Original Paper

High Glucose Promotes Tumor Invasion and Increases Metastasis-Associated Protein Expression in Human Lung Epithelial Cells by Upregulating Heme Oxygenase-1 via Reactive Oxygen Species or the TGF- β_1 /PI3K/Akt Signaling Pathway

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Key Words

HO-1 (heme oxygenase-1) • Human lung epithelial cells • CD147 • MMP-9 • High glucose (HG) • Reactive oxygen species (ROS)

Abstract

Background: Growing evidence indicates that heme oxygenase-1 (HO-1) is up-regulated in malignancies and subsequently alters tumor aggressiveness and various cancer-related factors, such as high glucose (HG) levels. HO-1 expression can be induced when glucose concentrations are above 25 mM; however, the role of HO-1 in lung cancer patients with diabetes remains unknown. Therefore, in this study, we investigated the promotion of tumor cell invasion and the expression of metastasis-associated proteins by inducing the up-regulation of HO-1 expression by HG treatment in A549 human lung epithelial cells. **Methods:** The expression of HO-1 and metastasis-associated protein expression was explored by western blot analysis. HO-1 enzymatic activity, reactive oxygen species (ROS) production and TGF- β_1 production were examined by ELISA. Invasiveness was analyzed using a Transwell chamber. **Results:** HG treatment of A549 cells induced an increase in HO-1 expression, which was mediated by the HG-induced generation of reactive oxygen species (ROS) and transforming growth factor- β_1 (TGF- β_1) in a concentration- and time-dependent manner. Following the increase in HO-1 expression, the enzymatic activity of HO-1 also increased in HG-treated cells. Pretreatment with N-acetyl-L-cysteine (NAC) or with phosphatidylinositol 3-kinase (PI3K)/Akt inhibitors attenuated the HG-induced increase in HO-1 expression. HG treatment

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of A549 cells enhanced the invasion potential of these cells, as shown with a Transwell assay, and increased metastasis-associated protein expression. However, HO-1 siRNA transfection significantly decreased these capabilities. **Conclusion:** this study is the first to demonstrate that HG treatment of A549 human lung epithelial cells promotes tumor cell invasion and increases metastasis-associated protein expression by up-regulating HO-1 expression via ROS or the TGF- β_1 /PI3K/Akt signaling pathway.

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Introduction

Epidemiological studies have shown an association between diabetes mellitus and cancer, and patients with diabetes mellitus are at a higher risk of liver, pancreatic, and breast cancer, irrespective of body mass index and of other confounding factors [1-4]. Diabetes may increase tumor cell proliferation and metastases in cancer patients [5]. It is possible that cCommon factors, including high levels of insulin and insulin-like growth factor [6], may have a role in cancer development in patients with diabetes mellitus. The present study indicated that inflammatory cytokines and oxidative stress were also primary causes of cancer development in patients with diabetes mellitus [7, 8]. In addition, glucose has an important role in cancer cell growth, and glucose metabolism selectively affects gene expression [9]. Cancer patients with diabetes, including lung cancer patients and those with other types of organ cancers, have a poorer prognosis [5, 10]. Moreover, diabetes was independently associated with a significantly higher risk of all-cause mortality in patients with non-small-cell lung cancer (NSCLC) [10]. Additionally, high glucose (HG) contributes to tumor formation, epithelial-mesenchymal transition, and oncofetal fibronectin production in cultured human epithelial lung cells (A549) [11]. These studies demonstrate an association between NSCLC development and progression and diabetes. Thus, a better understanding of these molecular signaling pathways could be critical for the development of therapeutic strategies against lung cancer with diabetes mellitus. Therefore, understanding the biological mechanisms by which diabetes or hyperglycemia affects NSCLC patient prognosis is necessary.

Previous studies have demonstrated that heme oxygenase-1 (HO-1) is up-regulated in malignancies and subsequently regulates tumor aggressiveness and various cancer-related factors [12, 13]. Inducible HO-1 belongs to a member of the heat shock protein family, which catalyzes the rate-limiting step of heme degradation into bilirubin, carbon monoxide (CO) and ferrous iron. Biliverdin and its metabolic products have important functions such as antioxidant, cytoprotective and anti-inflammatory effects [14, 15]. Furthermore, CO has anti-apoptotic and cytoprotective actions [16, 17]. HO-1 induction under stressful conditions protects cells against oxidative injury [18] caused by reactive oxygen species (ROS), reactive nitrogen species [19], heat shock [20], hypoxia [21], ultraviolet irradiation [22], and heavy metals [23, 24]. In addition, phosphatidylinositol 3-kinases (PI3Ks) and their downstream target Akt are a conserved family of signal transduction enzymes that play important roles in regulating HO-1 expression during many cellular processes. Furthermore, a recent study reported that the PI3K/Akt signaling pathway plays a major role in controlling HO-1 expression in different tumor cell systems [25]. HO-1 plays a positive role in cellular homeostasis, inflammatory responses and cell survival in numerous types of cells [26]. In the lung, as in many other tissues, HO-1 defends against oxidative and inflammatory insults in cells and tissues, thus maintaining cellular homeostasis [27]. HO-1 expression and enzymatic activity increase in various tumors, including renal cell carcinoma, hepatoma, and lung cancer, facilitating tumor growth, invasion, and metastasis. Several studies have reported that HO-1 expression promotes tumor growth lung cancer [28, 29], that HO-1 inhibition increased radiosensitivity in human NSCLC A549 cells [30] and that HO-1 overexpression may increase the metastatic potential of lung cancer and aid in predicting poor prognosis [12, 31-33]. Furthermore, NSCLC patients with a high HO-1 expression ratio exhibited a significantly poorer prognosis and a higher metastatic rate compared to those patients with a low HO-1 expression ratio in clinical experiments; HO-1 inhibition has been shown to have

an anti-tumor effect [12]. Interestingly, several lines of clinical evidence have demonstrated that the plasma HO-1 concentration is elevated in individuals with type 2 diabetes mellitus [34] and that lung cancer with diabetes mellitus has a negative prognosis [5, 10]. Moreover, HG induces ROS production and increases HO-1 expression in retinal endothelial cells [35]. These findings prompted us to consider the role of HO-1 in NSCLC patients with diabetes. Therefore, we investigated whether HG enhanced the tumor invasiveness capacity and increased metastasis-associated protein expression in A549 cells and determined the role of the HO-1 protein in these processes.

Tumor metastasis is a complicated pathological process that may involve many factors. The matrix metalloproteinase (MMP) family and cluster of differentiation 147 (CD147) have important roles in the processes of tumor invasion and metastasis. MMP-9, which is a member of the MMP family, is involved in breakdown of the extracellular matrix (ECM), which promotes tumor invasion and metastasis. CD147 is a highly glycosylated transmembrane protein that regulates many protein families and that affects tumor invasiveness and metastasis [12, 36, 37]. MMP-9 and CD147 expression is associated with the malignant potential of pancreatic cancer cells [38], NSCLC cells [12] bladder cancer cells [13] and breast cancer cells [39]. Several lines of evidence have indicated that HO-1 induces tumor invasiveness and metastasis by regulating these metastasis-associated proteins [18, 40]. HO-1 appears to regulate the expression of MMP-9 in NSCLC cells, which further enhances the metastatic potential of these cells [12]. Furthermore, prior studies have reported that HG affects CD147 and MMP-9 expression in other cell types [41, 42]. However, the association between HG and CD147 or MMP-9 expression in human lung epithelial cells has not yet been established.

In this study, we investigated the effects of HG on HO-1 expression and determined the role of HO-1 in HG-enhanced tumor invasiveness capacity in human lung epithelial cells. We reported that HG increased HO-1 expression and enzymatic activity, which were mediated by the HG-induced generation of ROS and transforming growth factor- β_1 (TGF- β_1). Additionally, HO-1 knockdown attenuated the HG-enhanced tumor invasiveness capacity and decreased metastasis-associated protein expression.

Materials and Methods

Cell culture

A549 cells were maintained in Dulbecco's modified Eagle's medium/5.5 mM glucose (normoglycemic; NG; HyClone, Thermo Fisher Scientific, Inc., Beijing, China). After reaching confluence, the cells were maintained under NG conditions, or in 25 mM glucose (hyperglycemic; HG), or in 25 mM D-Mannitol (M; Sigma-Aldrich), or in 2 ng/ml TGF- β_1 (kindly presented as a gift donated by Dr. Liu T). All cells were supplemented with 10% fetal calf serum (HyClone) and kept in a humidified 37 °C/5% CO₂ incubator.

Transfection of HO-1 siRNA in vitro

Small interfering RNA (siRNA) with a specific, double-stranded 21-nucleotide RNA sequence that was homologous to the target mRNA (AAC TTT CAG AAG GGC CAG GTG) was used to silence HO-1 expression. A negative control (NC) siRNA (GTT CTC CGA ACG TGT CAC GTA) was designed and synthesized using computer software (Invitrogen, USA). The cells were transiently transfected with HO-1 siRNA using X-treme Gene siRNA Transfection Reagent (Roche, Germany) according to the manufacturer's instructions. After incubation at 37 °C/5% CO₂ for 10 h, the culture solution was replaced, and the cells were further incubated for 48-72 h.

Western blotting

Western blots were performed using a standard protocol. Briefly, a cell lysate was prepared by lysing the cells for 30 min at 4–8 °C in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 1 mM PMSF (pH 7.4) and then centrifuging the lysate at 4000 g for 15 min at 4 °C. Then, the supernatant fractions were harvested. Aliquots of the cell lysates were subjected to 12% SDS-PAGE and transferred onto

PVDF membranes. Then, the membranes were blocked for 2 h at room temperature with 5% nonfat milk and probed with the following primary antibodies: polyclonal rabbit anti-CD147 antibodies (Cell Signaling Technology, USA), polyclonal rabbit anti-MMP-9 antibodies (Cell Signaling Technology, USA), mouse anti-HO-1 antibodies (Abcam, USA), or anti-actin antibodies (Santa Cruz Biotechnology, USA). The membranes were washed extensively and incubated with fluorescence-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies (Invitrogen). Western blot bands were quantified using an Odyssey infrared imaging system (Li-Cor Bioscience, USA).

Quantitative real-time PCR

RT-PCR was performed using standard methods as described previously. The primer sequences used to amplify HO-1 were as follows: *HO-1* sense, 5'-TTC TTC ACC TTC CCC AAC TA-3', and antisense, 5'-GCA TAA AGC CCT ACA GCA AC-3'. *GAPDH* was used as an internal control, and $\Delta\Delta C_t$ was calculated for each sample, with the expression levels indicated by values of $2^{-\Delta\Delta C_t}$.

Measurement of intracellular ROS

The peroxide-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; Sigma-Aldrich) was used to measure intracellular ROS accumulation. First, A549 cells were incubated for 45 min at 37 °C in phosphate-buffered saline (PBS) containing 10 μ M DCF-DA to label intracellular ROS, and then the cells were washed three times with PBS. DCF fluorescence was detected by fluorescence ELISA.

HO-1 enzymatic activity assay

The enzymatic activity of HO was determined according to a previous protocol. Briefly, A549 cells were treated under different conditions for 2 h or 48 h. After all treatments, the cells were solubilized in Tris buffer that was supplemented with a protease inhibitor cocktail. Cell extract was added to the reaction mixtures, and then the mixtures were incubated at 37 °C for 1 h in the dark. The reactions were terminated with ice. Bilirubin was extracted with chloroform; the absorbance at 464 nm (reference wavelength 530 nm) was measured with a spectrophotometer (Amersham Pharmacia Biotech) to determine bilirubin production. HO-1 activity was expressed as the fold change compared with the NG group.

TGF- β 1 production was detected by ELISA

A549 cells were exposed to NG conditions, HG treatment or mannitol treatment for 48 h or to H₂O₂ for 2 h. Next, the culture supernatants were assayed immediately using a human TGF- β ₁ ELISA kit (Shanghai Xin Yu Biotech Co., Ltd., China) and a spectrophotometer (Amersham Pharmacia Biotech). TGF- β ₁ production was expressed as ng per ml.

Invasion analyses

Invasiveness was analyzed using a Transwell chamber (sc-3422) according to the manufacturer's instructions. A549 cells were pretreated as indicated in our experiments, then suspended in serum-free medium, and seeded in the upper chamber (2×10^4). After 12 h of incubation, the chamber was briefly washed with PBS, fixed in 4% paraformaldehyde for 15 min, and stained with 0.1% crystal violet for 30 min. A549 invasiveness was evaluated by counting the cells in six randomly selected ($\times 10$) fields.

Statistical analysis

The data are presented as the mean $\pm$ SEM, with the number (*n*) of experiments indicated. Statistical analyses were performed using the unpaired *t*-test (SPSS 16.0 software). Graphs were prepared using Adobe Photoshop software or plotted in GraphPad Prism 5 software (GraphPad Software, Inc.). *p* < 0.05 was considered significant.

Results

HG levels increase HO-1 protein and mRNA expression

We quantified the effects of HG on HO-1 protein expression levels. Compared with NG, HG treatment for 72 h significantly increased HO-1 protein and mRNA expression by 49.67% and 56.35% (Fig. 1A and C), respectively. Furthermore, the effects of the glucose

Fig. 1. Influence of HG treatment on HO-1 mRNA and protein expression. A549 cells were treated with normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 25 mmol/L). HG treatment increased HO-1 protein levels in a time- (A) and dose-dependent (B) manner (n=6), which was accompanied by time- (C) and dose-dependent (D) increases in HO-1 mRNA levels (n=6). (E) A549 cells were treated with mannitol (25 mM) in place of glucose for 48 h to exclude osmotic effects; this treatment did not affect the HO-1 protein expression levels (n=3). *p<0.05, **p<0.01, ***p<0.01 with respect to NG.

levels on HO-1 protein and mRNA expression were dose-dependent (Fig. 1B and D) and time-dependent (Fig. 1A and C). Following the increase in HO-1 expression, the enzymatic activity of HO-1 (measured in pmol bilirubin/h/mg protein) was elevated in cells exposed to HG and H_2O_2 (Fig. 3). Then, 25 mM mannitol was administered in place of glucose for 48 h to exclude osmotic effects; this treatment did not significantly affect the levels of HO-1 protein expression and enzymatic activity, as shown in Fig. 1E.

HG-induced ROS generation mediates HO-1 expression

We investigated whether HG induced ROS production using the dye DCF-DA. Compared with cells in the NG and M groups, the cells treated with HG for 48 h showed significantly increased ROS production (see Fig. 2A). Cells were also treated with N-acetyl-L-cysteine (NAC, an antioxidant) to determine the role of ROS in the HG-induced up-regulation of HO-1 expression. Treatment with NAC decreased ROS production (Fig. 2A) and attenuated the HG-induced increase in HO-1 protein expression (Fig. 2B) compared with NG cells, which was consistent with the results of previous studies [30, 31]. We examined the effects of exogenous H_2O_2 (500 μ M) on HO-1 protein expression to provide more robust evidence for ROS-induced HO-1 expression. H_2O_2 treatment recapitulated the HG response and increased HO-1 protein levels (Fig. 2B). These data strongly suggested that HG was associated with ROS generation, which increased HO-1 expression.

Fig. 2. HG-induced ROS generation mediates HO-1 expression. A549 cells were treated with NG, HG, HG+NAC (100 μ M), or mannitol (25 mM) for 48 h or H₂O₂ (500 μ M) for 2 h. (A) HG and H₂O₂ treatments induced ROS generation, NAC pretreatment inhibited HG-induced ROS generation, and mannitol treatment did not induce ROS generation (n=6). (B) H₂O₂ treatment increased HO-1 protein expression (n=6), NAC pretreatment decreased the HG- or H₂O₂-induced up-regulation of HO-1 protein expression. **p<0.01, ***p<0.001 vs the NG group.

Fig. 3. Exposure to HG and to H₂O₂ increases HO-1 activity in A549 cells. Enzymatic activity was determined spectrophotometrically by measuring the formation of bilirubin. Enzymatic activity increased in cells that were exposed to HG (25 mM) for 48 h or to H₂O₂ (500 μ M) for 2 h compared with NG. The enzymatic activity observed in cells exposed to mannitol for 48 h was similar to that of the control. NAC pretreatment inhibited enzymatic activity. All the data that are presented in these figures were obtained from at least five independent experiments. ***p<0.001 vs the NG group.

PI3K/Akt pathways are involved in HO-1 induction by HG

The PI3K/Akt signaling pathway has been found to mediate HO-1 expression in different tumor cell systems [25]. We observed that selective inhibitors of PI3K (10 μ M) and Akt (100 nM) significantly decreased HG-induced HO-1 expression (Fig. 4A). However, when we treated A549 cells with PI3K/Akt inhibitors for 2 h in NG medium containing H₂O₂ (500 μ M), HO-1 expression was slightly inhibited; however, this relation did not reach statistical significance, as shown in Fig. 4B. These results indicated that the PI3K/AKT signaling

Fig. 4. The role of the PI3K/Akt pathway in the HG-induced expression of HO-1. A549 cells were pretreated with PI3K/Akt inhibitors for 30 min and then cultivated in HG media for 48 h or in H₂O₂ (500 μ M) for 2 h. (A) Selective inhibitors of PI3K (LY294002, 10 μ M) and Akt (API-59, 100 nM) significantly decreased HG-induced HO-1 expression (n=6). (B) H₂O₂-induced HO-1 expression was slightly inhibited without statistical significance (n=6) **p<0.01, ***p<0.001 vs the NG group.

Fig. 5. HG-induced TGF- β ₁ generation and the role of TGF- β ₁ in HG-induced HO-1 expression. The supernatants of A549 cells that were treated with HG or mannitol for 48 h or with H₂O₂ for 2 h were assayed using a TGF- β ₁ ELISA kit. (A) HG treatment increased TGF- β ₁ production compared with NG. H₂O₂ and mannitol treatments did not significantly affect TGF- β ₁ production (n=5). (B) Treating A549 cells with TGF- β ₁ (2 ng/ml) increased HO-1 expression for 2 h. TGF- β ₁-induced HO-1 expression was significantly inhibited in the presence of inhibitors of PI3K (10 μ M) and Akt (100 nM) (n=5). **p<0.01, ***p<0.001 vs the NG group.

pathway at least partially participated in the HG-induced increased expression of HO-1 in a ROS-independent manner.

HG-induced TGF- β ₁ generation and the role of TGF- β ₁ in HG-induced HO-1 expression

Several lines of evidence have shown that HG treatment increases TGF- β ₁ levels in A549 cells and that TGF- β ₁ induces HO-1 expression via the PI3K/AKT signaling

Fig. 6. The effect of H_2O_2 or TGF- β_1 treatment on HO-1 protein and mRNA expression. Cells were treated with H_2O_2 (500 μ M) or TGF- β_1 (2 ng/ml) for 2 h or with both H_2O_2 and TGF- β_1 simultaneously. Compared with NG, treatment with H_2O_2 or TGF- β_1 (2 ng/ml) for 2 h or both H_2O_2 and TGF- β_1 simultaneously significantly increased HO-1 protein and mRNA expression. ** $p < 0.01$, *** $p < 0.001$ vs the NG group.

pathway [43]. Therefore, we investigated the role of TGF- β_1 in HG-induced HO-1 expression to determine the upstream regulators of the PI3K/AKT signaling pathway. A549 cells were treated with HG or mannitol for 48 h or with H_2O_2 for 2 h, and then supernatants were assayed using a TGF- β_1 ELISA kit. As shown in Fig. 5A, HG treatment increased TGF- β_1 production compared with NG. However, H_2O_2 and mannitol treatments did not significantly affect TGF- β_1 production. A549 cells that were treated with TGF- β_1 (2 ng/ml) displayed increased HO-1 expression; in the presence of inhibitors of PI3K (10 μ M) and Akt (100 nM), TGF- β_1 -induced HO-1 expression was significantly inhibited (Fig. 5B). These results demonstrated that HG-induced HO-1 expression might be involved in TGF- β_1 production via the PI3K/Akt signaling pathway.

Inhibition of HO-1 activity attenuated HG-enhanced A549 cell invasiveness

Migration and invasion have important roles in the ability of cancer cells to form metastases. We investigated the effect of HG on the invasion and migration potential of A549 cells using Transwell chambers. HG treatment for 48 h enhanced the cell invasion potential by ~50% compared with the NG group (Fig. 8A). However, mannitol treatment for 48 h did not increase the cell invasion potential compared with the NG group (see Fig. 8A). We also evaluated the effect of ROS inhibition on HG-induced A549 cell invasiveness. Pretreatment with NAC (100 μ M) significantly attenuated the invasiveness potential of these cells (Fig. 8A). To further determine the role of H_2O_2 or TGF- β_1 in the HG-induced increase in the invasion potential of A549 cells, H_2O_2 and TGF- β_1 treatments increased the invasion potential of A549 cells compared with NG (Fig. 10). We employed two siRNA interference to assess the effect of HO-1 depletion on HG-induced A549 cell invasiveness. As shown in Fig. 7A, B, C and D and Fig. 9, HO-1 siRNA treatment significantly attenuated the HO-1 protein and mRNA expression under conditions in which HO-1 siRNA treatment did not affect HG-induced ROS and TGF- β_1 production. Accompanying HO-1 depletion, HG-induced increases in HO-1 enzymatic activity and cell invasiveness were significantly inhibited compared with

Fig. 7. Effects of HO-1 knockdown on the HG-induced generation of ROS and TGF- β_1 . (A and B) Western blot and RT-PCR experiments showed that transfection with HO-1 siRNA reduced HO-1 protein and mRNA expression. (C) HO-1 knockdown did not affect HG-induced TGF- β_1 generation. (D) HO-1 knockdown did not affect HG-induced ROS generation. (E) HO-1 knockdown inhibited HO-1 activity. *** $p < 0.001$ vs the NG group, ### $p < 0.001$ vs NCsi the group.

NC siRNA treatment (Fig. 7E and Fig. 8A). Taken together, these data indicate that HO-1 mediated the HG-induced increase in cell invasion.

The role of HO-1 in the HG-induced up-regulation of metastasis-associated protein expression

We assessed the effect of HG on the expression of the metastasis-associated proteins CD147 and MMP-9, which are associated with malignant potential [12]. As shown in Fig. 8B, HG treatment for 48 h increased the expression of CD147 and MMP-9 compared with that of the NG group. However, after HO-1 knockdown, the expression of CD147 and MMP-9 also decreased; a previous study described similar results in A549 cells [12]. Thus, these results strongly suggested that HO-1 mediated HG-induced metastasis-associated protein expression.

Discussion

In recent years, diabetes mellitus has been suggested to be a negative prognostic factor for lung cancer [5, 10]. One potential mechanism for this correlation is that hyperglycemia benefits advanced tumor propagation. However, the potential mechanism remains unclear. In the present study, our findings demonstrated that HG increased HO-1 protein expression via ROS or the TGF- β_1 /PI3K/Akt signaling pathway, which mediated tumor cell invasion and metastasis-associated protein expression, and indicated that HO-1 may serve as a potential therapeutic target in lung cancer. This result is also supported by previous reports [11-12, 35, 44, 45]. This conclusion was supported by four primary findings in the present study. First, we found that HG increased HO-1 protein and mRNA expression by the generation

Fig. 8. Effects of HO-1 knockdown and HG treatment on cell invasion and metastasis-associated protein expression in human lung epithelial cells. Invasiveness and migration characteristics were analyzed using Transwell chambers. (A) Cell migration and invasiveness increased by approximately 50% in the HG group (n=3). Mannitol treatment (instead of glucose) did not increase cell migration and invasiveness (n=3), and NAC pretreatment decreased cell invasiveness (n=3). HO-1 knockdown significantly attenuated the cell invasion potential. (B) HG treatment for 48 h increased the expression of metastasis-associated CD147 and MMP-9 proteins in human lung epithelial cells compared with the NG group (n=3). Additionally, HO-1 siRNA transfection significantly reduced HG-induced CD147 and MMP-9 protein expression compared with the NCsi group (n=3). **p<0.01 vs the NG group; ***p<0.001 vs the NG group; ###p<0.001 vs the NCsi group.

of ROS and TGF- β_1 and increased HO-1 enzymatic activity. Second, NAC or inhibitors for PI3K/Akt attenuated HG-induced HO-1 expression. Third, HG increased cell invasiveness and metastasis-associated protein expression. Fourth, HO-1 knockdown decreased HG-induced cell invasiveness and metastasis-associated protein expression and inhibited HO-1 enzymatic activity.

HO-1 plays a central role in cytoprotective and anti-inflammatory responses in vitro and in vivo. The present data revealed that HG increased HO-1 expression in A549 cells. Furthermore, following the increase in HO-1 expression, HO-1 enzymatic activity increased in cells exposed to HG; this finding was consistent with a previous observation in retinal endothelial cells [35]. A possible mechanism for increasing HO-1 protein expression is the increased generation of ROS, which are generated in response to hyperglycemia [8, 46]. In A549 cells, as in other types of cells [47, 48], HO-1 can be activated by ROS [49]. Moreover, growing evidence has indicated that HG can induce ROS generation in heterologous cell lines [35, 50]. Our results also demonstrated that HG induced ROS generation in A549 cells, and NAC, which is an antioxidant, significantly decreased the HG-induced generation of ROS and attenuated the HG-induced increased expression of the HO-1 protein. H₂O₂ (500 μ M) treatment also increased HO-1 protein levels. Several similar studies have been described in other cell types [50-52]. However, several possible mechanisms most likely exist for HG-induced ROS generation, which mediated the up-regulation of HO-1 expression. These possible mechanisms include glucose autooxidation, polyol pathway stimulation, protein kinase C-dependent NADPH oxidase activation, and advanced glycation end product

Fig. 9. Additional siRNA targeting HO-1 also decreased the invasion potential of A549 cells. We designed and synthesized another small interfering RNA (GGAGATTGAGCGCAACAAG) against HO-1 to reduce the possibility that off-target effects of the siRNA used. (A and B) Western blot and RT-PCR experiments showed that HO-1 siRNA-transfection reduced HO-1 protein and mRNA expression. (C) HO-1 knockdown significantly attenuated the cell invasion potential. ### $p < 0.001$ vs the NCsi group.

Fig. 10. H_2O_2 and TGF- $\beta 1$ treatments enhance the invasion potential of A549. We treated A549 cells with H_2O_2 or TGF- $\beta 1$ in place of HG, H_2O_2 or TGF- $\beta 1$ to further determine the role of H_2O_2 or TGF- $\beta 1$ in the HG-induced increase in the invasion potential of A549 cells. H_2O_2 and TGF- $\beta 1$ treatments increased the invasion potential of A549 cells compared with NG. ** $p < 0.01$, *** $p < 0.001$ vs the NG group.

production [53-55]. In this study, we did not investigate which of these possible mechanisms was responsible for the HG-induced up-regulation of HO-1 expression in A549 cells and awaited further investigation. In addition, we found that the PI3K/Akt signaling pathway had a role in the HG-induced up-regulation of HO-1 expression in this study through the generation of TGF- $\beta 1$, which, unexpectedly, was independent of ROS. This finding is supported by previous reports that HG induced TGF- β generation [11] and that TGF- $\beta 1$ up-regulated HO-1 expression via the PI3K/Akt signaling pathway [43]. However, our results are

inconsistent with previous observations that ROS induced an increase in HO-1 expression via the PI3K/Akt signaling pathway in other cells type [56, 57], which may be due to specific cell types or other factors. We cannot resolve this problem in the present study. Otherwise, TGF- β_1 induced the up-regulation of HO-1 expression via the nuclear factor- κ B [43] and p38 MAPK pathways [58]. Further studies will be required to clarify the potential mechanisms of this up-regulation. Taken together, these results suggested that HG treatment up-regulated HO-1, at least partly, via ROS or the TGF- β_1 /PI3K/Akt signaling pathway.

Several lines of evidence have indicated that HG enhances the invasiveness potential of MCF-7 breast cancer cells [44, 59] and of pancreatic cancer cells [45, 60]. Furthermore, previous studies have indicated that HO-1 positively correlates with tumor stage and lymph node metastasis and that HO-1 is associated with NSCLC progression [31]. Furthermore, NSCLC patients with a high HO-1 expression ratio exhibit a significantly poorer prognosis and a higher metastatic rate compared to those patients with a low HO-1 expression ratio in clinical experiments [12]. In our study, we investigated cellular invasion and the role of HO-1 using a Transwell chamber, and the results demonstrated that HG enhanced the capacity of A549 cell invasiveness. Similar studies have been described in A549 cells, pancreatic cancer cells and a human breast cancer cell line [12, 59, 60]. This finding was also supported by the report that HG levels contribute to tumor formation and progression in A549 cells [7]. However, this invasiveness potential was deterred by HO-1 knockdown, which suggested that the HG-enhanced invasiveness potential was mediated by HO-1. Moreover, this finding was consistent with prior studies that reported that HO-1 mediated cell invasion and migration in A459 cells [12] and in other types of tumor cells [61]. However, several reports have suggested that HO-1 overexpression suppresses angiogenesis and tumor invasion [39, 62]. This discrepancy may be due to the dual effect of HO-1 on tumor invasion depending on the specific type of cancer or other unknown factors. Unfortunately, we cannot exclude this discrepancy in the present study. We investigate the effect of HG on metastasis-associated protein (CD147 and MMP-9) expression and the role of HO-1 to further delineate the role of HO-1 in HG-induced tumor metastasis. Several previous studies have indicated that HG regulates CD147 and MMP-9 expression in other cell types [41, 42]. Furthermore, the expression levels of CD147 and MMP-9 are positively associated with HO-1 expression in NSCLC or other types of tumors and are associated with tumor metastasis and with poor clinical outcome [12]. In this study, we reported that HG treatment induced an increase in CD147 and MMP-9 expression and that this increase was decreased by HO-1 knockdown in A459 cells. This finding suggested that CD147 and MMP-9 were associated with HO-1 expression levels, thereby lending credence to previous reports indicating the involvement of HO-1 in CD147 and MMP-9 expression in A459 cells [12] and in other tumor cells [13, 39]. Based on our results, we propose that HG increases metastasis-associated protein (CD147 and MMP-9) expression by up-regulating HO-1 expression, which may promote tumor metastasis.

In summary, our results demonstrated that HG increased HO-1 expression and enzymatic activity via ROS or the TGF- β_1 /PI3K/Akt signaling pathway in A549 human lung epithelial cells and that HO-1 expression and enzymatic activity mediated tumor invasion and metastasis-associated protein expression. The pharmacological inhibition of HO-1 expression and the knockdown of HO-1 effectively inhibited the tumor invasion potential and metastasis-associated protein expression. This inhibition may become a powerful therapeutic strategy against lung cancer in patients with diabetes mellitus.

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Disclosure Statement

The authors declare that no conflict of interest exists.

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