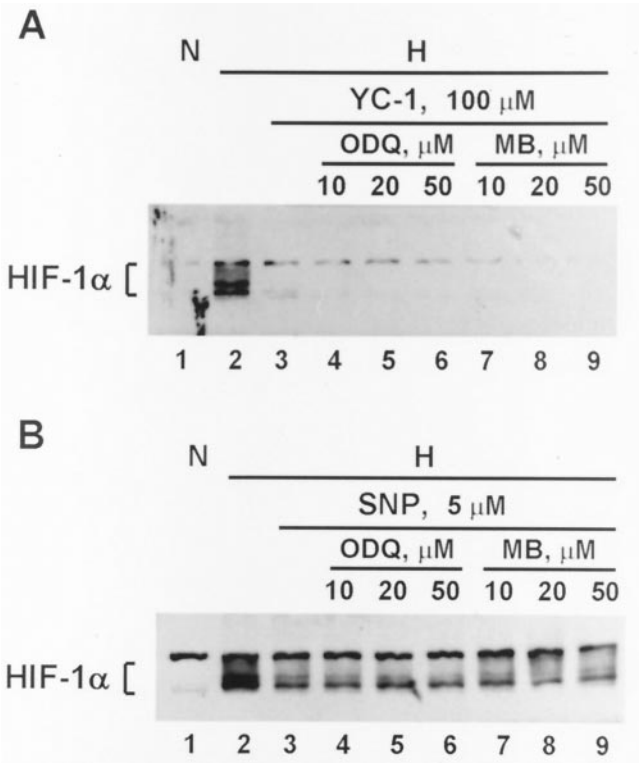

Fig. 4. Effects of sGC inhibitors on YC-1 and SNP suppression of HIF-1α. After a 4-hr hypoxia treatment of Hep3B cells in the absence and the presence of 100 μM YC-1 (**A**), 5 μM SNP (**B**), HIF-1α protein is analyzed by Western blot. sGC inhibitors, ODQ and MB were added to the media 1 hr before the hypoxic incubation. The concentrations of the inhibitors are indicated in figures.

on the accumulation of HIF-1α in hypoxic cells (Fig. 5, lane 5 and 6). However, combining the two did not produce a further reduction in the HIF-1α level (Fig. 5, lane 7), which suggests that the synergistic increase associated with this combined treatment, in terms of the cGMP level, does not affect hypoxic HIF-1α accumulation. Hypoxic cells were also treated with 8-bromo-cGMP, a membrane-permeable cGMP analog, but no loss of HIF-1α was observed at concentrations up to 1 mM (Fig. 5, lane 10 and 11). Based on these results, it is believed unlikely that the inhibitory effects of YC-1 and SNP are mediated through a pathway involving sGC or cGMP.

4. Discussion

We tested the possibility that YC-1 blocks HIF-1α-mediated hypoxic response. YC-1 reduced the cellular level of EPO and VEGF mRNAs in hypoxic Hep3B cells. It suppressed DNA-binding activity of HIF-1 and the protein amount of HIF-1α. It also reduced HIF-1α accumulation induced by cobalt and desferrioxamine, but antioxidants did not recover the HIF-1α suppressed by YC-1. sGC inhibitors failed to block the effects of YC-1, and 8-bromo-cGMP did not mimic actions of YC-1. Most effects of YC-1 on the hypoxic responses were comparable with those of SNP. These results suggest that YC-1 and SNP inhibited HIF-1α accumulation and the induction of EPO and VEGF in hypoxic cells through a cellular process linked with the metal-related oxygen sensing pathway.

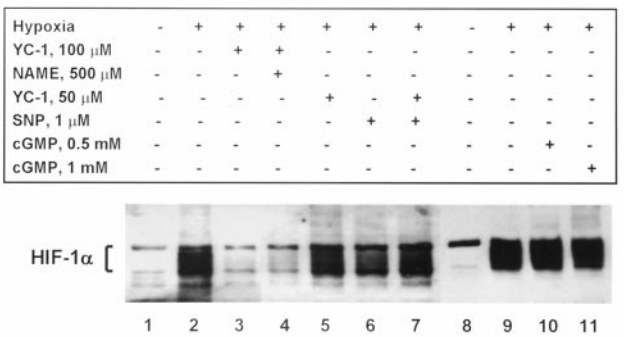

Fig. 5. Effects of cGMP and NAME on YC-1 suppression of HIF-1α. After a 4-hr hypoxia treatment of Hep3B cells, HIF-1α protein is analyzed by Western blot. YC-1 and SNP were added to the media 5 min prior to 4-hr incubation of hypoxia. NAME and 8-bromo-cGMP (cGMP) were added to the media 1 hr before the hypoxic incubation.

The inhibitory effect of NO on the hypoxic induction of EPO mRNA has been examined [12,13]. They used SNP at a concentration of 100 μM to show the inhibitory effect of NO on EPO mRNA in hypoxic Hep3B cells. This concentration of SNP is about 10-fold higher than was used in our study, as shown in Fig. 1A. We found 10 μM SNP was enough to suppress the hypoxic induction of EPO mRNA. Normally, 10 μM SNP is sufficient to show a dramatic increase in cGMP level, which suggests that this concentration of SNP is enough to achieve a biological effect through NO-specific receptors such as sGC. In contrast, because of the high reactivity of NO, a higher concentration of NO increases the chance that NO or its metabolites nonspecifically bind to intracellular molecules and modify their structures. Thus, the observation that 10 μM SNP suppresses hypoxic EPO induction suggests that the NO effect may be related to some cellular event linked with a specific NO receptor. In contrast, Kimura et al. [23] observed that lower concentrations of NO donors increased the expression of the VEGF reporter gene in normoxic cells even though higher concentrations of NO donors inhibited hypoxic expression of VEGF reporter gene. Moreover, lower concentrations of NO donors induced HIF-1α accumulation and HIF-1 activity in normoxic cells. For this reason, we examined YC-1 and SNP effects over a wide range of concentrations, from 2 nM to 200 μM. However, no evidence of HIF-1α accumulation due to NO in normoxic cells was obtained, as shown in Fig. 2D. This discrepancy in the effect of NO in normoxic cells remains to be explained.

YC-1 is known to increase cGMP level by direct stimulation of sGC. However, it has been suggested that YC-1 has an indirect effect to stimulate sGC. Wohlfart et al. [24] argued that YC-1 stimulates the production of NO in endothelial cells, and that the released NO may stimulate sGC and increase the intracellular cGMP level. They observed that incubation of bovine aortic endothelial cells with YC-1 produced a concentration-dependent NO synthesis and release, and that this YC-1 effect was decreased by NAME, an inhibitor of NO synthase (NOS). It was therefore, suggested that the YC-1 stimulation of NO synthesis is due to the activation of NOS. To rule out this indirect action of YC-1, we pre-incubated Hep3B cells with NAME to block this YC-1 effect, and then treated the cells with YC-1 under the hypoxic condition. The NAME pretreatment did not change the inhibitory effect of YC-1 on the hypoxic HIF-1α accumulation, as shown in Fig. 5 (lane 4). Therefore, the YC-1 effect seems not to be due to this indirect action of YC-1 via NOS.

In this study, the exact mechanism of the YC-1 effect was not determined. Bearing in mind, that the behavior of YC-1, in terms of hypoxic response, is similar to that of SNP, YC-1 and NO appears to share a common oxygen sensing pathway. In turn, NO is suggested to share a common oxygen sensing pathway with carbon monoxide (CO). It has been reported that CO also suppresses the hypoxic expression of EPO through the inhibition of HIF-1α accumulation [13]. Taken together, it is possible that the three molecules (CO, NO, and YC-1) target a common oxygen sensing pathway. Although they commonly activate sGC and increase the cGMP level, this signal transduction system is not likely to be responsible for the suppression of hypoxic responses. The other candidate common pathway may be the heme-containing oxygen sensor. It has been suggested that both CO and NO inhibit hypoxic responses, presumably by binding to the heme structure of the putative oxygen sensor [13]. This explanation for the mechanism in the case of CO and NO is reasonable because both molecules are proven to bind to heme iron. Recently, the possibility that YC-1 also directly binds to the heme structure has been suggested. Kharitonov et al. [25] observed that YC-1 shifts the wavelength of the Soret band provided by the heme of sGC. Denninger et al. [26] also observed the direct interaction of YC-1 with the heme of sGC using kinetic and Resonance Roman studies. If so, all three molecules (CO, NO, and YC-1) can interact with the heme structure of the oxygen sensor, and thereby also interfere with its ability to sense reduced oxygen tension. We observed that YC-1 inhibited HIF-1α induced by the addition of cobalt ion and the removal of iron, which suggests that YC-1 interferes with the metal-related oxygen sensing pathway. Whether or not the target in the metal-related pathway is the heme iron of the oxygen sensor is still unclear. If so, it is possible that YC-1 and NO disturb the function of the oxygen sensor by binding to its heme. However, because both the heme binding of YC-1 and the mechanism of oxygen sensing are still uncertain, the mode of action of YC-1 remains to be resolved.

In conclusion, our experiments show that YC-1 and SNP suppressed the induction of EPO and VEGF mRNAs in hypoxic Hep3B cells by inhibiting hypoxic HIF-1α accumulation at the post-translational level. The YC-1 effect seems to be linked with the metal-related oxygen sensing pathway, and not with either the cellular redox state or the activation of cGMP-dependent signal transduction. The observation that YC-1 suppresses the HIF-1-mediated hypoxic response, suggests that it might represent a reasonable starting compound for the development of novel pharmacological approaches for the treatment of EPO-overproducing tumors or to the inhibition of tumor angiogenesis. Moreover, it has potential as a new pharmacological tool for the study of the oxygen sensing mechanism.

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