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Hemin-induced Erk1/2 activation and heme oxygenase-1 expression in human umbilical vein endothelial cells

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Abstract

Hemin has been reported to be protective in the pathological process, but its protective mechanisms have not been precisely defined. Hemin could induce Erk1/2 phosphorylation in astrocyte. Erk1/2 phosphorylation has been proved to be involved in many growth signals cellular transduction. However, little study has been conducted as to the relationship between hemin and Erk1/2 activation in human umbilical vein endothelial cells (HUVECs). The present study aimed to investigate the relationship between hemin and Erk1/2 phosphorylation in HUVECs. The results showed that low concentration of hemin induced and sustained phosphorylation of Erk1/2 for a long time. The HO inhibitor protoporphyrin IX zinc (II) abrogated phosphorylation of Erk1/2 induced by hemin. Biliverdin, one of the metabolites of hemin, obviously induced the Erk1/2 phosphorylation in HUVECs. Both hemin and biliverdin promoted HUVEC cell growth. The results strongly suggested that hemin could induce and sustain Erk1/2 phosphorylation in HUVECs by way of HO-1 induction and biliverdin produced from HO-1 catalysing hemin degradation.

Keywords: *Endothelial cells, hemin, Erk*

Abbreviations: *HUVECs, human umbilical vascular endothelial cells; HO-1, heme oxygenase-1; ECs, endothelial cells; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinases; ZnPP, protoporphyrin IX zinc (II); Erk1/2, extracellular signal regulated kinases 1/2; Hb, haemoglobin; DFO, deferoxamine mesylate salt; CORM, tricarbonyldichlororuthenium (II) dimer (a kind of exterior carbon monoxide-releasing molecule); FAC, ferric ammonium citrate; ECL, chemiluminescence detection; BrdU, Bromodeoxyuridine.*

Introduction

It is well known that haemoglobin is a key component of erythrocytes, with release of haemoglobin from senescent erythrocytes broken down into heme and globin moieties. Heme rapidly intercalated into the plasma membrane of endothelial cells (ECs), where it can catalyse oxidative injury and cause cell death, which are involved in the pathogenesis of atherogenesis, reperfusion injury and the organ injury accompanying haemoglobinemia or myoglobinemia [1–3]. In contrast, Balla et al. [3] and Juckett et al. [4] found that more prolonged contact of ECs with heme rendered them remarkably resistant to subsequent oxidant challenges caused by exposure to peroxyl (H_2O_2), activated neutrophils or oxidized low density lipoprotein. Incubation of ECs with heme induced

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the production of hemedegrading enzyme, Heme Oxygenase-1 (HO-1). HO-1 is the key enzyme in heme catabolism, which degraded heme to CO, Fe and biliverdin. HO-1 protein has been considered to play an important role in the protection of the vasculature against heme [5].

Hemin has been found to lower blood pressure in spontaneously hypertensive rats [6] and prevented hypoxia-induced pulmonary hypertension [7]. Some research also reported that hemin had prevented vascular smooth muscle cells against Balloon injury [8] and prevented rat carotid artery against Balloon injury [9]. However, at present it remains unclear whether protection against heme is afforded by the induction of HO-1.

Erk1/2 belonged to the mitogen-activated protein kinases (MAPK) family [10]. In mammalian cells, three important groups of kinases, Erk1/2, c-jun NH_2-terminal kinase (JNK)/SAPK and p38 MAPK, composed the MAPK family [11]. Erk1/2 cascade appeared to be a main mediator signal promoting cell proliferation, differentiation or survival, whereas JNK and p38 MAPK cascades appeared to be involved in cell responses to stresses [12]. Erk1/2 could be activated by the upstream kinases Ras, MEK and then phosphorylated many downstream protein kinases or regulated some genes transcription [10,13].

Although research has shown that hemin induced Erk1/2 phosphorylation in astrocyte [14], to our knowledge phosphorylation of Erk1/2 in vascular endothelial cells has not been reported. Hence, in this present study, we aimed to elucidate the relationship between hemin and Erk1/2 phosphorylation in HUVECs.

Materials and methods

Reagents

Hemin, Protoporphyrin IX zinc (II) (ZnPP, HO inhibitor), Bovine haemoglobin (Hb, CO scavenger), Deferoxamine Mesylate salt (DFO, iron chelator), Tricarbonyldichlororuthenium (II) dimer (CORM, a kind of exterior carbon monoxide–releasing molecule) [15], Ferric ammonium citrate (FAC, iron donor) and MTT were all purchased from Sigma-Aldrich company (St. Louis, MO). Biliverdin was purchased from MP Biomedicals (Cat. No. 194886, Irvine, CA). Bromodeoxyuridine (BrdU) were purchased from the Institute of Biochemistry and Cell Biology (SIBCB, Shanghai 200031, China).

Cell culture and treatment

HUVECs (a gift from Dr Chang Bian, Zhejiang University) were cultured in RPMI 1640 (Genom Biomed Technology. Inc, Hangzhou, China) medium supplemented with 50 μg/ml Gentamycin and 10% foetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Hangzhou, China).

After serum-free culture for 16 h, HUVECs were incubated with 1, 5, 10, 25, 50 μmol/L of hemin or 100 μmol/L of H_2O_2 in a 37°C humidified environment containing 5% CO_2 and the phosphorylation of Erk1/2 in HUVECs was detected by Western blot assay.

After pretreatment with either ZnPP, Hb or DFO for 120 min, HUVECs were incubated with hemin for 120 min. Phosphorylation of Erk1/2 in HUVECs was then detected by Western blot.

HUVECs were separately incubated with 14.29 μmol/L of CORM [15], 10 μmol/L of FAC or 10 μmol/L of biliverdin for 120 min to investigate the induction of Erk1/2 phosphorylation.

Western blot assay

Cell lysates were subjected to a sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred to a nitrocellulose filter membrane. After blocking with blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v non-fat dry milk), nitrocellulose membranes were incubated with primary antibodies against total-Erk1/2 and phospho-Erk1/2 (Cell Signaling Technology, Inc. Danverse, MA) at 1:1000 dilution or primary antibody against HO-1 (Stressgen Biotechnologies Corporation, Victoria, Canada) at 1:2500 dilution. Membranes were then washed and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies. Immune complexes were exposed using the enhanced chemiluminescence detection system (Cell Signaling Technology, Inc. Danverse, MA).

Real-time PCR

Total RNA was extracted by Trizol (Invitrogen, Carlsbad, CA) and mRNA was reverse-transcribed with Avian Myeloblastosis Virus reverse transcriptase (BBI Diagnostics, MA). To quantify the expression of *HO-1* mRNA, real-time PCR was performed with Light Cycler system (Roche Diagnostics Biochemical products, IN). The sequences of primers were: HO-1 (NM_002133) sense, 5′-GCAACCC-GACAGCATGC-3′, antisense: 5′-TGCGGTGCA GCTCTTCTG-3′; β-actin sense, 5′-CACCACAC CTTCTACAATGAG-3′, antisense: 5′-CACCACAC CTTCTACAATGAG-3′.

Since the difference in the primer efficiency between the gene of interest primer set and endogenous control primer set was less than 5%, comparative CT method was used. The ΔCts of the target genes were normalized to the levels of β-actin as an endogenous control. The degree of change in each targeted gene was calculated and compared with the control. The

results were expressed as the means ± SE. The PCR experiments were repeated three times.

MTT assay

HUVECs were seeded into 96-well plates and were incubated in medium containing 10 μmol/L of hemin or biliverdin for 24 h at 37°C. MTT was dissolved in DMSO at 5 mg/ml. The stock solution was added to the culture medium at a dilution of 1:10. The plates were incubated at 37°C for 4 h. The purple formazan crystals formed after MTT metabolization were dissolved in DMSO and, after centrifugation, the optical density of the supernatant was read at 570 nm.

DNA synthesis

HUVECs were seeded in slides with 5% FBS culture medium. On the 2nd day, the culture medium was replaced with serum-free medium for 16 h so as to synchronize the cells in their G0 phase of the cell cycle. Then these cells were cultured with hemin or biliverdin dissolved in the serum-free medium for 24 h, followed by 120 min incubation of 10 μmol/L of BrdU in the 5% FBS culture medium. Non-specific sites were blocked for 30 min using 5% bovine serum albumin (BSA). The cells were incubated with mouse monoclonal anti-BrdU antibody (Boster Co. Ltd, China) at 37°C for 60 min. After 3-times of rinse with PBS, the slides were incubated with Biotin-conjugated second antibody for 20 min at room temperature, followed by washing and incubation with Streptavidin-Peroxidase for 20 min at room temperature. The DAB substrate was used for 10 min at room temperature. The BrdU positive cells were counted under a microscope and the percentage of S-phase nuclei in HUVECs was calculated by dividing the number of BrdU positive cells by the total number of cell nuclei. Experiment was repeated three times.

Statistical analysis

Data were expressed as means ± SE and one-way ANOVA was conducted to compare multiple groups. For two groups with non-normally distributed data, the Wilcoxon rank sum test was used. *P*-values less than 0.05 were regarded as statistically significant. These analyses were performed using SPSS 11.0.

Results

Hemin induced and sustained the phosphorylation of Erk1/2 in HUVECs

HUVECs were incubated with 25 μmol/L of hemin for 24 h and then the phosphorylated Erk1/2 and total Erk1/2 were detected by Western blot. 100 μmol/L of H_2O_2 was used as the positive control. At 25 μmol/L of hemin, phosphorylated Erk1/2 was induced, but was less than that induced by H_2O_2 (Figure 1A). To investigate the relationship between hemin and Erk1/2 phosphorylation, HUVECs were incubated with serial concentrations of hemin (1, 5, 10, 25 or 50 μmol/L) for 24 h. Western blot results revealed that at the concentration of 1–10 μmol/L, hemin could induce the Erk1/2 phosphorylation in HUVECs, but no change was found when 50 μmol/L was used (Figure 1B). At the following experiments we chose 10 μmol/L hemin and the time course of phosphorylation of Erk1/2 was investigated. At the same time, 100 μmol/L of H_2O_2 was used as the control. Interestingly, we found that the phosphorylation of Erk1/2 was induced even after 30 min incubating with 10 μmol/L of hemin and this increased phosphorylation lasted at least for 120 min. We also observed the phosphorylation of Erk1/2 sustained on a higher level, even at the 180 min time point (Figure 1C). Meanwhile, we tested the phosphorylation of Erk1/2 by using 100 μmol/L of H_2O_2 with 10 min incubation. It was found that phosphorylation of Erk1/2 was sustained at a high level after 30 min incubation and then it decreased 60 min after the treatment (Figure 1D).

Hemin induced HO-1 expression and ZnPP abrogated Erk1/2 phosphorylation induced by hemin

Hemin was an inducer of HO-1 protein, which was confirmed in our experiments on HUVECs. We treated the HUVECs with hemin for 120 min, the cellular RNA was extracted for real-time PCR analysis. The results showed that *HO-1* mRNA was significantly up-regulated (Figure 2A). Western blot assay also showed that the HO-1 protein was also induced after hemin treatment for 120 min (Figure 2B). To further elucidate whether HO-1 or its three metabolites participated in regulating the Erk1/2 phosphorylation in HUVECs, we pre-treated HUVECs with HO inhibitor ZnPP, CO scavenger Hb or iron chelator DFO for 120 min, then incubated with hemin for 120 min. These experiments data showed that ZnPP could obviously abrogate the phosphorylated Erk1/2 induced by hemin. Interestingly, Hb or DFO itself could also induce the phosphorylation of Erk1/2 in HUVECs. No difference was found between the administration of Hb or DFO alone and the combined use with hemin (Figure 2C).

Effect of three metabolites of hemin on the phosphorylation of Erk1/2

Because Hb or DFO itself could also induce the phosphorylation of Erk1/2 in HUVECs (Figure 2C), we are not sure whether CO, Fe^{2+} or biliverdin could induce the phosphorylation of Erk1/2 in HUVECs. To make sure which metabolite(s) of hemin contribute(s) to the phosphorylation of Erk1/2 induced by

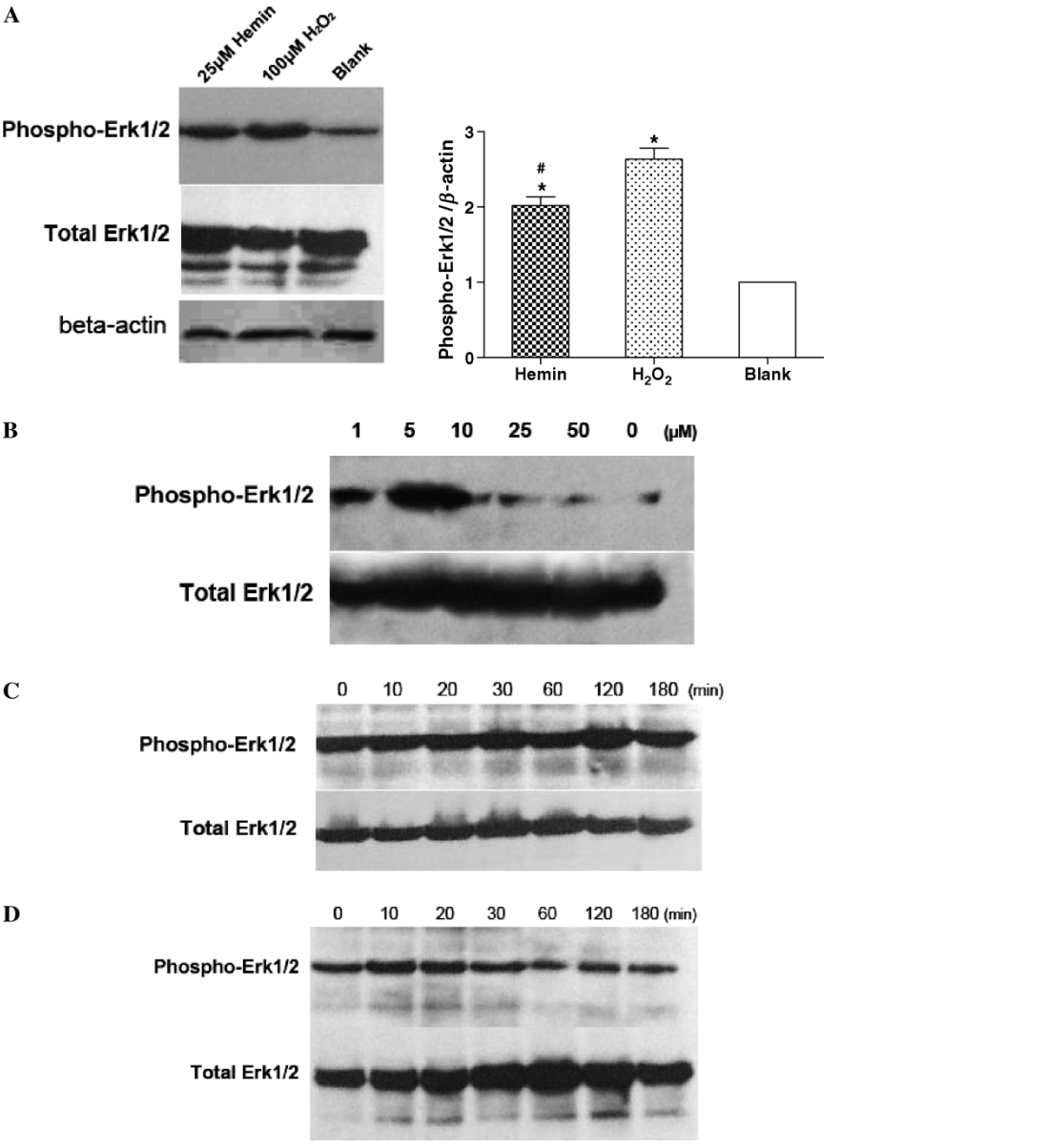

Figure 1. Hemin induced and sustained phosphorylation of Erk1/2 in HUVECs. (A) Hemin induced Erk1/2 phosphorylation. HUVECs were incubated with 25 μmol/L of hemin or 100 μmol/L of H_2O_2 for 24 h. Western blot analysis of Erk1/2 phosphorylation. Exposure time of Western blot was 10 s. The histogram represents the ratio between optical density of phosphorylated Erk1/2 and that of β actin ($n=3$, means $\pm$SE, $^{\star}p<0.01$ for hemin or H_2O_2 group vs Blank group; $^{\#}p<0.05$ for hemin group vs H_2O_2 group). (B) Relationship between hemin concentration and Erk1/2 phosphorylation. HUVECs were incubated with different concentrations of hemin for 24 h. Western blot was used to determine the Erk1/2 phosphorylation ($n=3$). (C) Time course of phosphorylation of Erk1/2 induced by 10 μmol/L of hemin. HUVECs were incubated with 10 μmol/L of hemin for serial time. Erk1/2 phosphorylation was determined by Western blot assay ($n=3$). (D) Time course of phosphorylation of Erk1/2 induced by 100 μmol/L of H_2O_2. HUVECs were incubated with 100 μmol/L of H_2O_2 for serial time. Phosphorylation of Erk1/2 was determined by Western blot assay ($n=3$).

hemin, we incubated the HUVECs separately with CORM, FAC or biliverdin. Finally, it was clear that biliverdin could significantly induce the Erk1/2 phosphorylation in HUVECs, whereas CO releasing molecule CORM and iron donor FAC didn't induce the phosphorylation of Erk1/2 (Figure 3).

Hemin promoted the growth of HUVECs

DNA synthesis determined by BrdU assay indicated that both 10 μmol/L of hemin and biliverdin increased the DNA synthesis in HUVECs (Figure 4A). MTT assay was employed to determine HUVECs growth. All the data showed that both 10 μmol/L of hemin and 10 μmol/L of biliverdin promoted HUVECs growth (Figure 4B).

Discussion

It has been reported that hemin was protective against tissue injuries [6,7]; however, the protection mechanism was poorly understood since there were many factors that were involved in the pathogenesis.

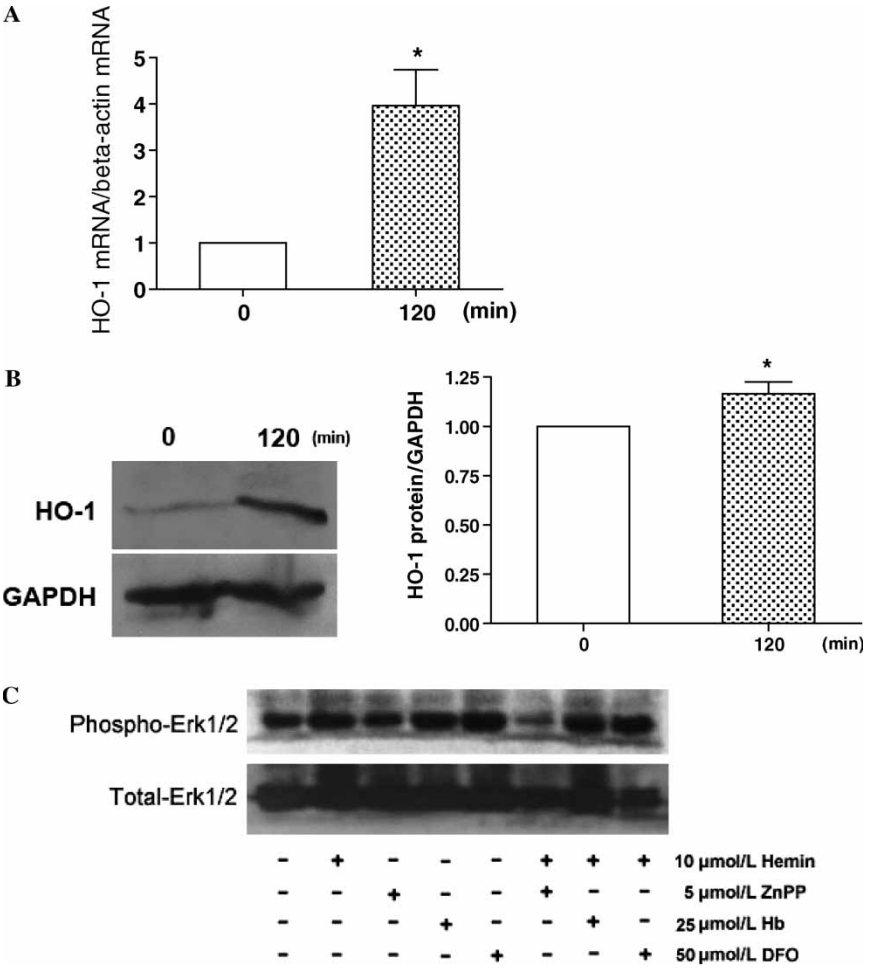

Figure 2. Hemin induced HO-1 expression and ZnPP abrogated Erk1/2 phosphorylation induced by hemin. (A) 10 μmol/L of hemin induced *HO-1* mRNA expression. HUVECs were incubated with 10 μmol/L of hemin for 120 min. *HO-1* mRNA was relatively quantified by real-time PCR ($n=3$, means ± SE, $^{\star}p<0.05$ for 120 min group vs 0 min group). (B) 10 μmol/L of hemin induced HO-1 protein expression after incubation HUVECs for 120 min. The typical graph of Western blot assay was present. The histogram represents the ratio between optical density of HO-1 protein and that of GAPDH ($n=3$, means ± SE, $^{\star}p<0.05$ for 120 min group vs 0 min group). (C) ZnPP abrogated Erk1/2 phosphorylation. HUVECs were separately pretreated with HO inhibitor ZnPP, CO scavenger Hb or iron chelator DFO for 120 min, then incubated with 10 μmol/L of hemin for another 120 min. Phosphorylation of Erk1/2 was determined by Western blot assay ($n=3$).

In our present study, we investigated the potential protective mechanism of hemin on human endothelial cells.

It has been reported that hemin induced Erk1/2 phosphorylation and cell proliferation in K562 cells [16,17]. Erk1/2 could mediate many signals transduction from outside cell to nuclear, which result in cell growth or proliferation. Some reports showed that hemin at low concentration can protect hepatocytes from glucose deprivation-induced cytotoxicity [18]; while at high concentration it can induce apoptosis in the PC12 cell line [19]. It seems hemin at low concentration was protective. Our results strongly demonstrated that hemin at low concentration could induce Erk1/2 phosphorylation in HUVECs. We then further investigated the mechanism of how the phosphorylated-Erk1/2 was induced in HUVECs. It has been reported that reactive oxygen species (ROS) played an important role in cell signalling pathway [20] and H_2O_2 has also been identified to be a cell signal molecule and served as a second messenger activated after EGF receptor activation [21]. We confirmed Guyton et al.'s [22] study that H_2O_2 at low concentration can immediately induced the Erk1/2 phosphorylation but failed

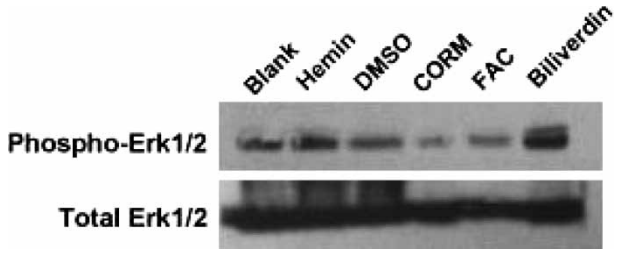

Figure 3. Effect of three metabolites of hemin on the phosphorylation of Erk1/2. HUVECs were separately incubated with 14.29 μmol/L of CORM, 10 μmol/L of FAC or 10 μmol/L of biliverdin for 120 min. Phosphorylation of Erk1/2 was analysed by Western blot assay ($n=3$).

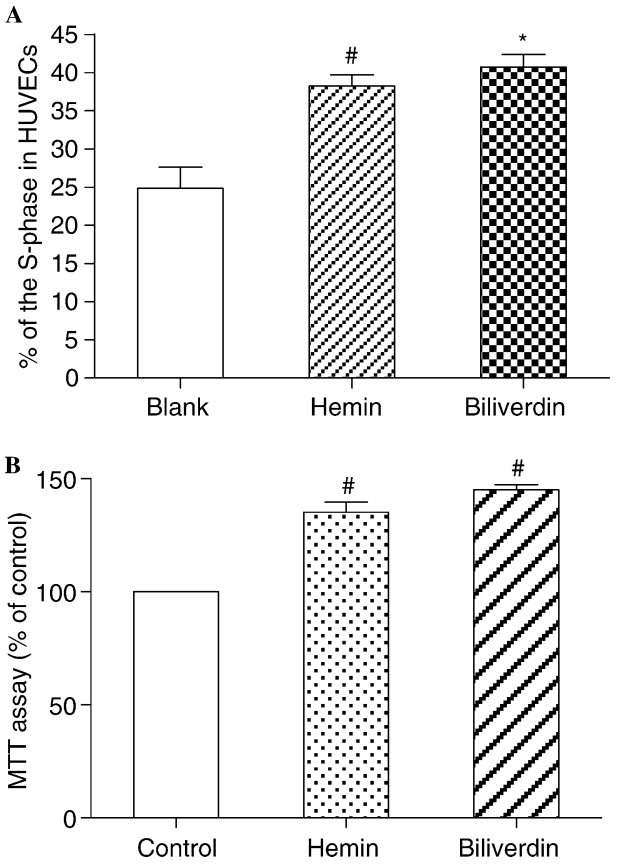

Figure 4. Hemin promoted the growth of HUVECs. (A) Percentage of the S-phase nuclei in HUVECs. HUVECs were cultured for 16 h in serum-free medium, then incubated with 10 μmol/L of hemin or 10 μmol/L of biliverdin for 24 h. BrdU assay determined the DNA synthesis in HUVECs ($n=3$, means ± SE, $^{\#}p<0.05$ for hemin group vs control group; $^{\star}p<0.01$ for biliverdin group vs control group). (B) HUVECs were incubated with 10 μmol/L of hemin or 10 μmol/L of biliverdin for 24 h. MTT assay determined the HUVEC cell growth ($n=3$, means ± SE, $^{\#}p<0.05$ for hemin or biliverdin group vs control group).

to sustain the Erk1/2 phosphorylation for a long time. Meanwhile, we found that the time course of Erk1/2 phosphorylation induced by hemin was significantly different from that induced by H_2O_2. We presumed H_2O_2 may be not involved in the hemin induction of Erk1/2 phosphorylation in HUVECs.

We treated the cells with 10 μmol/L of hemin for 120 min, *HO-1* mRNA and protein were obviously up-regulated in HUVECs. HO has three isoforms: HO-1, an inducible isoform; HO-2, constitutively synthesized; HO-3, a less efficient heme catalyst, being considered likely a pseudogene derived from HO-2 transcripts [23]. Treatment with hemin or by heat shock (42°C) led to a remarkable increase in the *HO-1* mRNA levels, while *HO-2* mRNA expression was not induced at all [24]. In our study, hemin markedly induced HO-1 protein expression and Erk1/2 phosphorylation, HO inhibitor ZnPP obviously abrogated the phosphorylated Erk1/2 induced by hemin in HUVECs. These results strongly suggested that it was HO-1, not HO-2 that played a key role in the Erk1/2 phosphorylation induced by hemin in HUVECs.

HO-1 can degrade hemin into CO, Fe^{2+} and biliverdin. Biliverdin could rapidly convert to bilirubin which was protective against cellular injury induced by ROS [25,26]. Taille et al. [27] found that biliverdin reduced Erk1/2 phosphorylation in airway smooth muscle (ASM) cells. In our study, biliverdin induced the phosphorylation of Erk1/2 in HUVECs, which indicated that in HUVECs both HO-1 protein and biliverdin took part in the Erk1/2 phosphorylation induced by hemin. Based on our data, we got the conclusion that hemin could induce and sustain Erk1/2 phosphorylation in HUVECs by way of HO-1 induction and biliverdin produced from HO-1 catalysing hemin degradation.

Heme could rapidly intercalate into the plasma membrane of ECs, where it can catalyse oxidative damage and cause cell death [1,2]. Taille et al. [27] found that hemin inhibited proliferation of ASM cells through biliverdin-mediated Erk1/2 phosphorylation reduction. Our results showed that both 10 μmol/L of hemin and biliverdin promoted HUVEC DNA synthesis and cell growth. We presumed hemin at low concentration might promote cell growth by induction of Erk1/2 phosphorylation which was mediated by HO-1 induction and biliverdin production in HUVECs.

We still don't know the downstream target of phosphorylated Erk1/2 induced by hemin. Fernando et al. [28] found that estradiol induced the phosphorylation of Erk1/2, which then phosphorylated the Ser112 and Ser136 at Bad protein to abrogate apoptosis in breast cancer cells. Bad protein may be the downstream target of Erk1/2 phosphorylation induced by hemin, which will be investigated in our next study.

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