Novel insights into the role of HSP90 in cytoprotection of H_2S against chemical hypoxia-induced injury in H9c2 cardiac myocytes

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Abstract. The present study evaluated potential mechanisms of hydrogen sulfide (H_2S)-mediated cardioprotection using an *in vitro* chemical hypoxia-induced injury model. We have demonstrated that H_2S protects H9c2 cardiomyoblasts (H9c2) against chemical hypoxia-induced injuries by suppressing oxidative stress and preserving mitochondrial function. The aim of this study was to investigate the role of heat shock protein 90 (HSP90) in cardioprotection of H_2S in H9c2 cells. The findings of the present study showed that cobalt chloride ($CoCl_2$), a chemical hypoxia agent, significantly enhanced the expression of HSP90 and that 17-allylamino-17-demethoxy geldanamycin (17-AAG), a selective inhibitor of HSP90, aggravated concentration-dependent cytotoxicity induced by $CoCl_2$. Exogenous administration of NaHS (a donor of H_2S) augmented not only HSP90 expression under normal conditions, but also $CoCl_2$-induced overexpression of HSP90. Pre-treatment with 17-AAG significantly blocked the cardioprotection of H_2S against $CoCl_2$-induced injuries, leading to increases in cytotoxicity and apoptotic cells. Furthermore, pre-treatment with 17-AAG also antagonized the inhibitory effects of NaHS on overproduction of reactive oxygen species (ROS), a loss of mitochondrial membrane potential (MMP) and ATP depletion induced by $CoCl_2$. In conclusion, these results demonstrate that the increased expression of HSP90 may be one of the endogenous defensive mechanisms for resisting chemical hypoxia-induced injury in H9c2 cells. We also provide novel evidence that HSP90 mediates the cardioprotection of H_2S against $CoCl_2$-induced injuries by its antioxidant effect and preservation of mitochondrial function in H9c2 cells.

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Introduction

Heat shock proteins (HSPs) are a family of protective proteins, which constitute an endogenous cellular defense mechanism against hostile environmental stress, and they can be induced by many stressful stimuli, such as ischemia, hypoxia and inflammation (1,2). Adaptation to stress leads to increases in the expression of some heat shock genes, which act as molecular chaperones to prevent protein misfolding and to assist in their refolding to the native state (3). Accumulating evidence suggests that overexpression of HSPs can protect myocardial cells against ischemia-reperfusion injury (4). HSP90, one of the most abundant cytosolic HSPs, comprises 1-2% of the total proteins in cells (5). A previous study showed that HSP90 can bind to the pro-apoptotic protein Apaf-1, thus preventing apoptosome formation and apoptosis (6). Under hypoxic conditions, HSP90 expression is enhanced (7), which may efficiently reduce myocardial reperfusion damage (8). Inhibition of HSP90 function with the HSP90 inhibitor geldanamycin or HSP90 si-RNA completely diminished the protection of hypoxic pre-conditioning against prolonged hypoxia/reoxygenation-induced injury in rat heart-derived H9c2 cells (9). Griffin *et al* have shown that radicicol protects neonatal rat myocardial cells against ischemic injury via augmenting HSP90 protein levels (10). However, whether HSP90 mediates the cardioprotection of hydrogen sulfide (H_2S) against chemical hypoxia-induced injury is unclear.

Similarly to nitric oxide (NO) and carbon monoxide (CO), H_2S is an endogenous gasotransmitter, which can be produced by catalysis of three enzymes, cystathionine γ-lyase (CGL or CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST). These enzymes are tissue-specific, for CGL is expressed in the cardiovascular system and smooth muscle cells, while CBS and 3MST are found in the brain (11). H_2S has been recognized as an important biological signaling molecule, and exerts a variety of cytoprotective actions in various models. H_2S protects astrocytes and neurons against oxidative insult (12,13). Our previous study also showed that H_2S possessed antioxidant and anti-apoptotic effects

against injury induced by the $A\beta_{25-35}$ peptide in PC12 cells (14). Similarly, in numerous cell and animal models of heart diseases, it has been demonstrated that the mechanisms of H_2S cytoprotection underlie the inhibition of myocardial inflammation and preservation of both mitochondrial structure and function (15-19). H_2S also protects the heart against isoproterenol induced-arrhythmias by scavenging reactive oxygen species (ROS) (20). Additionally, it is worth noting that H_2S protects the liver against injury induced by ischemia-reperfusion, and during this hepatic protection H_2S enhances HSP90 expression (21). However, it remains to be elucidated whether the up-regulated HSP90 is an epiphenomenon or a mediation of H_2S protection. Thus, the present study was conducted to determine whether H_2S up-regulates HSP90 expression in H9c2 cells. More importantly, we decided to further elucidate the roles of HSP90 in the cytoprotection of H_2S against chemical hypoxia-induced injury in H9c2 cells.

Cobalt chloride ($CoCl_2$) is a chemical hypoxia agent, which can mimic hypoxic/ischemic responses by increasing ROS generation, activating hypoxia inducible factor 1α (HIF-1α) and inducing the expression of many genes, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glycolytic enzymes during normoxia (23). In PC12 neuronal cells and H9c2 cells, $CoCl_2$ reduces cell viability and induces cellular apoptosis (14,22). The molecular mechanisms may be linked to intracellular ROS accumulation, disruption of mitochondrial membrane potential (MMP), release of cytochrome C from mitochondria to the cytosol and activation of caspase-9 as well as of caspase-3 (22,24,25). In the present study, H9c2 cells were exposed to $CoCl_2$ to establish a chemical hypoxia-induced insult model (22), to investigate i) the effect of H_2S or $CoCl_2$ on the expression of HSP90 in H9c2 cells, ii) whether HSP90 mediates the cardioprotection of H_2S against chemical hypoxia-induced injuries and if so iii) to investigate whether HSP90 plays role in the H_2S-induced anti-oxidative effect and preservation of mitochondrial function.

Materials and methods

Materials. Sodium hydrogen sulfide (NaHS), 17-allylamino-17-demethoxy geldanamycin (17-AAG), $CoCl_2$, dichlorofluorescin diacetate (DCFH-DA) and Rhodamine123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The SOD kit and the Cell Counter kit-8 (CCK-8) were purchased from Dojindo Lab. (Japan). DMEM-F12 medium and fetal bovine serum were supplied by Gibco-BRL. H9c2 cells were obtained from the Sun Yat-sen University Experimental Animal Centre.

Cell culture and treatments. The H9c2 cell line, a subclone of the original clonal cell line, is derived from embryonic rat heart tissue. H9c2 cells were cultured in DMEM-F12 medium supplemented with 15% fetal bovine serum at 37˚C under an atmosphere of 5% CO_2 and 95% air.

$CoCl_2$, a chemical hypoxia-mimetic agent, was added into the medium at the indicated concentrations to set up a chemical hypoxia-induced cellular model of cardiomyocyte injury. According to our previous study, the cytoprotective effects of H_2S were observed by administering 400 μM NaHS (a donor of H_2S) for 30 min before exposure to $CoCl_2$ (22). In order to clarify the effects of HSP90 on the cellular injury elicited by $CoCl_2$ or the cytoprotection induced by H_2S, cells were co-incubated with 17-AAG, a selective inhibitor of HSP90, and $CoCl_2$.

Cell viability assay. Cell viability was detected by using the CCK-8 kit. H9c2 cells were cultured in 96-well plates. When the cells were about 70% confluent, indicated conditioned-mediums were administered. After the treatments, 10 μl CCK-8 solution was added into each well and then the plates were incubated for 3 h in the incubator. Absorbance at 450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the optical density (OD) measurements from 4 wells of the indicated groups were used to calculate the percentage of cell viability according to the formula: percentage of cell viability = OD treatment group/OD control group x 100%. The experiment was repeated 3 times.

Western blot analysis for protein expression. After different treatments, H9c2 cells were harvested and lysed with ice-cold cell lysis solution, and the homogenate was centrifuged at 12,000 rpm for 10 min at 4˚C. Total protein in the supernatant was quantitated with a BCA protein assay kit. Total protein (30 μg from each sample) was separated by 12% SDS-PAGE. The protein in the gel was transferred on a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% free-fat milk in TBS-T for 2 h at room temperature, and then incubated with the primary antibodies specific to HSP90 (Cell Signaling Technology, Beverly, MA, USA), or β-actin with gentle agitation at 4˚C overnight and subsequently with the secondary antibodies for 1 h at room temperature. Following three washes with TBS-T, membranes were developed using enhanced chemiluminescence and exposed to X-ray films. For quantifying protein expression, the X-ray films were scanned and analyzed with ImageJ 1.41o software (National Institutes of Health, USA).

Measurement of intracellular ROS generation. Intracellular ROS generation was determined by the oxidative conversion of cell-permeable DCFH-DA to fluorescent DCF. H9c2 cells were cultured on a slide in DMEM-F12 medium. After indicated treatments, slides were washed twice with phosphate-buffered saline (PBS). DCFH-DA solution in serum-free medium was added at a concentration of 10 μM and co-incubated with H9c2 cells at 37˚C for 60 min. The slides were washed 3 times, and DCF fluorescence was measured over the entire field of vision by using a fluorescent microscope connected to an imaging system (BX50-FLA, Olympus, Tokyo, Japan). The mean fluorescence intensity (MFI) from 4 random fields was analyzed by using ImageJ 1.41o software and the MFI of DCF was used to indicate the amount of ROS.

Measurement of MMP. MMP was monitored by using a fluorescent dye, Rh123, a cell-permeable cationic dye that preferentially enters into mitochondria based on the highly negative MMP. Depolarization of MMP results in a loss of Rh123 from mitochondria and a decrease in intracellular green fluorescence. In the present study, Rh123 (100 mg/l) was added into cell cultures for 60 min at 37˚C and fluorescence was

measured over the entire field of vision by using a fluorescent microscope connected to an imaging system (BX50-FLA, Olympus). The MFI of Rh123 from 4 random fields was analyzed using the ImageJ 1.41o software and the MFI was taken as an index of the level of MMP.

ATP content measurement with HPLC. H9c2 cells were collected in 0.5 ml PBS and crushed by repeated freezing and thawing (3 times for 10 min each). Then 0.5 ml $HClO_4$ solution (0.5 M) were added and the pH of 1 ml of the supernatant was adjusted to ~7.0 using 2 M NaOH. After centrifugation at 12,000 rpm for 15 min at 4˚C, the supernatant was collected for ATP measurement. The above manipulation was performed in an ice bath. The ATP content was measured by high-performance liquid chromatography (HPLC) (HP 1100, Agilent Technologies, USA) using a mobile phase of KH_2PO_4 buffer at a speed of 0.5 ml/sec. A wavelength of 254 nm was used for the diode-array detector (DAD).

Measurement of SOD activity. The intracellular SOD activity was determined by using an SOD assay kit according to the manufacturer's protocol. WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is a soluble tetrazolium salt that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2^- is linearly related to xanthine oxidase activity and is inhibited by SOD. Cell lysis solution (20 μl) from the indicated sample and three blank solutions (water blank 1, sample blank 2 and water blank 3) were transferred in triplicate into microplate wells and 200 μl WST working solution and 20 μl enzyme working solution supplied by the manufacturer were added (except for blank 2 and 3, to which 20 μl dilution buffer was added). The reaction agents were incubated at room temperature for 30 min and the absorbance was read at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). SOD inhibition (%) was calculated using the equation: SOD inhibition (%) = $[(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})]/(A_{blank1} - A_{blank3}) \times 100$.

Nuclear staining for assessment of apoptosis. Chromosomal condensation and morphological changes in the nucleus of H9c2 cells were observed with the chromatin dye Hoechst 33342 followed by photofluorography. The cells were fixed with 4% paraformaldehyde in PBS for 10 min. After three washes with PBS, cells were stained with 5 mg/l Hoechst 33342 for 5 min, and then washed briefly with PBS and air-dried again. The cells were visualized under a fluorescent microscope (BX50-FLA, Olympus). Viable cells displayed normal nuclear size and uniform fluorescence, whereas apoptotic cells showed condensed, fractured or distorted nuclei.

Data analysis and statistics. All data are presented as the mean ± SEM. The assessment of differences between groups was analyzed by one-way ANOVA with SPSS 13.0 (SPSS Inc.). $P<0.05$ was considered to be statistically significant.

Results

Adaptive HSP90 overexpression protects H9c2 cells against cytotoxicity induced by $CoCl_2$. After H9c2 cells were exposed to 600 μM $CoCl_2$, the expression of HSP90 was detected at the indicated times. An significant increase in the expression of HSP90 was first observed at 12 h, and reached a peak at 24 h (Fig. 1A and B). Further study showed that inhibition of HSP90 by 2 μM 17-AAG, a selective inhibitor of HSP90, aggravated the concentration-dependent cytotoxicity induced by $CoCl_2$, whereas 17-AAG alone had no effect on the cell viability in H9c2 cells (Fig. 1C). These results suggest that adaptive HSP90 overexpression is a self-defense mechanism against the cytotoxicity induced by chemical hypoxia.

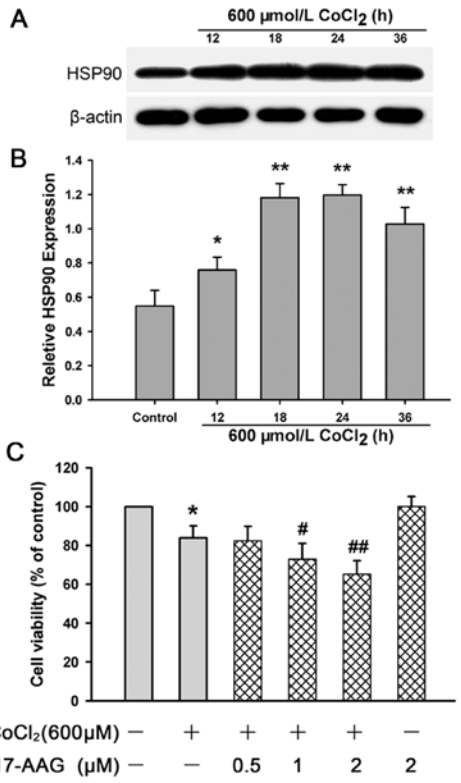

Figure 1. Effect of HSP90 on the cytotoxicity caused by $CoCl_2$ in H9c2 cells. (A,B) H9c2 cells were treated with 600 μM $CoCl_2$ for 12, 18, 24 and 36 h, respectively. (A) Expression of HSP90 was detected by Western blot analysis and (B) quantified by densitometric analysis with the ImageJ 1.41o software. (C) H9c2 cells were treated with 600 μM $CoCl_2$ for 24 h in the presence or absence of 17-AAG, a selective inhibitor of HSP90, at the indicated concentrations. The CCK-8 assay was performed to measure cell viability. $^*P<0.05$, $^{**}P<0.01$ vs. the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. the 600 μM $CoCl_2$ group.

H_2S-induced HSP90 overexpression mediates the cytoprotective effect of H_2S. As H9c2 cells were treated with 400 μM NaHS for 5-60 min, a time-dependent increase in HSP90 expression was found (Fig. 2A and B). Importantly, treatment with NaHS not only increased HSP90 expression under normal conditions, but also facilitated a significant up-regulation of HSP90 expression induced by 600 μM $CoCl_2$ treatment for 24 h (Fig. 2C and D). Moreover, our findings show that exposure of H9c2 cells to 600 μM $CoCl_2$ obviously attenuated cell viability and that NaHS at 400 μM significantly blocked this inhibitory effect of $CoCl_2$ (Fig. 2E). Inhibition of HSP90 by 2 μM 17-AAG statistically significantly eliminated the cytoprotection of NaHS against the cellular insult induced by $CoCl_2$ (Fig. 2E). These data indicated that HSP90 mediates the inhibitory effect of H_2S on the $CoCl_2$-triggered cytotoxicity.

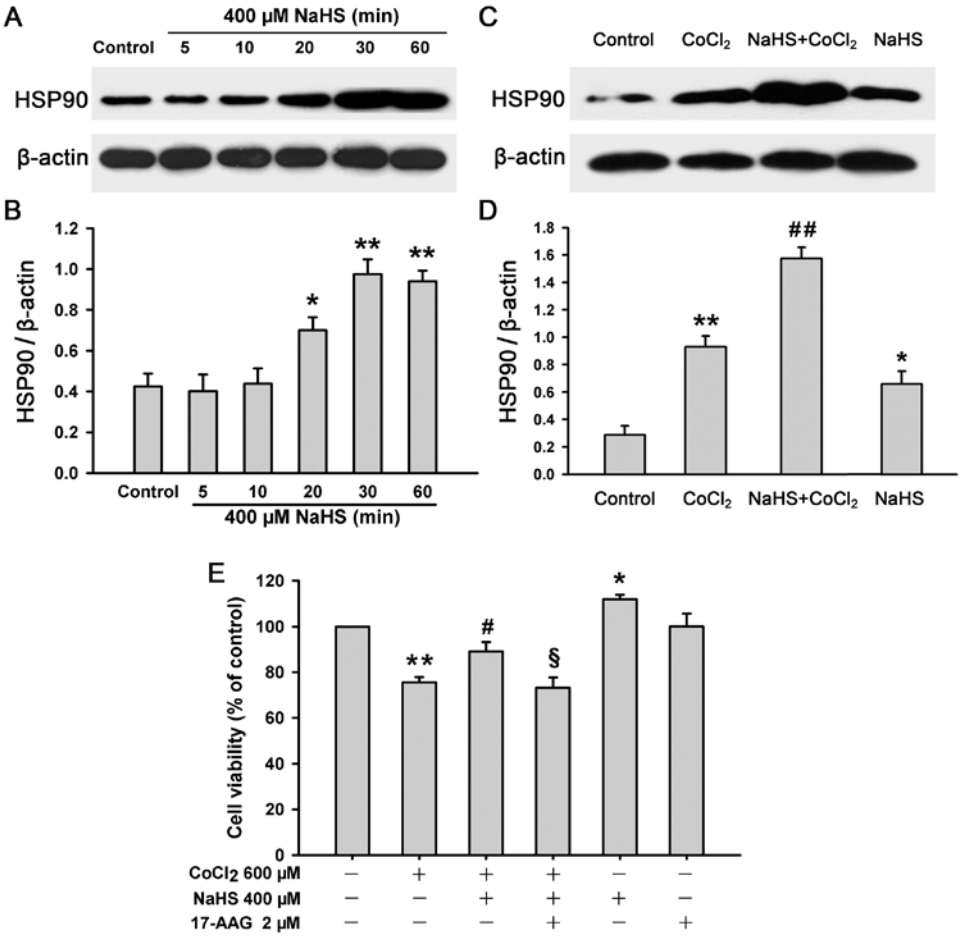

Figure 2. Role of HSP90 in the cytoprotection of NaHS against the cytotoxicity induced by $CoCl_2$ in H9c2 cells. (A,B) H9c2 cells were treated with 400 μM NaHS for the indicated time. (C,D) H9c2 cells were treated with 600 μM $CoCl_2$ for 24 h in the presence or absence of pre-treatment with 400 μM NaHS for 30 min. (A,C) Expression of HSP90 was detected by Western blot analysis and (B,D) quantified by densitometric analysis with the ImageJ 1.41o software. (E) H9c2 cells were treated with 600 μM $CoCl_2$ for 24 h, in the presence or absence of pre-treatment with 400 μM NaHS for 30 min. To inhibit HSP90, cells were co-treated with 17-AAG (2 μM) in the presence or absence of 400 μM NaHS for 30 min followed by 600 μM $CoCl_2$ treatment for 24 h. The CCK-8 assay was performed to detect cell viability. *P<0.05, **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the 600 μM $CoCl_2$ group; §P<0.05 vs. the 600 μM $CoCl_2$ pre-treated with 400 μM NaHS.

HSP90 is involved in the H_2S-elicited prevention against apoptosis induced by $CoCl_2$. Exposure of H9c2 cells to 600 μM $CoCl_2$ for 36 h caused a significant increase in the percentage of apoptotic cells (Fig. 3B and G). $CoCl_2$-induced apoptosis was considerably decreased by pre-treatment with 400 μM NaHS for 30 min (Fig. 3C and G). Importantly, the anti-apoptotic action of NaHS was obviously suppressed by inhibition of HSP90 with 17-AAG at 2 μM (Fig. 3D and G). In addition, NaHS or 17-AAG alone did not alter apoptosis in H9c2 cells (Fig. 3E-G).

HSP90 is implicated in the antioxidant effect of H_2S in H9c2 cells. Treatment with 600 μM $CoCl_2$ for 24 h led to an obvious increase in DCF-derived fluorescence, manifesting an accumulation of intracellular ROS (Fig. 4B). Treatment with $CoCl_2$ also resulted in a statistical decrease in SOD activity (Fig. 4H). Pre-treatment with 400 μM NaHS for 30 min prior to $CoCl_2$ exposure not only reduced the intracellular ROS level, but also ameliorated SOD activity (Fig. 4C and H). Moreover, 17-AAG (2 μM), a selective inhibitor of HSP90, significantly blocked the inhibition of ROS levels caused by NaHS pre-treatment. However, 17-AAG did not statistically alter the NaHS pre-treatment-induced amelioration of SOD activity.

HSP90 participates in the mitochondrial protection elicited by H_2S in H9c2 cells. As shown in Fig. 5, after H9c2 cells were subjected to 600 μM $CoCl_2$ for 24 h, mitochondria were obviously damaged, resulting in a decrease in the uptake of Rh123, indicating dissipation of MMP (Fig. 5B). The loss of MMP was ameliorated by pre-treatment with 400 μM NaHS for 30 min (Fig. 5C). Inhibition of HSP90 by 17-AAG (2 μM) statistically attenuated the mitochondrial protection of H_2S, leading to severe loss of MMP (Fig. 5D). These data suggest that H_2S can cause a preservation of mitochondrial function by up-regulation of HSP90 expression.

Furthermore, assessment of the intracellular ATP levels indicated that H_2S preserved the mitochondrial function of H9c2 cells by increasing ATP production. The exposure of H9c2 cells to 600 μM $CoCl_2$ for 24 h significantly inhibited ATP generation, which was reversed by NaHS pre-treatment for 30 min (Fig. 5H). Additionally, 17-AAG at 2 μM abrogated the NaHS-induced increase in ATP generation. NaHS or 17-AAG alone did not alter the ATP levels (Fig. 5H).

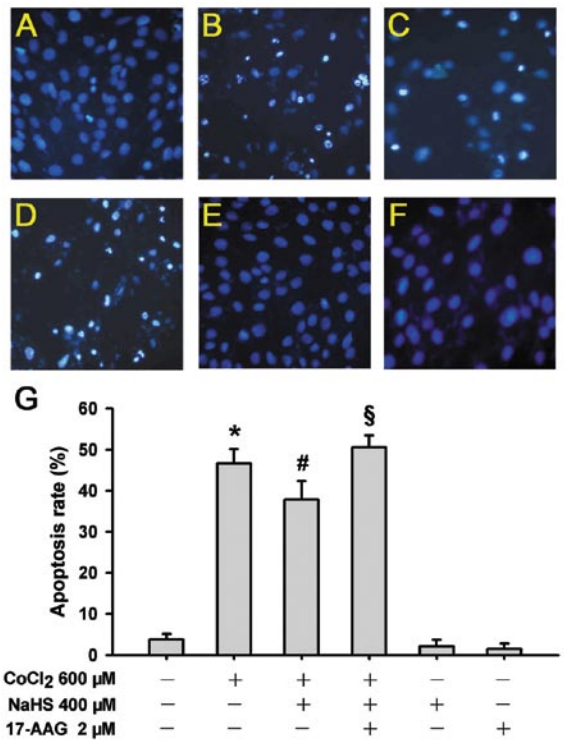

Figure 3. Role of HSP90 in the protection of NaHS against apoptosis-induced by $CoCl_2$ in H9c2 cells. (A-F) Hoechst 33342 nuclear staining followed by photofluorography to observe cellular apoptosis. (A) Control group. (B) H9c2 cells were exposed to 600 μM $CoCl_2$ for 36 h. (C) H9c2 cells were pre-treated with 400 μM NaHS for 30 min prior to $CoCl_2$ treatment. (D) H9c2 cells were treated with 2 μM 17-AAG and 400 μM NaHS for 30 min followed by exposure to 600 μM $CoCl_2$ for 24 h. (E) H9c2 cells were treated with 400 μM NaHS for 30 min followed by 36 h culture. (F) H9c2 cells were treated with 2 μM 17-AAG for 36 h. (G) The apoptotic rate was analyzed with a cell counter of the ImageJ 1.41o software. *P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the 600 μM $CoCl_2$ group; §P<0.05 vs. the 600 μM $CoCl_2$ group pre-treated with 400 μM NaHS for 30 min.

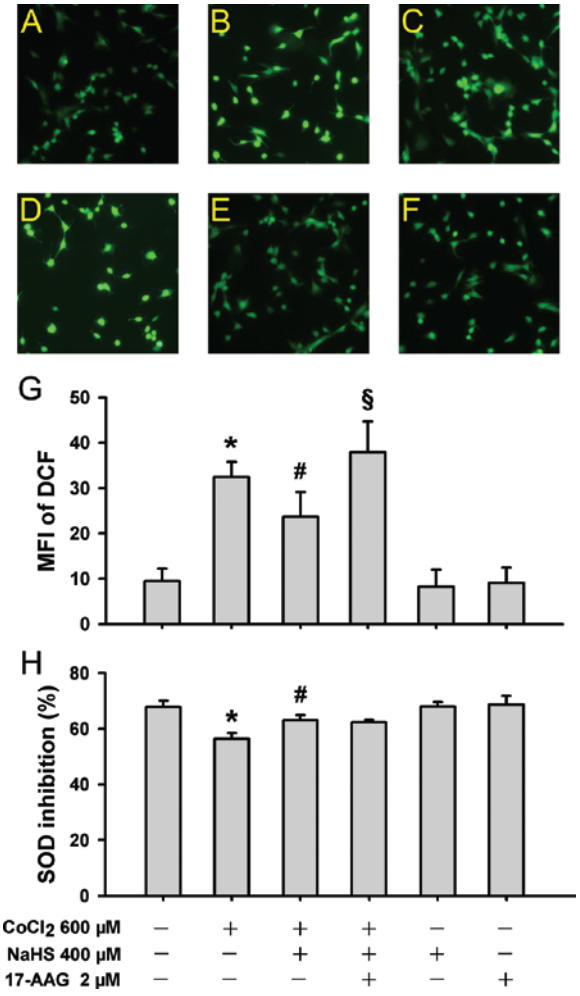

Figure 4. Involvement of HSP90 in the anti-oxidation of NaHS in H9c2 cells. (A-F) DCFH-DA staining followed by photofluorography to observe the intracellular ROS in H9c2 cells. (A) Control group. (B) H9c2 cells subjected to 600 μM $CoCl_2$ treatment for 24 h. (C) H9c2 cells were pre-treated with 400 μM NaHS for 30 min prior to $CoCl_2$ treatment. (D) H9c2 cells were treated with 2 μM 17-AAG and NaHS for 30 min followed by $CoCl_2$ treatment. (E) H9c2 cells were treated with 400 μM NaHS for 30 min followed by 24 h culture. (F) H9c2 cells were treated with 2 μM 17-AAG for 2.5 h followed by 24 h culture. (G) Quantitative analysis of the mean fluorescence intensity (MFI) of DCF with the ImageJ 1.41o software. (H) H9c2 cells were treated with the protocol indicated in (A-F). Intracellular SOD activity was detected with a commercial kit. *P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the 600 μM $CoCl_2$ group; §P<0.05 vs. the 600 μM $CoCl_2$ pre-treated with 400 μM NaHS group.

Discussion

Increasing evidence shows that H_2S may serve as an important cardioprotective agent. H_2S has been reported to reduce cardiomyocyte apoptosis *in vitro* and *in vivo* after myocardial ischemic-reperfusion (18,19). Inhibition of endogenous H_2S production by H_2S synthesis inhibitors obviously attenuated the protective effect of ischemic pre-conditioning in both the isolated heart and isolated cardiac myocytes (26). Our more recent study indicated that H_2S protects H9c2 cells against $CoCl_2$-induced cytotoxicity and apoptosis by its antioxidant effect (22). In the present study, we found that HSP90 mediates the cardioprotection of H_2S by suppressing oxidative stress and improving mitochondrial function.

HSP90 is an ubiquitous and highly abundant molecule that contributes to cell survival and protection by regulating the folding and stability of various cellular client proteins including survival and apoptotic factors (27). It was reported that hypoxia up-regulated the expression of HSP90 (7), which can efficiently attenuate the myocardial ischemia/reperfusion-induced myocardial dysfunction (8). In this study, it was shown that under normal conditions, HSP90 expression was moderate, revealing that HSP90 is needed in untreated H9c2 cells. We also observed that exposure of the cells to $CoCl_2$ markedly increased HSP90 expression, consistent with the previous study (7), but in contrast to a recent report that the expression level of HSP90 was considerably reduced by hypoxia ($Na_2S_2O_4$) in neurons (28). The discrepancy between the hypoxia-induced up-regulation vs. down-regulation effect on the expression of HSP90 revealed that the responses of HSP90 to various types of hypoxia may differ among different cell lines. Similarly to a previous study (7), our findings demonstrate that 17-AAG, a specific HSP90 inhibitor, enhanced the cytotoxicity induced by $CoCl_2$, suggesting that the increased expression of HSP90 may be one of the endogenous defensive mechanisms by which cells may become more resistant to chemical hypoxia-induced insults.

Importantly, we found that H_2S time-dependently induced an increase in HSP90 expression in H9c2 cells. In SH-SY5Y

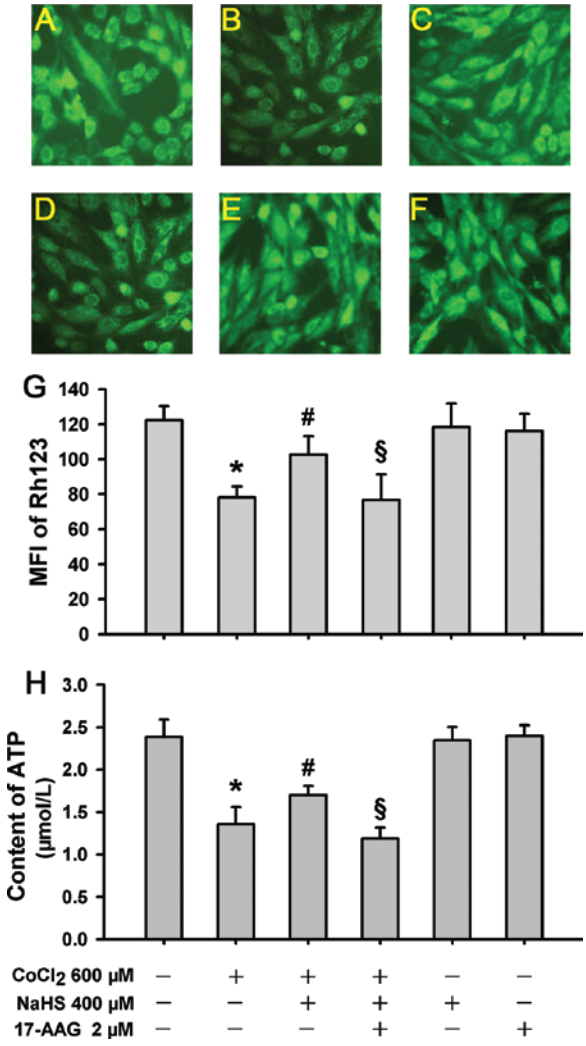

Figure 5. Role of HSP90 in the NaHS-induced mitochondrial protection. (A-F) Rh123 staining followed by photofluorography to detect mitochondrial membrane potential (MMP). (A) Control group. (B) H9c2 cells treated with 600 μM $CoCl_2$ for 24 h. (C) H9c2 cells were pretreated with 400 μM NaHS for 30 min prior to $CoCl_2$ treatment. (D) H9c2 cells were treated with 2 μM 17-AAG and NaHS for 30 min prior to $CoCl_2$ treatment. (E) H9c2 cells were treated with 400 μM NaHS for 30 min followed by 24 h culture. (F) H9c2 cells were treated with 2 μM 17-AAG for 24 h. (G) Quantitative analysis for MFI of Rh123 in (A-F) with the ImageJ 1.41o software. (H) H9c2 cells were treated according to the indicated protocol. The intracellular ATP levels were measured with HPLC. *P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the 600 μM $CoCl_2$ group; §P<0.05 vs. the 600 μM $CoCl_2$ pre-treated with 400 μM NaHS group.

cells (28) and liver (21), H_2S also enhanced HSP90 expression, which support our findings (21,28). Based on our results and the above mentioned data (21,28), we speculate that H_2S may be a novel inducer of HSP90. Interestingly, our findings showed that H_2S increased not only the HSP90 expression in untreated cells, but also the overexpression of HSP90 induced by $CoCl_2$ (600 μmol), further indicating that overexpression of HSP90 may be one of the molecular defensive mechanisms underlying the H_2S-induced cardioprotective effects against chemical hypoxia injury.

In order to explore whether HSP90 mediates the H_2S-induced cardioprotection against $CoCl_2$-induced injuries, including cytotoxicity, apoptosis, overproduction of ROS, dysfunction of mitochondria and a decrease in antioxidant ability, incubation with 17-AAG was performed 30 min prior to NaHS pre-treatment. It was shown that 17-AAG significantly blocked H_2S-induced inhibition of cytotoxicity and apoptosis evoked by $CoCl_2$, revealing involvement of HSP90 in the cardioprotection of H_2S against chemical hypoxia-induced injuries. Accumulating evidence indicates that HSP90 is cardioprotective and neuroprotective. Griffin *et al* have demonstrated that radicicol protects neonatal rat myocardial cells against ischemic injury via augmenting HSP90 protein levels (10). Inhibition of HSP90 function with the HSP90 inhibitor, geldanamycin, or HSP90 expression with siRNA, completely reduced the protection of hypoxic preconditioning against prolonged hypoxia/reoxygenation-induced injury in rat heart-derived H9c2 cells (9). Notably, in SH-SY5Y neuronal cells with two hypoxic models developed by subjecting cells to either oxygen-glucose deprivation or $Na_2S_2O_4$ (an oxygen scavenger), Tay *et al* observed that H_2S can up-regulate HSP90 expression and that blockade of HSP90 with a specific HSP90 inhibitor abolishes the neuroprotection (28). The findings of the present study are comparable with the results reported by Tay *et al* (28) suggesting that HSP90 may play an important role in cardioprotection of H_2S in H9c2 cells.

The results of another study have indicated that $CoCl_2$-induced apoptosis of PC12 cells may be associated with the mitochondrial-mediated apoptosis pathway (29). The mitochondrial-dependent apoptotic pathway may be activated under hypoxic/ischemia conditions, causing ROS production, disruption of MMP and release of cytochrome C from the damaged mitochondria into the cytoplasm (30). Our previous studies have shown that H_2S can protect H9c2 cells against $CoCl_2$-induced injuries by inhibiting the mitochondrial-mediated apoptotic pathway (22). The findings of the present study further demonstrate the involvement of HSP90 in the inhibitory effect of H_2S on the $CoCl_2$-induced mitochondrial-mediated apoptotic pathway. It was shown that 17-AAG obviously blocked the H_2S-induced inhibitory effect on over-production of ROS caused by $CoCl_2$, indicating that HSP90 may play a critical role in the antioxidant effect of H_2S, which contributes to scavenge ROS (22) and oxygen-derived free radicals (20).

Another possible role of HSP90 in the H_2S-induced cardioprotection may be associated with the preservation of mitochondrial function. In an *in vivo* model of myocardial ischemia-reperfusion (MIR), H_2S preserved mitochondrial function and membrane integrity after MIR injury (18). In the present study, exposure of H9c2 cells to $CoCl_2$ resulted in MMP loss and ATP depletion and the impaired mitochondrial function was ameliorated by H_2S. However, inhibition of HSP90 by 17-AAG significantly attenuated the mitochondrial protection of H_2S including the preservation of MMP and the improvement of ATP production, suggesting that HSP90 may participate in the preservation of the mitochondrial function by H_2S.

In conclusion, we have demonstrated that HSP90 is up-regulated by H_2S in rat heart-derived H9c2 cells. HSP90 mediates H_2S-induced cardioprotection against chemical hypoxia-induced injuries by inhibiting oxidative stress and preserving mitochondrial function. More studies will be required to elucidate the molecular mechanisms that underlie the anti-oxidative functions of HSP90.

Acknowledgements

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