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Iron released by SNP contributes to HO-1 induction via the cAMP–PKA–MAPK pathway in RAW 264.7 cells.

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Running title: SNP-mediated HO-1 induction through iron-PKA-ERK1/2 or JNK

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Abbreviations

ANOVA, analysis of variance; BSA, bovine serum albumin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DFO, deferoxamine; DMEM, Dulbecco's modified Eagles medium; ECL, enhanced chemoluminescence; ERK, extracellular regulated kinase; FAC, ferric ammonium citrate; FBS, fetal bovine serum; HCB, hydroxocobalamin; HO, heme oxygenase; JNK, c-Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PKA, protein kinase A; SDS, sodium dodecyl sulfate; SNAP, S-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside

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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 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 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 a PKA inhibitor 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 ERK1/2 or JNK 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

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SNP contributes to HO-1 induction via the cAMP–PKA–MAPK pathway.

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Introduction

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) (Maines, 1997; Durante et al., 1997a; Yet et al., 1997). Recent studies have shown that HO-1 expression plays a critical role in mediating antioxidant, anti-inflammatory, and antiapoptotic effects (Otterbein et al., 2000; Brouard et al., 2000). The beneficial effects of HO-1 induction might occur via several postulated mechanisms. Increased HO-1 activity results in degradation of the heme moiety, a potentially toxic prooxidant, and generates bilirubins, an antioxidant capable of scavenging peroxy radicals and inhibiting lipid peroxidation (Nath et al., 1998; Stocker et al., 1987; Llesuy and Tomaro, 1994).

NO is a free radical involved in the regulation of many physiological functions, including endothelium-dependent vasodilation, neurotransmission, and the cell-mediated immune 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 (Vane et al., 1994; Laskin 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 (Polte et al., 2000; Kim et al., 1995). Because NO is known as a potent inducer of HO-1, one may think with no doubt that 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 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, is 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, in particular the role of cAMP and MAPK.

Materials and Methods

Materials. Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were obtained from Gibco BRL (Rockville, MD). Anti-HO-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-p-MEK1/2, 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 (Rockville, MD). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Cell treatment. Cells were pretreated with H89 or KT5720 (a specific 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% naphthylethylenediamine 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% NP-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 phosphor-MAPKs, 30 μg of the total protein was electrophoresed on a 12% polyacrylamide gel. The gels were transferred to PVDF membranes by a semi-dry electrophoretic transfer at 15 V for 60–75 min. The PVDF membranes were blocked overnight at 4 °C in 5% BSA. The cells were incubated with the primary antibodies, diluted 1:500 in TBST containing 5% BSA for 2 h and then incubated with the secondary antibody at room temperature for 1 h. Antigoat IgG specific for HO-1 or antirabbit IgG specific for p-MEK1/2, p-JNK or p-p38 was used as the secondary antibody

(1:5000 dilution in TBST containing 1% BSA). The signals were detected by ECL (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 1,000 g for 10 min. The cAMP in the supernatant was analyzed with the cyclic AMP EIA kit (Cayman, 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 (ANOVA) and the Newman–Keuls test was used to locate any significant differences identified in the ANOVA. $P < 0.05$ or $p < 0.01$ 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 (Immenschuh et al., 1998a; Maines, 1997). 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 higher than that of SNP (Fig. 1A). SNAP also had a greater capability for nitrite production than did SNP in terms of concentration dependency (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. 2A, 2B). Treatment with 500 μM of 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 contributes more to SNP induction of HO-1.

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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. 3A, 3B), and pretreatment with DFO failed to decrease SNAP-induced HO-1 expression. DFO pretreatment caused a parallel increase in SNAP-induced HO-1 expression and decrease in SNAP-induced NO production (Fig. 3C, 3D). These results suggest that iron plays a critical role in the regulation of HO-1 by SNP.

Involvement of cAMP-dependent protein kinase A (PKA) pathway on the SNP-induced HO-1 expression. We next examined which of the two signaling pathways, PKG and 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 (Figure 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 increase in HO-1 expression. After SNP treatment, the HO-1 protein level decreased significantly after 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-induced HO-1 expression (Fig. 5A). To confirm the specificity of the inhibitors PD98059 and SP600135, 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.

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Phosphorylation of ERK and JNK by SNP through the cAMP-dependent PKA pathway. Treatment with MAPK inhibitors showed that upregulation 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. Preincubation 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 analogue 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 cAMP–

PKA–ERK/JNK pathway. The deposition of SNP ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$) in solution leads to the production of NO^+, ferricyanide, and cyanide (CN^-). We next investigated whether ferricyanide or iron in the form of ferric ammonium citrate (FAC) induces HO-1 via the PKA pathway and whether cyanide (CN^-) is involved 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). Additionally, the NO scavenger carboxy-PTIO (250, 500 μM) or hydroxocobalamin (HCB) (100, 300 μM), and the cyanide (CN^-) inhibitor, composed of 8 U/ml rhodanase with 5 mM $Na_2S_2O_3$ (Boullerne et al., 2000), did not inhibit the SNP-induced increase in HO-1 protein level (Fig. 7C,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 being 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 if there's 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. When compared nitrite production and HO-1 expression based on concentrations, SNP induced 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 in some degree. This suggests that iron rather than NO contributed to SNP induction of HO-1. The reason for 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 on iNOS expression (Weiss et al., 1994), thus, iron deprivation caused to enhance iNOS expression, which results in 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 hydroxyl radical and superoxide anion (Halliwell, 1989), which may increase the efficiency of NO, such as prolongation of half life without affecting change of concentration, so that it enhanced NO action in case of SNAP, which caused to increase HO-1 induction. Therefore, SNAP-induced HO-1 expression is NO-dependent. We believe that the inhibitory effects of DFO shown in SNP-induced HO-1 expression are related with chelatable iron released from SNP. Therefore, different effects of DFO on SNP- and SNAP-exposed cells suggest that induction of HO-1 by these chemicals is under different regulatory mechanisms. In deed, free iron is known to have effects on the gene expression of several proteins involved in iron metabolism, transferrin receptor and ferritin (Brenneisen et al., 1998; Raju and Maines, 1996; Suematsu et al., 1994), and on enzymes potentially involved in oxidant or inflammatory conditions-inducible nitric oxide synthase (Tenhunen et al., 1969), the aconitases (Tenhunen et al., 1970), and HO-1 (Ryter et al., 2000). Furthermore evidence from the results of carboxy-PTIO or hydroxocobalamin (HCB), a known scavenger of NO strongly indicates that iron is more critical than NO in SNP induction of HO-1 in RAW 264.7 cells. We found that any concentrations of carboxy-PTIO (250 ~ 500 μM) or 100 ~ 300 μM HCB failed to inhibit SNP-induced HO-1 protein level; even though 500 μM HCB showed weak inhibition of SNP

induction of HO-1. Cyanide does not appear to induce HO-1 *in vitro* (Motterlini et al., 1996) and is less likely to have contributed to the stimulation of HO activity by SNP, because the specific cyanide chelator rhodanese in $Na_2S_2O_3$ did not inhibited HO-1 induction by SNP. Thus, the present study emphasizes the metabolites of SNP other than NO are functionally important in some biological system, such as HO-1 induction. In fact, SNP generates ROS during the redox cycling of nitroprusside (Bates et al., 1991; Ramakrishna and Cederbaum, 1996) and it is metabolized to a number of products, such as NO, iron, cyanide, or oxygen free radicals (Ramakrishna and Cederbaum, 1996). It is well known that SNP forms a coordination complex of a ferrous ion (Fe^{2+}) with five cyanide anions (CN^-) and a nitrosonium ion (NO^+). We propose here that iron released by SNP plays a critical role for induction of HO-1 in RAW 264.7 cells. However, contribution of S-nitrosothiol which may have been produced by interaction of the NO^+ from SNP with thiol groups in HO-1 induction can not be excluded. Because thiols, by virtue of their ability to be reversibly oxidized, are recognized as key components involved in the maintenance of redox balance. Furthermore, increasing evidence suggests that thiol groups located on various molecules act as redox sensitive switches thereby providing a common trigger for a variety of ROS and RNS mediated signaling events. However, it is unlikely that SNAP can react directly with thiol groups to form S-nitrosothiol, because NO reacts with metals to form NO^+ (Stamler et al.,

1992), which then reacts with thiol groups to form S-nitrosothiols. We believe that the differences in iron homeostasis, and, to lesser extent, the formation of S-nitrosothiol which may differentially affect redox state of the cell. So this might explain the observed differences in HO-1 induction by SNP and SNAP.

The involvement of the MAPK in HO-1 induction has been highly variable, depending on stimuli and cell type. For instance, ERK and p38 MAPK are involved in NO-mediated and sodium arsenite-mediated induction of HO-1. (Elbirt et al., 1998; Chen and Maines, 2000). We, however, found that ERK1/2 and JNK, but not p38, are involved in HO-1 induction in the RAW 264.7 murine macrophage cell line. We also found that the PKA inhibitors blocked SNP-mediated HO-1 induction and that the cAMP activator forskolin induced HO-1, suggesting that, in addition to the ERK1/2 and JNK pathways, the cAMP-dependent protein kinase A (PKA) is also involved in SNP-induced HO-1 expression. Interestingly, DFO abolished SNP-activated ERK1/2 and JNK phosphorylation. FAC or ferricyanide increased the levels of HO-1 and cAMP, suggesting that iron released from SNP is a potent inducer of HO-1 through a cAMP-dependent pathway linked to the ERK1/2 and JNK pathways.

However, the inhibition of SNP-induced HO-1 expression by DFO was relatively modest compared to the inhibition by the inhibitor of cAMP or PKA pathway, which suggests that some other pathway is involved in the SNP-activated PKA pathway. Actually, the

inhibitor of sGC ODQ or LY83583 little inhibited the HO-1 induction by SNP, suggesting that SNP-generated NO maybe activate cGMP pathway which is linked to cAMP-PKA pathway. This possibility had been suggested by the report that cAMP mediates antioxidant protection by NO donors in endothelial cells, as a likely consequence of cGMP-dependent inhibition of cAMP breakdown (e.g. through blockade of phosphodiesterase III) (Polte and Schroder, 1998). The reports that HO-1 is also responsive to gene activation by cAMP (Durante et al., 1997b; Pizurki and Polla, 1994; Nakagawa et al., 1988), and HO-1 is also induced by cAMP and CRE activation (Immenschuh et al., 1998a) support firmly our results that SNP induces HO-1 by activation of iron-cAMP-PKA-MAPK.

We conclude that SNP changes cellular iron homeostasis by generating iron and NO^+. The increased cellular free iron level may activate the cAMP–PKA pathway, which is linked to the MAPK pathway, especially ERK1/2 or JNK, which regulates HO-1 expression via the cAMP response element/AP-1 site of the *HO-1* gene (Alam et al., 1994). To our knowledge, ours is the first study to report that the free iron–cAMP–ERK/1/2 or JNK signal 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.

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

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

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, Sakai H and Kohno S (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. The heme oxygenase system: a regulator of second messenger gases (1997) *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 Invest* **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 and Schroder H (1998). Cyclic AMP mediates endothelial protection by nitric oxide. *Biochem Biophys Res Commun* **251:**460-465

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.

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 Heart Circ Physiol* **279**:H2889-H2897.

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

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN and Ames BN (1987) Bilirubin is an antioxidant of possible physiological importance. *Science* **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 U S A* **91:**2046-2050.

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.

Footnotes

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Figure Legends

Figure 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 ± SEM values from three or more independent experiments.

Figure 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 in the 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 is presented as the mean ± SEM of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared to the control, $^{**}p < 0.01$) (control level = 1).

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Figure 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, B) or SNAP (C, 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 is presented as the mean ± SEM of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared to the control, $^{**}p < 0.01$; significance compared with SNP, †$p < 0.05$ or ‡$p < 0.01$) (control level = 1).

Figure 4. The involvement of the PKA pathway via cAMP on SNP-induced HO-1 expression. Cells were exposed to the specific PKA inhibitor (H89 or KT54720, 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 (A). 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 (B). The data is presented as the mean ± SEM of three independent

experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared to 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).

Figure 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 is presented as the mean ± SEM of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared to the control, $**p < 0.01$; significance compared with SNP, ‡$p < 0.01$) (control level = 1).

Figure 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

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inhibitor, H89, or the free iron chelator, DFO. (C) To confirm the direct activation of ERK1/2 or JNK pathway by a cAMP analogue, cells were treated with 8-Br-cAMP (100 μM) for 30 min with/without Rp-cAMPS. Each lane was loaded with 60 μg of the cell lysates. The data were confirmed in two experiments.

Figure 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 is presented as the mean ± SEM of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared to 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 in Materials and Methods. (C, 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, 500 μM) or hydroxocobalamin (HCB) (100, 300 μM), and the cyanide (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.

Figure 8. Possible mechanism by which SNP induces HO-1 expression. SNP-generated iron may activate the cAMP–PKA pathway, which is linked to MAPK pathway, resulting in HO-1 expression. SNP-donated NO maybe involve in the HO-1 expression partly through cGMP-mediated inhibition of cAMP breakdown (Polte and Schroder, 1998)

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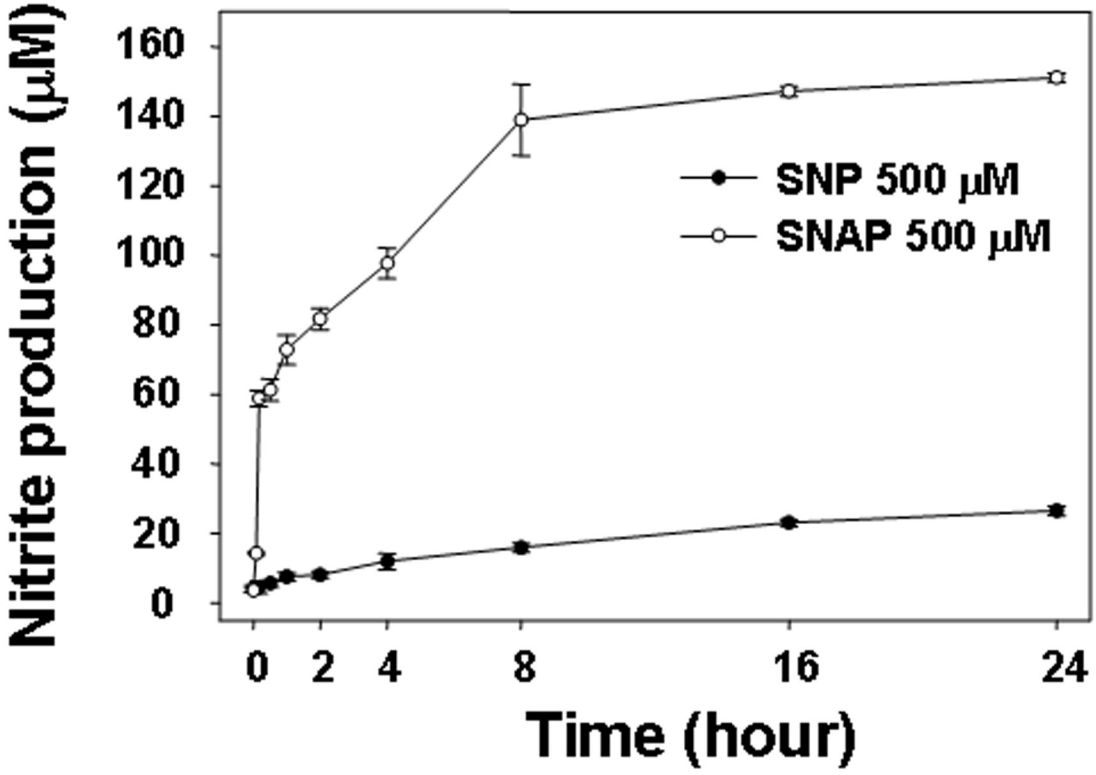

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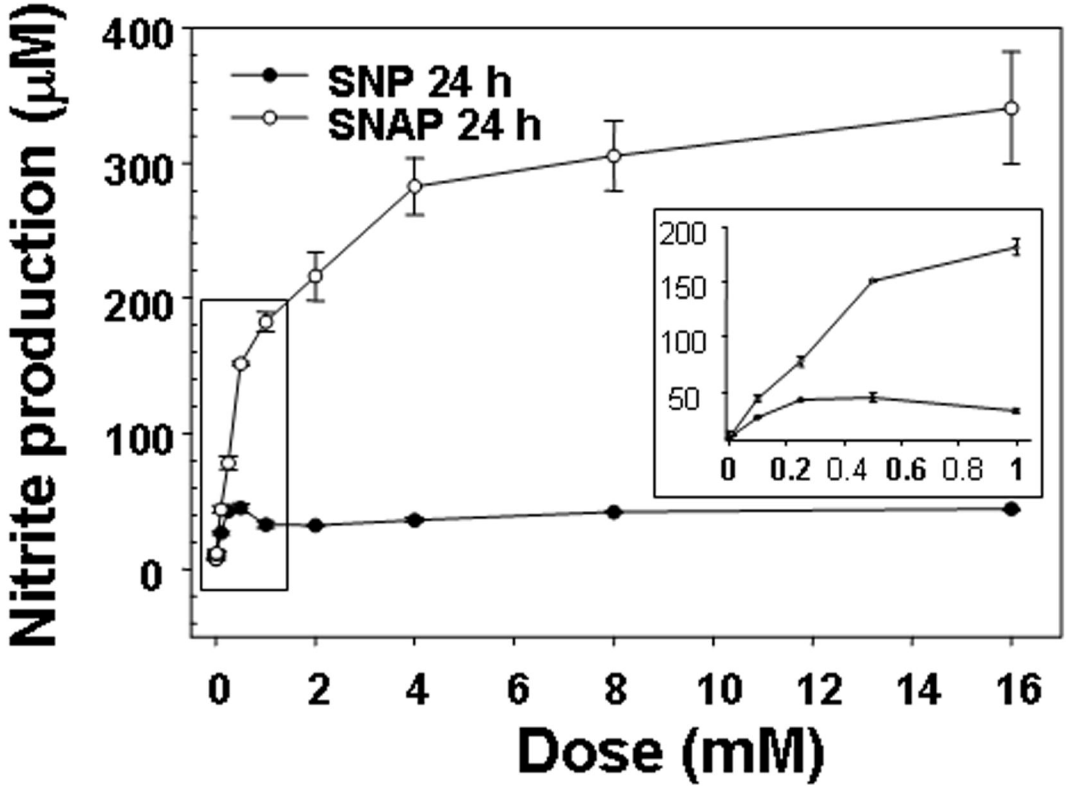

Figure 1

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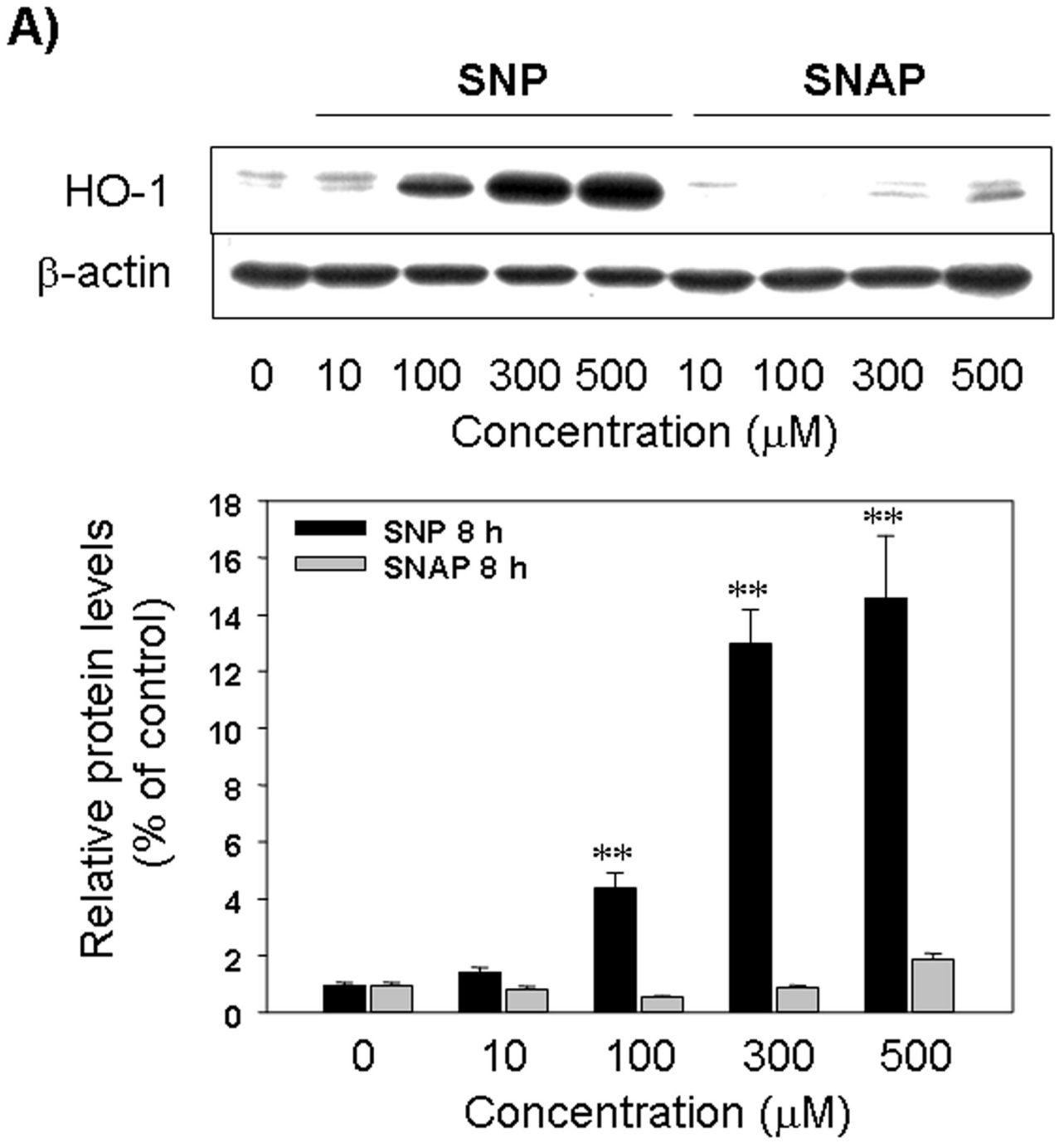

Figure 2-1

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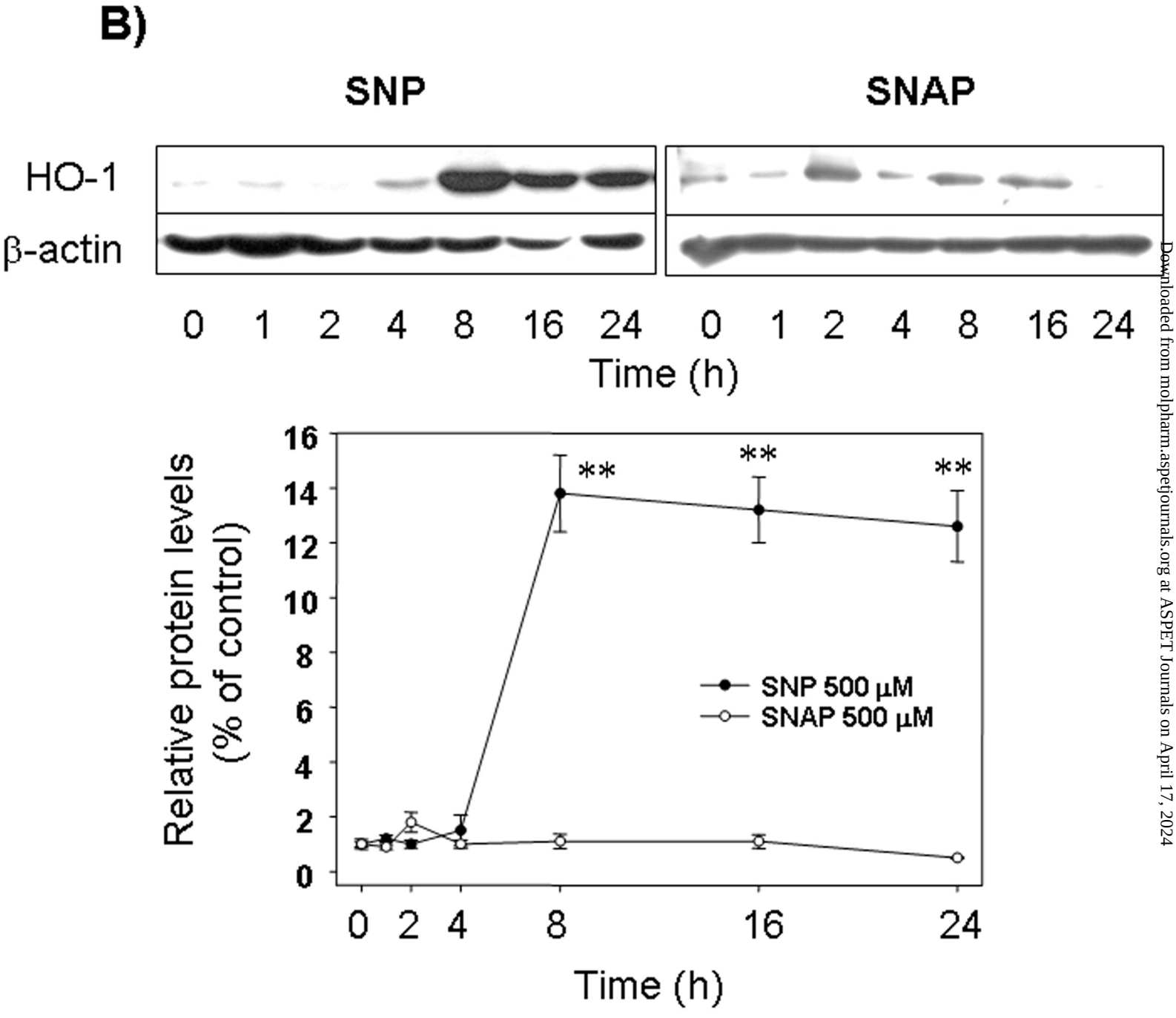

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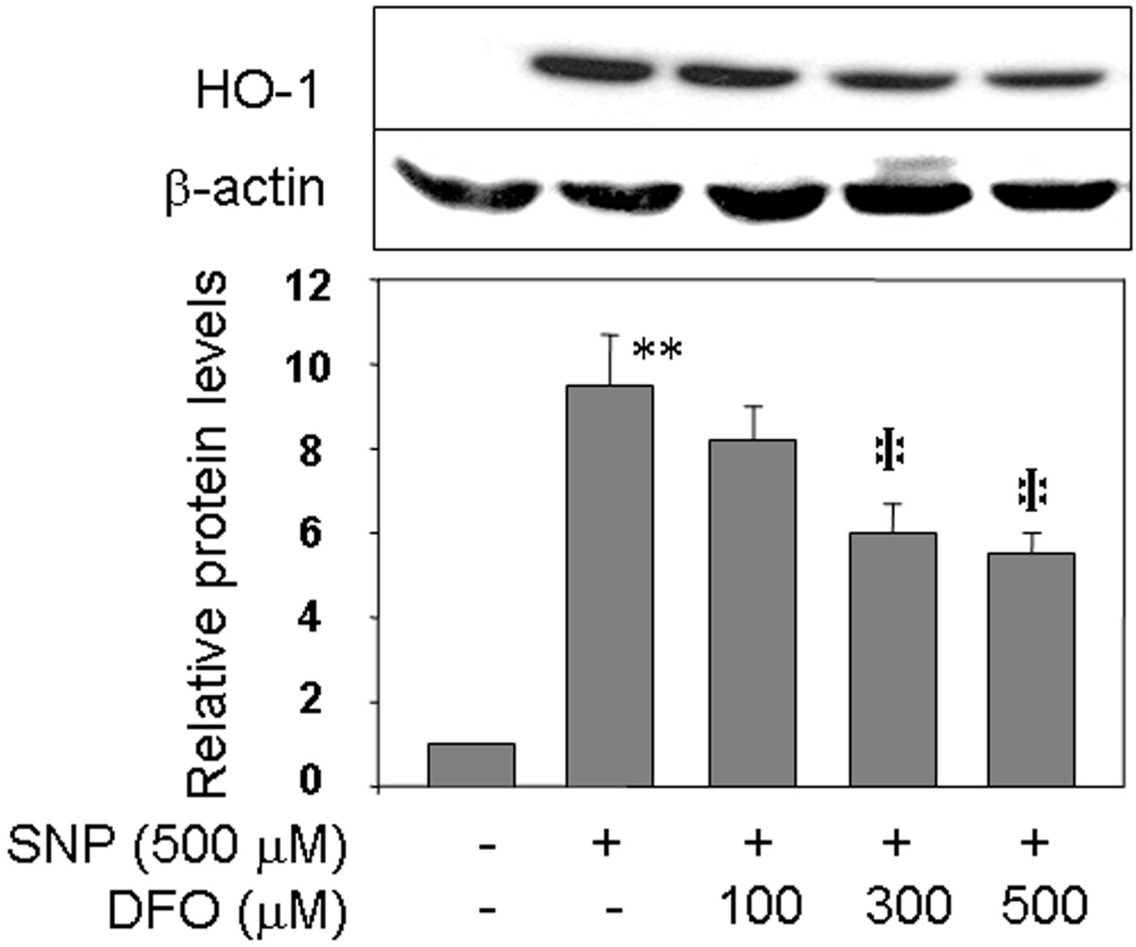

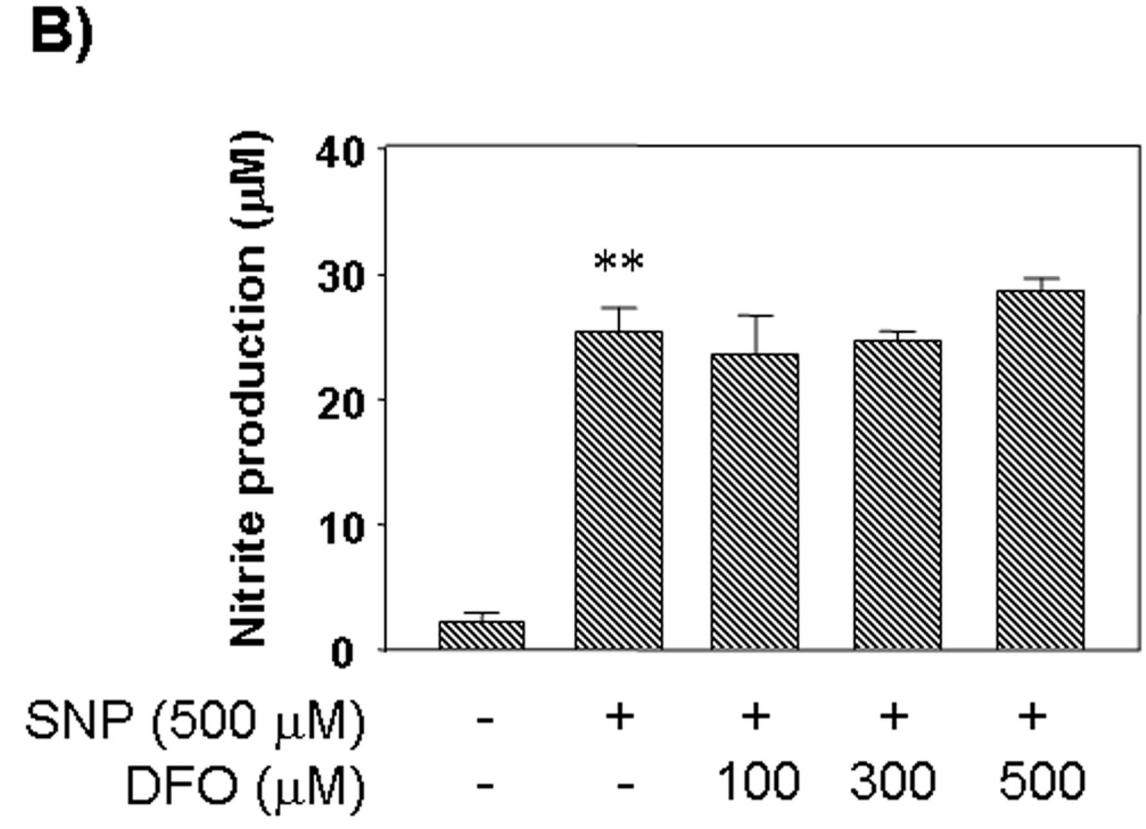

Figure 3-1

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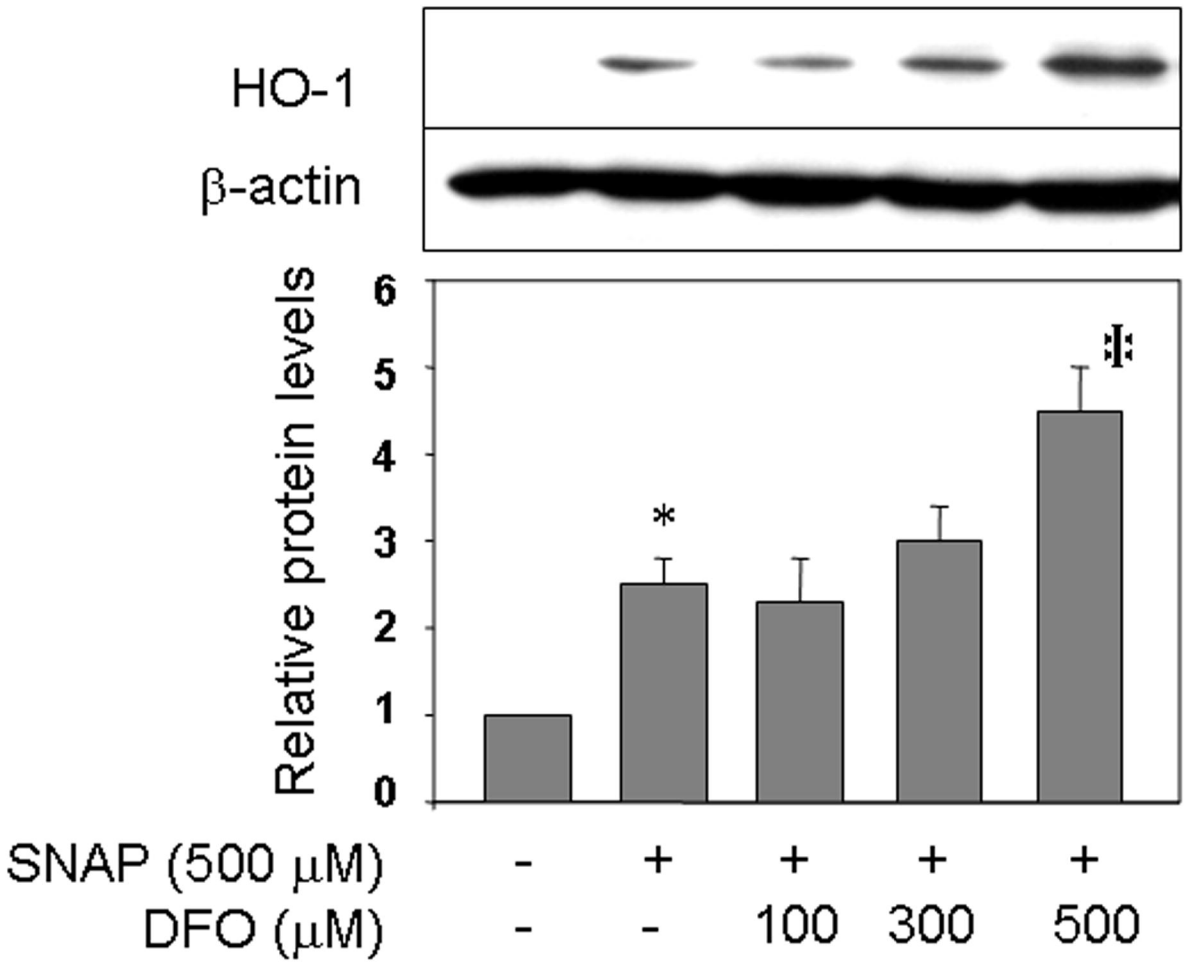

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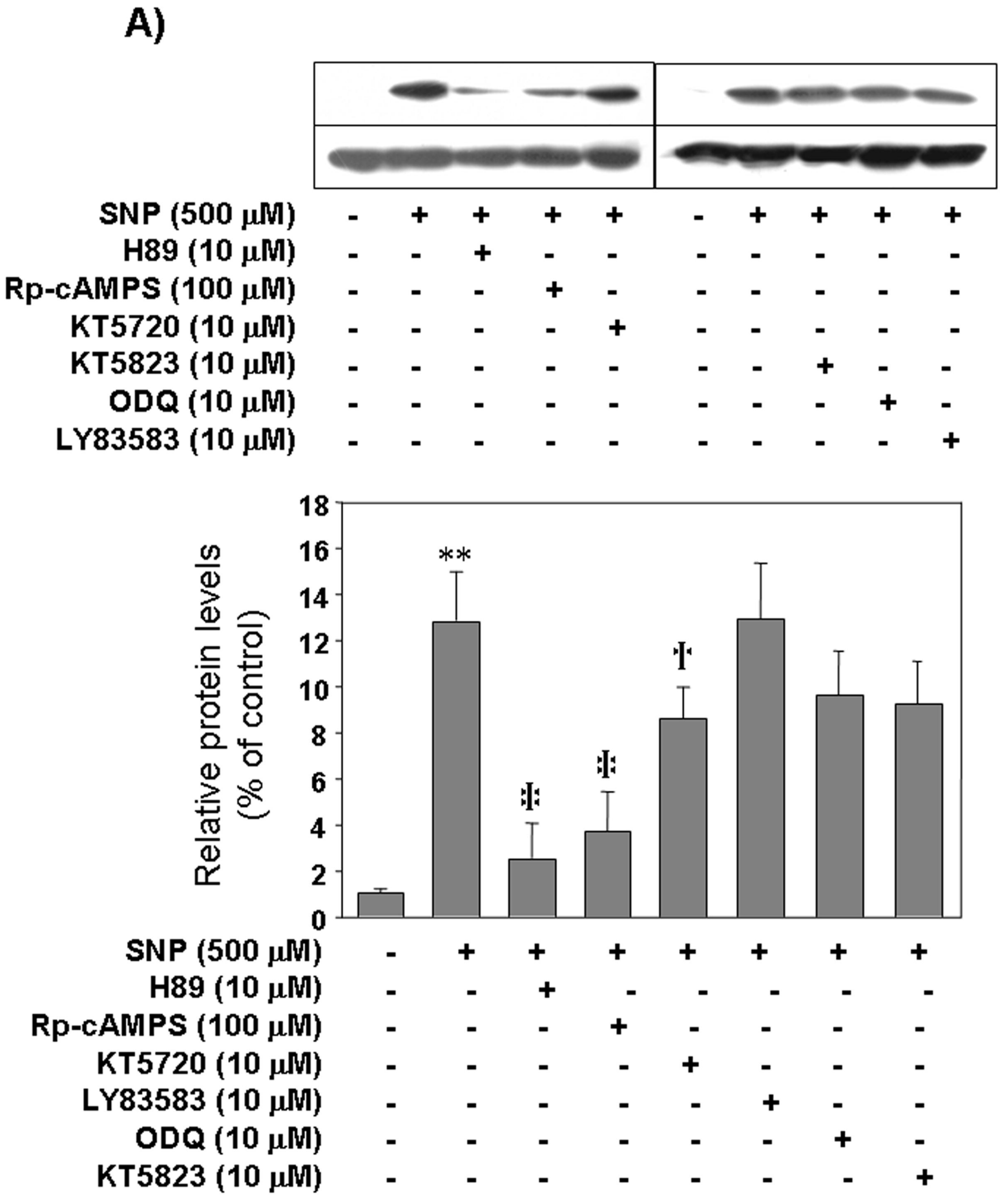

Figure 4-1

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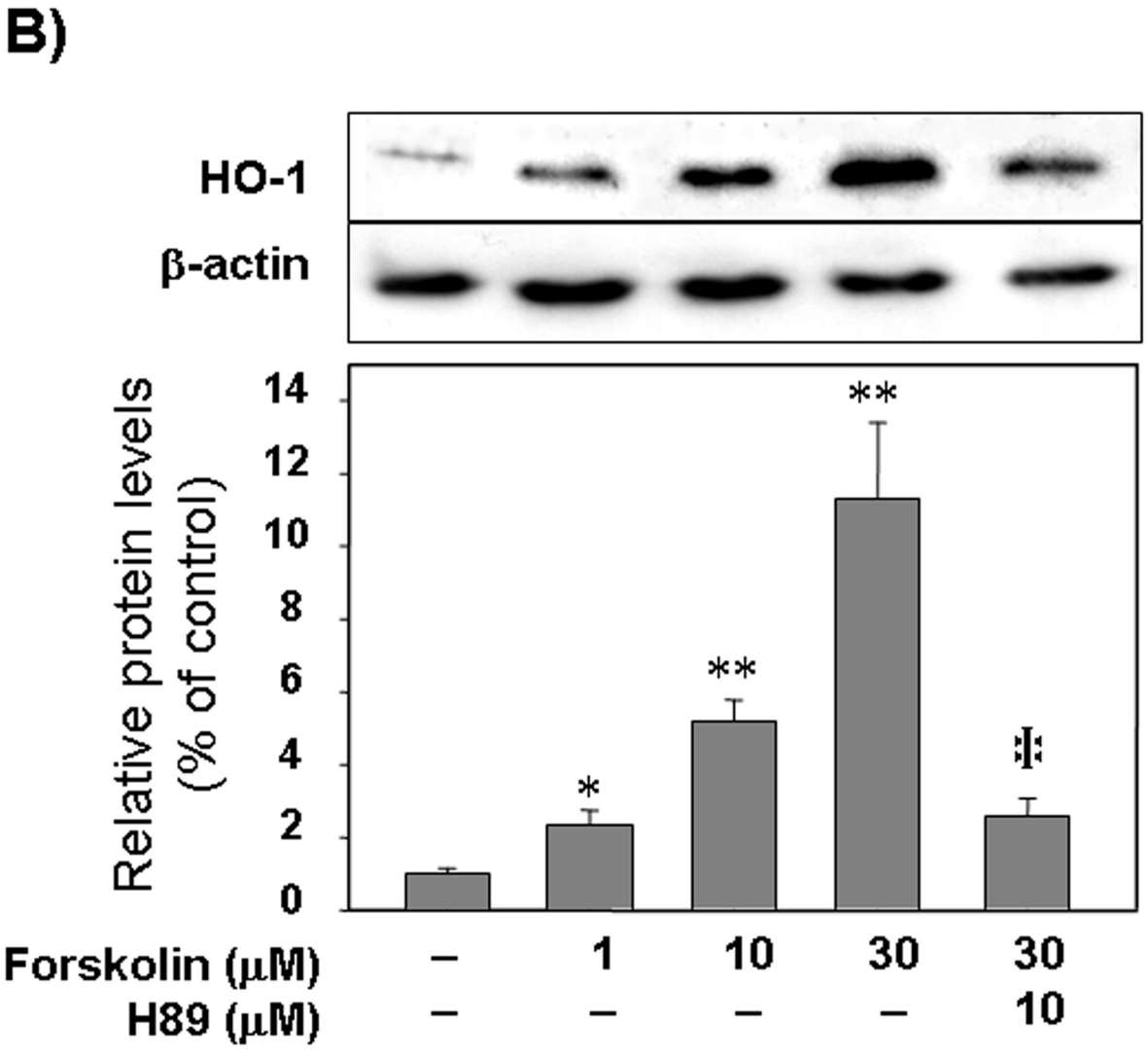

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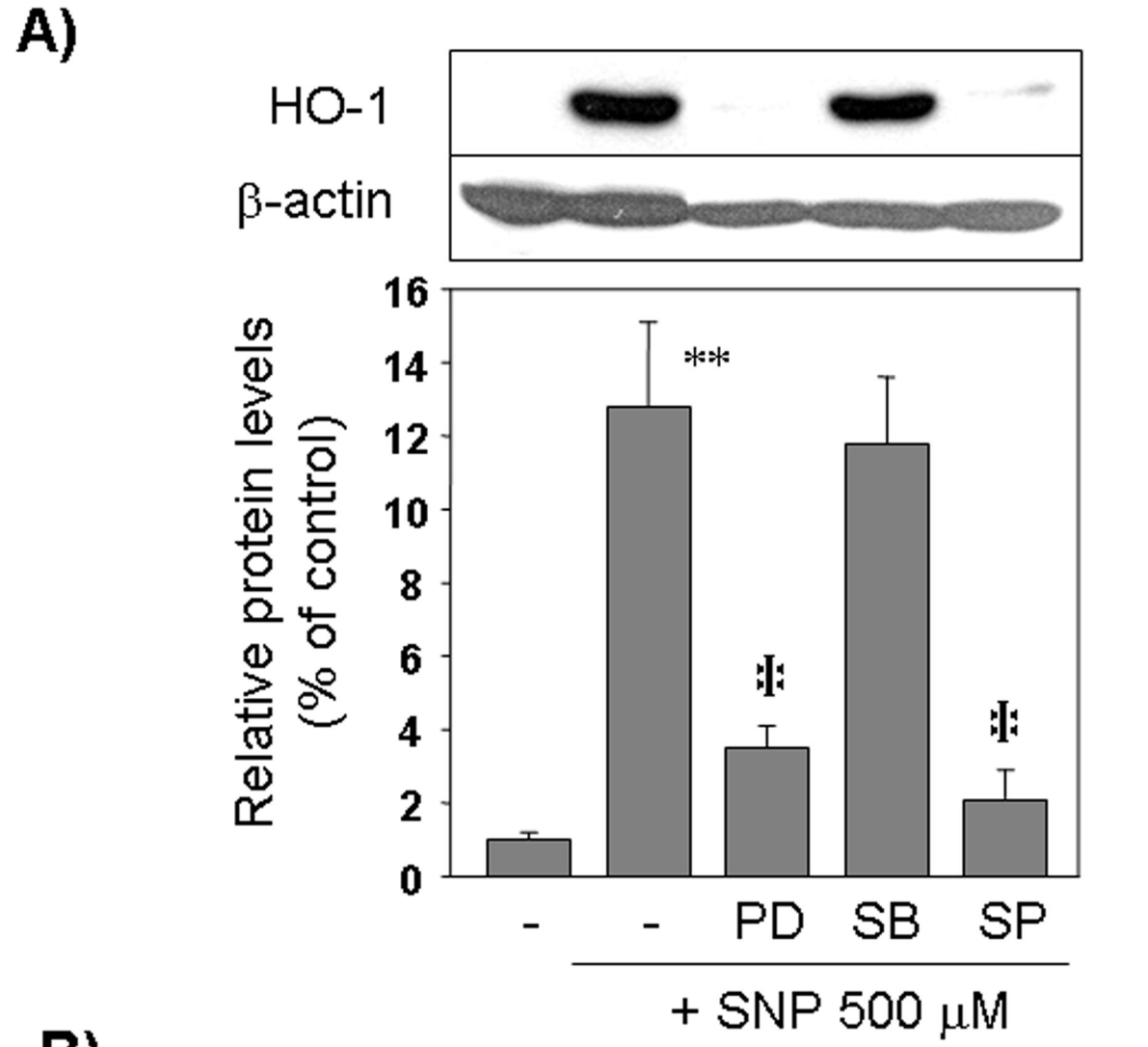

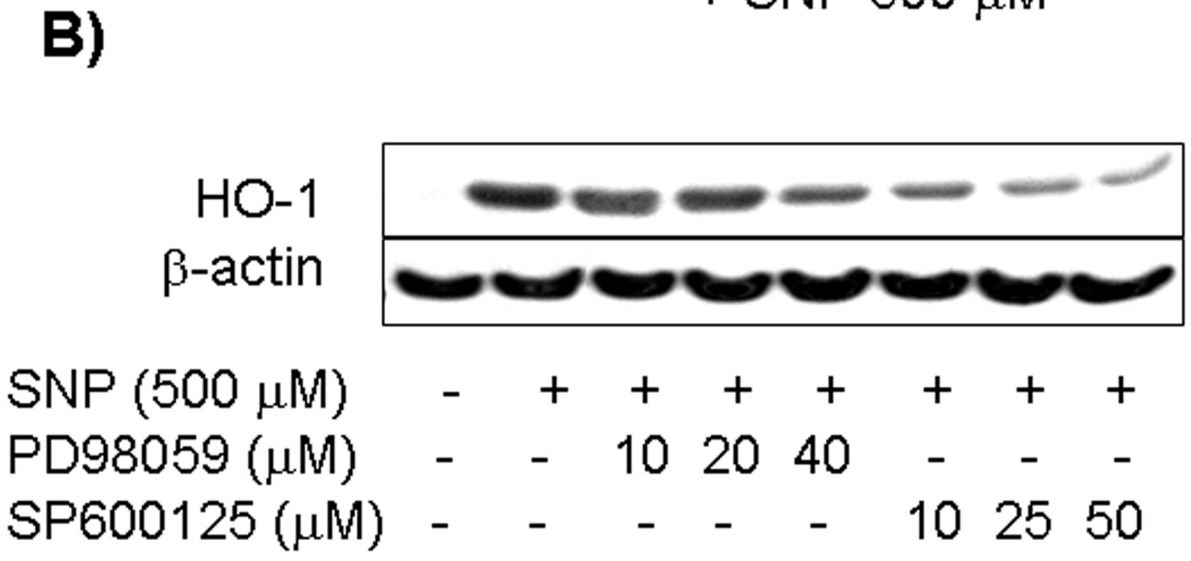

Figure 5

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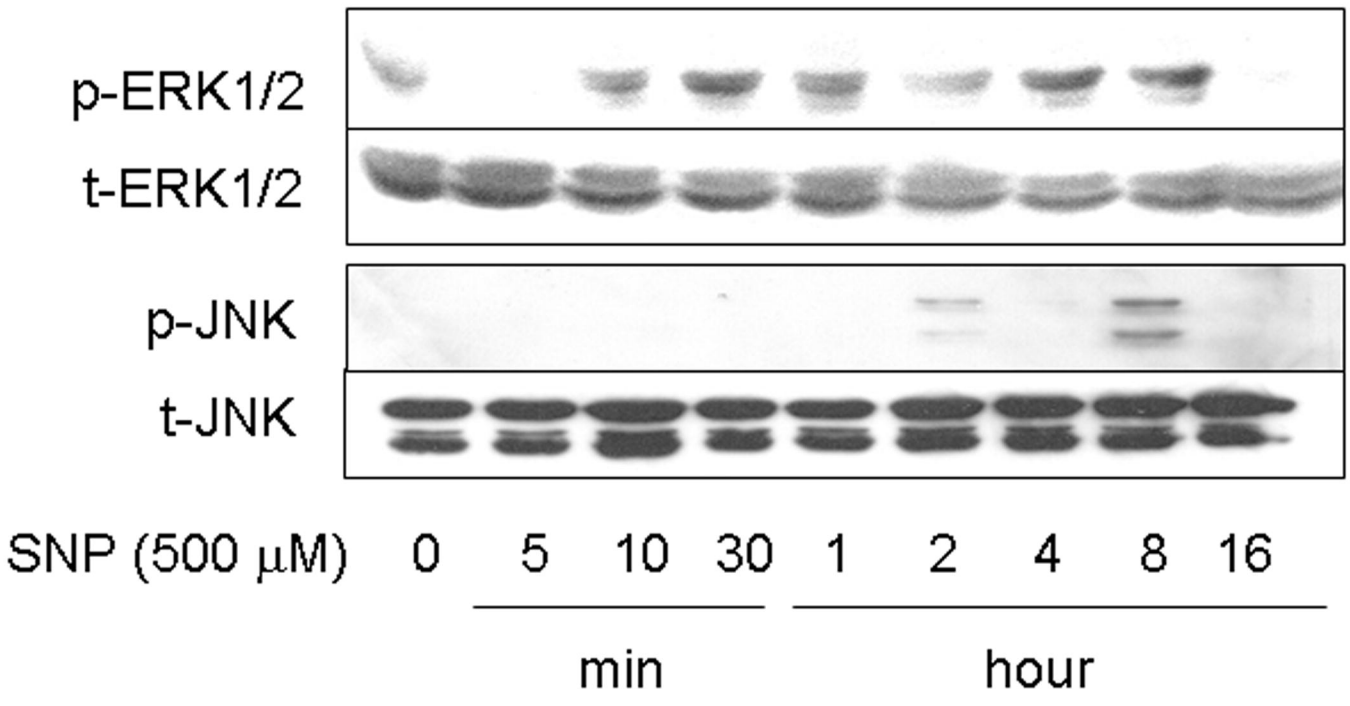

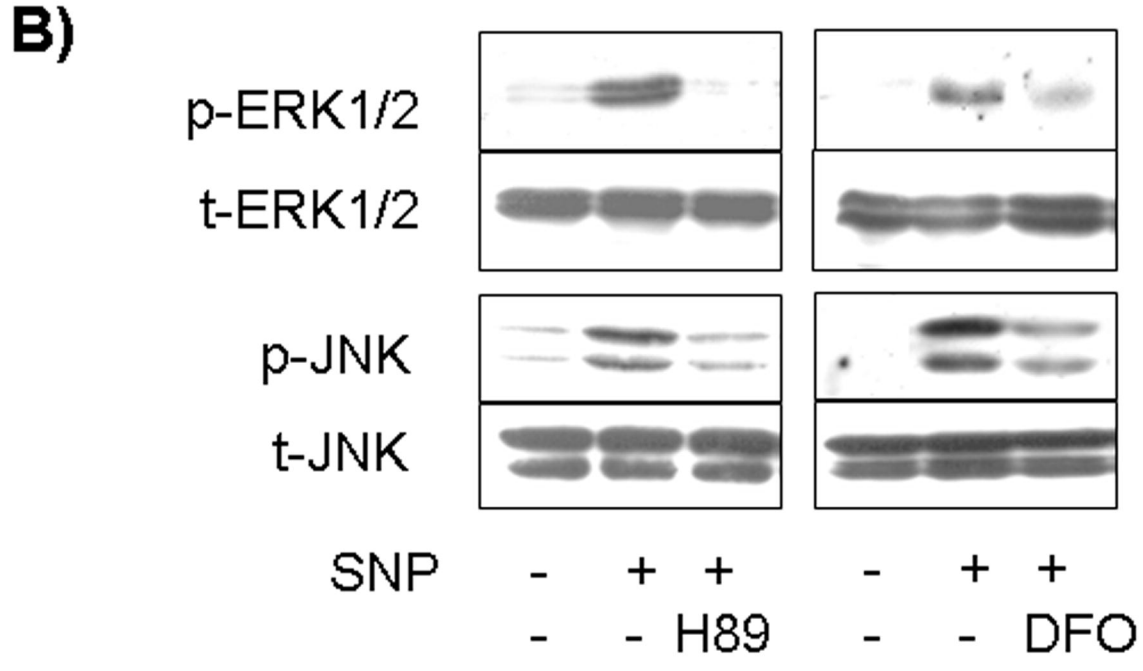

Figure 6-1

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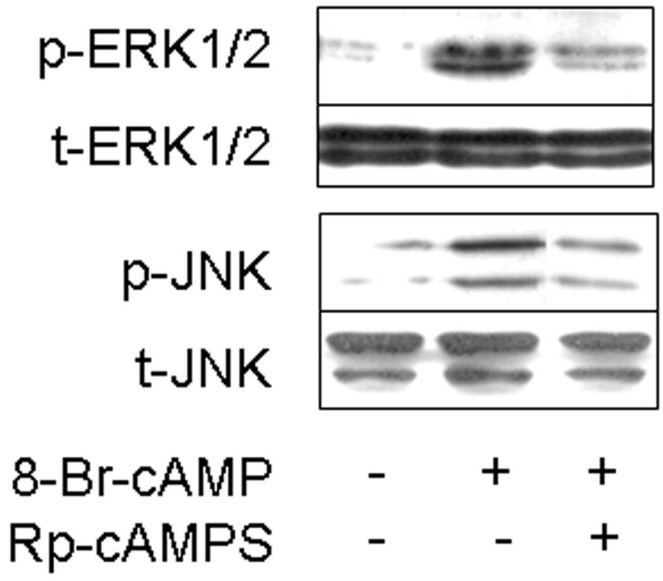

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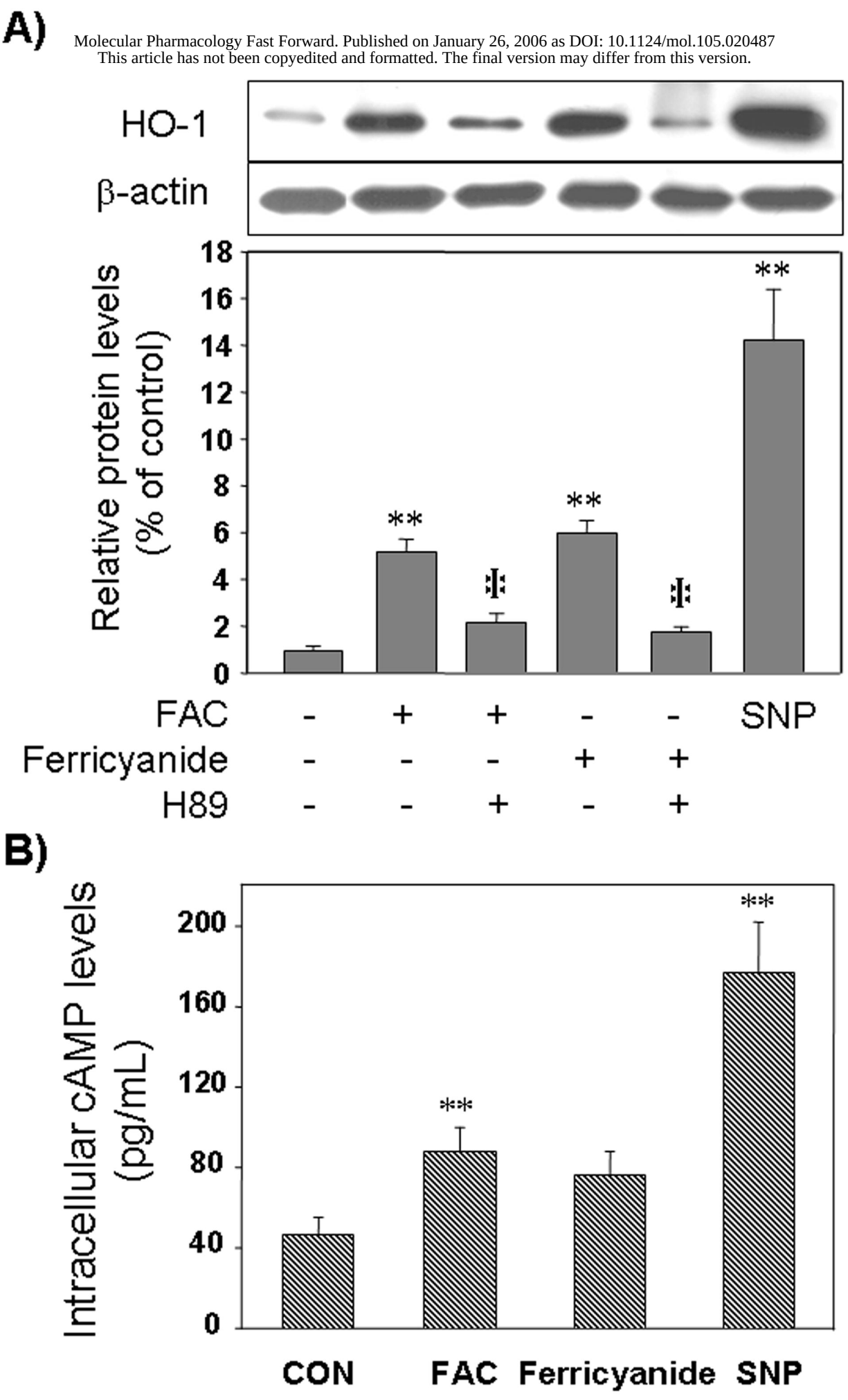

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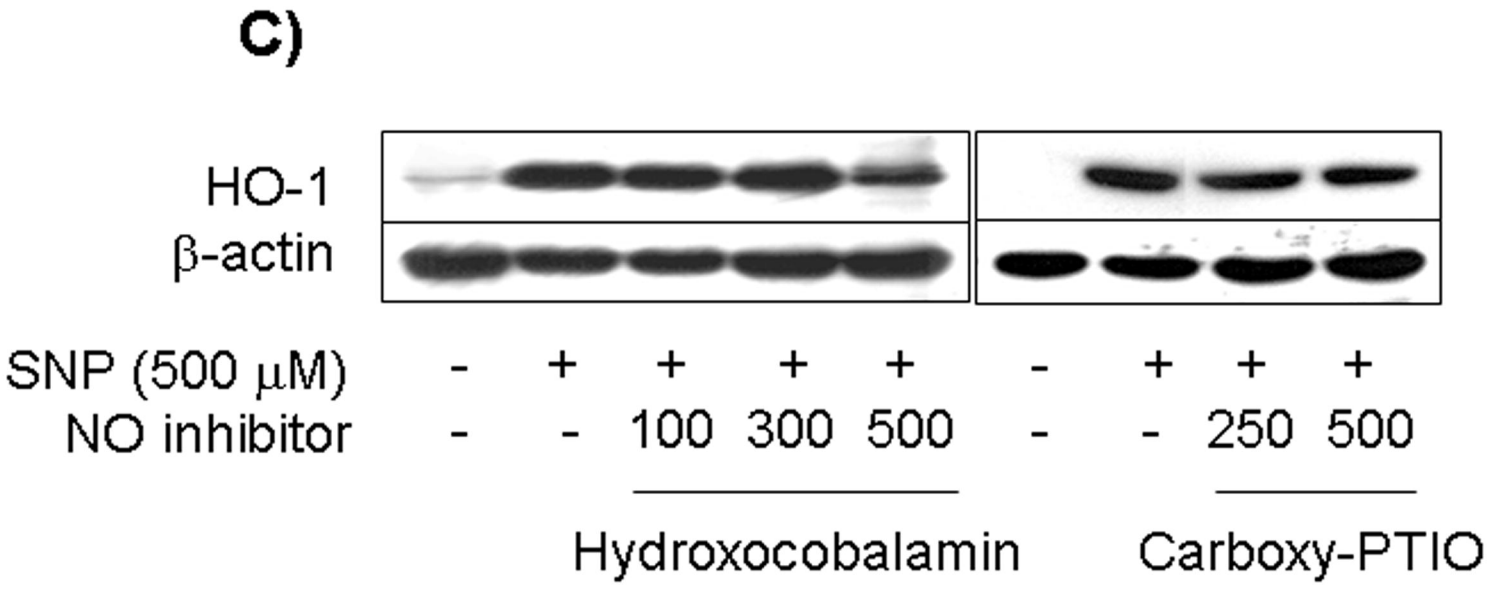

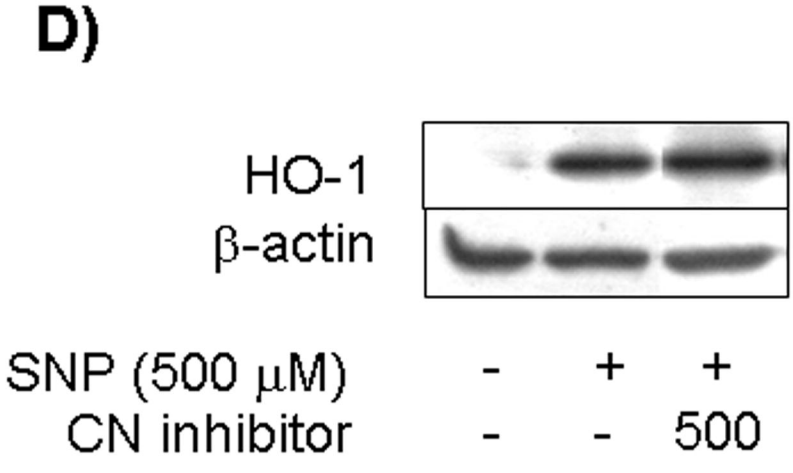

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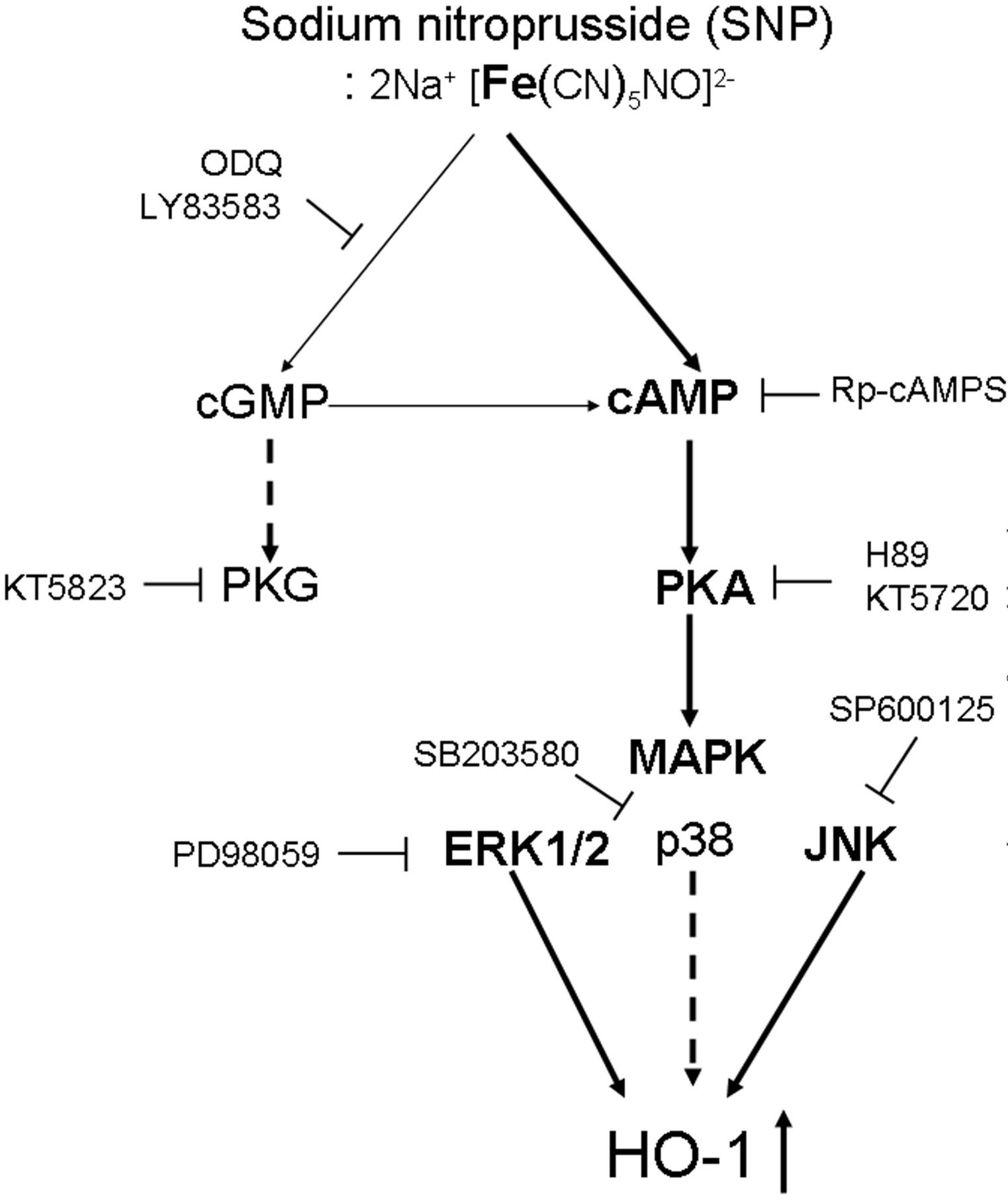

Figure 8