Iron Released by Sodium Nitroprusside Contributes to Heme Oxygenase-1 Induction via the cAMP-Protein Kinase A-Mitogen-Activated Protein Kinase Pathway in RAW 264.7 Cells

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ABSTRACT

Nitric oxide (NO) is a potent inducer of heme oxygenase (HO)-1, and NO-induced HO-1 expression is dependent on the cGMP-signaling pathway. Sodium nitroprusside (SNP) produces NO and iron. However, it is unclear whether NO is exclusively responsible for induction of HO-1 by SNP in RAW 264.7 cells. We tested our hypothesis that iron may contribute more to the SNP induction of HO-1 than does NO by comparing the HO-1 protein level and the production of NO in RAW 264.7 cells treated with SNP and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP). Although SNP induced less NO production than SNAP, SNP induced the production of more HO-1 protein than did SNAP. Deferoxamine (DFO) decreased SNP- but not SNAP-induced HO-1 expression but did not decrease the production of NO. SNP-induced HO-1 was significantly inhibited by specific protein kinase A (PKA) inhibitors or an antagonist of cAMP but not by guanylyl cyclase inhibitors. Exogenous iron (ferric ammonium citrate or ferricyanide) and forskolin increased the level of HO-1, which was inhibited by PKA inhibitor *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H89). These results indicate that iron and cAMP, but not cGMP, play crucial roles in the induction of HO-1 in RAW 264.7 cells. Moreover, DFO and inhibitors of extracellular signal-related kinases 1/2 or c-Jun NH$_2$-terminal kinase inhibited HO-1 production induced by SNP. This study illustrates that iron rather than NO from SNP contributes to HO-1 induction. Therefore, studies on the effects of SNP should consider the role of iron in some biological functions. We concluded that iron released by SNP contributes to HO-1 induction via the cAMP-PKA-mitogen-activated protein kinase pathway.

Heme oxygenase (HO) is an essential enzyme in heme catabolism that cleaves heme to release carbon monoxide, iron, and biliverdin (Tenhunen et al., 1968, 1969). HO-1 is induced by a variety of physiological stimuli, including heme, heavy metals, inflammatory cytokines, endotoxins, and nitric oxide (NO) (Durante et al., 1997b; Maines, 1997; Yet et al., 1997). Recent studies have shown that HO-1 expression plays a critical role in mediating antioxidant, anti-inflammatory, and antiapoptotic effects (Brouard et al., 2000; Otterbein et al., 2000). The beneficial effects of HO-1 induction might occur via several postulated mechanisms. Increased HO-1 activity results in the degradation of the heme moiety, a potentially toxic prooxidant, and generates bilirubins, an antioxidant capable of scavenging peroxy radicals and inhibiting lipid peroxidation (Stocker et al., 1987; Llesuy and Tomaro, 1994; Nath et al., 1998).

NO is a free radical involved in the regulation of many physiological functions, including endothelium-dependent vasodilation, neurotransmission, and the cell-mediated im-

ABBREVIATIONS: HO, heme oxygenase; BSA, bovine serum albumin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DFO, deferoxamine; ERK, extracellular signal-regulated kinase; FAC, ferric ammonium citrate; HCB, hydroxocobalamin; JNK, c-Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKG, protein kinase G; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; ROS, reactive oxygen species; p-, phospho-; Rp-cAMPS, adenosine-3′,5′-cyclic monophosphorothioate, Rp isomer; LY83583, 6-anilino-5,8-quinolinedione; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PD98059, 2′-amino-3′-methoxyflavone; SP600125, anthra(1,9-*cd*)pyrazol-6(2*H*)-one 1,9-pyrazoloanthrone; KT-5823, (9*S*,10*R*,12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo-[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid methyl ester; KT-5720, (9*S*,10*S*,12*R*)-2,3,9,10,11-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid hexyl ester.

mune response (Feldman et al., 1993; Moncada and Higgs, 1993; MacMicking et al., 1997). Alterations in NO synthesis are implicated in the pathophysiology of inflammation, septic shock, atherosclerosis, and glomerulonephritis (Laskin et al., 1994; Vane et al., 1994; Nathan, 1997; Furusu et al., 1998). NO is regarded as a pharmacologically active molecule of SNP; therefore, many of the biological actions of SNP are known to be mediated through the activation of guanylate cyclase and cGMP production (Kim et al., 1995; Polte et al., 2000). Because NO is known as a potent inducer of HO-1, one may think with no doubt that the induction of HO-1 by SNP is dependent on the cGMP-signaling pathway. However, it is quite conceivable that SNP may regulate HO-1 induction, a gene known to be sensitive to oxidative stress, via a change in the redox state by releasing free iron from SNP rather than only by the action of NO.

We investigated the possibility that a factor or factors other than NO, especially iron, are involved in the regulation of SNP-mediated HO-1 induction. We compared HO-1 induction in response to SNP, a NO^+ generator, with that induced by SNAP, a $NO^{\bullet}$ generator without any iron in its structure. We also investigated the possible signaling pathway involved in SNP-induced HO-1 expression, particularly the roles of cAMP and MAPK.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium, fetal bovine serum, and antibiotics (penicillin/streptomycin) were obtained from Gibco BRL (Rockville, MD). Anti-HO-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-p-ERK 1/2, anti-p-JNK, and anti-p-p38 antibodies were obtained from Cell Signaling Technology (Beverly, MA). LY83583, H89, carboxy-PTIO, SB203580, and PD98059 were obtained from Calbiochem (San Diego, CA). All other chemicals, including SNP, SNAP, deferoxamine (DFO), KT5720, KT5823, Rp-cAMPS, hydroxocobalamin, ferric ammonium citrate (FAC), and potassium ferricyanide [$K_3Fe(CN)_6$] were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell Treatment. Cells were pretreated with H89 or KT5720 [a specific protein kinase A (PKA) inhibitor], Rp-cAMPS (an antagonist of cAMP), LY83583 or ODQ (an inhibitor of soluble guanylyl cyclase), KT5823 (a specific PKG inhibitor), PD98059 (a selective ERK inhibitor), SB203580 (a p38 inhibitor), SP600125 (a selective JNK inhibitor), or DFO (a free iron chelator) for 1 h in serum-free medium, after which SNP or SNAP was added to the cells.

Assay for Nitrite Production. NO was measured as its stable oxidative metabolite, nitrite, as described previously (Green and Schaefer, 1981). After 18-h incubation, 500 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthyl-ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated with sodium nitrite standards.

Western Blot Analysis. The cells were harvested and lysed with buffer containing 0.5% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, and protease inhibitors. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, IL). To detect HO-1, 20 μg of the total protein was electrophoresed on a 10% polyacrylamide gel, and to detect phospho-MAPKs, 30 μg of the total protein

was electrophoresed on a 12% polyacrylamide gel. The gels were transferred to polyvinylidene difluoride membranes by a semidry electrophoretic transfer at 15 V for 60 to 75 min. The polyvinylidene difluoride membranes were blocked overnight at 4°C in 5% BSA. The cells were incubated with the primary antibodies, diluted 1:500 in Tris-buffered saline/Tween 20 containing 5% BSA for 2 h, and then incubated with the secondary antibody at room temperature for 1 h. Anti-goat IgG specific for HO-1 or anti-rabbit IgG specific for p-ERK 1/2, p-JNK, or p-p38 was used as the secondary antibody (1:5000 dilution in Tris-buffered saline/Tween 20 containing 1% BSA). The signals were detected by enhanced chemoluminescence (Amersham, Piscataway, NJ).

Determination of cAMP Content. The cells were treated with SNP and test substances for 4 h, and then medium was aspirated from plate. After adding 1 ml of 0.1 M HCl to the cells, the cells were incubated for 20 min at room temperature. The cells were scraped and centrifuged at 1000*g* for 10 min. The cAMP in the supernatant was analyzed with the cAMP EIA Kit (Cayman Chemical, Ann Arbor, MI) as described by the manufacturer.

Data Analysis. Scanning densitometry was performed using an Image Master VDS (Pharmacia Biotech Inc., San Francisco, CA). Treatment groups were compared using one-way analysis of variance, and the Newman-Keuls test was used to locate any significant differences identified in the analysis of variance. $P < 0.05$ or <0.01 was accepted as significant.

Results

The NO Donors SNP and SNAP Have Different Effects on the Production of Nitrite and HO-1 Level in RAW 264.7 Cells. NO is a potent inducer of HO-1, and NO-induced HO-1 induction is dependent on the GMP-signaling pathway (Maines, 1997; Immenschuh et al., 1998a). We compared the ability of SNP and SNAP to induce nitrite production in RAW 264.7 cells and investigated whether SNP- or SNAP-induced NO production coincides with HO-1 induction. The amount of nitrite produced after 24 h in the medium was 26.6 μM, with 500 μM SNP and 151.1 μM with SNAP, indicating that efficiency of generation of nitrite by SNAP was 5.7 times greater than that of SNP (Fig. 1A). SNAP also had a greater capability for nitrite production than did SNP in terms of concentration dependence (Fig. 1B). For example, at 24 h of treatment, SNAP-induced nitrite production (340.7 μM) was 7.7 times that induced by SNP (44.4 μM). In contrast, SNP was more effective at increasing the HO-1 protein level than SNAP, and SNAP showed only a weak ability to induce HO-1 in a time- and concentration-dependent manner (Fig. 2, A and B). Treatment with 500 μM SNP caused HO-1 production to reach a maximum level at 8 h, and this level was sustained until 24 h. These results suggest that the amount of NO is not proportional to HO-1 production in RAW 264.7 cells. Furthermore, it indicates that factor(s) other than NO possibly contribute more to SNP induction of HO-1.
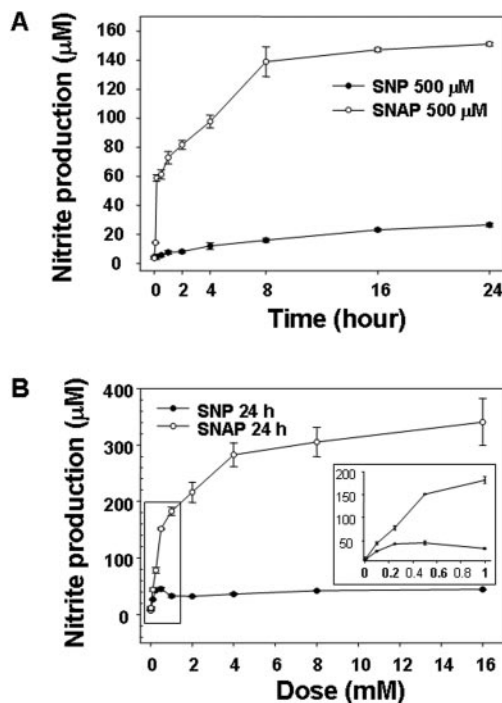
The Role of Iron in the Regulation of SNP-Induced HO-1. We next investigated the role of iron in the regulation of HO-1 by SNP. Because SNP is an iron complex in contrast to SNAP, we hypothesized that treating cells with SNP may donate its iron to cells, which induces HO-1. As expected, treating RAW 264.7 cells with increasing concentrations of DFO significantly decreased the SNP-induced HO-1 expression. In contrast, DFO treatment slightly increased NO production (Fig. 3, A and B), and pretreatment with DFO failed to decrease SNAP-induced HO-1 expression. DFO pretreat-

ment caused a parallel increase in SNAP-induced HO-1 expression and decrease in SNAP-induced NO production (Fig. 3, C and D). These results suggest that iron plays a critical role in the regulation of HO-1 by SNP.
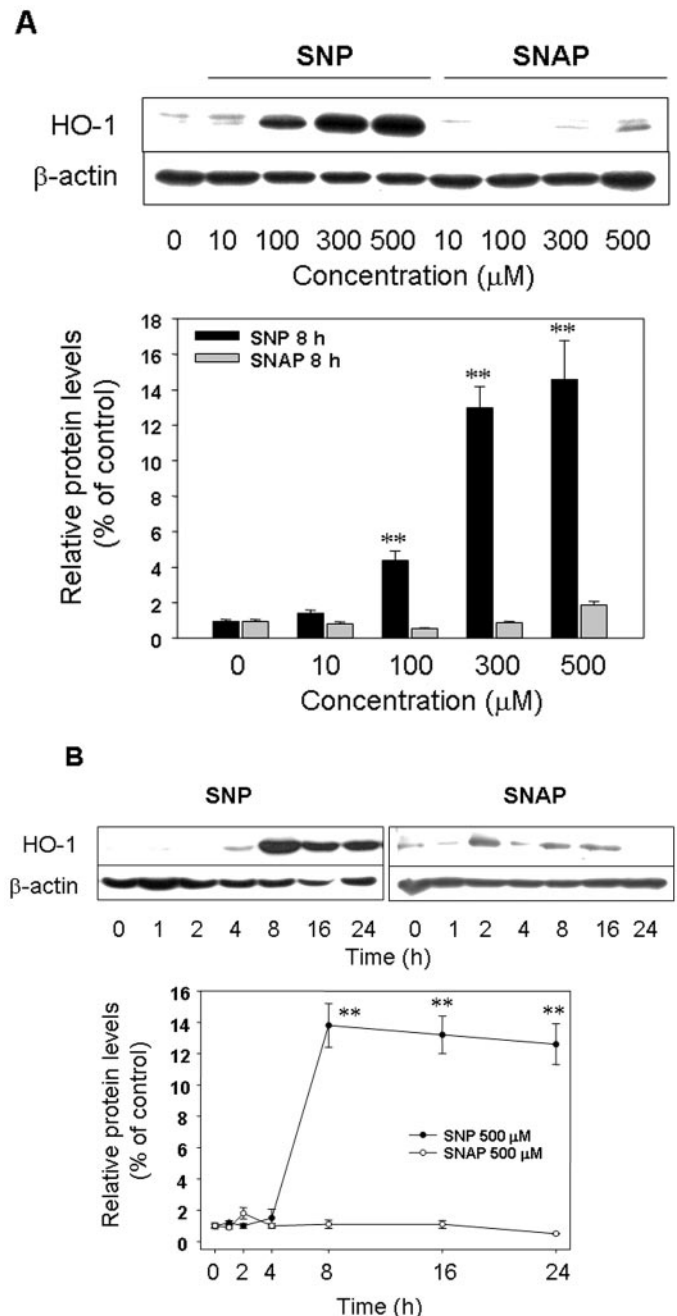
Involvement of cAMP-Dependent PKA Pathway on the SNP-Induced HO-1 Expression. We next examined which of the two signaling pathways, PKG or PKA, is involved in SNP-induced HO-1 expression. Cells were exposed to a specific PKA inhibitor (H89 or KT5720) and an antagonist of cAMP (Rp-cAMPS), or to an inhibitor of soluble guanylyl cyclase (ODQ or LY83583) and a specific PKG inhibitor (KT5823), for 1 h and then treated with 500 μM SNP for 8 h (Fig. 4A). Inhibition of cAMP or PKA pathway using H89, Rp-AMPS, or KT5720 efficiently inhibited SNP-induced HO-1 expression. In contrast, ODG and LY83583 little inhibited, but not significantly; however KT5823 (a specific PKG inhibitor) failed to inhibit the SNP-induced HO-1 (Fig. 4A). To confirm the involvement of the PKA pathway via cAMP on the SNP-induced HO-1 expression, cells were treated with forskolin, a cAMP generator. Forskolin increased the HO-1 protein level in a concentration-dependent manner, and this effect was inhibited by H89 (Fig. 4B). These data suggest that SNP regulates HO-1 via the cAMP-dependent PKA pathway rather than via the cGMP-dependent pathway.

Induction of HO-1 by SNP through the ERK and JNK Pathways. HO-1 is induced by many stimuli that also enhance the activity of MAPKs. We hypothesized that activation of MAPKs might be involved in the signaling pathways that induce *HO-1* gene expression. We investigated the signal-transduction pathway mediating the SNP-induced in-

crease in HO-1 expression. After SNP treatment, the HO-1 protein level decreased significantly after the addition of PD98059 (40 μM), a specific ERK inhibitor, and SP600125 (50 μM), a specific JNK inhibitor. In contrast, pretreatment with SB203580 (5 μM), a p38 inhibitor, did not alter SNP-

Fig. 2. SNP and SNAP regulate HO-1 induction differently and in a concentration- and time-dependent manner. RAW 264.7 cells were treated with SNP or SNAP at doses of 1, 10, 100, 250, or 500 μM (A) for 8 h and at 500 μM for 1, 2, 4, 8, 16, or 24 h (B). HO-1 protein levels were measured in SNP- or SNAP-treated cells by Western blot analysis as described under *Materials and Methods*. Thirty micrograms of protein extract from SNP- and SNAP-treated cells was loaded in same SDS-polyacrylamide gel to compare the level of HO-1 protein induced by SNP and SNAP. The band intensities were assessed by scanning densitometry. The data are presented as the mean $\pm$ S.E.M. of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared with the control, $**$, $P < 0.01$; control level = 1).

Fig. 1. The NO donors SNP and SNAP have different effects on nitrite production in the RAW 264.7 murine macrophage cell line. SNAP caused the production of more nitrite than SNP in RAW 264.7 cells. The kinetics of nitrite production by 500 μM SNP and 500 μM SNAP (A) and concentration-dependent nitrite production by SNP and SNAP after 24 h of treatment were measured in the culture medium using the Griess reagent (B). Data are mean $\pm$ S.E.M. values from three or more independent experiments.

induced HO-1 expression (Fig. 5A). To confirm the specificity of the inhibitors PD98059 and SP600125, we performed Western blot analysis using different doses of inhibitors and found that PD98059 and SP600125 inhibited specifically SNP-induced HO-1 expression (Fig. 5B). These results indicate that MAPKs differentially regulate SNP-induced HO-1 expression, which means that ERK and JNK, but not p38, are involved in the SNP-mediated induction of HO-1 protein.

Phosphorylation of ERK and JNK by SNP through the cAMP-Dependent PKA Pathway. Treatment with MAPK inhibitors showed that up-regulation of HO-1 by SNP occurs through the ERK and JNK pathways. We next examined whether SNP facilitates phosphorylation of ERK or JNK. Phosphorylation of ERK1/2 was first detected 10 min after SNP treatment, was sustained until 8 h after treatment, and then decreased thereafter (Fig. 6A). The phosphorylation level of JNK (p54/p46) was relatively weak and peaked 8 h after SNP treatment (Fig. 6A). These results

suggest that the ERK and JNK pathways are involved in HO-1 induction by SNP. Because the cAMP-dependent PKA pathway is involved in SNP-induced HO-1 expression, we investigated the relationship between PKA and MAPK. Pre-incubation of cells with H89 significantly inhibited the SNP-induced phosphorylation of ERK1/2 and JNK, indicating that SNP induces HO-1 via PKA-MAPK (Fig. 6B). Moreover, we confirmed that cAMP directly activates ERK1/2 and JNK phosphorylation by showing that a cAMP analog, 8-Br-cAMP (100 μM), activated ERK1/2 or JNK phosphorylation, which was inhibited by pretreatment of Rp-cAMPS, an antagonist of cAMP (Fig. 6C).

Involvement of Iron in the HO-1 Induction through cAMP. We showed that SNP induces HO-1 protein regardless of the level of NO production induced by SNP and that induction of the HO-1 protein by SNP is inhibited by the iron chelator, DFO, and by the specific PKA inhibitors. We hypothesized that SNP induces HO-1 expression via the
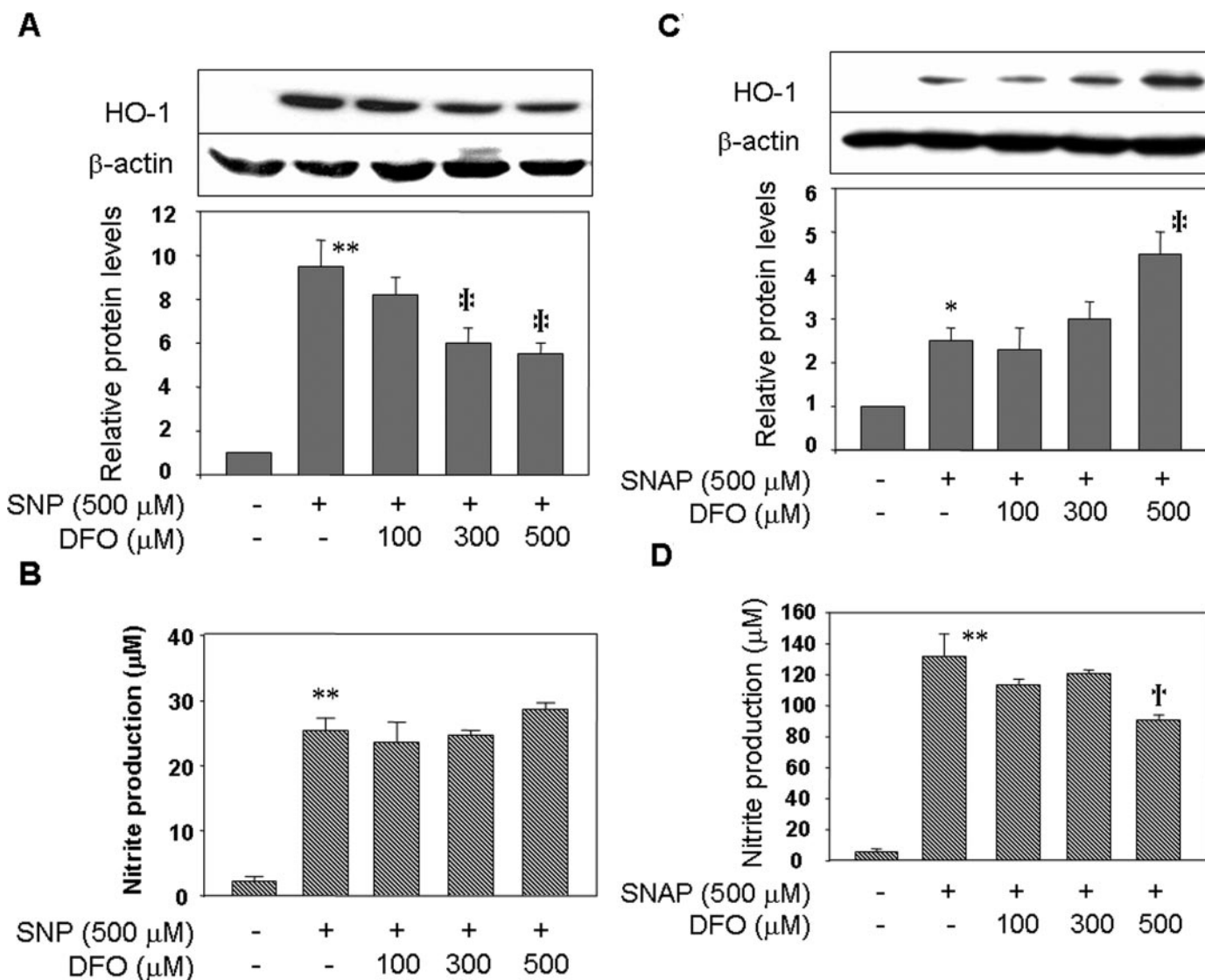
Fig. 3. The role of iron in the regulation of HO-1 and NO production by SNP and SNAP. Cells were pretreated with the iron chelator DFO (100, 300, or 500 μM) for 1 h before treatment with SNP or SNAP. After 8-h induction with SNP (A and B) or SNAP (C and D), the HO-1 protein level was measured in the cell extract using Western blot analysis, and nitrite production in the medium was measured using the Griess reagent. The band intensities were assessed by scanning densitometry. The data are presented as the mean $\pm$ S.E.M. of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared with the control, $\ast\ast$, $P < 0.01$; significance compared with SNP: $\dagger$, $P < 0.05$ or $\ddagger$, $P < 0.01$; control level = 1).

cAMP-PKA-ERK/JNK pathway. The deposition of SNP [Na$_2$[Fe(CN)$_5$NO] 2H$_2$O] in solution leads to the production of NO$^+$, ferricyanide, and cyanide (CN$^-$). We next investigated whether ferricyanide or iron in the form of FAC induces HO-1 via the PKA pathway and whether CN$^-$ is in-

volved in the induction of HO-1 protein by SNP. Treating RAW 264.7 cells with ferricyanide or FAC significantly increased the HO-1 protein level even though it was lower than SNP-induced HO-1 level, and this effect was inhibited by pretreatment with H89 (Fig. 7A). In cell lysates, the cAMP concentration increased significantly from 46 pg/ml in control cells to 88 pg/ml in FAC-treated cells and to 79 pg/ml in ferricyanide-treated cells (Fig. 7B). In addition, the NO scavenger carboxy-PTIO (250 and 500 μM) or hydroxocobalamin (HCB) (100 or 300 μM), and the CN$^-$ inhibitor, composed of 8 U/ml rhodanase with 5 mM Na$_2$S$_2$O$_3$ (Boullerne et al., 2001), did not inhibit the SNP-induced increase in HO-1 protein level (Fig. 7, C and D), suggesting that free iron released from SNP might induce HO via the PKA pathway.

Discussion

In general, it is well recognized that NO and cGMP are signal-coupling molecules that are responsible for many pharmacological actions manifested by SNP. Thus, NO is regarded as an exclusive molecule responsible for SNP-induced relaxation of vascular smooth muscle. However, apart from NO, SNP has been described as an NO$^+$ donor (Stamler et al., 1992). Thus, we wanted to address whether there is a possibility that SNP induces HO-1 in RAW 264.7 cells not by NO. The major finding of the present study was that SNP induced HO-1 expression in RAW 264.7 cells is via cAMP-MAPK pathways, which is mediated by iron, not by NO. By comparing nitrite production and HO-1 expression based on
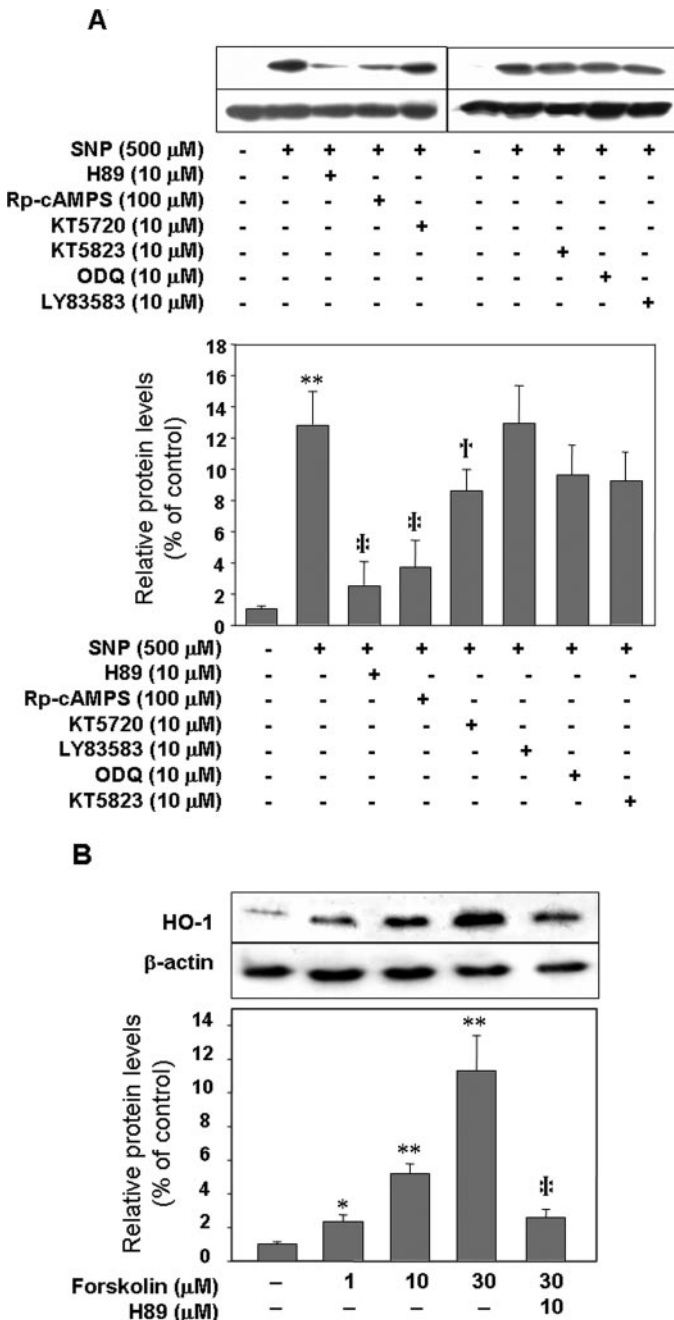
Fig. 4. The involvement of the PKA pathway via cAMP on SNP-induced HO-1 expression. A, cells were exposed to the specific PKA inhibitor (H89 or KT5720, 10 μM) and an antagonist of cAMP (Rp-cAMPS, 100 μM) or to an inhibitor of soluble guanylyl cyclase (ODQ or LY83583, 10 μM) and a specific PKG inhibitor (KT5823, 10 μM), for 1 h and then treated with 500 μM SNP for 8 h. B, to confirm the involvement of the PKA pathway via cAMP on SNP-induced HO-1 production, RAW 264.7 cells were treated with the cAMP generator forskolin. The data are presented as the mean ± S.E.M. of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared with the control, *, $P < 0.05$ or **, $P < 0.01$; significance compared with SNP or forskolin, †, $P < 0.05$ or ‡, $P < 0.01$; control level = 1).
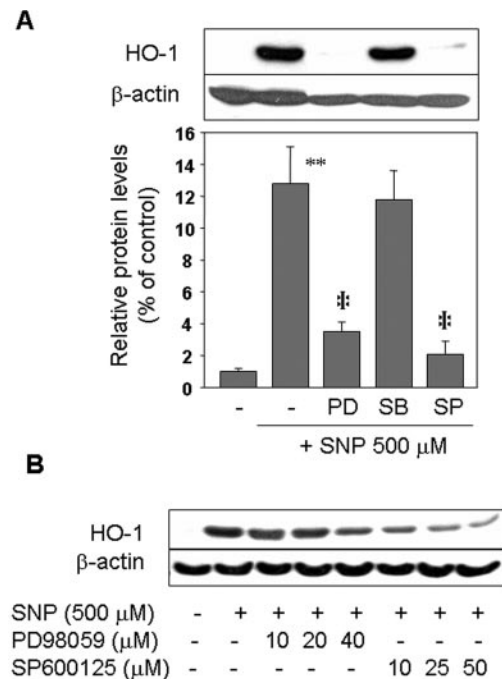
Fig. 5. SNP induction of HO-1 protein through the ERK and JNK pathways. A, the HO-1 protein level was measured in the cells treated with SNP (500 μM) for 8 h after pretreatment with PD98059 (40 μM), SB203580 (5 μM), or SP600125 (50 μM). B, PD98059 (10, 20, or 40 μM) and SP600125 (10, 25, or 50 μM) were added to specifically inhibit the ERK or JNK pathway in a concentration-dependent manner. The data are presented as the mean ± S.E.M. of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared with the control, **, $P < 0.01$; significance compared with SNP, ‡, $P < 0.01$; control level = 1).

concentrations, we found that SNP induced a much higher HO-1 protein level than SNAP, although the amount of nitrite donated from SNP in the medium was much lesser than SNAP. Furthermore, we found that HO-1 expression is dependent on cellular free iron. The iron chelator DFO inhibited the induction of HO-1 in response to SNP without reducing the nitrite production; rather, it increased SNP-mediated nitrite production to some degree. This suggests that iron rather than NO contributed to SNP induction of HO-1. The reason for the increase of nitrite by DFO in SNP-treated cells is not clear. However, it can be speculated that reduced cellular iron concentrations by DFO significantly influenced inducible nitric-oxide synthase expression (Weiss et al.,

1994). Thus, iron deprivation caused the enhanced inducible nitric-oxide synthase expression, which results in an increase of NO formation. However, it will require further investigation. On the other hand, DFO has been also suggested to directly scavenge reactive oxygen species (ROS), including
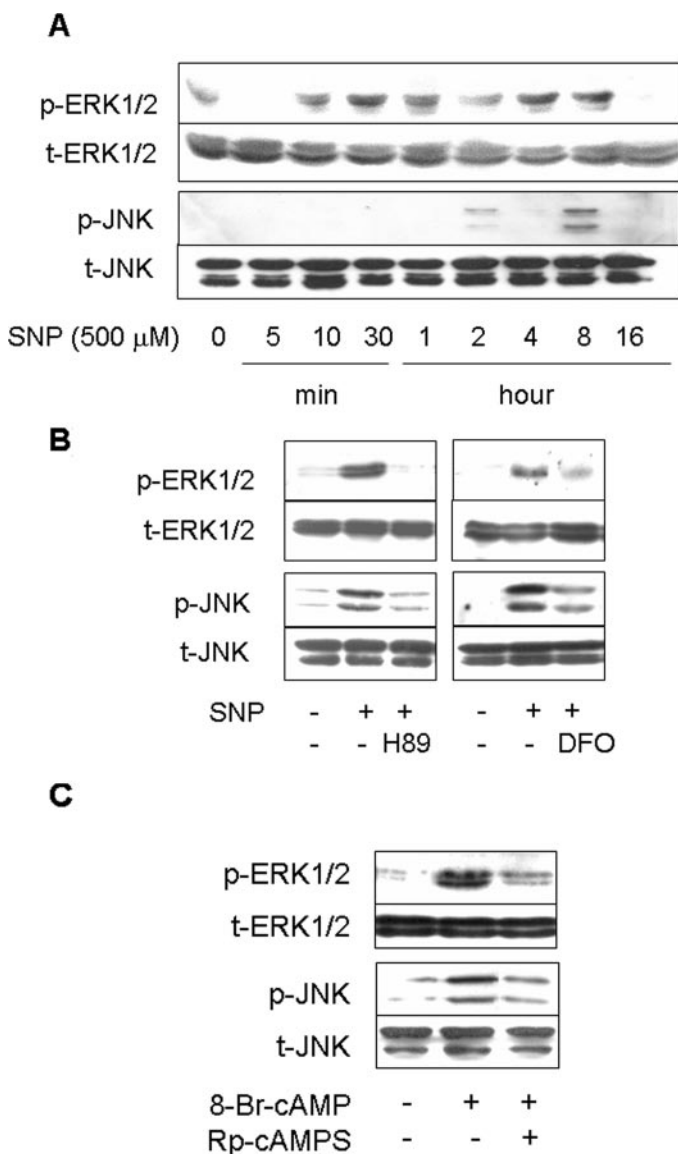
Fig. 6. SNP-induced phosphorylation of ERK and JNK through the PKA pathway. A, the cell lysate was extracted from the cells treated with SNP (500 μM) at the indicated times, and Western blot analysis was performed using anti-p-ERK1/2 and anti-ERK1/2 or anti-p-SAPK/JNK (p54/p46) and anti-SAPK/JNK antibodies (Thr183/Tyr185). B, the activation of the SNP-induced ERK or JNK pathway was prevented by pretreatment with the specific PKA inhibitor H89 or the free iron chelator DFO. C, TO confirm the direct activation of ERK1/2 or JNK pathway by a cAMP analog, cells were treated with 8-Br-cAMP (100 μM) for 30 min with or without Rp-cAMPS. Each lane was loaded with 60 μg of the cell lysates. The data were confirmed in two experiments.
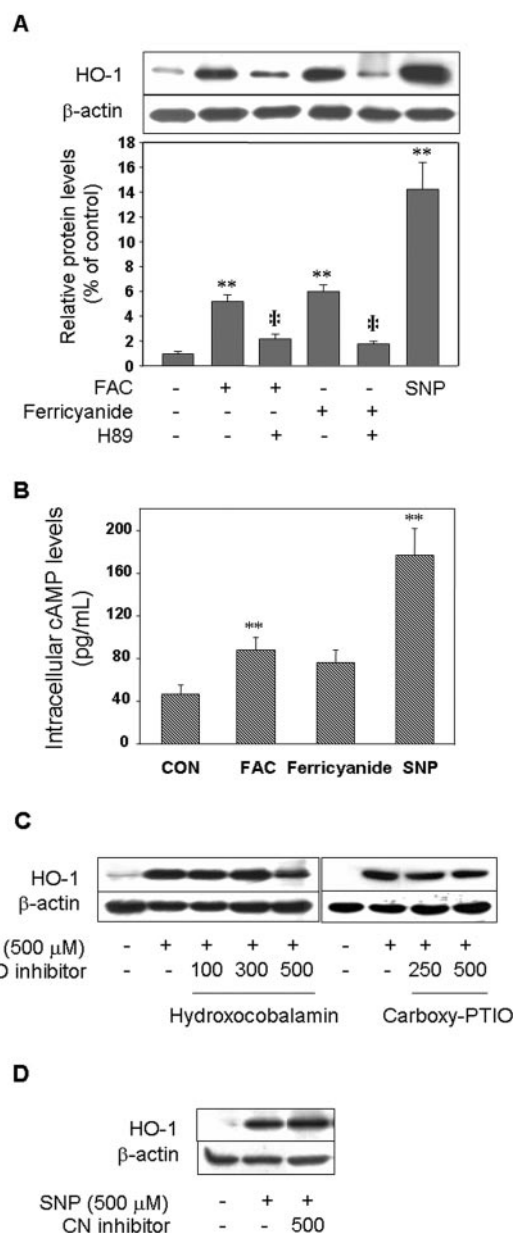
Fig. 7. The involvement of iron in HO-1 induction through cAMP. A, RAW 264.7 cells were treated with FAC (1 mM) or potassium ferricyanide (1 mM) with or without H89, a specific PKA inhibitor. After 8 h of treatment, the HO-1 protein level was determined by Western blot analysis. The data are presented as the mean ± S.E.M. of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared with the control, **, $P < 0.01$; significance compared with FAC or ferricyanide, ‡, $P < 0.01$; control level = 1). B, cell lysates were obtained from RAW 264.7 cells treated with FAC (1 mM) or potassium ferricyanide (1 mM) for 4 h, and the cAMP analysis was performed as described under *Materials and Methods*. C and D, to investigate the role of NO or cyanide in the SNP-induced HO-1 expression, cells were pretreated with the NO scavenger carboxy-PTIO (250 or 500 μM) or HCB (100 or 300 μM) and the CN$^-$ inhibitor, composed of 8 U/ml rhodanese with 5 mM $Na_2S_2O_3$, for 30 min and then treated with SNP for 8 h. The data were confirmed by repeated experiments.

free iron-cAMP-ERK1/2 or JNK signaling pathways are involved in SNP-mediated HO-1 induction. It should be noted, therefore, that care is needed when interpreting studies measuring the effects of SNP, because the role of iron molecules sometimes exceeds that of NO in some conditions such as HO-1 induction. After this article was accepted for publication, Wang et al. (2006) reported data supporting our conclusion that iron, not NO, is responsible for degradation of the iron regulation protein IRP2 by SNP. Therefore, SNP can no longer be regarded as an "NO-donor".

Acknowledgments

References

Alam J, Cai J, and Smith A (1994) Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5′ sequences are required for induction by heme or heavy metals. *J Biol Chem* **269:**1001–1009.

Bates JN, Baker MT, Guerra R Jr, and Harrison DG (1991) Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. *Biochem Pharmacol* **42:**S157–S165.

Boullerne AI, Nedelkoska L, and Benjamins JA (2001) Role of calcium in nitric oxide-induced cytotoxicity: EGTA protects mouse oligodendrocytes. *J Neurosci Res* **63:**124–135.

Brenneisen P, Wenk J, Klotz LO, Wlaschek M, Briviba K, Krieg T, Sies H, and Scharffetter-Kochanek K (1998) Central role of ferrous/ferric iron in the ultraviolet B irradiation-mediated signaling pathway leading to increased interstitial collagenase, matrix-degrading metalloprotease (MMP-1) and stromelysin-1 (MMP-3) mRNA levels in cultured human dermal fibroblasts. *J Biol Chem* **273:**5279–5287.

Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, and Soares MP (2000) Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med* **192:**1015–1026.

Chen K and Maines MD (2000) Nitric oxide induces heme oxygenase-1 via mitogen-activated protein kinases ERK and p38. *Cell Mol Biol* **46:**609–617.

Durante W, Christodoulides N, Cheng K, Peyton KJ, Sunahara RK and Schafer AI (1997a) cAMP induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle. *Am J Physiol* **273:**H317–H323.

Durante W, Kroll MH, Christodoulides N, Peyton KJ, and Schafer AI (1997b) Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ Res* **80:**557–564.

Elbirt KK, Whitmarsh AJ, Davis RJ, and Bonkovsky HL (1998) Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. *J Biol Chem* **273:**8922–8931.

Feldman PL, Griffith OW, and Stuehr DJ (1993) The surprising life of nitric oxide. *Chem Eng News* **71:**26–38.

Furusu A, Miyazaki M, Abe K, Tsukasaki S, Shioshita K, Sasaki O, Miyazaki K, Ozono Y, Koji T, Harada T, et al. (1998) Expression of endothelial and inducible nitric oxide synthase in human glomerulonephritis. *Kidney Int* **53:**1760–1768.

Green TR and Schaefer RE (1981) Intrinsic dichlorophenolindophenol reductase activity associated with the superoxide-generating oxidoreductase of human granulocytes. *Biochemistry* **20:**7483–7487.

Halliwell B (1989) Protection against tissue damage in vivo by desferrioxamine: what is its mechanism of action? *Free Radic Biol Med* **7:**645–651.

Immenschuh S, Hinke V, Ohlmann A, Gifhorn-Katz S, Katz N, Jungermann K, and Kietzmann T (1998a) Transcriptional activation of the haem oxygenase-1 gene by cGMP via a cAMP response element/activator protein-1 element in primary cultures of rat hepatocytes. *Biochem J* **334:**141–146.

Kim YM, Bergonia H, and Lancaster JR Jr (1995) Nitrogen oxide-induced autoprotection in isolated rat hepatocytes. *FEBS Lett* **374:**228–232.

Laskin JD, Heck DE, and Laskin DL (1994) Multifunctional role of nitric oxide in inflammation. *Trends Endocrinol Metab* **5:**377–382.

Llesuy SF and Tomaro ML (1994) Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. *Biochim Biophys Acta* **1223:**9–14.

MacMicking J, Xie QW, and Nathan C (1997) Nitric oxide and macrophage function. *Annu Rev Immunol* **15:**323–350.

Maines M (1997) The heme oxygenase system: a regulator of second messenger gases *Ann Rev Pharmacol Toxicol* **37:**517–554.

Moncada S and Higgs A (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* **329:**2002–2012.

Motterlini R, Foresti R, Intaglietta M, and Winslow RM (1996) NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am J Physiol* **270:**H107–H114.

Nakagawa J, von der Ahe D, Pearson D, Hemmings BA, Shibahara S, and Nagamine Y (1988) Transcriptional regulation of a plasminogen activator gene by cyclic AMP in a homologous cell-free system. Involvement of cyclic AMP- dependent protein kinase in transcriptional control. *J Biol Chem* **263:**2460–2468.

Nath KA, Grande JP, Croatt AJ, Likely S, Hebbel RP, and Enright H (1998) Intracellular targets in heme protein-induced renal injury. *Kidney Int* **53:**100–111.

Nathan C (1997) Inducible nitric oxide synthase: what difference does it make? *J Clin Investig* **100:**2417–2423.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, and Choi AM (2000) Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* **6:**422–428.

Pizurki L and Polla BS (1994) cAMP modulates stress protein synthesis in human monocytes-macrophages. *J Cell Physiol* **161:**169–177.

Polte T, Abate A, Dennery PA, and Schroder H (2000) Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. *Arterioscler Thromb Vasc Biol* **20:**1209–1215.

Polte T and Schroder H (1998) Cyclic AMP mediates endothelial protection by nitric oxide. *Biochem Biophys Res Commun* **251:**460–465.

Raju VS and Maines MD (1996) Renal ischemia-reperfusion upregulates heme oxygenase-1 (HSP32) expression and increases cGMP in rat heart. *J Pharmacol Exp Ther* **277:**1814–1822.

Ramakrishna Rao DN and Cederbaum AI (1996) Generation of reactive oxygen species by the redox cycling of nitroprusside. *Biochim Biophys Acta* **1289:**195–202.

Ryter SW, Si M, Lai CC, and Su CY (2000) Regulation of endothelial heme oxygenase activity during hypoxia is dependent on chelatable iron. *Am J Physiol* **279:**H2889–H2897.

Stamler JS, Singel DJ, and Loscalzo J (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science (Wash DC)* **258:**1898–1902.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, and Ames BN (1987) Bilirubin is an antioxidant of possible physiological importance. *Science (Wash DC)* **235:**1043–1046.

Suematsu M, Kashiwagi S, Sano T, Goda N, Shinoda Y, and Ishimura Y (1994) Carbon monoxide as an endogenous modulator of hepatic vascular perfusion. *Biochem Biophys Res Commun* **205:**1333–1337.

Tenhunen R, Marver H, and Schmid R (1969) Microsomal heme oxygenase, characterization of the enzyme. *J Biol Chem* **244:**6388–6394.

Tenhunen R, Marver HS, and Schmid R (1968) The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* **61:**748–755.

Tenhunen R, Ross ME, Marver HS, and Schmid R (1970) Reduced nicotinamide adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization. *Biochemistry* **9:**298–323.

Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J, and Willoughby DA (1994) Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc Natl Acad Sci USA* **91:**2046–2050.

Wang J, Fillebeen C, Chen G, Andriopoulos B, and Pantopoulos K (2006) Sodium nitroprusside promotes IRP2 degradation via an increase in intracellular iron and in the absence of S nitrosylation at C178. *Mol Cell Biol* **26:**1948–1954.

Weiss G, Werner-Felmayer G, Werner ER, Grunewald K, Wachter H, and Hentze MW (1994) Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med* **180:**969–976.

Yet SF, Pellacani A, Patterson C, Tan L, Folta SC, Foster L, Lee WS, Hsieh CM, and Perrella MA (1997) Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *J Biol Chem* **272:**4295–4301.

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