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Possible Implications of the Induction of Human Heme Oxygenase-1 by Nitric Oxide Donors[1]

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To explore the involvement of nitric oxide (NO) in the induction of heme oxygenase-1, an essential enzyme in heme catabolism, we studied the effects of NO donors on the expression of heme oxygenase-1 mRNA in HeLa human cervical cancer cells. Treatment with each of three NO donors, sodium nitroprusside, 3-morpholinosydnonimine, and *S*-nitroso-L-glutathione, caused noticeable increases in the expression levels of heme oxygenase-1 mRNA, but not heme oxygenase-2 mRNA. On the other hand, nitrite or 8-bromo cGMP exerted no noticeable effect on the levels of heme oxygenase-1 mRNA. We showed that sodium nitroprusside also increased the levels of heme oxygenase-1 protein. The sodium nitroprusside-mediated increase in heme oxygenase-1 mRNA levels was abolished by treatment with actinomycin D. The expression levels of heme oxygenase-1 mRNA were also increased by NO donors in human melanoma and neuroblastoma cell lines. Thus, the observed induction of heme oxygenase-1 may represent an important response to NO or NO-related oxidative stress. The half lives of heme oxygenase-1 and heme oxygenase-2 mRNAs were estimated to be about 3.2 h and more than 5 h, respectively.

Key words: carbon monoxide, heme oxygenase, nitric oxide, nitroprusside, stress protein.

Heme oxygenase [EC 1.14.99.3] is a rate-limiting enzyme in heme catabolism that cleaves heme to form biliverdin, carbon monoxide (CO), and iron (*1, 2*). Biliverdin is subsequently reduced to bilirubin (*3*). Two isozymes of heme oxygenase have been characterized in rat and human: heme oxygenase-1, an inducible form (*4, 5*) and heme oxygenase-2, a non-inducible form (*6-8*). Both isozymes possess similar catalytic activity and are widely distributed throughout the body. Heme oxygenase-1 is also categorized as a stress protein, because it is transcriptionally induced by various stimulants (reviewed in Ref. *9*), such as heat shock (*10*), cadmium (*11, 12*), and hemin (*11*). In addition, biliverdin and bilirubin have been shown to function as radical scavengers (*13*).

CO, generated by heme oxygenase, has been shown to function as a neural messenger by activating guanylate cyclase (*14-16*). CO is also an endothelium-independent vasorelaxant, although it is less than one thousandth as potent as nitric oxide (NO) (*17*). However, because of the continuous production of CO in the spleen and of its stability compared to the radical gas NO, it is conceivable that CO may play an important role in homeostasis in certain tissues. In this context, it is of interest that CO has been proposed to function as a modulator of sinusoidal tone in liver (*18, 19*).

It has been reported that treatment with NO donors induced the expression of heme oxygenase-1 mRNA and/or protein in human glioblastoma cell lines (*20, 21*), in rat hepatocytes (*22*), and in porcine aortic endothelial cells (*23*). The treatment of porcine aortic endothelial cells with cytokines also increased both heme oxygenase and inducible NO synthase (NOS) activities (*23*). These findings raised the possibility that the CO/heme oxygenase system may function in concert with the NO/NOS system. However, it should be noted that there is a species difference in the regulation of heme oxygenase-1 expression. For example, rat heme oxygenase-1 is a heat shock protein (*10*), whereas human heme oxygenase-1 is not induced by heat shock, except in a hepatoma cell line, Hep3B (*24, 25*), despite the presence of a heat shock element in its promoter region (*26, 27*). Thus, there are also differences in the regulation of heme oxygenase-1 expression among human cell lines.

We are currently considering two possible implications for the reported induction of human heme oxygenase-1 by NO donors: a cross-talk between the enzyme systems generating CO and NO and a defensive response (cytoprotection) against oxidative stress generated by NO. In relation to the latter possibility, NO was shown to mediate neurotoxicity caused by glutamate (*28*) or immunostimula-

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Abbreviations: CO, carbon monoxide; NO, nitric oxide; SIN-1, 3-morpholinosydnonimine; GSNO, *S*-nitroso-L-glutathione; NOS, nitric oxide synthase; SDS, sodium dodecyl sulfate; kb, kilobases; HSP-70, heat shock protein 70.

tion (*29*). It is also noteworthy that NO is non-enzymatically produced *in vivo*, especially in the mouth (*30*) and stomach (*31, 32*), and in certain pathological conditions, such as ischemic heart (*33, 34*).

In the present study, to explore the physiological significance of the NO donor-mediated induction of heme oxygenase-1, we examined whether or not the expression of heme oxygenase-1 is induced by NO donors in three human cell lines of different lineages, HeLa cervical cancer cells, MeWo melanoma cells, and SK-N-SH neuroblastoma cells. In addition, using HeLa cells, we have studied the mechanisms by which the expression of heme oxygenase-1 mRNA is regulated by NO donors.

MATERIALS AND METHODS

Materials—A human glioblastoma cell line, A172 was obtained from the American Type Culture Collection (Rockville, Maryland, USA); a human neuroblastoma cell line, SK-N-SH and a human cervical cancer cell line, HeLa from RIKEN cell Bank (Tsukuba). Sodium nitroprusside was purchased from Wako Pure Chemical (Osaka); 3-morpholinosydnonimine (SIN-1) and NO_2/NO_3 Assay Kit-C from Dojindo (Kumamoto); *S*-nitroso-L-glutathione (GSNO) from Alexis (Laufelfingen, Switzerland); actinomycin D, cycloheximide, 8-bromo cAMP, and 8-bromo cGMP from Sigma Chemical (St Louis, MO, USA); [α-^{32}P]-dCTP and monoclonal anti-72kDa heat shock protein from Amersham (Buckinghamshire, England); restriction endonucleases from Takara Shuzo (Otsu), Boehringer Mannheim (Mannheim, Germany), and New England BioLabs (Beverly, MA, USA).

Cell Culture—A172 cells were cultivated in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose, MeWo human melanoma cells in Dulbecco's modified Eagle's medium, SK-N-SH cells in RPMI medium, and HeLa cells in minimum essential medium at 37°C in 5% CO_2. All media were supplemented with 5% fetal calf serum. To examine the effects of NO on the expression of heme oxygenase-1 and -2 mRNAs, cells were cultivated in the fresh medium for 24 h, then exposed to 1 mM sodium nitroprusside at 37°C for 5 h, and then harvested for RNA extraction.

HeLa cells were exposed to one of the NO donors, sodium nitroprusside (0.01, 0.1, and 1 mM), SIN-1 (0.01 and 0.1 mM), and GSNO (0.01 and 0.1 mM). Cells were incubated at 37°C for 3-24 h and then harvested for RNA extraction and protein extraction. To estimate the amounts of NO generated by NO donors in 5 h, the concentrations of nitrite (NO_2^-), a metabolic product of NO, in the culture media were measured. The culture media (minimum essential medium without phenol red containing 5% fetal bovine serum) were collected 5 h after the addition of NO donors. The nitrite concentrations in the media were measured by use of the NO_2/NO_3 Assay Kit-C (Dojindo) following the manufacturer's protocols after the removal of proteins from the media using a Microcon (Amicon, Beverly, MA, USA).

To study the mechanisms of the induction of heme oxygenase-1 mRNA by NO donors, HeLa cells were incubated at 37°C for 5 h with 1 mM 8-bromo cyclic AMP, 1 mM 8-bromo cyclic GMP, or 1 mM sodium nitrite, and were harvested for RNA extraction. HeLa cells were also incubated with sodium nitroprusside (1 mM) and an RNA synthesis inhibitor, actinomycin D (1 μg/ml), or a protein synthesis inhibitor, cycloheximide (1 μg/ml), at 37°C for 5 h and then harvested for RNA extraction. The stability of heme oxygenase-1 mRNA was assessed as follows. HeLa cells were incubated with either sodium nitroprusside (1 mM), actinomycin D (1 μg/ml), or both agents at 37°C for 1.0, 2.5, and 5.0 h, and then harvested for RNA extraction. In another series of experiments, HeLa cells were incubated with sodium nitroprusside (1 mM) at 37°C for 5 h, followed by addition of actinomycin D (1 μg/ml). The cells were further incubated for 1.0, 2.5, and 5.0 h after the addition of actinomycin D.

Northern Blot Analysis—Total RNA was extracted from cultured cells by the guanidium thiocyanate-cesium chloride method, and subjected to Northern blot analysis (*35*). Total RNA (15 μg/lane) was electrophoresed on 1.0% agarose gel containing 2 M formaldehyde, transferred to a nylon membrane filter (Zeta-probe membrane, Biorad, CA, USA), and fixed with a UV-linker (Stratalinker 1800, Stratagene, CA, USA). The hybridization probes were the *Xho*I/*Xba*I fragment (−64/923) of human heme oxygenase-1 cDNA, pHHO1 (*5*), and the *Hin*fI/*Hin*fI fragment of human heme oxygenase-2 cDNA, pHHO2-1 (*36*). The probe for β-actin mRNA was the *Sma*I/*Sca*I fragment (124/1050) of a full-length human β-actin cDNA provided by Dr. T. Yamamoto (Tohoku University), and the probe for human heat shock protein 70 (HSP-70) mRNA was the *Hin*dIII/*Bam*HI fragment derived from a subclone pH 2.3 (*37*) provided by Dr. R. Morimoto (Northwestern University). These probes were labeled with [α-^{32}P]dCTP by the random priming method (*38*). The RNA blot was prehybridized at 42°C in a solution consisting of 5×SSC (0.75 M sodium chloride, 0.075 M sodium citrate), 1% sodium dodecyl sulfate (SDS), 50% formamide, 1×Denhardt's solution, and 0.2 mg/ml salmon testis DNA for 3 h, and then hybridized with radiolabeled DNA probe at 42°C for 16 h. The hybridized filter was extensively washed at 65°C with 0.1×SSC, 0.1% SDS. Radioactive signals were detected by exposing the filters to X-ray films (X-AR5, Kodak). The intensity of hybridization signals was quantified with a Bioimage analyzer (BAS 2000; Fuji Film, Tokyo).

Western Blot Analysis—HeLa cells were lyzed in triple detergent lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/liter phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml Nonidet P40, and 0.5% Na deoxycholate. The cell lysates were centrifuged at 15,000×g for 10 min, and the supernatant (100 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 Corporation, Bedford, MA, USA), 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 (*36*). The specific immunocomplexes were detected with a western blot kit (Smi Light, Sumitomo, Tokyo). Expression of HSP-70 was examined as an internal control using monoclonal anti-72 kDa heat shock protein.

RESULTS

We initially examined whether the expression levels of heme oxygenase-1 mRNA were increased in HeLa cells by treatment with NO donors, because this cell line has been extensively used for the study of the induction mechanism of heme oxygenase-1 gene expression (*12*). In addition, the expression of iNOS and constitutive NOS mRNAs was not detectable in HeLa cells by reverse-transcription polymerase chain reaction (data not shown), thus providing a suitable system to study the effects of exogenous NO. Treatment for 5 h with either sodium nitroprusside (0.1 and 1 mM), SIN-1 (0.01 and 0.1 mM), or GSNO (0.01 and 0.1 mM) caused a significant increase in the expression levels of heme oxygenase-1 mRNA of about 1.7 kilobases (kb), but had no noticeable effect on the expression levels of heme oxygenase-2 mRNA (about 1.4 kb), HSP-70 mRNA (about 2.7 kb), and β-actin mRNA (about 2.2 kb) (Fig. 1, A and B). The treatment with 0.1 mM sodium nitroprusside or GSNO increased the expression levels of heme oxygenase-1 mRNA by about sixfold, while SIN-1 caused only about a twofold increase. The nitrite concentrations in the media, which reflected the amounts of NO generated by NO donors in 5 h, were also measured (lower panel of Fig. 1B). Although 0.1 mM SIN-1 and 0.1 mM GSNO generated nitrite in larger amounts than did even 1 mM sodium nitroprusside, the greatest magnitude of induction was obtained with 1 mM sodium nitroprusside.

We then examined the effect of 8-bromo cGMP, as well as

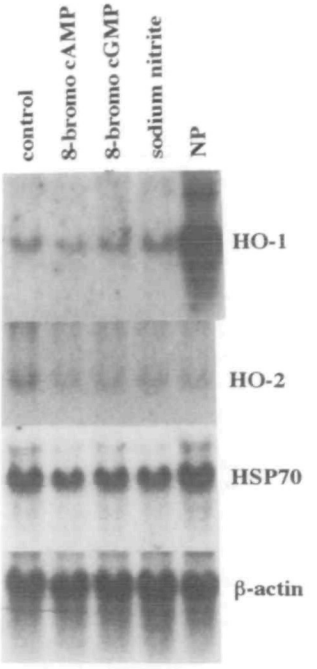
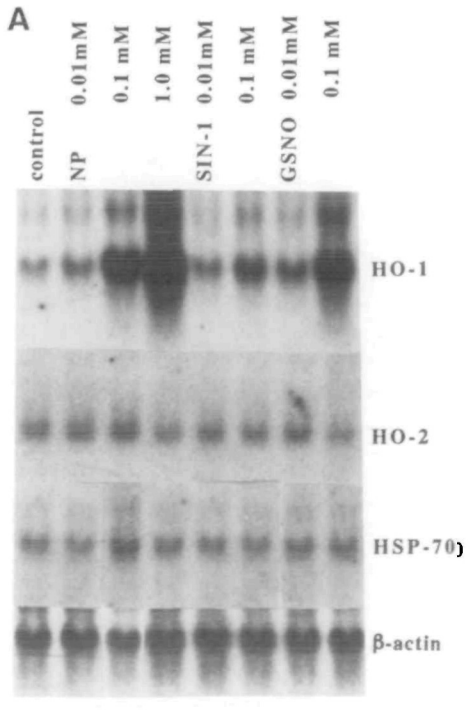


Fig. 2. **Effects of 8-bromo cyclic AMP, 8-bromo cyclic GMP, and sodium nitrite on the expression of heme oxygenase-1 and heme oxygenase-2 mRNAs.** HeLa cells were treated with each of the indicated reagents (1 mM) for 5 h, and then harvested for RNA preparation. The two bottom blots show the expression levels of HSP-70 and β-actin mRNAs.



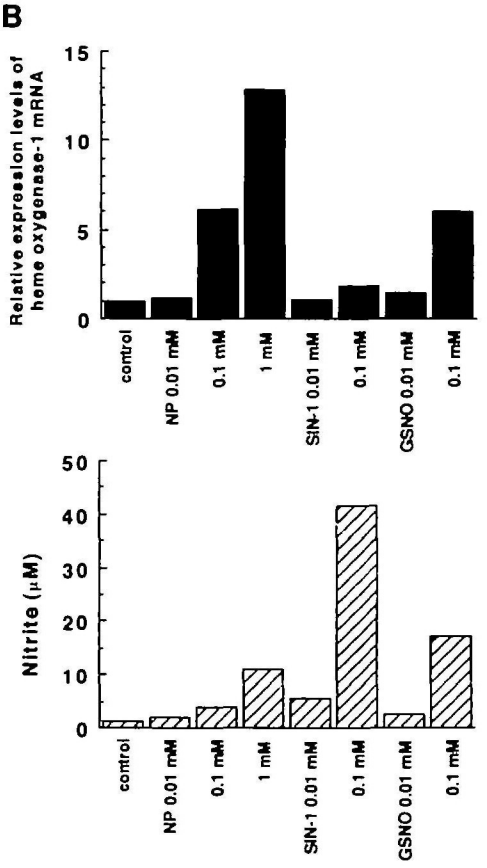


Fig. 1. **Dose-response effects of NO donors on the expression of heme oxygenase-1 and heme oxygenase-2 mRNAs.** A: Northern blot analysis showing the induction of heme oxygenase-1 mRNA in HeLa cells on treatment with NO donors for 5 h. The NO donors used were sodium nitroprusside (NP), 3-morpholinosydnonimine (SIN-1), and *S*-nitroso-L-glutathione (GSNO). Heme oxygenase-1 and heme oxygenase-2 mRNAs are indicated by HO-1 and HO-2, respectively. These blots were exposed to X-ray films for 2 days. The two bottom blots show the expression levels of HSP-70 and β-actin mRNAs, at exposures of 1 day and a few hours, respectively. (Note the differences in the exposure time among the blots.) The data shown are from one of two similar experiments. B: The relative expression levels of heme oxygenase-1 mRNA (top panel) and the nitrite concentrations in the culture media (the mean of three dishes) (bottom panel). 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 levels of heme oxygenase-1 mRNA.

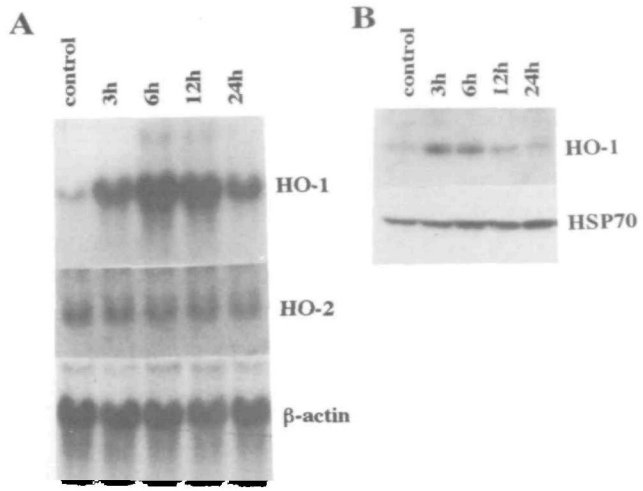


Fig. 3. **Time course of the effect of sodium nitroprusside on the expression of heme oxygenase-1.** HeLa cells were treated with 1 mM sodium nitroprusside for the indicated number of hours (h), and then harvested for RNA and protein preparation. A: Northern blot analysis of expression of heme oxygenase-1 and -2 mRNAs. The bottom blot shows the expression levels of β-actin mRNA as an internal control. B: Expression of heme oxygenase-1 protein (top) and HSP-70 protein (bottom), determined by Western blot analysis.

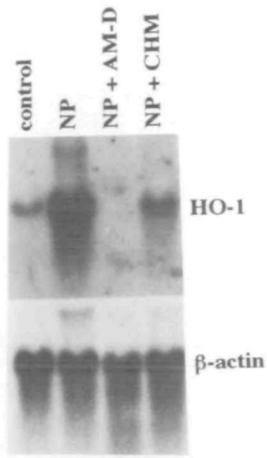


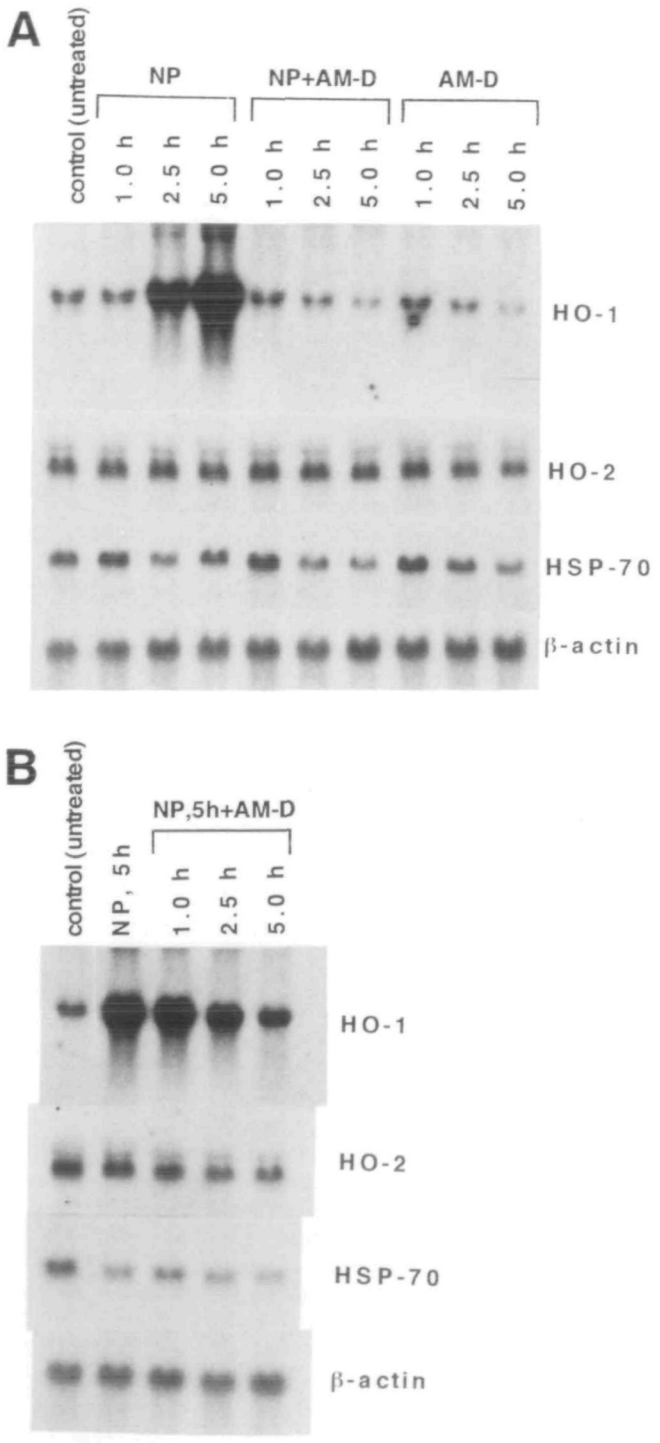
Fig. 4. **Northern blot analysis showing the effects of actinomycin D and cycloheximide on the induction of heme oxygenase-1 mRNA.** HeLa cells were treated with sodium nitroprusside for 5 h in the presence of actinomycin D (1 μg/ml) (NP+AM-D) or cycloheximide (1 μg/ml) (NP+CHM). The bottom blot shows the expression levels of β-actin mRNA. The exposure time for heme oxygenase-1 mRNA was 1 day and that for β-actin mRNA was 1 h.

8-bromo cAMP, on the expression of heme oxygenase-1 mRNA (Fig. 2), because NO activates soluble guanylate cyclase by binding tightly to the heme moiety of the enzyme (*39–41*). Neither 8-bromo cGMP nor 8-bromo cAMP was able to induce heme oxygenase-1 mRNA. In addition, treatment with nitrite, a metabolite of NO, had no significant effect on the expression of heme oxygenase-1 mRNA. Similarly, these reagents exerted no noticeable effect on the expression levels of heme oxygenase-2 and HSP-70 mRNAs.

To study the mechanism of induction of heme oxygenase-1 mRNA by NO donors, we used sodium nitroprusside (1 mM) in the subsequent experiments, since it gave rise to the highest magnitude of induction. A time course study revealed that the induction of heme oxygenase-1 mRNA was observed 3 h after the addition of sodium nitroprusside (6.5-fold increase) and reached the maximum (15-fold) at 6 h (Fig. 3A). Western blot analysis showed that heme oxygenase-1 protein was also increased about threefold at 3 h after the addition of sodium nitroprusside (Fig. 3B), whereas the levels of HSP-70 protein were unchanged throughout.

The presence of actinomycin D (1 μg/ml) completely inhibited the accumulation of heme oxygenase-1 mRNA caused by sodium nitroprusside (Fig. 4). Cycloheximide (1 μg/ml) noticeably reduced the magnitude of induction. In contrast, these inhibitors had no significant effect on the expression of β-actin mRNA. We next studied the effects of sodium nitroprusside on the stability of heme oxygenase-1 mRNA by using actinomycin D (Fig. 5). The addition of 1 mM sodium nitroprusside increased the expression levels of heme oxygenase-1 mRNA about sixfold at 2.5 h and about 13-fold at 5 h, while the addition of both 1 mM sodium nitroprusside and actinomycin D (1 μg/ml) decreased them with a half life of about 3.5 h (Fig. 5, A and C). The basal expression levels of heme oxygenase-1 mRNA were decreased by the addition of actinomycin D (1 μg/ml) with a half life of about 3.2 h. Thus, 1 mM sodium nitroprusside had no noticeable effect on the stability of heme oxygenase-1 mRNA. The expression levels of heme oxygenase-2 and HSP-70 mRNAs were decreased to about 75 and 55% of the control levels at 5 h after the addition of actinomycin D in either the presence or absence of sodium nitroprusside, while there was no significant change in the expression levels of β-actin mRNA. The expression levels of heme oxygenase-1 mRNA induced by the prior administration of sodium nitroprusside were decreased by the addition of actinomycin D with a half life of about 2.1 h (Fig. 5, B and C). Under these conditions, the half lives of heme oxygenase-2 and HSP-70 mRNAs were about 5 h, slightly shorter than those in Fig. 5A, but there was no significant change in the expression of β-actin mRNA (Fig. 5B). These results suggest that the sodium nitroprusside-mediated induction of heme oxygenase-1 mRNA expression occurs primarily at the transcriptional level.

To explore whether the induction of heme oxygenase-1 by NO donors may represent a general response, we next used two human cell lines of different lineages, MeWo melanoma and SK-N-SH neuroblastoma cells. A172 glioblastoma cells which express inducible NOS (*21, 42*) were also included as a control for sodium nitroprusside-mediated induction of heme oxygenase-1 mRNA (*21*). Treatment with 1 mM sodium nitroprusside for 5 h caused noticeable increases in the levels of heme oxygenase-1 mRNA in these cell lines, but no increases in the levels of heme oxygenase-2 mRNA, HSP-70 mRNA, and β-actin mRNA (Fig. 6). We also observed the induction of heme oxygenase-1 mRNA by SIN-1 or GSNO in A172 and SK-N-SH cells, in which the expression levels of heme oxygenase-2, HSP-70, and β-actin mRNA were not increased (data not shown). All these results were essentially identical to those observed in HeLa cells.



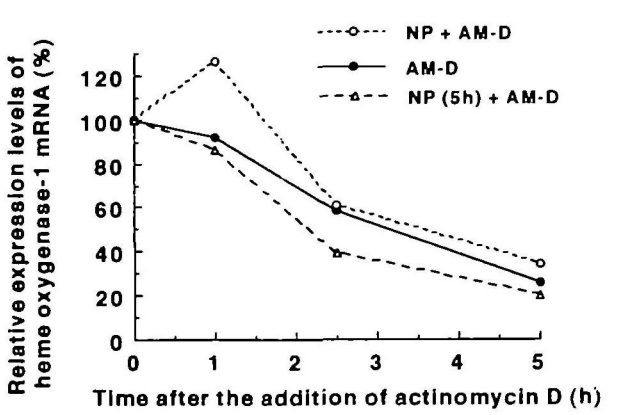


Fig. 5. **Effects of sodium nitroprusside on the stability of heme oxygenase-1 mRNA.** A: Northern blot analysis showing the expression levels of heme oxygenase-1 mRNA after addition of actinomycin D. HeLa cells were incubated for the indicated number of hours (1, 2.5, and 5 h) with either sodium nitroprusside (1 mM) (NP), sodium nitroprusside (1 mM) plus actinomycin D (1 μg/ml) (NP+AM-D), or actinomycin D (1 μg/ml) (AM-D). The blots for heme oxygenase-1 and heme oxygenase-2 mRNAs were exposed to X-ray film for 1 week to show small changes in the expression levels. The two bottom blots show the expression levels of HSP-70 and β-actin mRNAs. B: Northern blot analysis showing the stability of heme oxygenase-1 mRNA induced by sodium nitroprusside. HeLa cells were incubated with sodium nitroprusside (1 mM) for 5 h, followed by addition of actinomycin D (1 μg/ml). The cells were harvested at 1, 2.5, and 5 h after the addition of actinomycin D. The blots for heme oxygenase-1 and heme oxygenase-2 mRNAs were exposed to X-ray film for 1 day. C: Estimation of the half life of heme oxygenase-1 mRNA. The intensity of hybridization signals in A (AC-D and NP+AM-D) and B was quantified with a Bioimage analyzer as described in Fig. 1. The intensity representing heme oxygenase-1 mRNA in untreated cells (A) or the cells treated with 1 mM sodium nitroprusside for 5 h (B) was considered to be 100%. The data shown represent one series of experiments.

DISCUSSION

The present study has shown that the expression of heme oxygenase-1 mRNA is induced by treatment with NO donors in various human cell lines of different lineages. In HeLa cells, actinomycin D completely inhibited the accumulation of heme oxygenase-1 mRNA caused by sodium nitroprusside, while cycloheximide reduced the magnitude of induction. The half life of heme oxygenase-1 mRNA was estimated to be 3.2 h and the presence of 1 mM sodium nitroprusside had little effect on the stability of heme oxygenase-1 mRNA (3.5 h). These results suggest that sodium nitroprusside may act at the transcriptional level to increase the levels of heme oxygenase-1 mRNA, and protein synthesis is required for the full induction. The addition of actinomycin D decreased the expression levels of heme oxygenase-2 and HSP-70 mRNAs, but the half lives of these mRNAs (>5.0 h) were longer than the half life of heme oxygenase-1. In contrast, the expression levels of β-actin mRNA were not noticeably affected by treatment with either actinomycin D or cycloheximide, suggesting that β-actin mRNA is relatively stable.

It is noteworthy that 0.1 mM SIN-1 and 0.1 mM GSNO generated nitrite in larger amounts than did even 1 mM sodium nitroprusside in HeLa cells, although the greatest magnitude of induction of heme oxygenase-1 mRNA was obtained with 1 mM sodium nitroprusside. The dissociation between the nitrite production and the heme oxygenase-1 mRNA expression levels may be due to the differences in the chemical properties of these NO donors. Indeed, sodium nitroprusside possesses a strong nitrosonium ion character (NO^+) (*43*), while GSNO spontaneously releases NO. SIN-1 is known to generate peroxynitrite ($ONOO^-$) rather than NO, because decomposition of SIN-1 is accom-

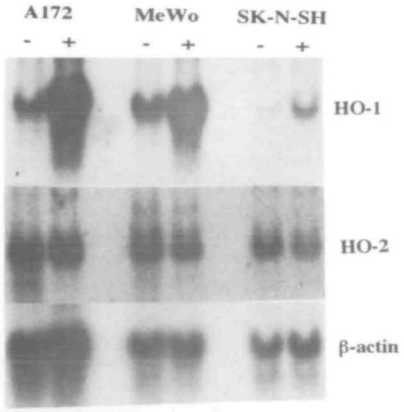


Fig. 6. **Effects of sodium nitroprusside on the expression of heme oxygenase-1 and heme oxygenase-2 mRNAs in human cell lines.** The indicated cell lines were treated with (+) or without (−) sodium nitroprusside (1 mM). The bottom blot shows the expression levels of β-actin mRNA as an internal control.

panied by virtually simultaneous release of superoxide anion (O_2^-) and NO, both of which immediately react with each other to generate peroxynitrite. It has recently been reported that nitrite is a stable product of peroxynitrite decomposition at physiological pH (*44*), and this seems consistent with the fact that the highest nitrite production was observed with 0.1 mM SIN-1 in HeLa cells (Fig. 1C). In this context, we excluded the possibility that the induction of heme oxygenase-1 mRNA by the NO donors is mediated by nitrite, a metabolite of NO (Fig. 2). It is therefore conceivable that peroxynitrite may be a weak inducer of heme oxygenase-1 under the conditions used.

The treatment with 8-bromo cGMP did not induce heme oxygenase-1 mRNA, suggesting that NO donors induce heme oxygenase-1 mRNA in a manner independent of cGMP. The expression levels of HSP-70 mRNA were also determined to evaluate the culture conditions, because the NO donors used in this study may generate not only NO, but also other reactive metabolites, such as peroxynitrite. We showed that the expression of HSP-70 mRNA was not induced by treatment with NO donors (Fig. 1).

The effects of sodium nitroprusside were also analyzed by means of the transient expression assays of the fusion gene, pHHOL15, containing a luciferase reporter gene under the human heme oxygenase-1 gene promoter (about −4.5 kb/+20 of the human heme oxygenase-1 gene). It should be noted that pHHOL15 contains a cadmium-responsive element which is required for the transcriptional activation caused by cadmium (*12*). The treatment with 1 mM sodium nitroprusside did not cause a significant increase in the relative luciferase activity in the transfected HeLa cells (data not shown), while the treatment with 5 μM cadmium increased it about threefold. These results suggest that the mechanism leading to the induction of heme oxygenase-1 by sodium nitroprusside may be different from that for the cadmium-mediated induction (*12*). On the other hand, heme oxygenase-1 activity may be increased secondary to heme liberation caused by NO, as proposed in the case of rat hepatocytes (*22*). Indeed, the doses of NO donors used in the present study may be high enough to release heme from hemoproteins, which would in turn induce heme oxygenase-1 mRNA. The *cis*-acting elements in the heme oxygenase-1 gene, mediating the response to NO donors, remain to be identified.

The present study has shown that the induction of heme oxygenase-1 mRNA by NO donors is a phenomenon which is observed in various human cell lines of different lineages. Increased expression of heme oxygenase-1 may result in the increased generation of CO and biliverdin/bilirubin, a radical scavenger (*13*), while NO was shown to mediate neurotoxicity caused by glutamate (*28*) or immunostimulation (*29*). It is therefore conceivable that induction of heme oxygenase-1 protects the cells against the oxidative stress, caused by NO and/or its metabolites derived from adjacent cells expressing NOS or by non-enzymatically produced NO (*30-34*). Alternatively, the induction of heme oxygenase-1 mRNA by NO donors may represent a coordinated regulation of the enzyme systems generating CO and NO. CO is more stable than the radical gas NO and possesses biological functions similar to those of NO, such as vasodilatation and neurotransmission (*14-19*). In this context, using A172 cells expressing inducible NOS, we recently showed that the induction of inducible NOS mRNA by cytokines was not accompanied by the induction of heme oxygenase-1 mRNA (*21*). These results suggest that the amounts of NO or NO metabolites endogenously produced may not be sufficient to induce heme oxygenase-1 mRNA in A172 cells. To help understand the physiological implications of the NO donor-mediated induction of heme oxygenase-1, we are currently investigating the molecular mechanisms of this induction.

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