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Up-regulation of Heme Oxygenase-1 Expression by cAMP-elevating Agents in RAW 264.7 cells

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Abstract–Heme oxygenase-1 (HO-1) is the inducible form of the rate-limiting enzyme of heme degradation; it regulates the cellular contents of heme. HO-1 is up-regulated by various stimuli including oxidative stress so that it is thought to participate in general cellular defense mechanisms against oxidative stress in mammalian cells. To investigate the role of the cAMP-dependent protein kinase A (PKA) signaling pathway on nitrogen oxidative stress-induced HO-1 gene expression, RAW 264.7 cell cultures were treated with sodium nitroprusside (SNP). SNP increased the expression of HO-1 mRNA and protein, time- and concentration-dependently. Treatment with H89, PKA inhibitor, but not LY83583, guanylate cyclase inhibitor, significantly diminished the HO-1 expression by SNP, indicating that cAMP plays a crucial role in the induction of HO-1. Incubation with cAMP-elevating agents, such as forskolin or isoproterenol resulted in up-regulation of the expression of HO-1. Forskolin-induced expression of HO-1 was inhibited by H89. Furthermore, propranolol, β–adrenoceptor blocker, inhibited the isoproterenol-induced HO-1 expression, supporting the importance of cAMP in the induction of HO-1 expression. Higenamine-S, but not higenamineR, enhanced the HO-1 expression induced by SNP. Furthermore, cellular toxicity induced by hydrogen peroxide was attenuated by the presence of SNP, which was further increased by the presence of ZnPPIX, HO-1 inhibitor. Collectively, these results strongly suggest that up-regulation of HO-1 expression in RAW 264.7 cells involves PKA signal pathway.

Key words: Cyclic AMP, heme oxygenase-1, macrophage, oxidative stress

Heme oxygenase (HO) is the rate-limiting enzyme for the degradation of proheme IX to biliverdin, which is a precursor of bililubin, the terminal heme-degradading product. Three isoforms of HO have been identified. HO-1 is inducible (Shibahara *et al.*, 1985), whereas HO-2 and HO-3 are constitutively expressed (Trakshel *et al.*, 1986, McCoubrey *et al.*, 1997). Carbon monoxide (CO) is another HO product of the HO reaction, which has well been recognized as a physiologically important vasoactive substance rather than a toxic waste product. This gaseous monoxide is necessary to maintain microvascular patency in the liver under both unstimulated and stress conditions (Suematsu *et al.*, 2000; Kyokane *et al.*, 2001). The exact functional role of HO-1 induction in response to oxidative stress is not fully understood. However, as HO-1 provides cytoprotection in various cell culture and animal models, HO-1 gene activation is considered to be an adaptive cellular defense mechanism (Lee *et al.*, 1996, Abraham *et al.*, 1995). Over expression of the HO-1 gene has been shown to attenuate the toxic effects of heme and hemoproteins in transfected coronary endothelial cells and to protect pulmonary epithelial cells against hyperoxia (Kyokane *et al.*, 2001). The important physiological function of HO-1 has been confirmed by observation in HO-1 knockout mice (Johnson *et al.*, 1995, Christou *et al.*, 2000). Cultured fibroblast cells from these animals are highly susceptible to heme- or hydrogen peroxide-mediated toxicity (Christou *et al.*, 2000).

In addition, exposure of HO-1 deficient mice to endotoxin results in increased hepatocellular necrosis and in higher mortality from endotoxic shock as compared to control animals (Christou *et al.*, 2000). The findings in HO-1 deficient mice were essentially confirmed in human HO-1 deficiency (Johnson *et al.*, 1996). Since the specific induction of heat shock

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proteins by pharmacological stimuli has significant clinical implications (Miano *et al.*, 1996, Abraham *et al.*, 1995). Targeted induction of HO-gene expression by non-stressful stimuli may serve as a novel approach to therapeutic intervention. Higenamine (racemic mixture) showed positive inotropic and chronotropic actions in experimental animals by cardiac β-adrenoceptor activation (Chang *et al.*, 1986). In isolated cardiac tissues, higenamine increased c-AMP contents, concentration-dependently, which are inhibited by the presence of propranolol (Chang *et al.*, 1994). Recently we developed a method to synthesize optical isomers of higenamine, higenamine-S and higenamine-R (Yun-Choi *et al.*, 2001). Higenamine-S shows positive inotropic and chronotropic action but R-isomer lacks such actions (Yun-Choi *et al.*, 2001). In cultured endothelial cells, cAMP-elevating agents show to protect oxidative injury (Chang, unpublished data). One of the possible mechanisms of this can be due to over expression of HO-1 in endothelial cells. To test this hypothesis we used RAW 264.7 cells, in which HO-1 expression by cAMP-elevating agents was investigated. Furthermore cyto-protective effect was analyzed by cell survival test when oxidative stress was induced by hydrogen peroxide.

MATERIALS AND METHODS

Cell culture and stimulation

RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). RAW 264.7 cells were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphoneic acid (HEPES), 100 units/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated fetal calf serum.

Cell viability

Assay for MTT, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT to formazan (Gross SS. and Levi V, 1992). Cells in 96-well plates were incubated in a humidified 5% CO_2 incubator with MTT (1 mg/ml for 3.5 h). Culture medium was removed by aspiration and cells were solubilized in dimethyl sulfoxide (200 μl). The extent of reduction of MTT to formazan within cells was quantified by measurement of the absorbance at 570 nm.

Western blot

For Western blotting, Cells were lysed in PRO-PREP protein extract solution and sonicated. The sample was centrifuged at 13000 rpm 20 min at 4°C Protein concentration of the lysates was determined by BSA. An equal volume of 2×SDS sample buffer (0.1 M Tris-Cl, 20% glycerol, 4% SDS and 0.01% bromophenol blue) was added and the samples were boiled for 5min. The samples (70 μg) were subjected to electrophoresis on a 10% SDS-polyacylamide gel for 1.5 h at 130 V. The separated proteins were transferred to PVDF membrane for 2 h at 20 mA with Transblot SD Semi-dry Transfer Cell (Bio-Rad). Membranes were incubated for 1 h in TBS and 1% Tween 20 (TBS-T) containing 5% nonfat milk and were then incubated for 2 h with goat polyclonal antibody against HO-1 (1:1000) dilution. After three washes in TBS-T for 10 min each and were then incubated for 1h with horseradish peroxidase (HRP) conjugated a secondary antibody. The membranes were washed and then developed using a Western Blotting Luminol Reagent system.

RT-PCR

RT-PCR was performed using the RT-PCR kit (Perkin Elmer). Oligo (dT) was used as reverse transcription primers. Specific primers for HO-1 cDNA fragment were as follow; primer 1, 5CAGCATGCCCCAGGATTTGT3; primer 2, 5-ACTATGTAAAGCGTCTCCAC3 For each RT-PCR, a sample without reverse transcriptase was processed in parallel and served as a negative control. Cycling parameters for amplifying RT products were as follows: 95 for 30 sec, 54 for 40 sec, 72 for 1min, for 35 cycles and then extension at 72 for another 5 min. After amplification, PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Materials

HEPES, Boric acid, Polyclonal rabbit anti actin, Sodium bicarbonate, Sodium citrate, TEMED, Sodium nitroprusside (SNP), Tween 20, Sodium bicarbonate, Dimethylsulfoxide (DMSO) and Ethylenediaminetetraacetic acid (EDTA) were from Sigma (St. Louis, MO. USA). LY83583 and H-89 were purchased from Calbiochem, USA. Anti-goat or anti-rabbit IgG-HRP, Polyclonal goat anti-(HO-1) antibody and Western Blotting Luminol Reagent were from Santa Cruz Biotechnology, CA, USA. Fetal bovine serum, Penicillin-Streptomycin, Penicillin, RPMI 1640 and Streptomycin were purchased from GibcoBRL, NY, USA. Agarose was purchased from FMC Bioproducts, ME, USA. ECL western blotting detection system was from Amersham Life Science, Buckinghamshire,

UK. PRO-PREP (protein extraction solution) was from iNtRon Biotechnology.

RESULTS

Induction of HO-1 gene expression by SNP in RAW 264.7 cells.

In order to induce HO-1 gene expression in RAW 264.7 cells, one of reactive nitrogen species, NO, was utilized by employing SNP. RAW 264.7 cells were treated with various concentrations of SNP (10, 100, 250 and 500 mM) for 6 h and proteins were isolated. Afterward, Western blot analysis was performed. The results show that SNP induces HO-1 protein expression in RAW 264.7 cells, which was dose dependent. (Fig. 1). To characterize further as to whether the expression of HO-1 is regulated at the transcriptional level, RT-PCR was performed. As shown in Fig. 1, HO-1 mRNA was clearly expressed by SNP (500 mM), the expression of HO-1 mRNA was detected as early as 40 min and reached peak expression at 4 hour and then reduced thereafter (data not shown).

Protein kinase A pathway involves in HO-1 induction

Cyclic nucleotides are important signal molecules which are related to many gene expression. To investigate the relative importance of cyclic nucleotide in the NO-induced HO-1 expression, we used H89, a PKA inhibitor, and LY83583, a guanylate cyclase inhibitor. As shown in Fig 1, H-89, not LY83583, significantly inhibited the expression of HO-1, indicating that PKA signal pathway is involved in the regulation of HO-1 induction in RAW 264.7 cells..

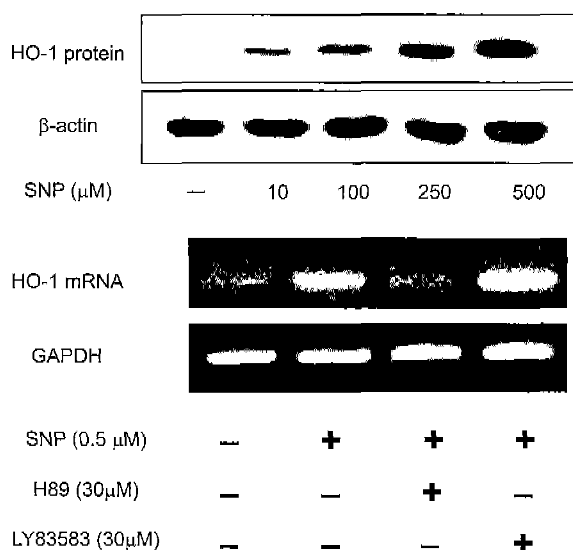

Fig. 1. Concentration-dependent induction of HO-1 protein by SNP in RAW 264.7 cells. Cells were exposed to different concentrations of SNP (0, 10, 100, 250 and 500 μM) for 6 h (upper panel). Effects of PKA inhibitor, H89, and soluble guanylate cyclase inhibitor, LY83583, on HO-1 mRNA expression by SNP (lower panel). Cells were pretreated with H89 or LY83583 for 30 min before incubation with SNP.

Effect of forskolin on HO-1 protein expression.

To understand the relationship between the HO-1 inductions and cAMP directly, RAW 264.7 cells were treated with forskolin (1, 10 and 30 μM) for 6 h, in which H-89 (30 μM) was added 30 min before treatment with forskolin. The result showed that expression of HO-1 protein was up-regulated in a concentration-dependent manner, an effect which was inhibited by H89 (Fig. 2) supporting that PKA signal pathway may be the common route for induction of HO-1 regardless of the stimulant, NO or cAMP.

Effect of isoproterenol and propranolol on HO-1 protein expression.

To confirm further that cAMP can induce the expression of HO-1, cells were treated with isoproterenol (1, 10 and 100 μM) for 6 h, and in some experimental settings propranolol was introduced. Western blot analysis showed that isoproterenol induced HO-1 protein expression in a concentration-dependent manner, although the intensity of the expression

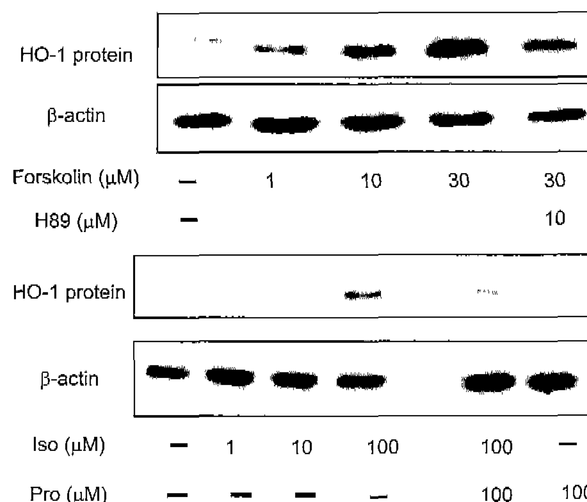

Fig. 2. Induction of HO-1 protein by cyclic AMP-elevating agents, forskolin, higenamine-S and isoproterenol. Forskolin, concentration-dependently increased expression of HO-1, which was inhibited by H89 (upper). Isoproterenol expressed HO-1 protein concentration-dependent manner, which was inhibited by propranolol (lower).

was smaller than that of forskolin. The enhanced expression was antagonized by a β-antagonist propranolol (100 μM), confirming that cAMP is important in the induction of HO-1 at least in RAW 264.7 cells (Fig. 2.)

Effect of higenamine on HO-1 protein expression.

As we are trying to develop more potent and selective β-adrenoceptor acting drug among the isoquonoline alkaloids, we recently established the method to synthesize the optical isomers of isoquinoline alkaloids. When compared the pharmacological effects on β-adrenoceptor activating action, higenamine-S isomer is more selective than R-isomer in cardiac muscles (Chang *et al.*, 2001). We therefore investigated whether higenamine isomers differently affect the expression of HO-1, because S-isomer is related to cAMP but R-form is not. As shown in Fig. 3, S-isomer but not R-isomer, up-regulated the expression of HO-1 in NO-stress stimuli. Higenamine, furthermore, is a potent antioxidant which is almost equipotent as glutathione in quenching the peroxinitrite radicals (Lee *et al.*, 2001). To investigate whether the induction of HO-1 by higenamine was related to antioxidant action, PDTC was employed, where PDTC did not affect SNP-induced HO-1, indicating that antioxidant action can be ruled out for the induction of HO-1. Addition of higenamine-S along with SNP enhanced the expression of HO-1, which was also concentration-dependent as shown in Fig. 4.

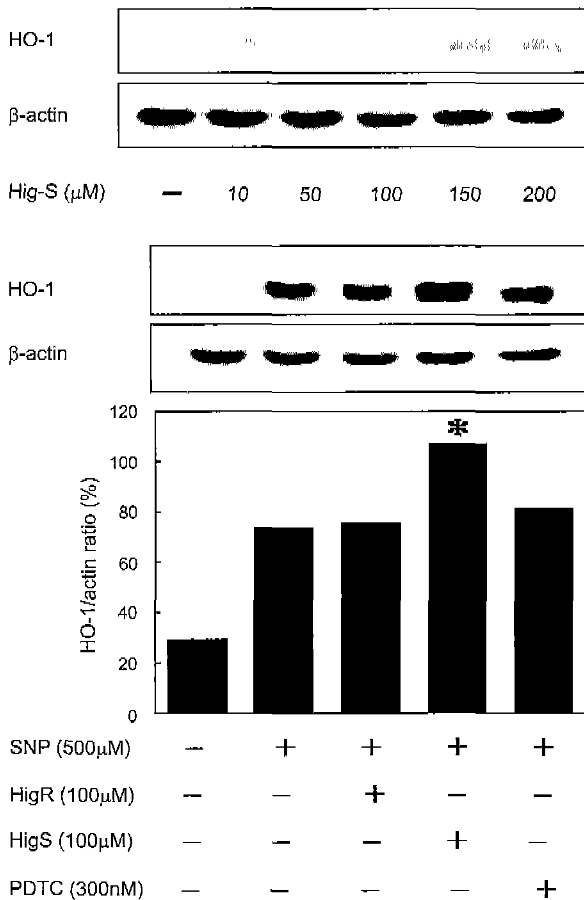

Fig. 3. Effects of higenamine isomers (R and S) and PDTC on SNP-induced HO-1 protein expression. Higenamine-S increased HO-1 protein concentration-dependently (upper). Higenamine-S significantly augmented the expression of HO-1 protein by SNP, but higenamine-R and PDTC were without effect (lower). *indicates significantly different (P<0.05) from treatment with SNP alone.

Cytoprotection effect of SNP from oxidant injury

Finally, we tested the cell survival rate by MTT assay in the presence of HO-1 inducing agent, SNP, or HO-1 inhibitor, ZnPPIX, where high concentration of hydrogen peroxide was incubated for 6 h. Results indicated that SNP concentration-dependently increased the survival rate. In contrast, in the presence of ZnPPIX, the increased cell survival rate by the same concentration of SNP was significantly decreased (Fig. 5). Higenamine and forskolin also increased the survival rate in hydrogen peroxide-induced cellular injury (data not shown).

DISCUSSION

The present study showed that for the first time that HO-1

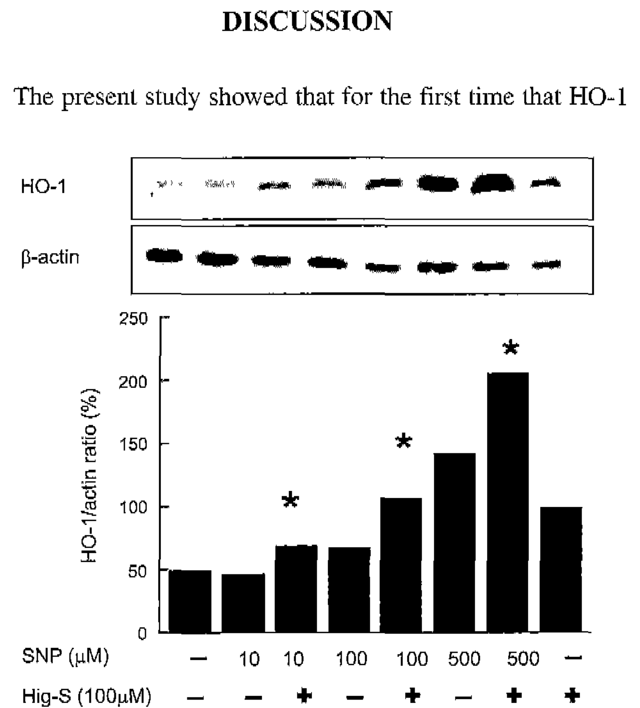

Fig. 4. Comparison of HO-1 protein expression by SNP in the presence or absence of different concentrations of higenamine-S. Higenamine-S, concentration-dependently up-regulated the SNP-induced HO-1 protein expression. *indicates significantly (P<0.05) different from each corresponding controls.

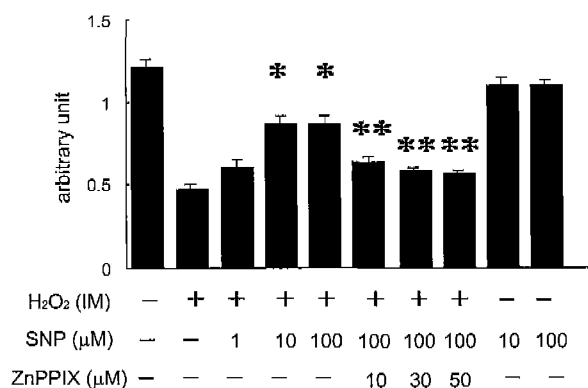

Fig. 5. Protective effects of SNP by hydrogen peroxide-induced cellular injury. Cells were treated high dose of hydrogen peroxide for 6 h, in which different concentrations of SNP were pretreated and cell viability was measured by MTT. In some cases, ZnPP, HO-1 enzyme inhibitor, also co-incubated with SNP 30 min before H_2O_2-treatment. *, and ** indicate that significantly different from H_2O_2-treated controls at $P<0.05$ and $P<0.01$, respectively.

gene was up-regulated in RAW'264.7 cells by way of PKA signal pathway. A protective role of HO-1 has been reported in vitro for a variety of stress inducers such as hemoglobin, hypoxia, glutathione depletion, cytokines. NO is known to induce HO-1 in many cells. Induction of HO-1 gene by SNP in RAW 264.7 cells was not affected by soluble guanylate cyclase inhibitor LY83583 or ODQ (data not shown) but was inhibited by a PKA inhibitor, H89. The present results raise a question how NO released from SNP activates PKA signal pathway. Some report, however, showed that induction of HO-1 mRNA by NO is independent of cGMP signaling pathway in smooth muscle cell. At the present time, we can not explain how NO signal relates with PKA pathway. Or there may be some other molecule than NO can be released from SNP, such as Fe, or CN, which may contribute to induction of HO-1. In the present study we would like to focus on the cAMP and PKA signal pathway on the HO-1 induction in RAW 264.7 cells. To investigate the involvement of cAMP on HO-1 expression, we used forskolin, isoproterenol and higenamine. Forskolin, cAMP activator, clearly up-regulated HO-1 expression in RAW 264.7 cells, indicating that cAMP by itself, not stressful stimuli, can induce HO-1 expression in RAW 264.7 cells like as in hepatocyte cells (Immenschuh *et al.*, 1998). To clarify the signal pathway up-regulating HO-1 by forskolin relates to PKA, the effect of H89 was investigated. As with SNP-induced HO-1 suppressed by H89, forskolin-stimulated up-regulation of HO-1 also reduced by H89, further supporting that PKA pathway is important in expression of HO-1. It has been reported that expression of HO-1 gene was via protein kinase C (PKC) or prostaglandin or via activator protein-1 (AP-1) (Camhi *et al.*, 1998), and via mitogen-activated protein kinases, ERK and p38, in Hela cells (Chen *et al.*, 2000). Therefore, the induction of HO-1 gene by PKA signaling pathway in RAW 264.7 cells seems to be a cell type-specific response. The cAMP-dependent induction of the HO-1 gene is of physiological and pharmacological significance for several reasons. The increase of HO-1 activity and mRNA expression seems to be a protective response against oxidative stress in *in vivo* (Applegate *et al.*, 1991) and *in vitro* (Lautier *et al.*, 1992). The cytoprotective effect of HO-1, the toxicity caused by heme and hemoglobin was attenuated efficiently when the HO-1 gene was over-expression in coronary endothelial cell cultures (Ahmed *et al.*, 2000). Therefore, the induction of the HO-1 gene may be significant for the general endogenous cellular protection during inflammation (Ahmed *et al.*, 2000). Higenamine was reported to inhibit NO production and expression of inducible nitric oxide syntase (iNOS) in endotoxin-stimulated RAW 264.7 cells and also posseses an antioxidant action (Kang *et al.*, 1999, Lee *et al.*, 2001). Recently, we developed a method to synthesize optical isomers of higenamine and effect of each isomer on various cell systems is actively investigating by us. So far, in general, S-isomer is more potent than R-isomer in anti-platelet aggregation and inhibition of NO production. The most strikingly different action is on cardiac tissue. The S-form activates cardiac β-adrenoceptor in isolated rat atrial tissue thereby accelerating heart rate and positive inotropic effect, while the R-isomer is lacking such activity (Yun-Choi *et al.*, 2001). To investigate the effect of higenamine-S on HO-1 expression in RAW 264.7 cells, treatment with higenamine alone or with SNP was made. Higenamine-S concentration-dependently increased HO-1 expression, which effect was clearly demonstrated in the presence of SNP. In addition, this synergic effect was inhibited by PKA inhibitor (data not shown), further confirming that PKA signal pathway is operating in this system. Although we did not measure cAMP content in the present experiment, higenamine-R isomer did not induce HO-1 expression in the presence of SNP, which can be explained why R-isomer shows no cardiac stimulatory effect. At the present time the functional significance of HO-1 expression is not clear. However, many reports indicate that HO-1 plays some role in cellular adaptive defense mechanisms. The present results indicate that oxidant-injury by hydrogen peroxide was attenuated by the presence of SNP, which was more

deteriorated by the presence of ZnPPIX, HO-1 inhibitor. Of course, higenamine and forskolin protected hydrogen peroxide-induced cellular toxicity (in preparation). Based on the result of PDTC on HO-1 expression, antioxidant chemical may not induce HO-1 expression, so protective effect of higenamine on oxidant injury can be expected through its antioxidant action and HO-1 induction. In conclusion, present study provides evidence that expression of HO-1 gene in RAW 264.7 cells is regulated by PKA signaling pathway. Since the specific induction of HO-1 by pharmacological stimuli has significant clinical implications, targeted induction of HO-1 gene expression by non-stressful stimuli, such as higenamine-S, may serve as a novel approach to therapeutic intervention.

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