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The protective effect of magnesium lithospermate B against glucose-induced intracellular oxidative damage

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ABSTRACT

Objectives: To investigate the effects of magnesium lithospermate B (LAB) on intracellular reactive oxygen species (ROS) production induced by high dose of glucose or H_2O_2, we explored the influences of LAB on the expression of heme oxygenase-1 (HO-1) and nuclear factor E2-related factor-2 (Nrf2) in HEK293T cells after treatment with high dose of glucose.

Materials and methods: The total nuclear proteins in HEK293T cells were extracted with Cytoplasmic Protein Extraction Kit. The ROS level was determined by flow cytometry. The mRNA and protein expression of HO-1 and Nrf2 were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot.

Results: LAB reduced the ROS production in HEK293T cells cultured under oxidative stress. High dose of glucose enhanced the expression of HO-1 mRNA and HO-1 protein in a time-dependent manner. LAB enhanced the expression of HO-1 mRNA and HO-1 protein in a dose-dependent manner treated with high dose of glucose. The amount of Nrf2 translocation was enhanced after cells were pretreated with 50 μmol/L or 100 μmol/L LAB. Silencing of Nrf2 gene eliminated the enhanced expression of HO-1 protein induced by high dose of glucose plus LAB.

Conclusions: LAB plays an important role against glucose-induced intracellular oxidative damage. The enhanced expression of HO-1 mRNA and HO-1 protein caused by LAB is regulated via Nrf2 signal pathway.

1. Introduction

Nowadays, type 2 diabetes mellitus (T2DM) has become a major public health and economic problem in the world. It has been well known that prevalence of T2DM is increasing all over the world. High blood sugar induced oxidative stress is the common pathophysiological pathway in diabetic complications, especially in vascular disease [1,2]. Recent studies suggest that excessive reactive oxygen species (ROS) produced by mitochondrial electron transport chain after stimulation of high dose of glucose may be the first step for diabetes caused endothelial dysfunction [3]. Therefore, the treatment of oxidative stress prevention for patients with diabetes accompanied by vascular disease has become a new research hotspot.

Heme oxygenase-1 (HO-1) is the initial and rate-limiting enzyme of heme metabolism and heme can be decomposed to release free iron, carbon monoxide, and biliverdin, which can be quickly converted to bilirubin. There are three HO isozymes in human, which are HO-1, HO-2, and HO-3 [4]. HO-1, also known as heat shock protein 32 with molecular weight of 32 kDa, has low expression in most organs in human. However, the expression of HO-1 obviously increased after the body is induced by stimuli such as stress, hypoxia, ultraviolet radiation, heavy metals and a variety of disease states including diabetes, high cholesterol, smoking and

Abbreviations: LAB, magnesium lithospermate B; ROS, reactive oxygen species; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor2; GSTs, glutathione *S*-transferases; CAT, hydrogen peroxidase; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; O^{2-}, superoxide anion; MDA, malondialdehyde; ARE, antioxidant response element; EpRE, electrophile response element; NF-κB, nuclear factor kappa B; Keapl, Kelch-like ECH-associated protein 1; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorodihydrofluorescein; CPK, creatine phosphokinase; AP-1, activator protein 1; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; GSH-Px, glutathione peroxidase.

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other lesions [5]. The molecular structures of HO-2 and HO-3 are similar, but they are less powerful for the decomposition of hemoglobin [4–6]. Bilirubin is an endogenous antioxidant and an effective superoxide scavenger. It can inhibit the formation of oxidized low density lipoprotein and make a synergistic effect with other antioxidant defense system such as superoxide dismutase and vitamin E in human body. The antioxidant capacity is even more powerful than vitamins C and E [7]. Morita et al. [8] found that suitable level of endogenous bilirubin reduces the damage on body caused by oxidative stress. HO-1 is one of the most important endogenous protection system and plays an important role in the cell protection through anti-inflammation, anti-oxidation, inhibition of apoptosis, and improvement of microcirculation [9]. Induction of HO-1 gene is regulated primarily at the transcriptional level. A number of antioxidant response elements (ARE) or electrophilic response elements (EPRE) have been identified. ARE in the promoter regions contains binding sites for heat shock factor, NF-κB, and nuclear factor E2-related factor-2 (Nrf2) [10]. ARE is similar to the Maf recognition element and specifically combines with basic leucine zipper transcription factor including Jun, Fos and Nrf2 [11].

Nrf2 is basic leucine zipper (bZIP) family of white matter [12]. In non-active state, it is located in the cytoplasm and binds with cytoplasmic protein chaperone Keapl (Kelch-like ECH-associated protein 1). Under the stimulation of ROS or other nucleophilic agents, Nrf2 is activated and transferred into the nucleus after uncoupling with Keapl. Nrf2 regulates ARE-dependent gene expression of phase II detoxification enzymes and antioxidant genes including glutathione *S* transferase (GSTs), hydrogen peroxidase (CAT), superoxide dismutase (SOD), and HO-1[11]. Nakasolz et al. [12] reported that the expression of Nrf2 protein is both time and dose dependent, and reaches the peak value at 50 μmol/L at 6 h when neuroblastoma cells were treated with HO-1 classic inducer hemin. Recently, Chen's [13] study showed that LAB promotes Nrf2 translocation into the nucleus in aortic smooth muscle cells at the normal and glucose stress state. Many natural dietary antioxidants such as salvia magnesium acetate, curcumin, and bioflavonoids enhance the expression of HO-1.

Sal B is the most active component of Danshen water-soluble substances and has a very strong antioxidant activity due to a number of phenolic hydroxyl components. Previous studies showed that Sal B inhibits the production of superoxide anion (O^{2-}), prevent the formation of erythrocyte hemolysis induced by hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) [14]. In addition, Sal B could delay the occurrence of renal failure in diabetic rats [15] and inhibited the activity of aldose reductase in vascular smooth muscle cell induced by high dose of glucose and endometrial hyperplasia, which result in a decreased damage of smooth muscle cell by high dose of glucose [16]. LAB, as the main form of Sal B, is the main component of salvianolate injection and its content is more than 80%. At present, LAB was widely used in the treatment of many diseases such as atherosclerosis, diabetes, and coronary heart disease in clinic. However, its mechanism for anti-oxidative damage is still unclear.

In this study, we aimed to assess the effect of LAB against high dose of glucose-induced intracellular oxidative damage and its action mechanism.

2. Materials and methods

2.1. Cell culture

HEK293T cells were maintained in Dulbecco's modified eagle's medium (DMEM, Invitrogen, CA, USA) containing 10% fetal bovine serum (Every Green, Hangzhou, China), 100 U/mL penicillin, and 100 μg/mL streptomycin before initiating differentiation.

2.2. Determination of ROS generation in treated cells

Differentiated HEK293T cells were grown in 6-well plates at 1×10^5–2×10^6 cells per well. When the culture reached 90% confluence, cells were collected and divided into 5 experimental groups. The groups were set as follows: (1) normal control, (2) treated with 30 mmol/L glucose, (3) pretreated with 50 μmol/L LAB for 30 min, then 30 mmol/L glucose was added to cells, and cells were continuously cultured for an hour, (4) treated with 100 mmol/L H_2O_2 (5) pretreated with 50 μmol/L LAB for 30 min, then 100 mmol/L H_2O_2 was added to cells, and cells were continuously cultured for 15 min. After the cells of each group were treated, the cell culture medium was removed, 1 mL of 10 μmol/L DCFH-DA (diluted concentration) was added to cover the cells completely, and the cells were cultured for another 30 min. After rinsing with serum-free cell culture medium three times to remove free DCFH-DA, cells were centrifuged at 13,000*g* for 5 min and then resuspended in 0.5 mL PBS. The level of ROS in HEK293T cells was determined by flow cytometry with excitation wavelength at 488 nm and emission wavelength at 525 nm. Each experiment was repeated three times ($n = 3$).

2.3. RNA isolation and semi-quantitative RT-PCR on HO-1

Total RNA was extracted by using trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA concentration was determined spectrophotometrically at 260 nm. Genomic DNA from total RNA was removed by using DNaseI (Promega, Wisconsin, USA) before reverse transcription. Semi-quantitative RT-PCR was performed as described previously to determine the mRNA levels of β-actin and HO-1. The primer pairs used in the amplification of the HO-1 were: forward primer, 5′-TTGCCAGTGCCAC-CAAGTTC-3′; reverse primer, 5′-TCAGCAGCTCCTGCAACT-CTCC-3′. The primer pairs of β-actin were: forward primer, 5′-TGGCACCCAG-CACAATGAA-3′; reverse primer, 5′-CTAAGTCATAGTCCGCCTA-GAAGCA-3′. Polymerase chain reactions (PCR) were carried out using 2 μL cDNA, 12.5 μL SYBR ®Premix Ex TaqTM (Takara, Dalian, China), 0.5 μL Rox Reference Dye II (50) (Takara, Dalian, China), 0.4 μL of each primer (10 μM) (Takara, Dalian, China). The total reaction volume was brought up to 25 μL with H_2O. PCR was initiated by heating to 95 °C for 2 min, then amplified through 40 cycles: denaturing at 95 °C for 15 s, annealing at 59 °C for 30 s for HO-1.

2.4. Construction and transfection of pRNAT-U6.1/Neo-siNrf2 plasmid

The primer pairs used in the amplification of the Nrf2 shRNA were: forward primer, 5′-GATCCCGAGTATGAGCTGGAAAAACTTGA-TATCCGGTTTTTCCAGCTCATACTCTTTTTTCCAAA-3′: Reverse primer, 5′-AGCTTTTGGAAAAAAGAGTATGAGCTGGAAAAACCGGATATCAAG TTTTTCCAGCTCATACTCGG-3′ (Genescript, Guangzhou, China). pRNAT-U6.1/Neo vector (Genescript , NJ , USA) was double digested by *BamH* I and *Hind* III (Takara, Dalian, China). Double-stranded DNA and pRNAT-U6.1/Neo vector were connected at the ratio of 4:1. The constructed pRNAT-U6.1/Neo-siNrf2 plasmid was identified by PCR with forward primer: 5′-TACGATACAAGGCTGTTAGAG-3′ and reverse primer: 5′-TAGAAGGCACAGTCGAGG-3′. The pRNAT-U6.1/Neo-siNrf2 and non-specific control shRNA (Invitrogen, CA, USA) were transferred into HEK293T cells using lipofectamine 2000 (Invitrogen, CA, USA) by following the manufacturer's instruction.

2.5. Nuclear protein preparation and Western blot

Protein extraction reagent A of 200 μL was added to each 20 μL collected cell precipitation and then violently vortexed for 5 s. The mixture was incubated in ice bath for 15 min. Then, 10 μL protein extraction reagent B was added to the mixture,

violently vortexed for 5 s, and incubated in ice bath for another 1 min. After that, solution was centrifuged at 12,000*g* for 10 min at 4 °C. After removal of supernatant, 50 μL nuclear protein extraction reagent was added to nuclear precipitation and mixed. The mixture was centrifuged at 12,000*g* for 15 min at 4 °C. The supernatant was saved and protein concentration was determined using Bradford Protein Assay Kit (Beyotime, Shanghai, China) [17]. Western blot analyses were performed as described previously [18]. Briefly, the supernatants were separated by 5% SDS–PAGE for 3 h and transferred to a polyvinylidene difluoride (PVDF) membrane followed by a 12 h incubation in 5% non-fat milk in PBST (0.1% Tween 20 in TBS) at 4 °C overnight. The blot was probed with polyclonal antibody against HO-1 (Abcam, MASS, USA) or antibody against Nrf2 (Santa cruz, CA, USA). The reaction was performed with infrared-labeled goat anti-mouse secondary antibody (Li-COR, NEBR, USA). The signal was detected by ECM (Boster, Wuhan, China).

2.6. Statistical analysis

The SPSS software package (Version 13.0 for Windows; SPSS, Chicago, IL, USA) was used for statistical analysis. All data are expressed as mean ± SD. ANOVA test was used to detect differences between groups and Student–Newman–Keuls (SNK) test was used

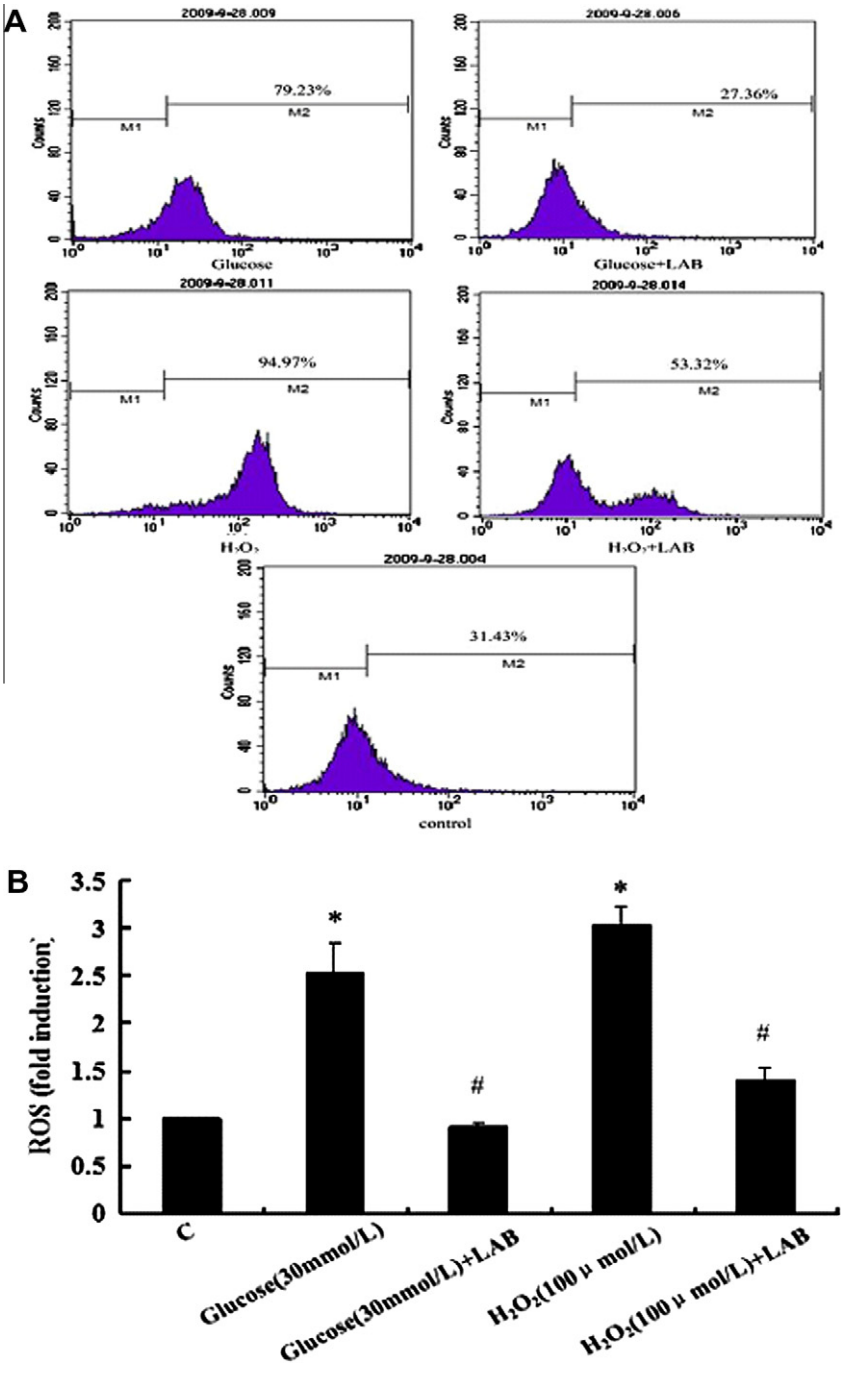

Fig. 1. Protective effect of LAB on ROS generation induced by glucose and H_2O_2 in HEK293T cells. Cells were divided into five groups: (1) control; (2) 30 mmol/L glucose; (3) 100 μmol/L H_2O_2; (4) 30 mmol/L glucose plus 50 μmol/L LAB; (5) 100 μmol/L H_2O_2 plus 50 μmol/L LAB. (Panel A) Flow cytometry charts of intracellular ROS levels in different groups. (Panel B) Fold changes of different groups compared to control. $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with high dose of glucose or H_2O_2.

for multiple comparisons. Statistical significance was accepted when $P < 0.05$.

3. Result

3.1. Effects of LAB on intracellular ROS production induced by high dose of glucose or H_2O_2

Flow cytometry analysis showed that the fluorescence intensity in the HEK293T cells treated with 30 mmol/L glucose or 100 μmol/L H_2O_2 were 2.54 times (79.00 ± 5.8% vs. 30.72 ± 0.47%) and 3.01 times (92.83 ± 8.9% vs. 30.72 ± 0.47%) compared with normal control, respectively. After cells were pretreated with 50 mmol/L LAB, the fluorescence intensity of cells decreased 1.7 times ($P < 0.05$) and 2.18 times ($P < 0.01$) compared with high dose of glucose treatment only group and H_2O_2 treatment only group (Fig. 1).

3.2. The time effect of high dose of glucose induction on the expressions of HO-1 mRNA and protein

In this experiment, cells were divided into five groups: control group and four glucose treatment groups (cells were treated with 30 mmol/L of glucose in the medium for 2 h, 6 h, 24 h, and 48 h, respectively).We found that the expression of HO-1 mRNA was significantly increased, reached a peak value at 24 h, and dropped sharply at 48 h after cells were treated with high dose of glucose. There were significant differences in the HO-1 mRNA expression in HEK293T cells treated with high dose of glucose for 2, 6, and 24 h compared to control group (3.96 ± 0.71, $P < 0.05$; 9.57 ± 1.39, $P < 0.01$; 12.29 ± 2.54, $P < 0.001$, respectively) (Fig. 2). Our data showed similar results on the expression level of HO-1 protein. There were significant differences in the relative ratios of HO-1 protein/β-actin expression in cells treated with high dose of glucose for 2 h, 6 h, and 24 h compared to control group (0.33 ± 0.09, $P < 0.05$; 0.72 ± 0.21, $P < 0.01$; 0.89 ± 0.13, $P < 0.01$, respectively) (Fig. 2).

3.3. The effect of LAB on the expressions of HO-1 mRNA, protein and Nrf2 protein in HEK293T cells induced by high dose of glucose

Cells were pretreated with 10 μmol/L, 50 μmol/L, and 100 μmol/L LAB for 30 min respectively before the addition of 30 mmol/L glucose. There was a significant increase in the expression level of HO-1 mRNA in all LAB + glucose treatment groups compared with glucose only group ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). There was also a significant increase in the expression level of HO-1 protein in 50 μmol/L LAB + glucose ($P < 0.05$) and 100 μmol/L LAB + glucose ($P < 0.01$) treatment groups compared with glucose only group (Fig. 3A and B).

The relative ratio of Nrf2 protein/Lamin A in different groups for 24 h were 0.25 ± 0.03, 0.40 ± 0.06, 0.46 ± 0.06, 0.62 ± 0.09, 0.74 ± 0.11, respectively (Fig. 3C and D). There was a significant increase in the expression levels of Nrf2 protein in 30 mmol/L glucose group compared with control ($P < 0.05$) and 30 mmol/L glucose + 50 μmol/L LAB group and 30 mmol/L glucose + 100 μmol/L LAB group compared with 30 mmol/L high dose of glucose group ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 3C and D).

3.4. The effect of LAB on the expression of Nrf2 protein in HEK293T cells stimulaed by high dose of glucose

Cells were pretreated with 10 mol/L, 50 mol/L, and 100 mol/L LAB for 30 min, respectively before the addition of 30 mmol/L glucose. The relative ratio of Nrf2 protein/Lamin A in control group, 30 mmol/L glucose group, 30 mmol/L glucose + 10 mol/L LAB group, 30 mmol/L glucose + 50 mol/L LAB group, and 30 mmol/L glucose + 100 mol/L LAB group for 24 h were 0.25 ± 0.03, 0.40 ± 0.06, 0.46 ± 0.06, 0.62 ± 0.09, 0.74 ± 0.11, respectively. We found that there was a significant increase in the expression levels of Nrf2 protein in 30 mmol/L glucose group compared with control ($P < 0.05$) and 30 mmol/L glucose + 50 mol/L LAB group and 30 mmol/L glucose + 100 mol/L LAB group compared with 30 mmol/L high dose of glucose group ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 4).

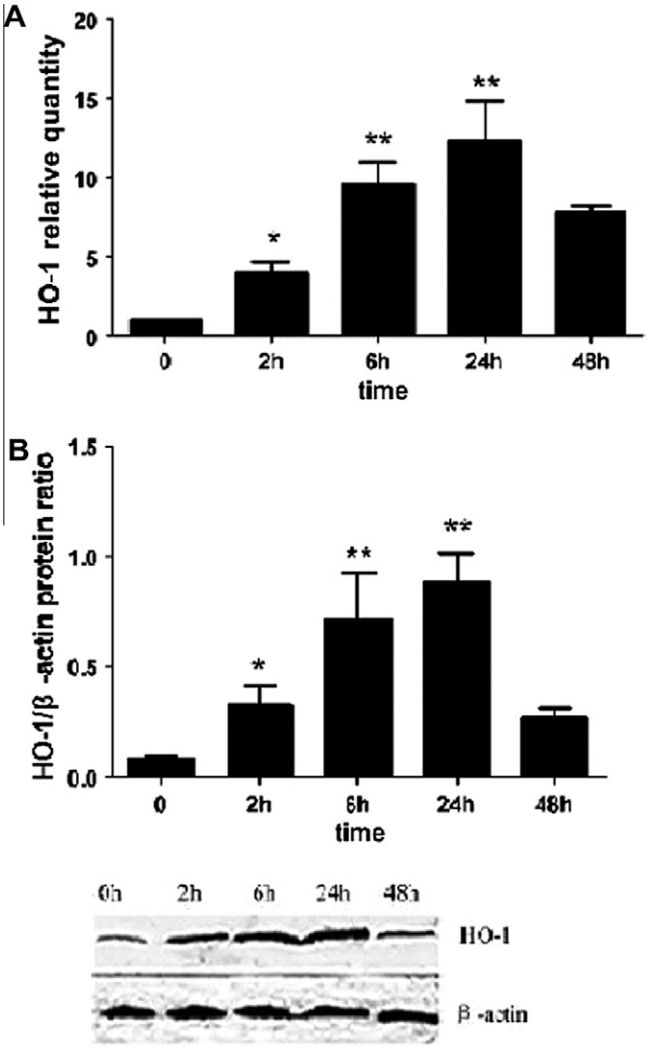

Fig. 2. Time-dependent effect of high-dose of glucose on the expression of HO-1 mRNA and protein in HEK293T cells. Cells were divided into five groups: control group and 30 mmol/L of glucose induction for 2 h, 6 h, 24 h, and 48 h, respectively. (Panel A) The change of HO-1 mRNA after treated with 30 mmol/L of glucose for different endurance of time. (Panel B) The change of HO-1 protein after treated with 30 mmol/L of glucose for different endurance of time. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

3.5. The role of Nrf2 gene in the regulation of HO-1 protein expression in HEK293T cells treated with LAB and high dose of glucose

We successfully constructed RNA interference eukaryotic expression vector pRNAT-U6.1/Neo-siNrf2, which targets Nrf2 gene (Fig. 5A–C). The pRNAT-U6.1/Neo-siNrf2 and non-specific control shRNA plasmids were transiently transfected into HEK293T cells by lipofectamine 2000. The results showed that the expression levels of Nrf2 protein in non-specific shRNA transfection cells and non-specific shRNA transfection cells pretreated with 50 μmol/L LAB for 30 min were significantly more than that in

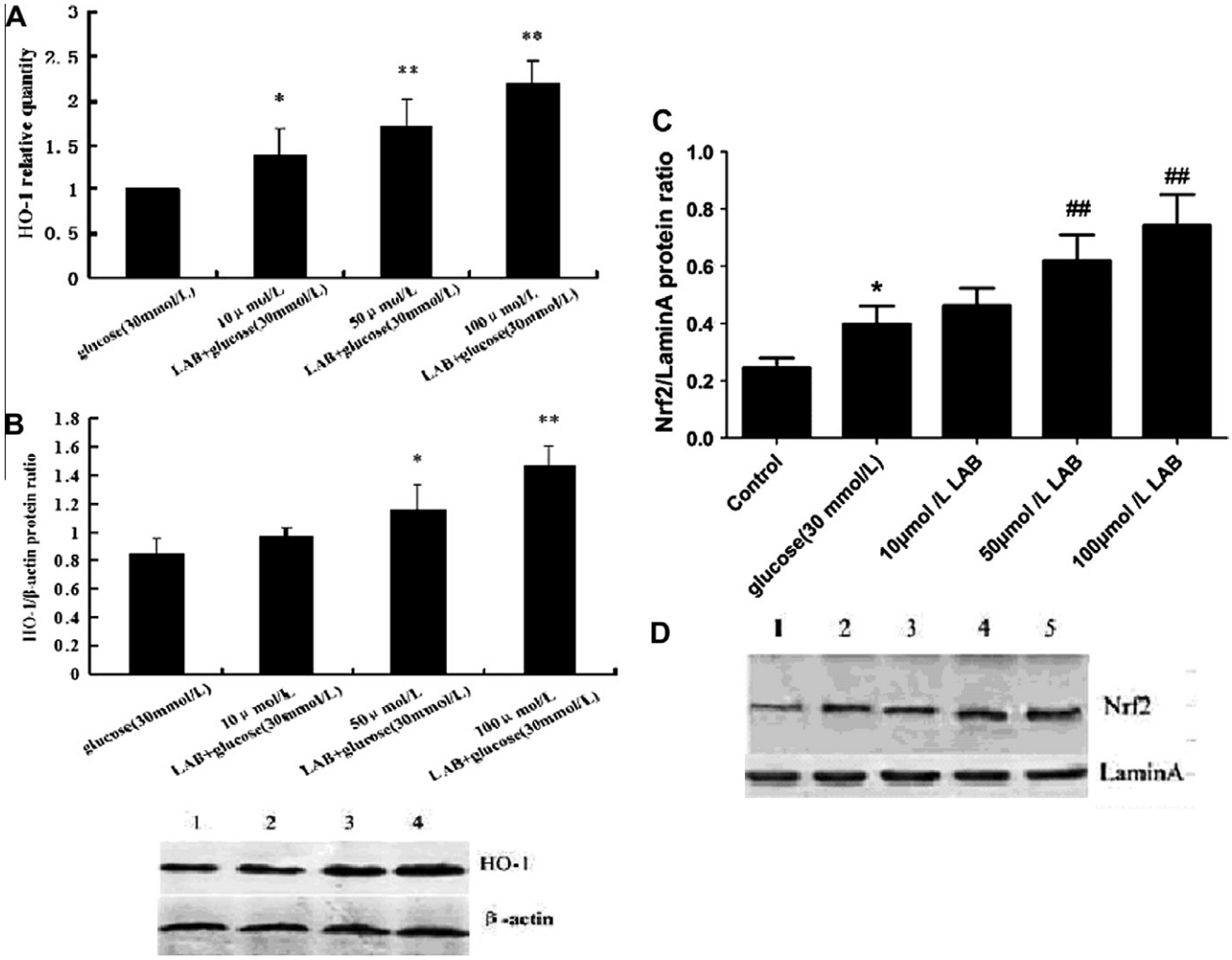

Fig. 3. The effect of LAB on the expressions of HO-1 mRNA, protein and Nrf2 protein induced by high dose of glucose in HEK293T cells. Cells were pretreated with 10 μmol/L, 50 μmol/L, and 100 μmol/L LAB for 30 min, respectively before the addition of 30 mmol/L glucose. (Panel A) The relative quantitation of HO-1 mRNA in different groups. (Panel B) The change of HO-1 protein in different groups. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. (Panel C) Quantitative expression of Nrf2 protein in different groups. $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with glucose only group. (Panel D) Western blot image for Nrf2 protein expression.

pRNAT-U6.1/Neo-siNrf2 transfection cells (Fig. 5D and E). In addition, the expression of HO-1 protein in pRNAT-U6.1/Neo-siNrf2 transfection cells induced by 30 mmol/L glucose for 24 h and pRNAT-U6.1/Neo-siNrf2 transfection cells pretreated with 50 μmol/L LAB for 30 min and then induced by 30 mmol/L glucose for 24 h were significantly less than that in non-specific shRNA transfection cells induced by 30 mmol/L glucose for 24 h and non-specific shRNA transfection cells pretreated with 50 μmol/L LAB for 30 min and then induced by 30 mmol/L glucose for 24 h (Fig. 4D and E). These findings suggest that the enhanced expression of HO-1 protein in cells induced by high dose of glucose plus LAB vanished after Nrf2 gene in cells was silenced.

4. Discussion

Oxidative stress is a state of tissue damage resulted from imbalance between the ROS production of free radicals and antioxidant defense system. Excessive ROS will damage the tissue, and ROS can activate the downstream pathway which can further increases peroxide production. Zhang et al. [19] reported that Sal B could inhibit ROS generation in human arterial smooth muscle cells stimulated by TNF-α. We are interested in whether or not LAB can directly wipe out the excessive ROS in HEK293T cells to protect cells. In this study, HEK293T cells were treated separately with 30 mmol/L glucose only or 100 μmol/L H_2O_2 only or together with LAB pretreatment, results showed that 30 mmol/L glucose or 100 μmol/L H_2O_2 markedly increased the production of ROS in HEK293T cells and LAB can inhibit the ROS production in HEK293T cells, which indicate LAB may play a protective role in the oxidative damage caused by high dose of glucose and H_2O_2 (Fig. 1). In physiological conditions, there are a series of antioxidant defense systems to reduce ROS damage to the body. There are some free radical scavengers in cells such as superoxide dismutase (SOD), quinone oxidoreductase (NQO1), heme Oxygenase -1 (HO-1) and vitamin E.

Heme oxygenase is the rate-limiting enzyme catalyzing oxidation degradation of heme in vivo, and it plays an important role in the anti-oxidative stress and anti-apoptosis [5,6]. It is found to be a stress protein induced by many factors. Hill-Kapturczak et al. [20] found that the expression levels of HO-1 mRNA and HO-1 protein significantly increased after TGF-β1 stimulation for 4 h in human renal proximal tubule cells. In the current study, the expression level of HO-1 mRNA markedly increased after treatment with high dose of glucose, reached the peak value at 24 h and reduced at 48 h in HEK293T cells. The change of HO-1 protein expression level was similar to that of HO-1 mRNA (Fig. 2). Our

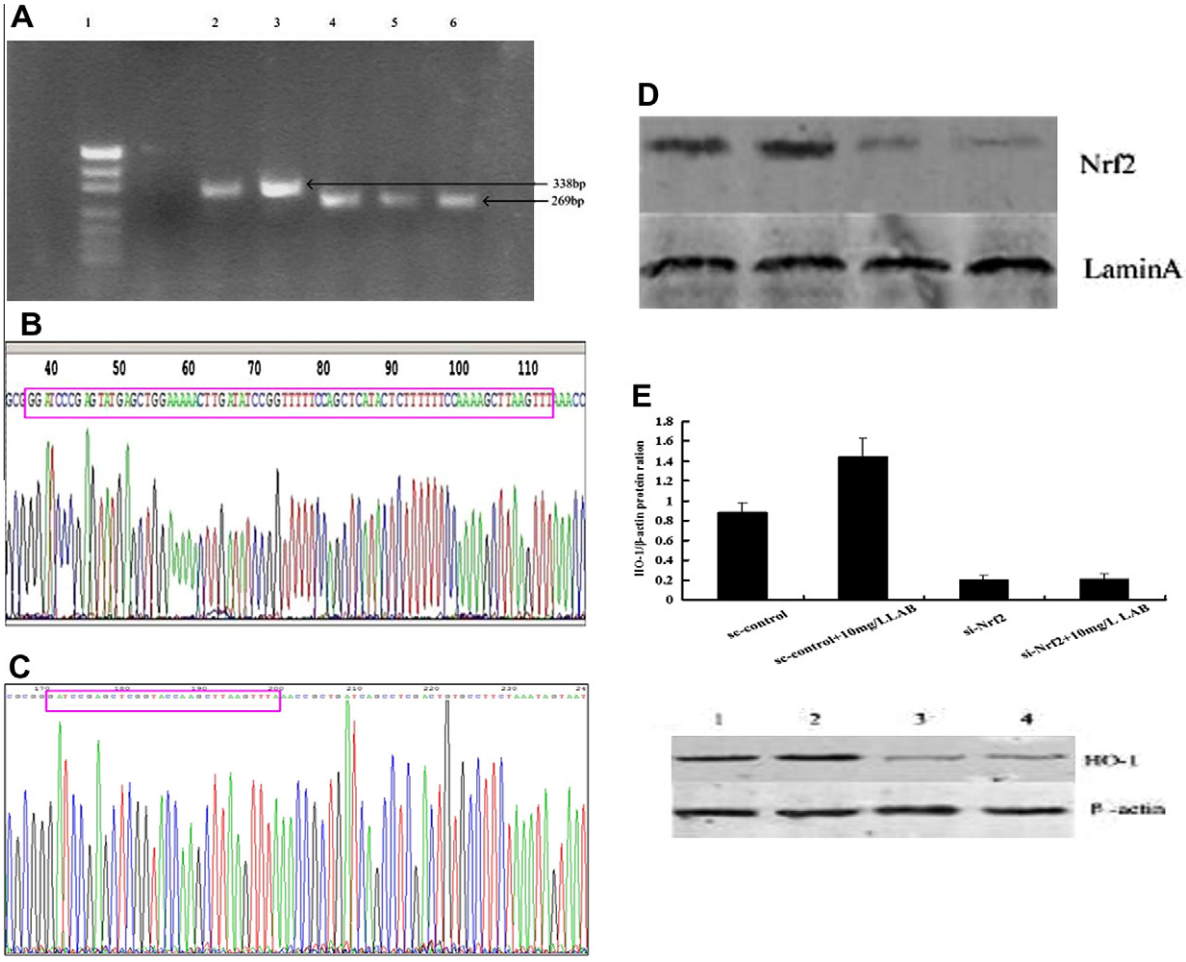

Fig. 4. The construction and identification of eukaryotic expression vector pRNAT-U6.1/Neo-siNrf2 and the effects of Nrf2 gene silence on the expression levels of Nrf2 protein and HO-1 protein after treatment with LAB in HEK293T cells. (Panel A) The PCR products of recombinant plasmid and control empty vector. lane 1: 500 bp marker, lanes 2 and 3: PCR product of recombinant vector; lanes 4–6: PCR product of empty vector. (Panel B) Partial DNA sequence of recombinant plasmid after insertion of Nrf2 interference fragment. (Panel C): Partial DNA sequence of empty vector to show the insertion site. (Panel D) The Western blot of Nrf2 protein. (Panel E) The relative quantitation and Western blot of HO-1 protein.

results are in accordance with Hill-Kapturczak's study result. These findings suggest that the body self-protective systems are activated once the body is involved in stress condition such as the stimulation of high dose of glucose. Therefore, we think that the enhanced expression levels of HO-1 mRNA and protein can protect body from oxidative stress injury. Many previous studies reported that Sal B play an important role in anti-inflammation, anti-myocardial and cerebral ischemia, anti-atherosclerosis, anti-apoptosis, anti-liver fibrosis, anti-aging, anti-tumor and so on [15–16]. In this study, our data showed that 50 μmol/L and 100 μmol/L LAB significantly increased the expression levels of HO-1 mRNA and protein in HEK293T cells, which suggest that LAB have an obvious protective effect on glucose-induced intracellular oxidative damage (Fig. 3A and B).

Nrf2 protein was widely expressed in various tissues and cells [11]. When the body is stimulated by oxidative stress, the activity of Nrf2 is increased and it regulates many downstream gene transcriptions and protein expressions of a variety of antioxidant enzymes and detoxifying enzymes of phase II, thereby cell protective ability is enhanced in the body via removal of ROS, maintenance of intracellular redox state, and reduction of oxidative damage [11]. In type 2 diabetic rat model, the vascular complications were obviously increased due to a decrease in the ability of Nrf2 protein switching to the nucleus [21]. In our study, we found that 50 μmol/L and 100 μmol/L LAB significantly increased the Nrf2 protein translocation in HEK293T cells, which suggests that LAB may play a very important role in the Nrf2 nuclear transfer in cells under high dose of glucose stimulation (Fig. 3C and D).

Previous studies revealed that the HO-1 transcriptional regulation is controlled by the flanking regulatory elements of HO-1 gene promoter, which are binding sites for oxidoreductase transcription factors such as Nrf2, NF-κB, and AP-1 [19–21]. In this study, we found that the expression level of Nrf2 protein markedly increased when the expression level of HO-1 protein was induced by LAB and high dose of glucose (Fig. 3C and D). We postulate that the overexpression of Nrf2 protein may cause enhanced HO-1 expression after stimulation with high dose of glucose in HEK293T cells. To test this hypothesis, we constructed RNA interference eukaryotic expression vector pRNAT-U6.1/Neo-siNrf2, which targets Nrf2, and transiently transfected it into HEK293T cells. Our results showed that the enhanced expression level of HO-1 protein induced by high dose of glucose plus LAB vanished after the Nrf2 gene was silenced (Fig. 5D and E). Therefore, the further induction by LAB on HO-1 gene expression, which is stimulated by high dose of glucose, is at least partly due to the activation of Nrf2 signaling pathway.

In summary, in this study, we found that LAB not only directly reduced excessive ROS induced by high dose of glucose

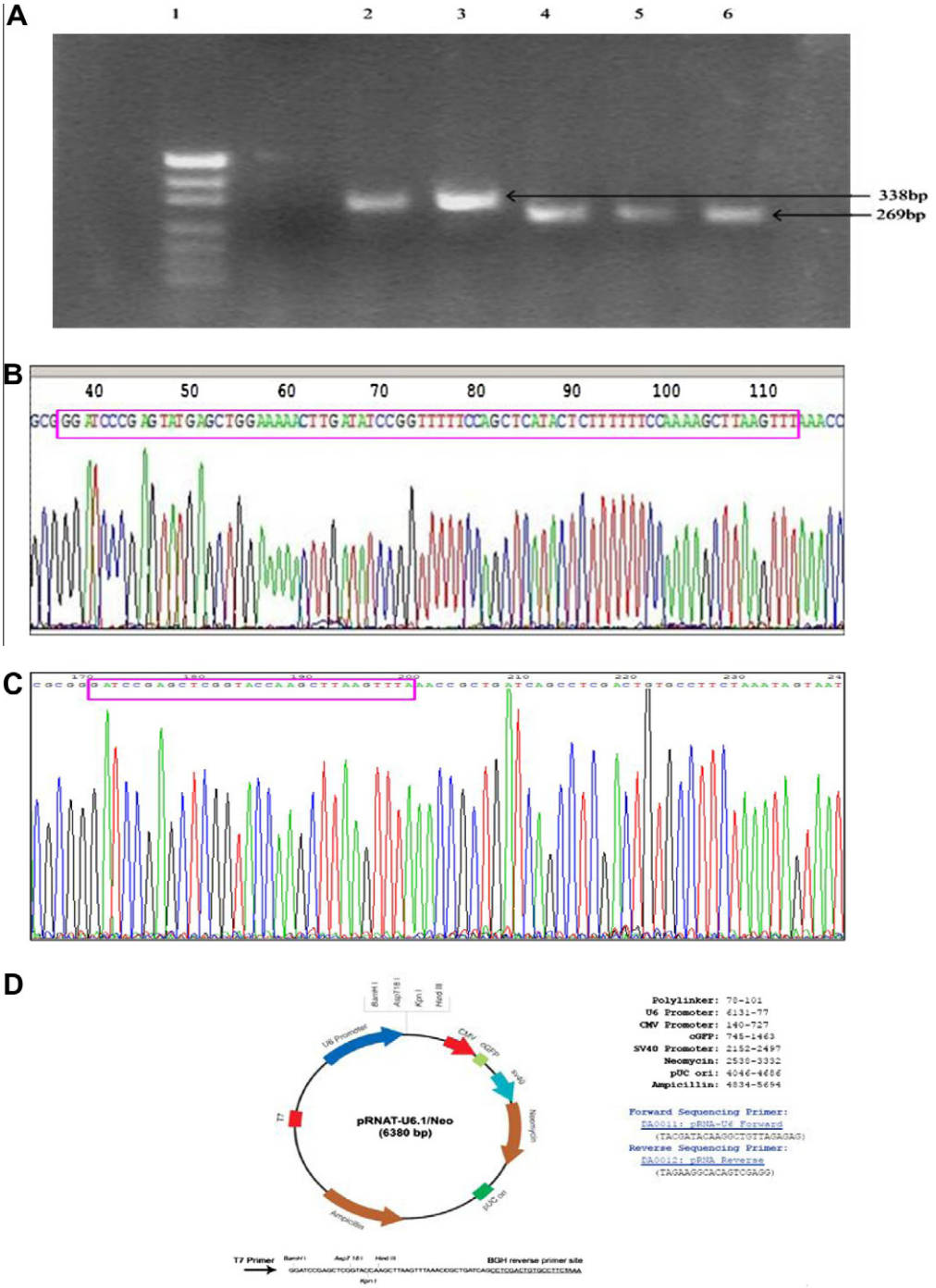

Fig. 5. The construction and identification of eukaryotic expression vector pRNAT-U6.1/Neo-siNrf2. (Panel A) The PCR products of recombinant plasmid and control empty vector. lane 1: 500 bp marker, lanes 2 and 3: PCR product of recombinant vector; lanes 4–6: PCR product of empty vector. (Panel B) Partial DNA sequence of recombinant plasmid after insertion of Nrf2 interference fragment. (Panel C) Partial DNA sequence of empty vector to show the insertion site. (Panel D) The structure of empty vector.

stress, but also enhanced the expression levels of intracellular antioxidant enzyme HO-1 and transcription factor Nrf2 in HEK293T cells. However, further efforts are needed to elucidate the entire Nrf2-ARE signaling pathway and whether or not other antioxidant enzymes are involved in the antioxidant effect of salvia magnesium acetate on the cellular and animal level.

5. Conflict of interest

None declared.

Acknowledgments

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