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Induction of Heme Oxygenase-1 as a Response in Sensing the Signals Evoked by Distinct Nitric Oxide Donors

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ABSTRACT. To gain insights into the cellular responses evoked by nitric oxide (NO), we have studied the effects of NO donors with distinct chemistries on the expression of heme oxygenase-1 mRNA by northern blot analysis. The expression levels of heme oxygenase-1 mRNA were increased significantly in DLD-1 human colorectal adenocarcinoma cells by treatment with each of three NO donors: sodium nitroprusside (SNP), *S*-nitroso-L-glutathione (GSNO), and 3-morpholinosydnonimine (SIN-1). A combination of SIN-1 plus SNP or GSNO additively increased heme oxygenase-1 mRNA expression, whereas synergistic induction was seen with SNP plus GSNO. The SNP-mediated induction was not affected noticeably by extracellular superoxide dismutase, catalase, or mannitol, while the induction by SIN-1 was attenuated by superoxide dismutase. Thus, the SNP-mediated induction of heme oxygenase-1 mRNA expression may be independent of reactive oxygen species, and the induction by SIN-1 is mediated partly by peroxynitrite, which is generated by immediate reaction of NO and superoxide anion. Transient transfection assays suggested that treatment with SNP, but not with GSNO or SIN-1, increased the expression of a reporter gene through a *cis*-acting element, including the cadmium-responsive element, of the human heme oxygenase-1 gene. These results suggest that SNP induces heme oxygenase-1 mRNA expression through a mechanism different from that for GSNO or SIN-1. We therefore propose that induction of heme oxygenase-1 represents a common cellular response in sensing the signals evoked by distinct NO donors. BIOCHEM PHARMACOL **58**;2:227–236, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. heme oxygenase; sodium nitroprusside; nitric oxide; oxidative stress; bilirubin

NO‖ is a free radical gas and plays a role as a signaling molecule regulating a wide range of biological functions, such as smooth muscle relaxation, neurotransmission, and immune cell-mediated cytotoxicity [for review see Refs. 1 and 2]. NO is able to react with oxygen (O_2), transition metals, and a superoxide anion (O_2^-) [3, 4], which in turn may generate various redox forms of NO and may cause secondary oxidative stress. Certain redox-activated forms of NO, which possess the character of a nitrosonium ion (NO^+), can react with thiols on proteins [3, 4]. As molecular tools to mimic the functions of NO, various structurally dissimilar reagents, including SNP, GSNO, and SIN-1, have been used as NO donors. However, it has not necessarily been established whether these reagents with different chemical properties exert their effects via NO.

The metabolism of SNP has been studied extensively, because SNP has been used successfully to control hypertensive emergencies [5, 6]. Nitroprusside anion is a coordination complex of a ferrous ion (Fe^{2+}) with five cyanide anions (CN^-) and a nitrosonium ion (NO^+), and may undergo redox cycling in the presence of reducing agents, accompanied by generation of NO and reactive oxygen species, such as O_2^-, hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) [7, 8]. GSNO represents a major NO carrier in biologic systems and may undergo either homolytic cleavage of the S—N bond with release of NO or heterolytic decomposition to generate NO^+ [9]. SIN-1 is known to generate peroxynitrite ($ONOO^-$) rather than NO, because spontaneous decomposition of SIN-1 is accompanied by virtually simultaneous release of O_2^- and NO, which immediately react with each other to generate $ONOO^-$ [10]. Peroxynitrite may produce oxidant injury by itself or through the formation of a hydroxyl-like radical [11, 12].

Heme oxygenase (EC 1.14.99.3) is an essential enzyme in heme catabolism that cleaves heme to release carbon monoxide, iron, and biliverdin [13, 14]. There are two types

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‖ *Abbreviations*: NO, nitric oxide; SNP, sodium nitroprusside; GSNO, *S*-nitroso-L-glutathione; SIN-1, 3-morpholinosydnonimine; and CdRE, cadmium-responsive element.

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of heme oxygenase isozymes, inducible heme oxygenase-1 [15] and constitutively expressed heme oxygenase-2 [16–18], both of which are expressed ubiquitously. Human heme oxygenase-1 is remarkably induced in response to various environmental factors [19, 20], such as its substrate heme [21, 22] and heavy metals [23–25]. Induction of heme oxygenase-1 has been considered as cellular protection against oxidative stress, because the bile pigment produced possesses radical scavenging activity [26, 27]. We have reported that treatment with either SNP, GSNO, or SIN-1 causes a significant increase in the expression levels of heme oxygenase-1 mRNA and/or protein in various human cell lines [28–30]. Therefore, it is conceivable that induction of heme oxygenase-1 may represent a defensive response to ameliorate cytotoxicity caused by NO and/or secondary oxidative stress. Among SNP, GSNO, and SIN-1, SNP is the most efficient inducer for heme oxygenase-1, but the mechanisms by which these NO donors manifest their effects have not been studied fully.

In this study, to better understand the physiological significance of heme oxygenase-1 induction in NO cytotoxicity, we compared the effects of the three NO donors (SNP, GSNO, and SIN-1) in DLD-1 human colorectal adenocarcinoma cells. This cell line expresses the endogenous inducible NO synthase gene, whose expression is increased by treatment with cytokine mixtures [31]. In addition, the gastrointestinal tract may be exposed continuously to NO or its related metabolites, which are produced locally or derived from either food or NO produced nonenzymatically in the mouth [32] and stomach [33, 34]. Thus, DLD-1 cells could provide a suitable system to study the regulation of heme oxygenase-1 gene expression in response to NO. Analyzing the induction properties of heme oxygenase-1 mRNA expression caused by SNP, GSNO, and SIN-1, we provide evidence that induction of heme oxygenase-1 mRNA may represent a cellular response to the stresses caused by distinct redox-interrelated forms of NO.

MATERIALS AND METHODS

Materials

A human colorectal adenocarcinoma cell line, DLD-1, was obtained from Dainippon Pharmaceutical. SNP, potassium ferrocyanide, potassium ferricyanide, superoxide dismutase, and catalase were purchased from the Wako Pure Chemical Co. SIN-1 and NO_2^-/NO_3^- Assay Kit-C were obtained from Dojindo; GSNO from the Alexis Co.; ascorbate from the Sigma Chemical Co.; and biotinylated molecular weight markers from APRO Science.

Cell Culture

DLD-1 human colorectal adenocarcinoma cells were cultivated at 37° under 5% CO_2 in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum. To examine the effect of NO donors, DLD-1 cells were cultivated in fresh medium for 24 hr, and then were exposed to either SNP, GSNO, or SIN-1 for 5 hr. The cultured cells and medium were collected 5 hr after the addition of NO donors. In some experiments, DLD-1 cells were incubated with one of the three NO donors and an RNA synthesis inhibitor, actinomycin D (1 μg/mL), or a protein synthesis inhibitor, cycloheximide (1 μg/mL), at 37° for 5 hr. The nitrite concentrations in the medium were measured by the NO_2^-/NO_3^- Assay Kit-C following the manufacturer's protocols after the removal of proteins from the medium with a Microcon (Amicon, Inc.). The effects of extracellular catalase, glutathione, or superoxide dismutase on the heme oxygenase-1 mRNA expression induced by treatment with each NO donor were examined. The effects of exogenous H_2O_2 also were studied by incubating cells with 500 μM H_2O_2 with or without catalase for 60 min, followed by a 5-hr recovery in fresh medium.

Northern Blot Analysis

Total RNA was extracted from DLD-1 cells by the guanidinium thiocyanate–cesium chloride method [35], and was subjected to northern blot analysis. Total RNA (10 or 15 μg/lane) was electrophoresed on a 1% agarose gel containing 2 M formaldehyde, transferred to a nylon membrane filter (Zeta-Probe membrane, Bio-Rad), and fixed with a UV-linker (Stratalinker 1800, Stratagene). The filter was prehybridized at 42° in a solution consisting of 5x SSC (0.75 M sodium chloride, 0.075 M sodium citrate), 1% SDS, 50% formamide, 5x Denhardt's solution, and 0.2 mg/mL of salmon testes DNA for 2 hr, and was hybridized with radiolabeled cDNA probe at 42° for 16 hr. The hybridized filter was washed extensively at 65° with 1x SSC and 0.1% SDS. Radioactive signals were detected by exposing the filters to X-ray films (X-AR5, Kodak) and also were quantified with a Bioimage Analyzer (BAS 2000, Fuji Film Co., Ltd.). Each cDNA probe was labeled with [α-^{32}P]dCTP (Amersham), using a random primer labeling kit (Takara, Tokyo). The hybridization probe for heme oxygenase-1 mRNA was the *Xho*I/*Xba*I fragment (-64/923) derived from the human heme oxygenase-1 cDNA, pHHO1 [21]. A β-actin probe was prepared as described previously [28]. In this study, the blots for β-actin mRNA were exposed to X-ray films for about 5 hr. The exposure time for the blot of heme oxygenase-1 mRNA expression varied depending on the experiments and thus was described in the figure legends. Northern blot analyses were performed at least two times for each series of experiments, and one representative blot is shown.

Plasmid Construction

The pHHOL constructs, containing the promoter region of the human heme oxygenase-1 gene [36] upstream from the firefly luciferase gene, were described previously [24]. The constructs, pHHOL5, pHHOL14, and pHHOL15, contain the *Pst*I/*Xho*I fragment (−282 bp/+20), the *Xba*I/*Xho*I

fragment (−4 kb/+20), and the *Pst*I/*Xho*I fragment (about −4.5 kb/+20) of the human heme oxygenase-1 gene, respectively. For analysis of the enhancer function with a heterologous promoter, pSVLE(-) containing the simian virus 40 promoter upstream from the luciferase gene [24] was used. Synthetic double-stranded oligonucleotides, Cd6, Cd7, and AP-1, were designed (see Fig. 5) and inserted by blunt end ligation between the *Bam*HI and *Bgl*II sites of pSVLE(-), yielding pSVLCd6, pSVLCd7, and pSVLAP1, respectively. The cloning sites are located upstream from the simian virus 40 promoter. The AP-1 oligonucleotide, 5′-CTAGTGATGAGTCAGCCGGATC-3′, contains the binding site for the transcription factor AP-1, as underlined [37].

Transient Expression Analysis

DLD-1 cells (6×10^5) were seeded in a 6-cm diameter dish 20 hr before DNA transfection. Cells were transfected by the calcium phosphate method [38] with modifications; namely, cells were incubated with the plasmid DNA precipitated with calcium phosphate for 5 hr, then treated with glycerol for 5 min, and incubated in fresh medium supplemented with 10% fetal bovine serum for 24 hr. The DNA used for cotransfection was 6 μg of each fusion gene and 1 μg of the β-galactosidase expression vector pCH110 (Pharmacia LKB Biotechnology Inc.), containing the simian virus 40 early promoter. Following the 24-hr incubation, cells were left untreated or treated with cadmium or each NO donor for 5 hr, harvested, and lysed as described previously [24, 25]. The supernatants were assayed for luciferase activity and for β-galactosidase activity as an internal control. Luciferase activity was divided by β-galactosidase activity to calculate the normalized luciferase activity. Under the conditions used, the maximum relative luciferase activity was obtained with each fusion construct.

Western Blot Analysis

DLD-1 cells were cultivated in fresh medium for 24 hr, and then were left untreated for up to 24 hr or treated with either cadmium (50 μM) or SNP (1 mM). DLD-1 cells were lysed in a triple detergent lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/mL of phenylmethylsulfonyl fluoride, 1 μg/mL of aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate. The cell lysates were centrifuged at 15,000 *g* for 10 min, and the supernatant (30 μg protein) was analyzed on a SDS-polyacrylamide gel (12%). The proteins in the gel were treated with 10% methanol buffer containing 48 mM Tris, 39 mM glycine, and 0.037% SDS and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.), which was also pretreated with the same buffer. Expression of heme oxygenase-1 protein was detected with a rabbit anti-human heme oxygenase-1 antibody [18]. The specific immunocomplexes were detected with a western blot kit (ECL Plus). The same blot was reused to detect the expression of heat shock protein 70 with an anti-72-kDa heat shock protein monoclonal antibody (Amersham).

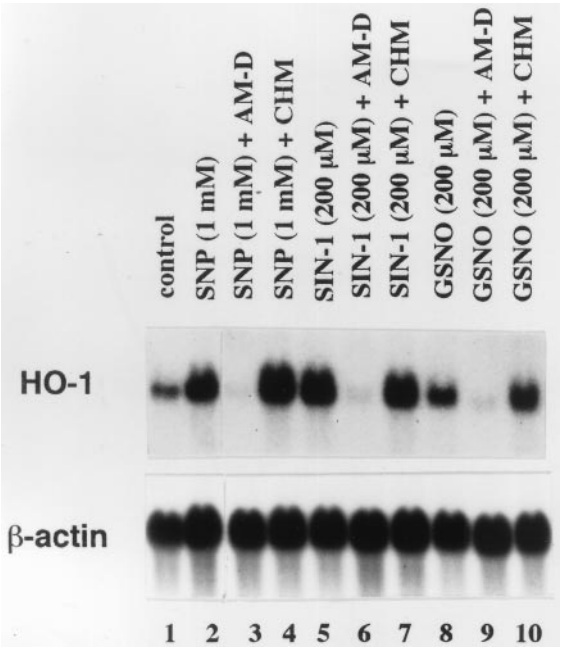


FIG. 1. Induction of heme oxygenase-1 mRNA in DLD-1 cells by NO donors. Shown is the autoradiogram of the RNA blot hybridized with ^{32}P-labeled heme oxygenase-1 or β-actin cDNA probe. DLD-1 cells were treated with the indicated concentrations of each NO donor for 5 hr in the presence or absence of actinomycin D (AM-D) or cycloheximide (CHM). Each lane contained RNA (10 μg) prepared from DLD-1 cells, untreated (control) or treated. The top panel shows the expression of heme oxygenase-1 mRNA, indicated by HO-1. The blot was exposed to an X-ray film for 2 days. The bottom panel shows the expression of β-actin mRNA as an internal control. The data shown are from one of two similar experiments.

RESULTS

Induction of Heme Oxygenase-1 mRNA by NO Donors in DLD-1 Colorectal Adenocarcinoma Cells

We initially performed the concentration–response experiments in DLD-1 cells by using various concentrations of SNP (10 μM to 2 mM), SIN-1 (10 μM to 1 mM), and GSNO (10 to 300 μM). The greatest induction level of heme oxygenase-1 mRNA was obtained with 1 mM SNP, 200 μM SIN-1, and 200 μM GSNO, respectively. The levels of heme oxygenase-1 mRNA expression reached a maximum at 3 hr and maintained the highest levels up to 8 hr. In contrast, expression levels of heme oxygenase-2 mRNA were not changed (data not shown). We noticed that GSNO at 300 μM showed strong cell toxicity.

Treatment for 5 hr with either SNP (1 mM), SIN-1 (200 μM), or GSNO (200 μM) caused a significant increase in the expression levels of heme oxygenase-1 mRNA (Fig. 1). The accumulation of heme oxygenase-1 mRNA caused by each NO donor was inhibited completely by actinomycin D (1 μg/mL), but apparently was not affected by cyclohexi-

mide (1 μg/mL). Expression levels of heme oxygenase-1 mRNA in cells treated with actinomycin D were rather lower than those of untreated cells (lane 1), whereas actinomycin D had no significant effect on the expression of β-actin mRNA.

Synergistic Effect of SNP Plus GSNO in Increasing Heme Oxygenase-1 mRNA Levels

We then analyzed the effects of a combination of NO donors on the expression of heme oxygenase-1 mRNA (Fig. 2, A and B). Each combination of two NO donors caused a remarkable increase in the induction levels of heme oxygenase-1 mRNA. The combination of SNP plus GSNO, especially, caused a synergistic increase (22-fold) in the expression levels of heme oxygenase-1 mRNA (lane 5), and this induction was much greater than the additive increase obtained with the combination of SNP plus SIN-1 (lane 6) or GSNO plus SIN-1 (lane 7). A hybridization signal of larger size may represent the alternatively spliced transcripts of the heme oxygenase-1 gene (lanes 5–8), which frequently are detectable when transcription of the heme oxygenase-1 gene is increased remarkably [18, 24, 39]. The effect of a combination of GSNO plus potassium ferricyanide, a control reagent for SNP, on the expression levels of heme oxygenase-1 mRNA was similar to that of treatment with GSNO alone (lane 9). Treatment with either 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide (data not shown), or sodium nitrite (see Fig. 3B, lanes 10 and 11) had no noticeable effects on the expression of heme oxygenase-1 mRNA. Thus, the observed synergistic effect with GSNO was specific to SNP.

The concentrations of nitrite, a stable metabolite of NO, were measured in the culture medium, because nitrite represents the amount of NO generated. The nitrite concentrations accumulated for 5 hr in the culture medium of cells treated with SNP, GSNO, and SIN-1 were about 8, 26, and 67 μM, respectively (Fig. 2C). These differences may reflect the chemical properties of the NO donors. It is noteworthy that 200 μM GSNO and 200 μM SIN-1 generated nitrite in larger amounts than did 1 mM SNP, although the greatest level of induction of heme oxygenase-1 mRNA was obtained with SNP. Any combination of the two NO donors always showed additive effects on the nitrite production in culture medium.

Possible Mechanisms of Induction of Heme Oxygenase-1 mRNA by SNP

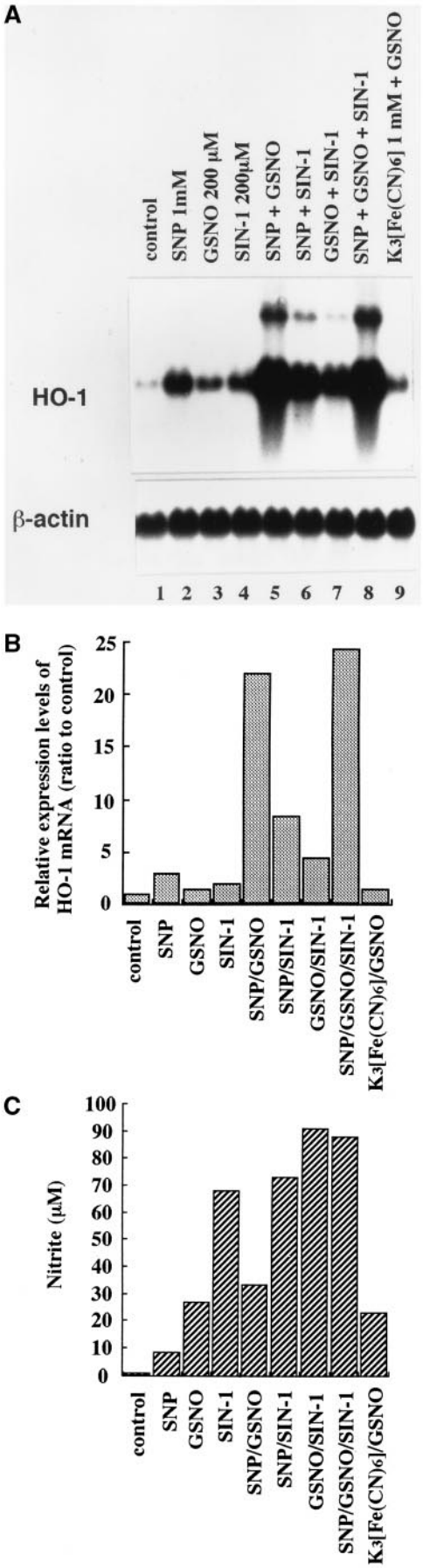
To evaluate the involvement of reactive oxygen species in the SNP-mediated induction of heme oxygenase-1 mRNA, we examined the effects of catalase (100 U/mL), glutathione (200 μM), and superoxide dismutase (100 U/mL). Glutathione was reported to increase the release of NO from SNP [7]. Coincubation with each reagent did not affect the induction levels caused by 1 mM SNP (Fig. 3A, lanes 3–5). The nitrite concentrations in the culture medium were increased 2-fold by glutathione (10 μM nitrite with SNP and 21 μM nitrite with SNP plus glutathione). H_2O_2 increased heme oxygenase-1 mRNA expression in DLD-1 cells, and the accumulation of heme oxygenase-1 mRNA caused by H_2O_2 was inhibited by catalase (lanes 6 and 7). Thus, a mechanism for the SNP-mediated induction of heme oxygenase-1 mRNA may be different from that for the H_2O_2-mediated induction. We next examined the effects of ascorbate (400 μM) on the induction of heme oxygenase-1 mRNA by SNP, because ascorbate could increase the formation of NO from SNP [7] or the formation of reactive oxygen species, such as $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$, from SNP [8]. In this series of experiments, a lower concentration of SNP (200 μM) was used to detect the enhancing effects of ascorbate [29]. The presence of ascorbate with 200 μM SNP caused greater induction of heme oxygenase-1 mRNA (Fig. 3B, lane 4). The addition of catalase abolished this stimulatory effect of ascorbate (lane 6). Superoxide dismutase or mannitol, which scavenges hydroxyl radical, apparently did not affect heme oxygenase-1 mRNA expression (lanes 5 and 7). Treatment with either ascorbate (data not shown), superoxide dismutase (lane 8), catalase (lane 9), or sodium nitrite (lanes 10 and 11) alone had no noticeable effects on the expression of heme oxygenase-1 mRNA. Therefore, it is conceivable that the augmented effect of ascorbate on the SNP-mediated induction of heme oxygenase-1 is mediated, in part, by H_2O_2 (see Fig. 7).

Heme Oxygenase-1 mRNA Induction by SIN-1

We examined the effect of catalase or superoxide dismutase on the expression of heme oxygenase-1 mRNA induced by treatment with SIN-1 (Fig. 4), because superoxide dismutase usually is added along with SIN-1 to increase the half-life of NO [40]. In this series of experiments, higher concentrations of SIN-1 were used, although the magnitude of induction of heme oxygenase-1 mRNA obtained with 300 or 500 μM SIN-1 was similar to that obtained with 200 μM SIN-1. The presence of superoxide dismutase attenuated the SIN-1-mediated induction (lanes 3 and 5), whereas addition of catalase apparently did not affect this induction (lane 6). Thus, O_2^- and/or $ONOO^-$ may be responsible for the SIN-1-mediated induction of heme oxygenase-1 mRNA (see Fig. 7). In contrast, the GSNO-mediated induction of heme oxygenase-1 mRNA was not affected by superoxide dismutase or catalase (data not shown).

Effect of NO Donors on the Promoter Activities of the Heme Oxygenase-1 Gene

Transient transfection assays were performed in DLD-1 cells to compare the effects of NO donors on the promoter activity of the human heme oxygenase-1 gene. The basal promoter activities of pHHOL15, containing the 5′-flanking region of about 4.5 kb, and pHHOL14, containing



about 4 kb of this region, were about 8- and 4-fold higher, respectively, than the activity of pHHOL5 containing the 282-bp promoter region (Fig. 5A). Treatment with SNP led to a 2-fold increase in the relative luciferase activities in the cells transfected with pHHOL15, whereas the expression of pHHOL14 was not increased. In this series of experiments, the effect of cadmium was examined as a positive control, since pHHOL15 contains the CdRE located at the −4.1 kb region of the heme oxygenase-1 gene [24]. In contrast, treatment with GSNO or SIN-1 caused a small increase (~1.4- to 1.8-fold) in the relative luciferase activities in the cells transfected with either pHHOL14 or pHHOL15. Thus, the *Pst*I/*Xba*I segment (−4.5 kb/−4 kb), carried by pHHOL15, contains a *cis*-acting element that is required for the induction of reporter gene expression by SNP. Furthermore, using fusion plasmids that contain various deletions in the *Pst*I/*Xba*I segment (−4.5 kb/−4 kb), we found that the CdRE and/or its adjacent element are required for the induction of luciferase activity caused by SNP (data not shown). Incidentally, the potential AP-1 site is located immediately downstream from CdRE [24].

To examine the possible involvement of the CdRE or the potential AP-1 site in the SNP-mediated induction of relative luciferase activity, we used a series of fusion genes containing a synthetic oligonucleotide as a putative enhancer element upstream of the simian virus 40 promoter (Fig. 5B). Basal expression of pSVLCd7 containing both CdRE and the AP-1 site was twice as high as that of pSVLCd6 containing CdRE or pSVLAP1 containing the AP-1 consensus sequence. SNP treatment induced luciferase activity by about 1.7-fold in the cells expressing pSVLCd7, but not pSVLCd6 and pSVLAP1. An enhancerless plasmid, pSVLE(-), was not induced by SNP (data not shown). Furthermore, GSNO did not affect the expression of pSVLCd6 or pSVLCd7. Cadmium treatment increased luciferase activities by about 2.2- and 3.1-fold in the cells transfected with pSVLCd6 and with pSVLCd7, respectively. Thus, both CdRE and the AP-1 site are needed for the induction by SNP.

FIG. 2. Effects of a combination of NO donors on heme oxygenase-1 mRNA expression. (A) Northern blot analysis. DLD-1 cells were treated for 5 hr with the indicated NO donors. The NO donors used were 1 mM SNP, 200 μM GSNO, and 200 μM SIN-1. As a control for SNP, 1 mM potassium ferricyanide ($K_3[Fe(CN)_6]$) was used with GSNO. Each lane contained 10 μg RNA, and the exposure time was 16 hr for the top panel. The data shown are from one of two similar experiments. (B) The relative expression levels of heme oxygenase-1 mRNA and (C) the nitrite concentrations in the culture medium. The intensity of hybridization signals in A was quantified with a Bioimage analyzer, and the intensity representing heme oxygenase-1 mRNA was normalized with respect to the intensity for β-actin mRNA in each experiment. The ratio of each normalized value to that of the control is shown as the relative expression level of heme oxygenase-1 mRNA. Thus, the data shown at top represent one of two similar experiments, while the data shown at bottom are the means of three dishes.

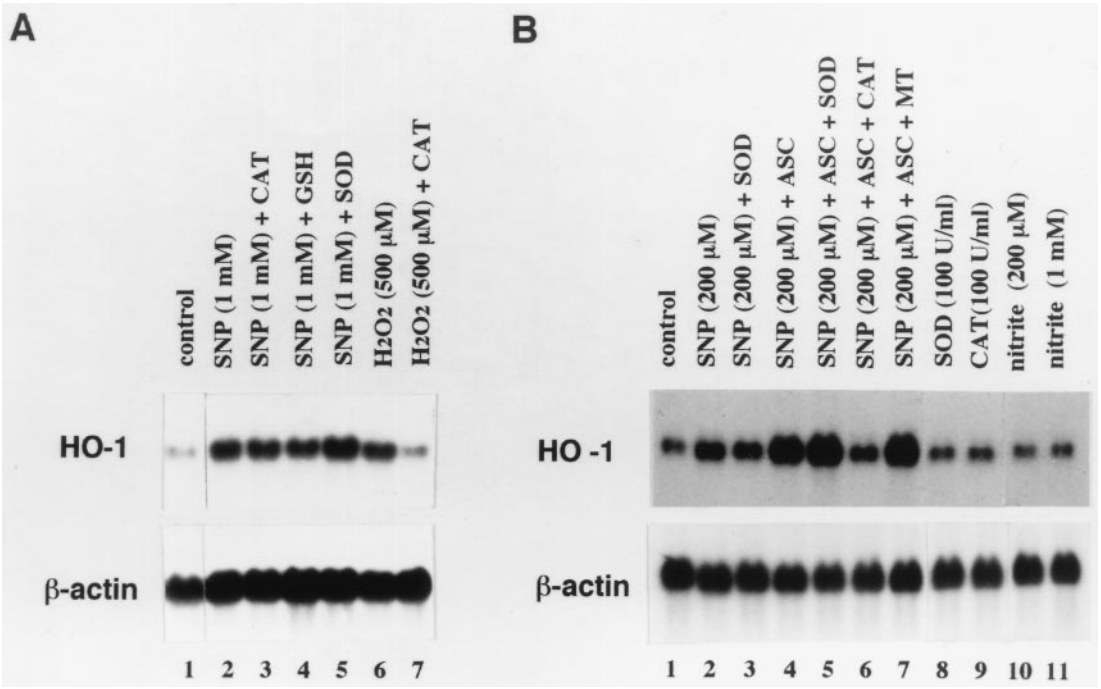


FIG. 3. Induction properties of heme oxygenase-1 mRNA expression mediated by SNP. Shown is the autoradiogram of the RNA blot hybridized with ^{32}P-labeled heme oxygenase-1 or β-actin cDNA probe. The amounts of RNA loaded in each lane were 10 μg in A and 15 μg in B. (A) Effects of catalase (CAT, 100 U/mL), glutathione (GSH, 200 μM), or superoxide dismutase (SOD, 100 U/mL) on expression of heme oxygenase-1 mRNA induced by SNP. DLD-1 cells were treated for 5 hr with 1 mM SNP in the presence or absence of the indicated scavengers. Cells were treated with 500 μM hydrogen peroxide as described in Materials and Methods. The blot was exposed to an X-ray film for 16 hr (top panel). (B) Effects of ascorbate (ASC, 400 μM), superoxide dismutase, or catalase on the induction of heme oxygenase-1 mRNA caused by SNP. DLD-1 cells were treated for 5 hr with SNP in the presence or absence of the indicated chemicals. The concentrations of reagents were the same as in A, except for 40 μM mannitol (MT). The exposure time was 2 days for the top panel. These experiments were performed twice.

Induction of Heme Oxygenase-1 Protein by SNP

We then determined whether SNP increases the expression levels of heme oxygenase-1 protein in DLD-1 cells (Fig. 6). The effect of cadmiun also was examined, since the presence of CdRE in the potential SNP-responsive element prompted us to compare the effects of cadmiun and SNP on the expression of heme oxygenase-1 protein. The 33-kDa heme oxygenase-1 protein was increased in a time-dependent manner by treatment with either cadmium (50 μM) or SNP (1 mM), with the maximum levels at 6 hr (Fig. 6). A similar pattern of induction of heme oxygenase-1 protein was observed with a 200 μM concentration of GSNO or SIN-1 (data not shown). Moreover, expression levels of heat shock protein 70 were increased by cadmium, but were not changed noticeably by SNP (Fig. 6).

DISCUSSION

Intravenous infusion of SNP has been accepted as an effective therapy for hypertensive emergencies [5, 6]. A possible mechanism for its hypotensive action may involve nitrosylation of iron in the heme at the active site of guanylate cyclase, which in turn stimulates cyclic GMP formation [2, 3]. Moreover, SNP could generate reactive oxygen species during the redox cycling of nitroprusside [7, 8]. The first step in the reduction of nitroprusside is the formation of the pentacyano form $[Fe(CN)_5NO]^{3-}$ of nitroxide radicals, which is reduced rapidly to the tetracyano form $[Fe(CN)_4NO]^{2-}$ [8]. Reductive metabolism of nitroprusside may generate H_2O_2, O_2^-, and $^{\cdot}OH$ as by-products. Indeed, ascorbate increased the magnitude of induction of heme oxygenase-1 mRNA expression caused by SNP, and this augmentation was abolished by the addition of catalase. Thus, the stimulatory effect of ascorbate is mediated in part by H_2O_2 (Fig. 7), which is consistent with the observation that H_2O_2 induced heme oxygenase-1 mRNA expression (Fig. 3). On the other hand, the induction mediated by SNP alone was not affected by the presence of catalase, superoxide dismutase, or mannitol in the culture medium (Fig. 3), suggesting that the SNP-mediated induction may be independent of H_2O_2, O_2^-, and $^{\cdot}OH$. Moreover, treatment with a 1 mM concentration of potassium ferricyanide or potassium ferrocyanide had no noticeable effects on the expression of heme oxygenase-1 mRNA (data not shown). Therefore, it is conceivable that the Fe-NO moiety of SNP is responsible for the induction of heme oxygenase-1 mRNA, suggesting that induction of heme oxygenase-1 mRNA expression may be mediated by nitrosylation of transition metals. Alternatively, nitrosylation of thiols on certain regulatory proteins may be responsible for the induction, since SNP possesses strong NO^+ character [3].

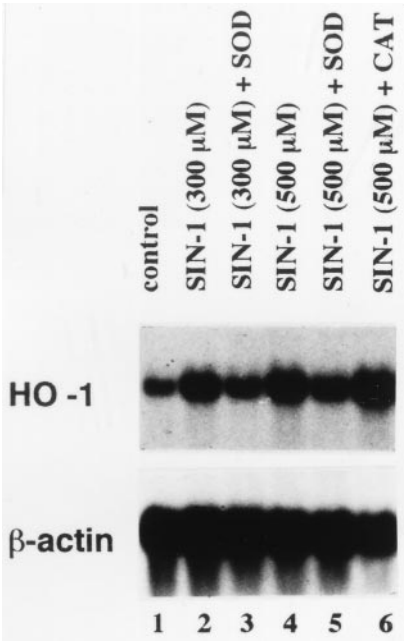


FIG. 4. Effects of superoxide dismutase or catalase on expression of heme oxygenase-1 mRNA induced by treatment with SIN-1. DLD-1 cells were treated for 5 hr with SIN-1 in the presence or absence of the indicated chemicals, catalase (100 U/mL) and superoxide dismutase (100 U/mL). Shown is the autoradiogram of the RNA blot hybridized with ^{32}P-labeled heme oxygenase-1 or β-actin cDNA probe. Each lane contained 15 μg RNA, and the exposure time was 2 days for the top panel. These experiments were performed twice.

Transient transfection assays indicate that the *cis*-acting element of the heme oxygenase-1 gene responsive to SNP includes CdRE, which has been established to confer induction by cadmium on a reporter gene [24, 25, 39]. Both CdRE and a putative AP-1 site are required for the induction of a reporter gene by SNP, but neither CdRE nor the AP-1 consensus sequence alone is sufficient for induction by SNP (Fig. 5). These results suggest that proteins that bind to both elements or cooperative interactions between the proteins bound to CdRE and to the potential AP-1 site are required for induction by SNP. This notion is of interest in view of the significance of CdRE, because cadmium is unlikely to function as a physiological regulator for heme oxygenase-1 gene transcription. Thus, CdRE may represent a binding site for factors that could sense the signals evoked by NO–transition metal complexes or nitrosylation of thiols. In this context, heme oxygenase-1 is suitable to function under redox stress, since heme oxygenase-1 is not a hemoprotein [41] and lacks cysteine residues [15, 21]. For comparison, we also analyzed the expression of metallothionein IIA mRNA, because metallothionein is enriched in cysteine residues and its expression is induced by heavy metals, including cadmium [25]. However, metallothionein IIA mRNA expression was not affected significantly by SNP, GSNO, or SIN-1 under the conditions used (data not shown). Moreover, expression of heat shock protein 70 was induced by cadmium, but not by SNP (Fig. 6), SIN-1 (200 μM), or GSNO (200 μM) (data not shown).

GSNO may undergo homolytic cleavage of the S—N bond with release of NO in the presence of reducing agents, such as ascorbate [42], but the presence of ascorbate with GSNO had no noticeable effects on the expression of heme oxygenase-1 mRNA (data not shown). In addition, superoxide dismutase did not noticeably affect the GSNO-mediated induction of heme oxygenase-1 mRNA (data not shown), suggesting that $O_2^{\cdot}$ is not directly involved in this induction process. Another possibility is that NO^+ is generated during heterolytic decomposition of GSNO and is transferred to thiols on certain regulatory proteins, some of which may be involved in heme oxygenase-1 mRNA expression. In this context, transient transfection assays have shown that the effect of GSNO on the promoter function of the heme oxygenase-1 gene is different from the effect of SNP (Fig. 5B). Interestingly, a combination of SNP and GSNO caused a synergistic increase in the heme oxygenase-1 mRNA levels. Taken together, we suggest that GSNO may induce heme oxygenase-1 mRNA expression through nitrosylation of certain regulatory proteins, while SNP may induce it through the Fe—NO complex (Fig. 7).

Decomposition of SIN-1 results in virtually simultaneous release of O_2^- and NO, which immediately react with each other to generate $ONOO^-$ [10]. The highest nitrite concentration seen with SIN-1 is consistent with the report that nitrite is a stable product of peroxynitrite decomposition at physiological pH [43]. Thus, SIN-1 generates peroxynitrite rather than NO in culture medium of DLD-1 cells. SIN-1 could release detectable amounts of NO, if superoxide dismutase is present in amounts sufficient to react with O_2^- concomitantly produced during SIN-1 decomposition [43]. However, the presence of superoxide dismutase significantly decreased the magnitude of SIN-1-mediated induction of heme oxygenase-1 mRNA. These results suggest that $ONOO^-$ rather than NO is responsible for the SIN-1-mediated induction (Fig. 7).

In summary, we have provided evidence that SNP induces heme oxygenase-1 mRNA expression in DLD-1 human colorectal adenocarcinoma cells through a mechanism different from that for GSNO or SIN-1; namely, SNP, GSNO, and SIN-1 may induce heme oxygenase-1 mRNA expression through Fe-nitrosyl complex, nitrosylation of thiols, and $ONOO^-$, respectively. These mechanisms are also different from those for induction by cadmium or H_2O_2. We therefore propose that heme oxygenase-1 plays a role as a sensor in response to multiple cellular signals evoked by the redox-activated forms of NO and by oxidative stress.

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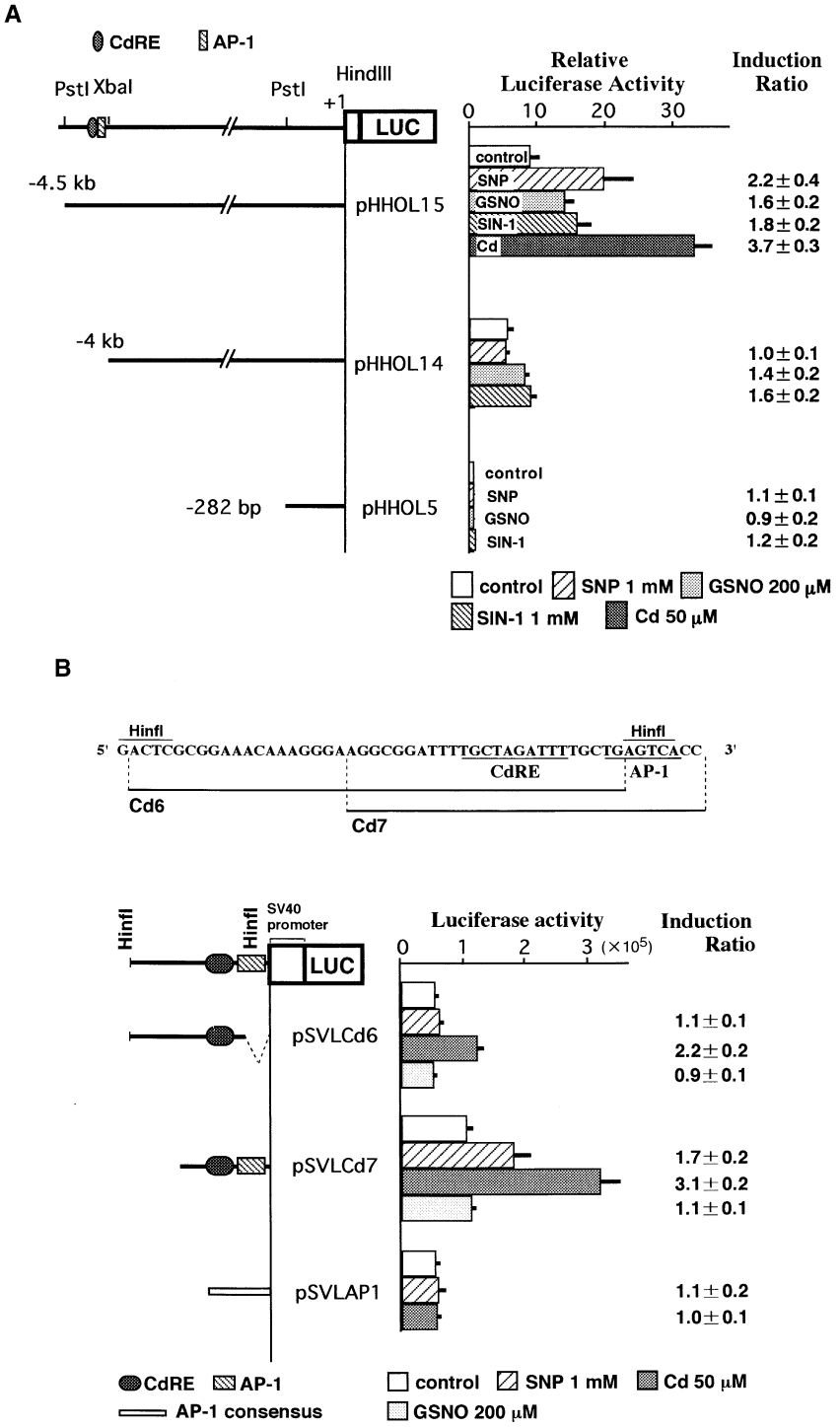

FIG. 5. Effect of NO donors on the promoter function of the human heme oxygenase-1 gene. (A) DLD-1 cells were cotransfected with the fusion genes containing the 5′-flanking region of the human heme oxygenase-1 gene and a β-galactosidase expression vector pCH110. The transfected cells were left untreated or treated for 5 hr with the indicated reagents. The promoter regions used for construction of fusion genes are schematically shown to the left. The cadmium-responsive element (CdRE) and a putative AP-1 binding site (AP-1) are indicated. The data, shown as relative luciferase activity, are the ratio to the value obtained with pHHOL5. The magnitude of induction of each normalized luciferase activity is also shown to the right. The data shown are means ± SD of three independent experiments. (B) Functional analysis of CdRE and a potential AP-1 binding site. The nucleotide sequence shown at the top is the enhancer element containing CdRE and a potential AP-1 binding site, which is located at the −4.1 kb region of the human heme oxygenase-1 gene [24]. The synthetic oligonucleotides, Cd6 and Cd7, are indicated by a thick line. The bottom panel shows functional analysis of the fusion plasmids, pSVLCd6, pSVLCd7, and pSVLAP1, each of which contains a respective double-stranded oligonucleotide in the upstream region of pSVLE(-), lacking a simian virus 40 enhancer. The data shown are normalized luciferase activity. The magnitude of induction of each normalized luciferase activity is also shown to the right. The data shown are means ± SD of three independent experiments.

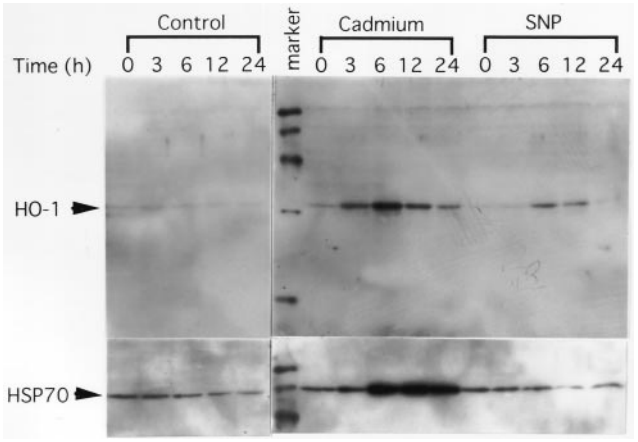

FIG. 6. Time course of the effect of cadmium or SNP on the expression of heme oxygenase-1 protein. DLD-1 cells were left untreated (control) or treated with cadmium (50 μM) or SNP (1 mM) for the indicated hours, and were harvested for protein preparation. Expression of heme oxygenase-1 protein (top) and heat shock protein 70 (bottom) was determined by western blot analysis (performed once). The biotinylated size markers (marker) were rabbit phosphorylase *b* (97,200), bovine serum albumin (66,000), ovalbumin (45,000), bovine carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), and chick lysozyme (14,300).

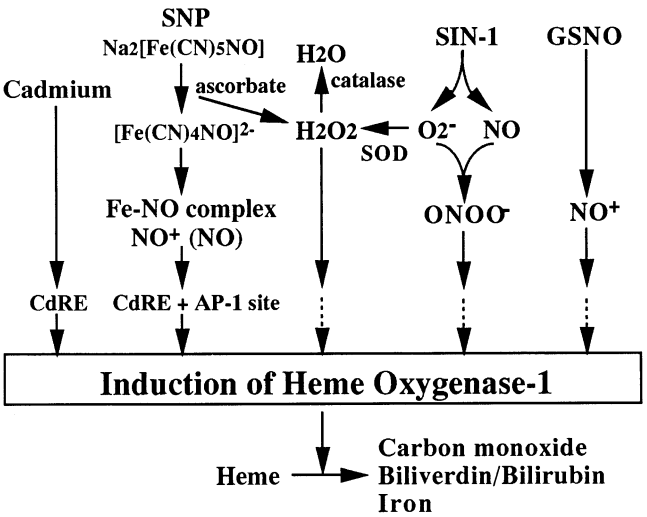

FIG. 7. Proposed mechanisms for the induction of heme oxygenase-1 mRNA expression by SNP. For details, see Discussion.

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