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Protection of Primary Cultured Mouse Hepatocytes from Chemical Hypoxia-induced Injury by Hydrogen Sulfide

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We examined the effect of hydrogen sulfide (H_2S) in chemical hypoxia-induced injury in mouse hepatocytes. Cell viability was significantly decreased by cobalt chloride ($CoCl_2$), a well-known hypoxia mimetic agent in a time- and dose- dependent manner. Sodium hydrosulfide (NaHS, a donor of H_2S) pretreatment before exposure to $CoCl_2$ significantly attenuated the $CoCl_2$-induced decrease of cell viability. $CoCl_2$ treatment resulted in an increase of intracellular ROS generation, which is inhibited by NaHS or *N*-acetyl-cysteine (NAC, a ROS scavenger), and p38 MAPK phosphorylation, which is also blocked by NaHS or NAC. The $CoCl_2$-induced increase of the Bax/Bcl-2 ratio was attenuated by NaHS, NAC, and SB 203580 (p38 MAPK inhibitor). The $CoCl_2$-induced decrease of cell viability was also attenuated by NaHS, NAC, and SB 203580 pretreatment. Additionally, NaHS inhibited the $CoCl_2$-induced COX-2. Similar to the effect of NaHS, NAC blocked $CoCl_2$-induced COX-2 expression. Furthermore, NS-398 (a selective COX-2 inhibitor) attenuated not only the $CoCl_2$-induced increase of the Bax/Bcl-2 ratio, it also decreased cell viability. Taken together, H_2S protects primary cultured mouse hepatocytes against $CoCl_2$-induced cell injury through inhibition of the ROS-activated p38 MAPK cascade and the COX-2 pathway.

Key words : Hydrogen sulfide, cobalt chloride, chemical hypoxia, mouse hepatocytes

Introduction

Hydrogen sulfide (H_2S), an endogenous gaseous mediator, is produced by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine-γ-lyase, cystathionine-β-synthase and 3-mercaptopyruvate sulfurtransferase, during cysteine metabolism [6, 46]. H_2S was known as a toxic gaseous material, but it is now a signaling gasotransmitter, which exerts physiological or pathological roles both *in vivo* and *in vitro* [36, 53]. H_2S promotes vascular smooth muscle relaxation and induces vasodilation of isolated blood vessels [9, 32]. It has been also evident that H_2S exerts their role as a potent antioxidant [27, 60]. Additionally, previous studies show that H_2S shows a protective role against various stimuli-triggered injuries in many organs including heart, kidney and so forth [3, 49]. However, the mechanisms of H_2S attenuated hepatic ischemic injury remains to be elucidated.

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Hepatic tissue is quite vulnerable to hypoxic injury compared with other organs [34]. In general, hypoxia generates reactive oxygen species (ROS) and excessive ROS generation is known to cause oxidative damage to DNA, lipids and proteins [5, 52]. This results in hypoxic cell injury, which is mediated by ROS-induced intracellular signaling molecules, including mitogen-activated protein kinases (MAPKs) [10]. MAPKs participate in varioius cellular functions such as cell proliferation, cell differentiation, cell motility, and cell death [37]. There are three major MAPK family subgroups: extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun *N*-terminal of stress-activated protein kinase (JNK), and p38 protein kinase. Especially, signal cascade involving p38 MAPK, activated by extracellular stress signals, are involved in cell differentiation and death and is known to be activated by a variety of environmental stresses or chemicals [40, 55]. Previously, it was reported that $CoCl_2$ activates p38 MAPK, which is involved in $CoCl_2$-induced apoptosis in other cell types [65]. These findings suggest that ROS-induced p38 MAPK can be involved in $CoCl_2$-induced hepatic injury. It has also been reported that ROS can stimulate cyclooxygenase (COX)-2 expression in $CoCl_2$-induced chemical hypoxic condition [56, 58]. At present, at least two COX isoenzymes, COX-1 and COX-2, have been identified [59].

COX-1 is usually expressed constitutively, whereas COX-2 remains undetectable in most tissues in the physiological state but rapidly accumulates under pathological conditions [11]. The roles of COX-2 overexpression are complicated and ambivalent in different models, mediating either cytotoxicity or cytoprotection [4, 7, 21, 28, 50, 62]. Some studies indicated that inhibition of COX-2 activation attenuated ischemic hepatic injury [21] while other reports showed that during ischemic cardiotoxicity, overexpression of COX-2 play its protective role [4, 28].

In the present study, we investigated the cytoprotective effect of H_2S in primary cultured mouse hepatocytes treated with cobalt chloride ($CoCl_2$), a widely used hypoxia mimetic agent that promotes the accumulation of hypoxia-inducible factor-1 α (HIF-1α), a critical regulator for the cellular response to hypoxia [18], and induces oxidative stress [23, 64] and apoptosis in various cells [26, 64]. Therefore, this study assessed the effect of H_2S on hypoxic cellular injury induced by $CoCl_2$ and its associated mechanisms in primary cultured mouse hepatocytes.

Materials and Methods

Materials

Eight-week-old male ICR mice were purchased from Daehan Bio Link Co. (Incheon, Korea). All animal management procedures were conducted in accordance with the standard operation protocols established by Kyungpook National University. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan). $CoCl_2$, *N*-acetyl-l-cysteine, SB 203580, and type IV collagenase were obtained from Sigma-Aldrich (St. Louis, USA). The 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) were purchased from Life Technologies (Grand Island, USA). Fetal bovine serum (FBS) was acquired from Thermo scientific (USA). The phospho-p38 MAPK antibodies were obtained from New England Biolabs (Hertfordshire, UK). The HIF-1α, Bcl-2, caspase-3, COX-1, COX-2, goat anti-rabbit IgG, and goat anti-mouse IgG antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, USA).

Isolation of mouse hepatocytes

Primary mouse hepatocytes were isolated from mouse liver using the two-step EDTA and collagenase perfusion method. After the mouse was anesthetized, the liver was perfused with Krebs-Henseleit buffer without Ca^{2+} and SO_4^{2-} (115 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 1.18 mM $MgCl_2$, 1.23 mM NaH_2PO_4, 6 mM glucose, 0.1 mM EDTA) through the hepatic portal vein to rinse the blood out (flow: at 7~9 ml/min for 5 min). Then, the liver was perfused with Krebs-Henseleit buffer without Ca^{2+} and SO_4^{2-} containing 0.02% collagenase and 0.1 mM $CaCl_2$ until the liver appeared soft. The liver was then removed and gently minced, and the obtained cells were dispersed in medium (DMEM; Life Technologies, NY, USA) containing 10% FBS and 1% penicillin/streptomycin (Life Technologies, NY, USA). The solution containing the mixed cells and debris was filtered through a 100-μm filter. Subsequently, the filtrate was centrifuged at 50 *g* for 3 min at 4°C; the cells were washed with DMEM three times and then seeded in collagen-coated plates. The cells were maintained in DMEM high glucose (4.5 g/l) supplemented with 10% FBS, 1% penicillin/streptomycin, 1 μg/ml insulin, and 10^{-12} M dexamethasone for 24 h at 37°C in a humidified atmosphere (5% CO_2). The cells were incubated with fresh Williams' E medium without FBS 24 h prior to the experiments.

Cell viability assay

Cell viability was detected using CCK-8 assay system. Mouse hepatocytes were cultured in 96-well plates with three triplicate wells in each group. The cells were treated with conditioned medium as indicated. The CCK-8 solution was added to each well at a 1:10 dilution followed by further incubation at 37°C for 3 h. Absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., VT, USA). All values are expressed as the mean (± standard error, SE) of triplicate experiments. The values were converted from absolute counts to a percentage of the control.

Western blot analysis

The cell homogenates (30 μg of protein) were separated using 10% or 12% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) transfer membranes. The blots were then washed with Tris-buffered solution containing Tween-20 (TBST, 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween-20), blocked with 5% skimmed milk powder in TBST for 1 h, and incubated for 12 h with the appropriate primary antibody at the dilutions as recommended by the supplier (1:1,000). The membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody

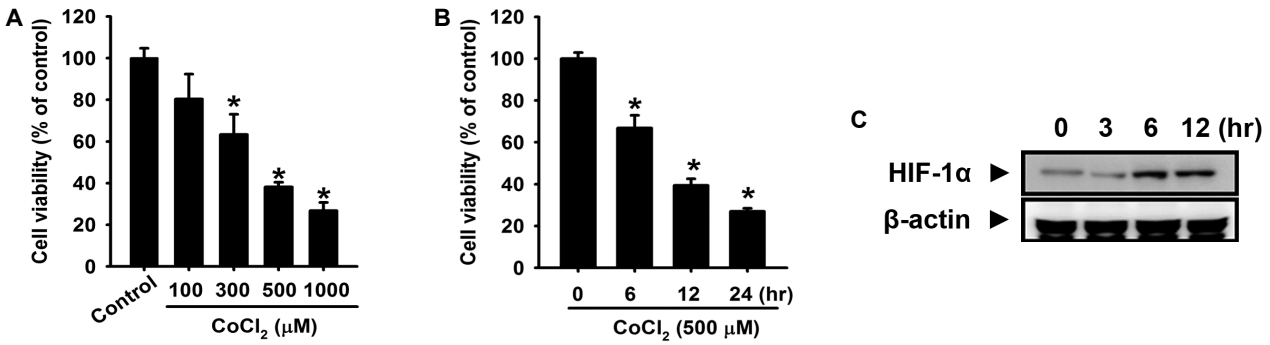

Fig. 1. Effect of $CoCl_2$ in cell viability in primary cultured mouse hepatocytes. (A) Mouse hepatocytes were incubated with $CoCl_2$ at indicated concentrations for 12 h. (B) Cells were incubated with 500 μM $CoCl_2$ for various times (0-24 h). Cell viability was measured by CCK-8 assay. (C) Cells were incubated for various times (0-12 h), and the level of cellular HIF-1α expression was detected through western blotting. The values are expressed as the mean ± SE of three independent experiments with triplicate dishes. *p<0.05 vs. control.

(1:5,000) for 12 h. The bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, USA) according to the manufacturer's protocols.

Detection of intracellular ROS

CM-H_2DCFDA (DCF-DA), which functions as a ROS-sensitive fluorophore, was used to detect the intracellular ROS. The cells were plated on 35-mm cell culture dishes and incubated under the conditions as described previously. The cells were then kept in the dark and treated with 5 μM DCF-DA for 30 min at 37°C. After all the treatments were completed, the cells were washed three times with PBS and were imaged using fluorescence microscopy (100×; DM IL LED Fluo, Leica, Germany).

Statistical analysis

The results are expressed as the mean ± SE. The difference between the two mean values was analyzed by Student's t test. A P value of <0.05 was considered significant.

Results

H_2S inhibits $CoCl_2$-induced cytotoxicity in primary cultured mouse hepatocytes

To investigate the effect of $CoCl_2$, the level of cell viability was examined with the CCK-8 assay system. As shown in Fig. 1A, $CoCl_2$ significantly decreased the level of cell viability in a time-dependent manner. Fig. 1B shows that treatment of $CoCl_2$ to mouse hepatocytes at concentrations ranging from 100 to 1,000 μM for 12 h led to a decrease in cell viability in a dose-dependent manner. Additionally, the effect of $CoCl_2$ treatment on the level of hypoxia-inducible factor-1α (HIF-1α) expression was analyzed in order to verify whether the hypoxic cell responses were induced by $CoCl_2$. The expression of HIF-1α was increased in a time-dependent manner (Fig. 1C). To determine the effect of hydeogen sulfide (H_2S) on $CoCl_2$-induced cell injury, cells were pretreated with various concentrations of sodium hydrosulfide (NaHS, a donor of H_2S) for 30 min and then treated with 500 μM $CoCl_2$. NaHS significantly attenuated the decreased cell viability and these results indicate that NaHS pretreatment protects against $CoCl_2$-induced hypoxic cell injury in primary cultured mouse hepatocytes (Fig. 2).

Effect of H_2S on $CoCl_2$-induced oxidative stress and p38 MAPK in primary cultured mouse hepatocytes

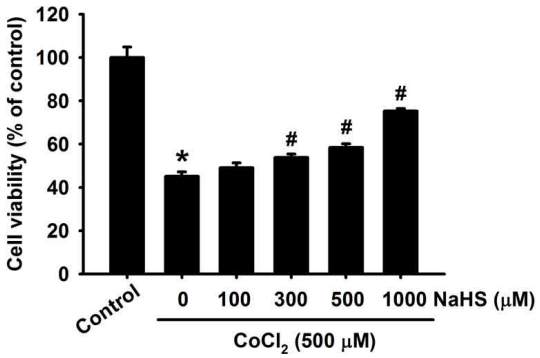

Fig. 2. Effect of H_2S on $CoCl_2$-elicited cytotoxicity. Mouse hepatocytes were incubated with 500 μM $CoCl_2$ for 12 h in the absence or presence of pretreatment with NaHS at the indicated concentrations for 30 min. Cell viability was measured by CCK-8 assay. The values are expressed as the mean ± SE of three independent experiments with triplicate dishes. *p<0.05 vs. control, $^{\#}p$<0.05 vs. $CoCl_2$ only.

In order to determine whether the H_2S-induced protective effect was associated with its antioxidation in $CoCl_2$-treated mouse hepatocytes, intracellular reactive oxygen species (ROS) levels were observed. As shown in Fig. 3A, $CoCl_2$-increased ROS were attenuated by NaHS (1,000 μM) or *N*-acetyl cysteine (NAC), a common ROS scavenger (1,000 μM) . In addition, the signaling molecule associated with $CoCl_2$-induced cell injury, p38 MAPK, was elevated. The maximum level of p38 MAPK activation was observed at 90-120 min after treatment with $CoCl_2$ (Fig. 2B), and NaHS and NAC attenuated $CoCl_2$-induced phosphorylation of p38 MAPK (Fig. 3C). Furthermore, we observed that pretreatment with NaHS, NAC, and SB 203580 (p38 MAPK inhibitor, 1 μM) obviously attenuated the $CoCl_2$-induced decrease of Bcl-2 expression and increase of Bax expression. Additionally, increase of Bax/Bcl-2 ratio by $CoCl_2$ treatment was significantly decreased by NaHS, NAC and SB 203580 (Fig. 3D). Consistent with these results, decreased cell viability induced by $CoCl_2$ treatment was partially recovered by pretreatment with NaHS, NAC, and SB 203580 (Fig. 3E).

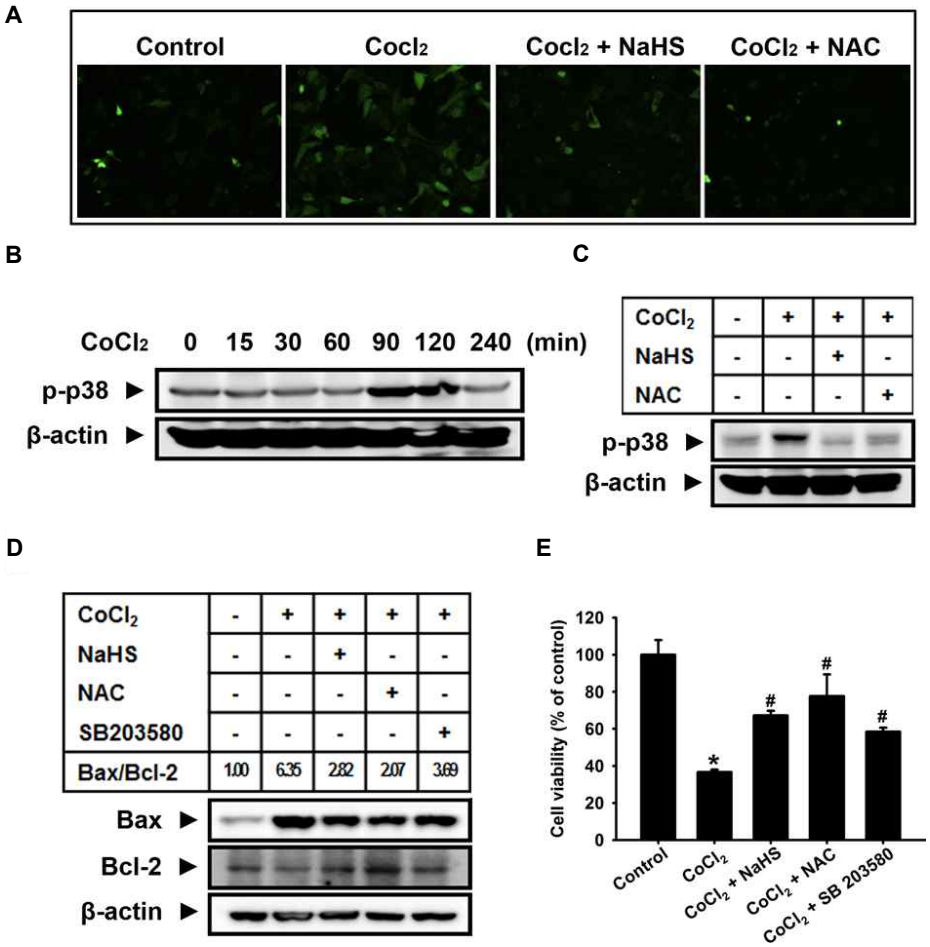

Fig. 3. Effect of H_2S on the $CoCl_2$-induced ROS generation, p38 MAPK activation, and cell injury. (A) Dichlorofluorescein (DCF)-sensitive cellular ROS was assessed. (a) Control. (b) Treatment with 500 μM $CoCl_2$ for 2 h (c) Pretreatment with 1,000 μM NaHS for 30 min before exposure to 500 μM $CoCl_2$ for 2 h (d) Pretreatment with 1,000 μM *N*-acetyl-cysteine (NAC) for 30 min before exposure to 500 μM $CoCl_2$ for 2 h. (B) Cells were incubated for various times (0-240 min) with 500 μM $CoCl_2$. (C) Cells were pretreated with 1,000 μM NaHS or 1,000 μM NAC for 30 min and then treated with 500 μM $CoCl_2$ for 2 h. Cell lysates were subjected to western blotting to determine the levels of phospho-p38 MAPK. (D) Cells were pretreated with 1,000 μM NaHS, 1,000 μM NAC or 1 μM SB 203580 (p38 MAPK inhibitor) for 30 min and then treated with 500 μM $CoCl_2$ for 12 h. Cell lysates were subjected to western blotting to determine the levels of Bax and Bcl-2 expression and the Bax/Bcl-2 ratio was calculated. (E) Cells were pretreated 1,000 μM NaHS, 1,000 μM NAC, or 1 μM SB 203580 for 30 min followed by incubation in the presence or absence of 500 μM $CoCl_2$ for 12 h. Cell viability was measured by CCK-8 assay. The values are expressed as the mean ± SE of three independent experiments with triplicate dishes. *p<0.05 vs. control, $^{\#}p$<0.05 vs. $CoCl_2$ only.

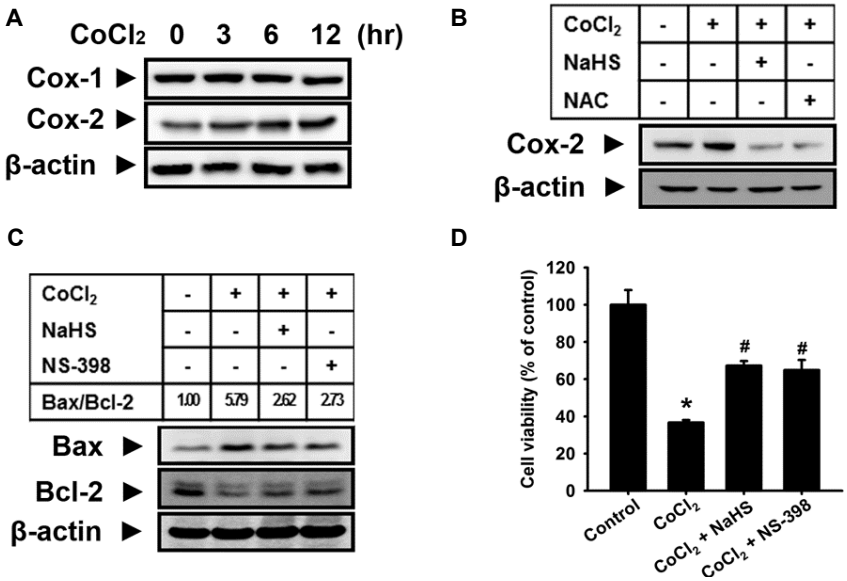

Fig. 4. Effect of H_2S on $CoCl_2$-induced COX-2 expression and cell injury. (A) Mouse hepatocytes were incubated with 500 μM $CoCl_2$ for various times (0-24 h). (B) Cells were pretreated with 1,000 μM NaHS or 1,000 μM NAC for 30 min and then treated with 500 μM $CoCl_2$ for 12 h. Cell lysates were subjected to western blotting to determine the level of COX-2 expression. (C) Cells were pretreated with 1,000 μM NaHS or 10 μM NS-398 (COX-2 inhibitor) for 30 min and then treated with 500 μM $CoCl_2$ for 12 h. Cell lysates were subjected to western blotting to determine the levels of Bax and Bcl-2 expression and the Bax/Bcl-2 ratio was calculated. (D) Cells were pretreated 1,000 μM NaHS or 10 μM NS-398 for 30 min followed by incubation in the presence or absence of 500 μM $CoCl_2$ for 12 h. Cell viability was measured by CCK-8 assay. The values are expressed as the mean ± SE of three independent experiments with triplicate dishes. $^*p<0.05$ vs. control, $^{\#}p<0.05$ vs. $CoCl_2$ only.

Effect of H_2S on $CoCl_2$-induced COX-2 expression

After treatment of mosue hepatocytes with $CoCl_2$, COX-2 expression was significantly augmented, while COX-1 expression was not significantly changed (Fig. 4A). Pretreatment with different concentrations of NaHS and NAC markedly attenuated the increased COX-2 expression by $CoCl_2$ (Fig. 4B). We observed that pretreatment with NaHS or NS-398 (a selective COX-2 inhibitor, 10 μM) obviously inhibited the $CoCl_2$-induced decrease of Bcl-2 expression and increase of Bax expression and increase of Bax/Bcl-2 ratio by $CoCl_2$ treatment was significantly decreased by NaHS or NS-398 (Fig. 4C). In addition, decreased cell viability by $CoCl_2$ was partially recovered by pretreatment with NaHS or NS-398 (Fig. 4D).

Discussion

Hydrogen sulfide (H_2S), an endogenous gaseous mediator, exerts various physiological a pathophysiological effects *in vivo*, including anti-oxidative or anti-inflammatory effects in heart, liver, kidney and other organs [3, 13, 22, 49]. Although H_2S has long been considered as a toxic environmental pollutant emerging from sewers, marshes, and volcanic eruptions, H_2S has been recently recognized along-side nitric oxide and carbon monoxide as an endogenously produced gaseous signaling molecule [22, 33, 47]. Previous studies suggested that H_2S is a potent antioxidant [27, 60] and it has also been demonstrated that H_2S effectively inhibits apoptosis of a number of cell types [12, 39, 44] and this effect has been shown to promote cytoprotection. Cobalt chloride ($CoCl_2$) has been reported to take the place of ferrous ions in prolyl-4-hydroxylase (P4H), thereby causing a conformational change in the P4H protein that consequently leads to a hypoxic condition, characterized by intranuclear accumulation of hypoxia inducible factor-1α (HIF-1α) [15, 42, 61]. $CoCl_2$ has been also known to induce apoptosis in various types of cells [16, 29, 48]. In the current study, we demonstrated that chemical hypoxia-induced hepatocellular injury is markedly decreased by sodium hydrosulfide (NaHS, a donor of H_2S). The exposure of mouse hepatocytes to $CoCl_2$ induced cytotoxicity, evidenced by the decreased cell viability, and we observed that NaHS significantly attenuated $CoCl_2$-induced decrease of cell viability. These results indicate that hydrogen sulfide (H_2S) plays a role in suppress-

ing cell death induced by chemical hypoxia.

It has been known that hypoxia or hypoxia-mimicking $CoCl_2$-induced injury is mediated by the generation of reactive oxygen species (ROS) [17, 65]. In general, overproduction of ROS induced by oxidative stress may cause oxidative damage to DNA, lipids and proteins, resulting in apoptosis of cells including hepatocytes [25, 31]. In this study, exposure of mouse hepatocytes to $CoCl_2$ increased intracellular ROS and $CoCl_2$-induced ROS generations were attenuated by pretreatment of NaHS or *N*-acetylcystein (NAC), a common ROS scavenger. These results show that H_2S possesses scavenging activity of ROS, which induced by $CoCl_2$. Indeed, Geng et al. reported that H_2S directly scavenges superoxide anions and H_2O_2 [14]. Recent studies have demonstrated that ROS are important triggers to upregulate p38 MAPK activity and that antioxidants can be consequently applied to inhibit p38 activation [8, 30, 65]. In hepatocytes, the p38 signaling pathway is preferentially activated by inflammatory cytokines and various stresses, such as UV light or hypoxia [2, 31, 38, 54]. ROS also can activate p38 pathway [19, 31, 63]. In accordance with previous reports, our results showed that NaHS or NAC attenuated $CoCl_2$-induced p38 MAPK activation. These results suggest that H_2S blocks p38 MAPK through its antioxidant effect. Various studies have demonstrated that $CoCl_2$ elicits oxidative stress, which constibutes to apoptosis in other cell types [30, 57, 65]. Additionally, oxidative stress-induced p38 MAPK activation was involved in hepatocyte injury [1, 20, 45]. This is supported by our results in which $CoCl_2$-induced cytotoxicity was prevented by NaHS, NAC, and SB 203580 (a p38 MAPK inhibitor) treatment. In addition, NaHS, NAC, and SB 203580 blocked the $CoCl_2$-induced increase of Bax/Bcl-2 ratio. Of the Bcl-2 family of proteins, including Bcl-2 and Bcl2-related family members such as Bcl-xL, Bad and Bax, play an important role in the regulation of apoptosis [41]. In the present study, we showed that pretreatment of mouse hepatocytes with NaHS, NAC, and SB 203580 resulted in a significant decrease of increased expression of Bax, a proapoptotic Bcl-2 family member and potentiation of decreased expression of Bcl-2, an antiapoptotic Bcl-2 family member, by $CoCl_2$. Therefore, our results suggest that H_2S pretreatment plays a cytoprotective role via attenuation not only of $CoCl_2$-induced ROS, but also of p38 MAPK activated by $CoCl_2$-induced ROS.

Our current study showed that exposure of mouse hepatocytes to $CoCl_2$ elevated expression of COX-2. At present, COX is classified by at least two forms [24, 43]. COX-1 is constitutively expressing form, while COX-2 is induced by multiple factors including cytokines, hormones, mitogens, oxidative stresses, and so forth. In our results, enhanced COX-2 expression by $CoCl_2$ was suppressed by the pretreatment with NaHS and NAC. This result indicates that COX-2 expression is enhanced by $CoCl_2$-induced intracellular ROS and H_2S represses COX-2 expression by its antioxidant activity. Previous studies showed that inhibition of COX-2 exerts hepatoprotective effects on liver damage [51]. COX-2 expression is upregulated by ischemia or reperfusion in the rat liver, and the inhibition of COX-2 is found to improve liver function and viability [26, 35]. Through this study, we observed that NaHS and NS-398, a selective inhibitor of COX-2, significantly attenuated $CoCl_2$-induced increase of Bax/Bcl-2 ratio. In addition, NaHS and NS-398 attenuated $CoCl_2$-induced cytotoxicity. Overall, these findings suggest that oxidative stress plays a pivotal role in hepatocellular injuries induced by $CoCl_2$ and ROS mediate $CoCl_2$-induced injury through p38 MAPK and COX-2 pathway in mouse hepatocytes. In conclusion, the present study demonstrated that H_2S has a cytoprotective effect against chemical hypoxia-induced cell injury through inhibition of the ROS, p38 MAPK, and COX-2 pathway in primary cultured mouse hepatocytes.

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초록 : 화학적 허혈에 의해 손상된 마우스 간세포에 대한 hydrogen sulfide의 간세포 보호 효과

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본 연구는 화학적 허혈에 의해 손상된 마우스 간세포에서 hydrogen sulfide (H_2S)의 효과를 규명하기 위해 수행되었다. 본 연구에서 허혈 모방 화합물로 알려져 있는 cobalt chloride ($CoCl_2$)는 간세포 손상을 시간 및 농도 의존적으로 유의성 있게 증가 시켰다. $CoCl_2$에 의한 간세포 손상은 Sodium sulfide (NaHS, H_2S 공여제)의 전처리에 의해 유의적으로 감소 되었다. $CoCl_2$는 세포 내 활성산소(reactive oxygen species, ROS)의 농도를 증가시켰으며, 이는 NaHS 및 *N*-acetyl-cysteine (NAC, a ROS 제거제)에 의해 감소하였다. 또한, $CoCl_2$에 의해 증가된 p38 MAPK 인산화가 NaHS 및 NAC에 의해 억제되었다. $CoCl_2$에 의해 증가된 Bax/Bcl-2 비율은 NaHS, NAC 및 SB 203580 (p38 MAPK 저해제)에 의해 차단되었으며, $CoCl_2$에 의해 유발된 간세포의 손상 또한 NaHS, NAC 및 SB 203580의 전처리에 의해 억제되었다. NaHS는 $CoCl_2$에 의해 증가된 COX-2의 발현을 억제하였다. 또한, NaHS의 효과와 유사하게 $CoCl_2$에 의해 증가된 COX-2의 발현이 NAC에 의해 억제되었다. 더욱이, NS-398 (COX-2 선택적 억제제)는 $CoCl_2$에 의한 Bax/Bcl-2 비율의 증가를 억제하였을 뿐 아니라, 간세포의 세포 손상 또한 억제하였다. 결론적으로, H_2S는 초대배양 된 마우스 간세포에서 $CoCl_2$에 의해 유발된 간세포의 손상을 ROS에 의해 유발된 p38 MAPK 및 COX-2 경로의 활성화를 억제함으로써 세포보호효과를 수행하는 것을 알 수 있었다.