Inhibition of vascular smooth muscle cell proliferation by chronic hemin treatment

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Chang T, Wu L, Wang R. Inhibition of vascular smooth muscle cell proliferation by chronic hemin treatment. Am J Physiol Heart Circ Physiol 295: H999–H1007, 2008. First published July 11, 2008; doi:10.1152/ajpheart.01289.2007.—Hemin, an oxidized form of heme, is an essential regulator of gene expression and cell cycle progression. Our laboratory previously reported (34) that chronic hemin treatment of spontaneously hypertensive rats reversed the eutrophic inward remodeling of small peripheral arteries. Whether long-term treatment of cultured vascular smooth muscle cells (VSMCs) with hemin alters the proliferation status of these cells has been unknown. In the present study, hemin treatment at 5 μM for 4, 7, 14, and 21 days significantly inhibited the proliferation of cultured rat aortic VSMCs (A-10 cells) by arresting cells at Go/G1 phases so as to decelerate cell cycle progression. Heme oxygenase (HO) activity and inducible HO-1 protein expression were significantly increased by hemin treatment. HO inhibitor tin protoporphyrin IX (SnPP) abolished the effects of hemin on cell proliferation and HO activity. Interestingly, hemin-induced HO-1 expression was further increased in the presence of SnPP. Hemin treatment had no significant effect on the expression of constitutive HO-2. Expression of p21 protein and the level of reactive oxygen species (ROS) were decreased by hemin treatment, which was reversed by application of SnPP. After removal of hemin from culture medium, inhibited cell proliferation and increased HO-1 expression in VSMCs were returned to control level within 1 wk. Transfection with HO-1 small interfering RNA significantly knocked down HO-1 expression and decreased HO activity, but had no effect on HO-2 expression, in cells treated with or without hemin for 7 days. The inhibitory effect of hemin on cell proliferation was abolished in HO-1 silenced cells. It is concluded that induction of HO-1 and, consequently, increased HO activity are responsible for the chronic inhibitory effect of hemin on VSMC proliferation. Changes in the levels of p21 and ROS might also participate in the cellular effects of hemin.

HEMEN, A PROSTHETIC GROUP in heme-containing proteins, is involved in many cellular processes, such as cell metabolism, respiration, signaling, proliferation, and differentiation (32). Intracellular concentration of heme is regulated by the balance between its synthesis and degradation. The rate-limiting enzyme in the degradation of heme is heme oxygenase (HO), converting heme to carbon monoxide (CO), iron, and biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase (35, 37). HO is a microsomal enzyme with two generally acknowledged isozymes expressed in many types of cells, including vascular smooth muscle cells (VSMCs) (37). HO-1 isozyme is ubiquitously distributed and inducible by a variety of physiological and pathophysiological stimuli (15). HO-2 is a constitutive form expressed in many organs under physiological conditions (40).

HO/CO system plays an important role in blood pressure regulation (34, 35, 37). Induction of HO-1 by endotoxin contributes to severe hypotension associated with septic shock (40). Expression of HO-1 in blood vessels induced by hemin treatment in vivo reduces vasoconstriction and normalized blood pressure in spontaneously hypertensive rats (SHR) (23, 34, 35). Along the same line, HO inhibitors increase blood pressure and peripheral resistance, suggesting a critical vasoregulatory role for HO (13, 22). Accumulated evidence also indicates that HO-1 offers protection against many vascular disorders. Increased HO-1 expression decreases intimal thickening after arterial injury (9) and attenuates atherosclerosis in apolipoprotein E-deficient mice (14). Interestingly, increased activity of HO-1 inhibits proliferation of VSMCs (5, 18, 20) but promotes growth of endothelial cells (6). In our laboratory’s previous study, we observed that induction of HO-1 by chronic hemin treatment of SHR reversed the eutrophic inward remodeling of small peripheral arteries in these hypertensive rats (34). These findings raise the possibility that hemin treatment may contribute to vascular remodeling by inhibiting VSMC growth.

Hemin is an oxidized form of heme and an effective inducer of HO-1. In cultured VSMCs, 24-h incubation with hemin induced HO-1 expression (10, 19). In these short-period treatment experiments, VSMCs were incubated with hemin at concentrations as high as 100 μM (19). However, the effect of chronic hemin treatment (>4 days) at physiologically relevant concentration range (<10 μM) on the proliferation of VSMCs has not been studied yet. We now reported the effects of chronic hemin treatment on cell proliferation, expression of HO and p21, and production of reactive oxygen species (ROS) in VSMCs.

MATERIALS AND METHODS

VSMC preparation. Rat thoracic aortic smooth muscle cell line (A-10) was obtained from American Type Culture Collection. A-10 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), as previously described (1). Cultured cells were grown to 50–60% confluence before being starved in FBS-free DMEM for 24 h. Starved cells were cultured in DMEM containing 10% FBS alone or together with hemin (5 μM), tin protoporphyrin IX (SnPP, 10 μM), or hemin plus SnPP for 4, 7, 14, and 21 days. All manipulations were carried out in dim light, and the plates were protected from light by wrapping in aluminum paper. Cell culture media, with or without the addition of selective substances,

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were changed every other day. Cells were split and subcultured after cells grown to confluence. Treated cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested by trypsinization for cell number counting, protein extraction, or flow cytometry analysis. For the purpose of ROS production measurement, untreated and treated cells were cultured in phenol red-free DMEM containing 10% FBS and protected from light.

**Measurement of cell proliferation and apoptosis.** Cell proliferation was determined by cell number counting, as well as a colorimetric method using One Solution Cell Proliferation Assay kit (Promega). For cell number counting, cells from different treatments were trypsinized, suspended in 400 μL DMEM, and counted under microscope using a hemocytometer. In the colorimetric method, the intensity of absorbance at 450–540 nm is in linear response to cell numbers, and the absorbance at 492 nm was recorded using a Multiscan plate reader (Thermo Labsystems), according to the manufacturer’s protocol. For this purpose, cells were plated at 4 × 10^4 cells/well in 96-well plate and cultured in 200 μL medium overnight followed by deprivation of PBS for 24 h and then treated for 7 days, as described above. In apoptosis analysis, untreated and hemin-treated cells were seeded on glass coverslips for Hoechst 33258 staining. Apoptotic cells were identified by condensation and fragmentation of nuclei using fluorescence microscope (Olympus IX70, Tokyo, Japan). Percentage of apoptotic cells was calculated based on the number of condensed and fragmented nuclei from 10 random fields.

**Flow cytometry analysis.** Cell cycle distribution was analyzed by measuring the fluorescence of propidium iodide stained DNA using Beckman Coulter Epics XL (Mississauga, Ontario). Untreated and hemin-treated cells were harvested by trypsin digestion and washed two times with PBS and then suspended in 1 ml PBS (1 × 10^6 cells/ml) and fixed with cold 70% ethanol for at least 2 h on ice. Finally, fixed cells were stained in PBS containing 50 μg/ml propidium iodide and 20 μg/ml RNase at room temperature for 20 min. At least 5,000 cells were counted for each analysis. Histograms of DNA contents were analyzed using FlowJo software (version 7.0, Tree Star) to determine the percentage of cells in each phase of the cell cycle.

**HO-1 activity assay.** HO enzyme activity was quantified by measuring in vitro bilirubin production in cell lysates following reported methods with some modifications (38). Briefly, untreated and treated cells were harvested by trypsin digestion and sonicated on ice in 500 μL of 100 mM phosphate buffer (pH 7.4) containing 2 mM MgCl₂ and a cocktail of protein inhibitors. Cell lysates were centrifuged for 20 min at 15,000 g at 4°C. The supernatant was used to measure HO activity, and protein concentration of supernatant was determined by bichinchonic acid method (28). The reaction mixture, consisting of 100 μL of sample lysates, 100 μL of rat liver cytosol (source of biliverdin reductase), 40 μM hemin, and 1 mM NADPH, was incubated at 37°C for 1 h and protected from light. The reaction was stopped by placing the mixture on ice. For small interfering RNA (siRNA) transfected cells, HO activity was determined by measuring bilirubin released into culture media by cultured cells (12, 33). Cell culture media from wells in 24-well plates were collected after 7-day treatment. Collected media were centrifuged, and the resulting supernatant was used in bilirubin measurement. Production of bilirubin or released bilirubin into cell culture media was measured using a total bilirubin kit (Sigma Diagnostics, St. Louis, MO). A rat liver cytosol reaction was used as a baseline to correct the measured bilirubin production in cell lysates, expressed as picomoles of bilirubin formed per hour per milligram of proteins. Fresh media were used to correct the measurement of released bilirubin in cell culture media, expressed as percentage of control.

**Measurement of ROS level.** Level of ROS was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probes (1). DCFH-DA is oxidized by H₂O₂ and peroxynitrite (derived from superoxide anion and nitric oxide) to form dichlorofluorescein (DCF) with detectable fluorescence. Fluorescence of oxidized DCF was recorded with excitation/emission wavelength at 485/527 nm using fluorescence plate reader. Untreated and treated cells for 7 days were plated at 4 × 10^4 cells/well in 96-well plate with 200 μL normal media or media containing hemin (5 μM), SnPP (10 μM), or hemin/SnPP, and cultured for 12 h to allow cells to attach to the bottom of wells. Cells were loaded with DCF (5 μM) probe for 2 h. Excess probe was washed away, and probe-loaded cells were incubated in 200 μL normal media or media containing hemin (5 μM), SnPP (10 μM), or hemin/ SnPP for another 4 h. Intensity of fluorescence was recorded in the presence of 100 μL PBS/well after cells were washed three times with PBS.

**Western blot analysis.** Total proteins were extracted from untreated and treated cells with 500 μL of cell lysis buffer (in mM) composed of 20 Tris–HCl (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerolphosphate, 1 Na₃VO₄, 1 PMSF, as well as 1% Triton X-100, 1 μg/ml leupeptin, and a cocktail of protein inhibitors (Sigma Diagnostics, St. Louis, MO). Proteins (20 μg) were separated by Western blot analysis, according to our established procedure. The primary antibody dilutions were 1:5,000 for HO-1, 1:10,000 for HO-2, and 1:200 for p21 antibodies. Secondary antibody was used at a dilution of 1:10,000. Western blots were digitized with Chemi Genius² Bio Imaging System (SynGen, Frederick, MD), quantitated using the software of GeneTools from SynGene, and normalized with the quantity of loaded β-actin.

**HO-1 siRNA transfection.** Chemically synthesized HO-1 siRNA targeted against rat HO-1 mRNA (5′-UCAACAGCGCUCAAUCUGUCG3′) were transfected into VSMCs using DharmaFECT1 transfection regents following the manufacturer’s protocol (Dharmacon). The control scrambled siRNA was a proprietary nontargeting siRNA (Dharmacon), having at least four mismatches with all known human, mouse, and rat genes. Synchronized cells, 1.5 × 10^4 cells in 500 μL or 3 × 10^5 cells in 100 μL of antibiotic-free complete medium, were plated in each well of 24-well or 96-well plates and incubated overnight before transfection. HO-1 siRNA and scrambled control siRNA at a final concentration of 100 nM were used to transfect cells. DharmaFECT1 regents were used at 0.05 μl/well for 24-well plates or 0.01 μl/well for 96-well plates. For transfection, siRNA and DharmaFECT1 regents were separately diluted in serum-free and antibiotic-free medium, incubated 5 min at room temperature, and then combined and incubated for 20 min at room temperature. Cells were transfected for 24 h before the starting of hemin treatment. To secure the long-term silence of HO-1, hemin-treated or untreated cells were cultured in antibiotic-free complete medium and continually transfected and retransfected with HO-1 siRNA or scramble control siRNA every other day during the 7-day hemin treatment. After hemin treatment finished, cells cultured in 96-well plates were used to determine proliferation, according to the colorimetric method described above. Cells in 24-well plates were collected for HO proteins analysis by Western blot, and cell culture medium was collected for HO activity assay.

**Materials and data analysis.** FBS, DMEM, hemin, Hoechst 33258, propidium iodide, RNase, and G418 were purchased from Sigma. SnPP was from Porphyrin Products (Carnforth, UK). Antibodies against HO-1 and HO-2 were purchased form Stressgen Biotechnologies, and that against p21 (M-19) from Santa Cruz Biotechnology. DharmaFECT1 transfection regents, HO-1, and scramble control siRNA were purchased from Dharmacon (Chicago, IL). Probe DCFH-DA was purchased from Invitrogen. All data are expressed as means ± SE from at least three experiments, unless otherwise stated. Statistical analyses were performed using Student’s t-test or one-way ANOVA.

**RESULTS**

Hemin treatment inhibited the proliferation of VSMCs. VSMCs were cultured in medium supplemented with hemin (5 μM) for different periods. Hemin treatment significantly de-
creased cell populations by 10.2, 16.8, 11.7, and 18.3% at 4, 7, 14, and 21 days, respectively, compared with those of untreated cells at matching time points (Fig. 1A). The effects of 7- and 21-day hemin treatment were reversed by coapplication of SnPP (10 μM), an inhibitor for HO, but application of SnPP alone had no significant effect on cell populations (Fig. 1, B and C). Similar results after 7-day treatment were also observed using the colorimetric method to measure cell population (Fig. 1D). To test whether decreased cell population is resulted from an increased cell apoptosis, we examined the effect of hemin treatment on cell apoptosis. Cells undergoing apoptosis would show condensation and fragmentation of nuclei by Hoechst 33342 staining. We did not, however, observe a significant difference in the percentage of apoptotic cells between untreated and hemin-treated cells (Fig. 2).

**Hemin treatment inhibited cell cycle progression.** To understand the underlying mechanism of hemin-induced inhibition of VSMC proliferation, cell cycle distributions in untreated and treated cells were analyzed. In untreated cells, the percentages of cells in G0/G1, S, and G2/M phases were 64.6, 14.5, and 20.8%, respectively (Fig. 3). However, the percentage of cells in G0/G1 phases was increased to 69.1%, whereas the percentages of cells in S and G2/M phases were significantly decreased to 12.4 and 18.0%, respectively, in cells treated with hemin for 7 days (Fig. 3), indicating an inhibited cell growth. Coapplication of SnPP reversed the effects of hemin on cell cycle distribution by significantly decreasing the percentage of cells in G0/G1 phases to 65.1%, but increasing the percentage of cells in S and G2/M phases to 15.1 and 20.2%, respectively. Cell cycle distribution of VSMCs treated by coapplication of SnPP and hemin was not different from that of untreated cells. Furthermore, no significant effect on cell cycle distribution was detected in cells treated by SnPP alone, compared with untreated cells (Fig. 3). These results indicate that hemin treatment inhibited VSMC proliferation by slowing down cell cycle progression and arresting cells at G0/G1 phases.

**Effects of chronic hemin treatment on protein levels of HO-1 and HO-2.** To understand the relationship of HO expression and the inhibitory effect of hemin on cell proliferation, we determined protein levels of HO-1 and HO-2 in hemin-treated cells. Hemin treatments for 4, 7, 14, and 21 days significantly increased the level of HO-1 proteins in VSMCs (Fig. 4, A and B).

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Fig. 1. Effect of long-term hemin treatment on the proliferation of vascular smooth muscle cells (VSMCs). A: representative Hoechst 33342 staining of VSMCs with or without hemin (5 μM) treatment for 7 days. B and C: changes in cell population after 7- or 21-day treatment with hemin (5 μM), tin protoporphyrin IX (SnPP; 10 μM), or hemin with SnPP. Data were pooled from at least 4 experiments. D: changes in cell proliferation after different treatments for 7 days were determined by colorimetric method. Data were obtained from 8 experiments. *P < 0.05 vs. untreated group. #P < 0.05 vs. hemin-treated group.

Fig. 2. Effect of long-term hemin treatment on the apoptosis of VSMCs. A: representative Hoechst 33342 staining of VSMCs with or without hemin (5 μM) treatment for 7 days. B: summary of the apoptotic status of VSMCs with or without hemin treatment for 7 days. Results were obtained from 9 experiments.

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B). Coapplication of SnPP with hemin did not inhibit hemin-induced HO-1 expression, but significantly induced HO-1 to even higher levels compared with hemin treatment alone (Fig. 4, A and B). Furthermore, the presence of SnPP alone significantly increased HO-1 protein expression compared with that of untreated cells. The expression level of HO-2 proteins was not significantly affected by hemin treatments compared with untreated cells (Fig. 4, C and D).

**Increased HO activity and decreased p21 levels by chronic hemin treatment.** Consistent with the increased HO-1 protein level in hemin-treated cells, activities of HO were significantly increased in cells treated with hemin for 7 and 21 days compared with that of time-matched untreated cells (Fig. 5). Coapplication of SnPP with hemin decreased HO activities to the level in untreated cells. SnPP alone had no significant effects on basal HO activity (Fig. 5). The protein level of p21 in VSMCs treated with hemin for 7 and 21 days was significantly increased (Fig. 6). Application of SnPP with hemin significantly decreased p21 level and so did SnPP alone, compared with that of untreated cells (Fig. 6).

**Changes in ROS level in hemin-treated cells.** Hemin treatment for 7 days decreased oxidized DCF level, indicating a decrease in the production of ROS. Coapplication of SnPP with hemin reversed the effect of hemin by increasing oxidized DCF to the level in untreated cells (Fig. 7). However, application of SnPP alone also decreased oxidized DCF level compared with untreated cells (Fig. 7).

**The proliferation of VSMCs after hemin withdrawal.** To further elucidate whether the chronic hemin treatment causes a prolonged cell proliferation inhibition and HO-1 protein induction, cells treated with hemin for 21 days were released from hemin treatment. We found that these VSMCs, after a 7-day hemin withdrawal, had a proliferation rate similar to that of time-matched untreated cells (Fig. 8). Western blot results indicated that HO-1 protein level in 21-day hemin-treated cells, after 7-day hemin withdrawal, was decreased to a similar level in time-matched untreated cells (Fig. 8).

**Silencing of HO-1 in VSMCs abolished the effect of hemin on proliferation.** HO-1 siRNA transfection or scramble control siRNA did not alter the proliferation of cultured cells or the total HO activity of the unstimulated cells (Fig. 9). HO-1 protein expression, but not HO-2 protein expression, was significantly reduced by HO-1 siRNA transfection (Fig. 9, B).
and C). While hemin treatment (5 μM for 7 days) significantly decreased the proliferation of VSMCs transfected with scramble control siRNA, HO-1 siRNA transfection nullified hemin-suppressed cell proliferation (Fig. 9A). The same hemin treatment upregulated HO-1 expression in cells transfected with scramble control siRNA, but HO-1 siRNA transfection abolished the upregulation of HO-1 induced by hemin (Fig. 9C). Total HO activity in scramble control siRNA transfected cells, reflected by the bilirulin released into cell culture medium, was significantly increased by 160% after hemin treatment. In contrast, total HO activity in HO-1 siRNA transfected cells only increased by 17.5% after hemin treatment (Fig. 9D).

**DISCUSSION**

Hemin is an extremely versatile molecule that exhibits multifaceted effects, depending on cell types. For example, anti-proliferative effect of hemin was reported in VSMCs (19), hepatoma cells (7), and renal tubular epithelial cells (11). On the other hand, hemin promotes proliferation of human umbilical vein endothelial cells (12) and rabbit coronary microvessel endothelial cells (6). In our laboratory’s previous studies, administration of hemin for 3 consecutive weeks normalized blood pressure in SHR and reversed the eutrophic inward remodeling of small peripheral arteries of SHR (34). To understand the mechanism for the beneficial effect of long-term hemin treatment on blood pressure, we investigated the effect of long-term hemin treatment on the growth of VSMCs in vitro using a concentration of hemin close to the circulatory level in SHR reached by hemin daily injection at 15 mg·kg⁻¹·day⁻¹ (34). At 5 μM, hemin significantly inhibited VSMC proliferation over a 21-day treatment period. To our knowledge, this is the first study investigating the effect of hemin treatment for as long as 3 wk on VSMC proliferation. Cell population was decreased by an average of 14.2% over the 21-day treatment period. Hemin treatment for 7 days increased the percentage of cells in G0/G1 phases by 4.5%, while it decreased the percentage of cells in S and G2/M phases by 5%. Consistent with our results, inhibited proliferation measured by DNA synthesis was reported in cultured human airway smooth muscle cells by hemin treatment (10 μM) (31) and in rabbit VSMC treated with hemin (20–100 μM) (19).

Apoptosis and cytotoxicity effects of hemin were reported in cultured rat basilar artery VSMCs and hepatoma cells exposed to hemin for 24 h at 40 and 50 μM, respectively (7, 21). In our study, no significant effect of hemin on cell apoptosis was
observed in cells treated with hemin for 7 days. In cultured renal tubular epithelial cells, hemin (10 μM) treatment for 24 h had no effect on apoptosis (11). A variety of cells, including auditory cell (16), liver cell (39), endothelial cell (27), and human monocyte (17), were protected by hemin from cytotoxicity and apoptosis induced by several treatments. Thus the cytotoxic or cytoprotective effects of hemin might depend on its concentration and cell types. Our results suggest that chronic hemin (5 μM) treatment had no effect on VSMCs' apoptosis.

Hemin-induced HO-1 expression was reported both in whole animal (23, 34) and cultured cells, including rabbit VSMCs (19), bovine VSMCs (3), human HaCaT keratinocyte (12), rat renal tubular epithelial cell (11), and auditory cells (16). Consistent with previous reports, induced HO-1 expression was also observed in our study in cultured VSMCs treated with hemin at 5 μM from 4 to 21 days. To determine whether the inhibited cell proliferation by hemin is due to inducible HO-1, HO inhibitor SnPP was applied to cultured VSMCs. Coapplication of SnPP blocked the effects of hemin treatment on cell proliferation and cell cycle distribution, while SnPP alone had no significant effect on these cellular events. Interestingly, SnPP alone increased HO-1 protein expression, and coapplication of SnPP with hemin further upregulated HO-1 proteins. Similar effects of hemin/SnPP and SnPP alone on HO-1 mRNA and protein were reported in lung tissues of mice or liver from Sprague-Dawely rats (4, 26, 38). A transcriptional de-repression mechanism has proposed that heme activates HO-1 gene expression by binding and removing transcriptional repressor (29, 30). SnPP may bind and remove transcriptional repressors like heme (24), leading to HO-1 upregulation. On the other hand, synthetic protoporphyrin, such as SnPP, is a structural analog of heme but cannot be degraded by HO. Consequently, although HO-1 protein expression may be upregulated, HO proteins still cannot break down heme to produce CO and bilirubin. HO activity is thus inhibited. Therefore,
it is understandable that, despite increased HO-1 protein expression induced by hemin/SnPP or SnPP alone, HO activity was still blocked by SnPP to a basal level comparable with that in untreated cells.

Upregulation of p21 protein, HO-1-derived CO, and bilirubin-dependent modulation of ERK1/2 phosphorylation have been suggested to be responsible for HO-1-mediated inhibition of cell proliferation (19, 25, 31). Cyclin-dependent kinase inhibitor p21 is an important negative regulator for cell proliferation (8). In rat renal tubular epithelial cell, upregulation of p21 is dependent on hemin concentrations (5, 10, and 20 μM) (11). Deletion of p21 gene in mouse VSMCs abolished the inhibitory effect of HO-1 on cell proliferation (9). Consistent with previous reports, we found that inhibition of VSMC proliferation by hemin treatment for 7 and 21 days was accompanied by an increased expression of p21 protein. Application of SnPP successfully blocked the effect of hemin on p21 protein. Similarly, increase in p21 protein in rat renal tubular epithelial cells treated by 10 μM hemin for 24 h was abolished by zinc protoporphyrin (10 μM) (11). Because HO-2 expression is constitutive, hemin-induced HO-1 protein would be the main contributor for the change of HO activity. Therefore, HO-1 may be responsible for the induction of p21 protein and the inhibition of cell proliferation. Intriguingly, we found that SnPP decreased expression of p21 protein, with or without hemin treatment, to a level lower than that of untreated cells in the present study. It appears that SnPP may affect p21 level via other currently unknown pathways. We also examined the levels of p27, cyclin A, and phospho-Akt with or without hemin treatments, but no difference was observed (data not shown).

Hemin has been regarded as a prooxidant. Inducible HO-1 and its catalytic product bilirubin have been proposed to counteract oxidant-induced cell injury (3). On the other hand, oxidants, including ROS, are signals involved in the regulation of cell proliferation (36). Thus the level of oxidants was measured in hemin-treated and untreated cells using DCFH-DA probe. Oxidized DCF reflects the production of H2O2, peroxynitrite derived from superoxide anion and nitric oxide. Our study revealed that hemin treatment decreased oxidized DCF level in VSMCs, indicating a decrease in oxidants. This could be explained by increased bilirubin production from hemin-induced HO activity. In the same line, application of SnPP blocked the effect of hemin on oxidized DCF level. Our results indicate that HO activity may be responsible for the change of redox state in hemin-treated VSMCs. However, we found that oxidized DCF level was decreased in cells treated by SnPP alone compared with untreated cells. Although SnPP increased the expression of HO-1, HO activity was not significantly affected by SnPP treatment. Therefore, other pathways, in addition to HO activity, may be involved in the decreased oxidized DCF in cells treated by SnPP.

To avoid the nonspecific effect of SnPP on other proteins and to confirm the specific inhibition of HO-1 by SnPP, two approaches were taken. The first one was to use low concentration of SnPP. It should be known that SnPP at low concentration may be more specific for HO-1 inhibition and have the advantage that it will not affect cell proliferation at this low concentration. The second approach we used was to transfect cells with HO-1 siRNA to specifically inhibit the expression of HO-1. The results shown in Fig. 9 lead to the same conclusion as those using SnPP, demonstrating that the cellular and molecular effects of hemin treatment on VSMCs are mediated by the upregulated HO-1 expression. It has to be pointed out, however, that both approaches failed to affect total basal HO activity. This can be explained, in the presence of low concentration of SnPP, by the induction of HO-1 expression by SnPP, which compromised its inhibitory effect on total HO activity.
The unchanged total basal HO activity in HO-1 silenced cells may be related to unaffected HO-2 expression. Thus, at a given basal cellular heme level, HO-2 enzyme may increase its activity to compensate decreased HO-1 activity due to HO-1 silencing. This speculation also explains increased total HO activity in hemin-treated and HO-1 silenced cells.

In adult SHR treated with hemin (15 mg/kg) through implanted osmotic minipumps for 3 consecutive weeks, the systolic blood pressure was normalized, and this normalization was maintained for 9 mo after the removal of hemin minipumps (34). Furthermore, upregulation of HO-1 expression in vascular tissues and the reversed vascular remodeling were also sustained for 9 mo after withdrawal of hemin (34). One hypothesis for this sustained anti-hypertension effect of heme treatment regime is that, during the 3-wk treatment period, heme might turn on some long-term mechanisms, so that, even after heme withdrawal, the increased HO-1 expression and normalized vascular remodeling would not be turned off. This hypothesis was tested here by examining HO-1 expression and cell proliferation in cultured VSMCs after hemin withdrawal. To this end, cells were treated with heme for 21 days, and then the cells were continuously cultured in the absence of heme from the culture medium. Withdrawal of hemin treatment for 7 days was sufficient to reverse the proliferation rate and HO-1 expression of these cells to the levels similar to that of untreated cells. These in vitro results indicated that a 21-day hemin treatment could not sustain the elevated expression of HO-1 protein in cultured VSMCs after hemin withdrawal. Therefore, the sustained upregulation of HO-1 expression in vascular tissues and normalization of blood pressure of SHR after 3-wk hemin treatment (34) may still result from the residual heme accumulated within vascular wall or inside VSMCs.

In conclusion, long-term heme treatment at physiologically or therapeutically relevant concentrations inhibited VSMC proliferation by decelerating cell cycle progression through arresting cells at G0/G1 phases. Induction of HO-1 by heme, and thus increased HO activity, is responsible for the inhibitory effect of heme on cell growth. Changes in the level of p21 and ROS might also be involved in heme-mediated inhibition of VSMC proliferation.

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