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High glucose induced human umbilical vein endothelial cell injury: involvement of protein tyrosine nitration

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Abstract The dysfunction and further damage of endothelium play an important role in the development and progression of diabetic vascular complications. Protein tyrosine nitration is involved in endothelial cell injury induced by high glucose. Little is known about protein nitration in human umbilical vein endothelial cells (ECV304) induced by high glucose. In the present article, exposure of ECV304 to 30 mM high glucose (HG30) and 40 mM high glucose (HG40) or hemin–nitrite–H_2O_2 system for 72 h, the cell injury in ECV304 induced by high glucose and exogenous nitrating agent was studied. After 72 h treatment, it was found that high glucose stimulated ECV304 injury in a dose-dependent manner, including reducing cell viability, increasing malondialdehyde (MDA) content, decreasing glutathione (GSH) content, increasing intracellular reactive oxygen species (ROS), increasing the production of nitric oxygen (NO) (increased nitrite content in cell and nitrate content in medium) and generating protein tyrosine nitration. It was also found that protein tyrosine nitration could induce cell injury further. By comparison the protein tyrosine nitration induced by high glucose condition and extrinsic factors (hemin–nitrite–H_2O_2 system), it may be speculated that protein is nitrated selectively to generate nitrotyrosine in diabetic vascular complications.

Keywords Diabetic vascular complications · High glucose · Tyrosine nitration · ECV304 · Hemin

Abbreviations

GSH	Glutathione
HG30	30 mM high glucose
HG40	40 mM high glucose
MDA	Malondialdehyde
NG	Normal glucose
NO	Nitric oxygen
NT	Nitrotyrosine
PGI_2	Prostaglandin I2
PI3	Phosphatidyl inositol 3
ROS	Reactive oxygen species
S.D	Standard deviation

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Introduction

The endothelium is believed to be not only a semipermeable barrier between the blood and the interstitium to facilitate the exchange of water and small molecules, but also participates in metabolic, synthetic, and regulatory pathways [1]. Endothelial dysfunction is a form of vascular damage, which participates in type 1 and type 2 diabetes. The dysfunction and further damage of endothelium play

an important role in the development and progression of diabetic microangiopathy and macroangiopathy [2, 3]. The relationship between endothelium dysfunction and diabetic complications has been reviewed. Cosentino et al. summarized endothelial dysfunction in diabetic patients and animals, then they demonstrated that hyperglycemia may induce endothelial dysfunction [4], while Pănuş et al. [5] indicated that other factors also resulted in endothelial dysfunction besides hyperglycemia. Among those factors which contribute to endothelium dysfunction of type 2 diabetic patients, hyperglycemia [6–10] and oxidative stress [11–13] are found to be critically important. Hyperglycemia can produce oxidative stress and ultimately acute endothelial dysfunction in blood vessels of diabetic patients [14]. In the pathogenesis of diabetes and diabetic complications, high glucose promoted mitochondrial reactive oxygen species (ROS) production [15], then ROS participated in diabetic angiopathy [16].

Nitric oxide (NO) is the best-characterized vasodilator, which leads to physiological vasodilation and the relaxation of smooth muscle cells. NO participates in both type 1 [17] and type 2 diabetes [18, 19]. The overproduction of NO would react with oxygen-free radicals and produce more potent oxidants to damage proteins under oxidative stress condition, such as nitrating tyrosine residues to generate nitrotyrosine (NT), which has been found in diabetes [20–22] and other diseases [23–28]. The bioactivity of NO would decrease when endothelial dysfunction happens. The decrease of NO mostly comes from the increase of ROS production. ROS would react with NO to generate more active species, especially peroxynitrite, to damage proteins and lipids. Under high glucose condition, the presence of nitrotyrosine in endothelial cells of diabetes suggested a possible involvement of peroxynitrite in the development of diabetes and diabetic vascular complications [14, 20, 29].

It is generally accepted that hyperglycemia can generate ROS in diabetes and diabetic complications. A little is known about tyrosine nitration in endothelial cell under high glucose condition. Zou et al. [28] have demonstrated that high glucose could result in tyrosine nitration of prostaglandin I2 (PGI_2) synthase in human aortic endothelial cells (HAECs). Moreover, El-Remessy et al. [30] have studied high glucose-induced tyrosine nitration of PI3-kinase in retinal endothelial cells. Though human umbilical vein endothelial cells (HUVECs) are an abundant and easily accessible endothelial cell type, no investigation about tyrosine nitration has been found in HUVECs. The aim of this study is to evaluate: (1) whether high glucose induces human umbilical vein endothelial cell (ECV304) injury; (2) whether the nitrated proteins induced by high glucose are different from other exogenous species.

Materials and methods

Reagents

Rabbit polyclonal antibody against 3-NO_2-Tyr, D-glucose, ferriprotoporphyrin IX (hemin), *o*-phthalaldehyde (OPA), butylated hydroxytoluene (BHT), sodium nitrite ($NaNO_2$), and Glucose oxidase (GOx) were purchased from Sigma; newborn calf bovine serum (CBS), Dulbecco's Modified Eagle Medium (DMEM, high glucose, 5.6 mM, for cell culture), Trypsin, HEPES, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) were purchased from Gibco BRL (Gaithersburg, MD, USA); penicillin G and streptomycinsulfate were purchased from Amersco; ROS detection kit was purchased from Beyotime Company (Jiangsu, China).

Cell culture

Human umbilical vein endothelial cells (ECV304, purchased from CCTCC, Wuhan, China) was cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose, 5.6 mM) containing 10% (v/v) newborn calf bovine serum (CBS), 100 U/ml penicillin G, and 100 μg/ml streptomycinsulfate in a humidified atmosphere with 5% CO_2 at 37°C. Cells were exchanged medium and passaged every 3 days. Cells were used for experiments within eight passages to ensure cell line stability. Cells were passaged into 24-well culture dishes, 6-well culture dishes, 35 mm Petri dishes or 10 cm culture dishes, and were serum-starved for 24 h in DMEM when the cells had reached subconfluence. Cells were cultured in normal glucose (NG) (Blank group, 5.6 mM glucose), high glucose (Control group, NG supplemented with 30 mM glucose (HG30) and 40 mM glucose (HG40), respectively), and/or hemin–nitrite–H_2O_2 system (Control group) for 72 h. Hemin was dissolved in DMSO as 10 mM stock solution and diluted to 1 mM with culture medium before used. Glucose (G)-glucose oxidase (GOx) system was used to produce H_2O_2, in which H_2O_2 was generated in situ [31] (in this study, the H_2O_2 production was about 3 μM/min). Control cells received an equivalent amount of DMSO (the final concentration of DMSO did not exceed 0.2%).

Determination of cell viability evaluated as mitochondrial activity

Cell viability evaluated as mitochondrial activity was quantified by measuring dehydrogenase activity retained in the cultured cells by using MTT assay [32]. Cells were plated at a density of 1×10^5 cells/ml in 24-well plates for

4 parallels and incubated for 1 day. Cells were incubated for another day in serum-starved DMEM medium before treated with D-glucose and other drugs. After treatment for 72 h, cells were incubated with 5 mg/ml MTT (40 μl/well) in PBS for 4 h at 37°C. Then the MTT-containing medium was removed and the intracellular formazan product was solved in DMSO for quantification at A_{570} with a microplate reader.

Determination of lipid peroxidation

The extent of lipid peroxidation in ECV304 cells treated with D-glucose was assessed according to the methods published before [33]. ECV304 cells were seeded at 1×10^5 cells/ml in Falcon 6-well plates and D-glucose was added. After washing twice with cold phosphate buffer (pH 7.4) before harvest, cells were scraped into 1.0 ml of 0.1 M phosphate buffer (pH 7.4). The cell suspension was homogenized by gentle sonication at 0°C, then centrifuged for 10 min at 8,000*g* (4°C). The final assay mixture contained 80 μl of the supernatant, 80 μl 20% (w/v) trichloroacetic acid (TCA), 200 μl 0.6% thiobarbituric acid (TBA), 20 μl 1% butylated hydroxytoluene (BHT), and 20 μl 1% sodium dodecyl sulfate (SDS). After thorough mixing, the mixture was heated in boiling water bath for 60 min, cooled down by tap water, and then 1 ml of *n*-butanol was added, mixed vigorously for 30 s, centrifuged, the supernatant was recorded at 532 nm. 2-thiobarbituric acid-reactive substances values were calculated from the molar extinction coefficient of the MDA-TBA complex of 1.56×10^5 cm^{-1} M^{-1}.

Determination of the intracellular GSH content

Samples for measuring GSH were processed according to the methods in Hissin et al. [34] with some modifications by Jiao et al. [35]. Briefly, ECV304 cells were seeded at 1×10^5 cells/ml in Falcon 6-well plates and D-glucose was added. After washing twice with cold phosphate buffer (pH 7.4) before harvest, cells were scraped into 1.0 ml of 0.1 M phosphate buffer (pH 8.3) containing 5 mM ethylene diamine tetraacetic acid (EDTA). The cell suspension was homogenized by gentle sonication at 0°C, then centrifuged for 10 min at 8,000*g* (4°C). The final assay mixture contained 50 μl of the supernatant, 50 μl (final concentration 50 μg/ml) of the *o*-phthalaldehyde solution, and 900 μl of phosphate EDTA buffer (pH 8.3). After thorough mixed and incubated at room temperature for 30 min, diluted with 1.0 ml phosphate EDTA buffer (pH 8.3), the fluorescence was read at an emission wavelength of 420 nm and an excitation wavelength of 350 nm. The content of GSH in cells was gained by the standard curve of GSH.

Determination of intracellular reactive oxygen species (ROS)

The level of intracellular reactive oxygen species (ROS) was determined on the basis of the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) upon reaction with hydroxyl radical, hydrogen peroxide, or peroxynitrite [36]. Briefly, cells in 35 mm petri dishes were incubated with control media or HG30 and HG40 for 72 h. Then cells were washed twice with cold PBS (pH 7.4) and incubated with DCFH-DA at room temperature for 30 min in dark. Fluorescent signal was recorded by using a fluorescence microscopy (488 nm filter; OLYMPUS IX-71, Japan). The fluorescence intensity of eight fields per dish was measured and the reactive oxygen species (ROS) level was quantified by measurement of fluorescence intensity with WinView32 software. Three parallel experiments were performed. Results were shown as the mean value.

Determination of nitrite/nitrate concentration

The level of NO induced by D-glucose was determined indirectly as the content of nitrite and nitrate by using capillary electrophoresis (CE, Beckman P/ACETM MDQ, USA). Briefly, ECV304 cells were seeded at 1×10^5 cells/ml in Falcon 6-well plates and D-glucose was added for 72 h. Culture medium was precipitated with identical volume of TCA (20%). After mixed vigorously for 30 s, the mixture was centrifuged for 15 min at 8,000*g*. The supernatant was used to determine the content of nitrate in medium.

After D-glucose treatment, cells in dishes were washed twice with cold PBS (pH 7.4) and collected. The cell suspension was centrifuged for 15 min at 4,000g (4°C). The precipitate was washed twice with cold PBS (pH 7.4) and centrifuged. The precipitate was dissolved with 200 μl PBS. Then the cell suspension was applied to froze-thawing circle for three times, homogenized by gentle sonication at 0°C and centrifuged for 15 min at 4,000g (4°C). The supernatant was used to determine the content of nitrite in cells.

Protein nitration detected by Western blotting

Protein nitration in ECV304 cells induced by HG30 and HG40 and hemin–nitrite–H_2O_2 system was detected by western blotting. Briefly, after treatment cells in 10 cm petri dishes were collected, and lysed in cell lysate (100 mM Tris–HCl, pH 8.0; 0.15 mM NaCl; 1 mM EDTA; 1 mM phenylmethane sulfonate fluoride (PMSF); 10 μg/ml aprotinin;

10 μg/ml leupeptin; 1% Tween-20). The protein contents were measured as Peterson (Peterson, 1977) described. Then 80 μl aliquots were mixed with 20 μl 5 × sample loading buffer, heated at 100°C for 3 min, and loaded on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) for electrophoresis and staining. After electrophoresis, proteins were transferred to nitrocellulose membrane and immunoblotted with a rabbit polyclonal antibody against 3-nitrotyrosine. The antibody was detected using an anti-rabbit secondary antibody conjugated with horseradish peroxidase. Chemiluminescence was used to identify specific proteins according to the ECL system (Pierce).

Statistical analysis

Experimental values were means ± S.D. of the numbers of experiments indicated in the legends. Significance was assessed by using the one way ANOVA test ($P < 0.05$ as significant).

Results

Effects of high glucose on cell viability

Cell viability was detected by MTT assay. As shown in Fig. 1, HG30 and HG40 both significantly decreased ECV304 cell viability, indicating that high glucose could reduce cell viability.

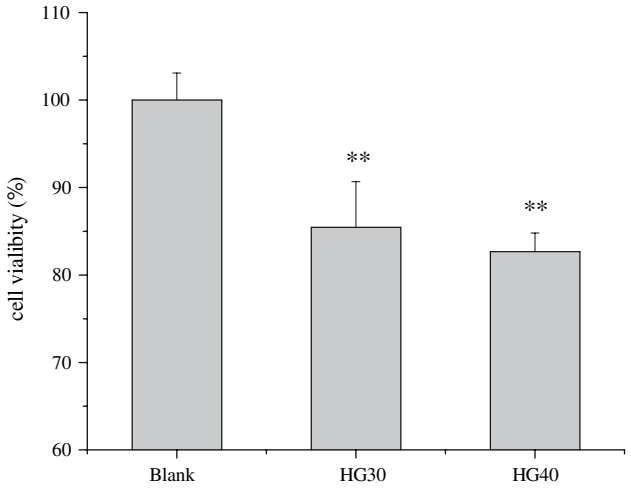

Fig. 1 Effect of high glucose medium on cell viability. Cells were cultured in culture medium containing different contents of glucose for 72 h, and then cell viability was evaluated by using MTT assay after treatments. Values are means ± S.D. of four determinations. Blank represents the group of cell with NG; Control (30 mM) and Control (40 mM) represent the group of cell with HG30 and HG40 respectively. $^{**}P < 0.01$ compared to Blank group

Effect of high glucose on cell lipid peroxidation

Lipid peroxidation was estimated by free 2-thiobarbituric acid-reactive substances production. Exposure of cells to HG30 and HG40 led to a significant increase in MDA content (Fig. 2). The increase of MDA content was concomitant with the increase of D-glucose concentration ($MDA_{40mM} > MDA_{30mM}$).

Effect of high glucose on intracellular GSH level

GSH is an important cellular antioxidant. The GSH depletion predicates the redox imbalance which would induce oxidative injury. As shown in Fig. 3, high glucose induced a distinct decrease in intracellular GSH content, and the max decrease reached almost 100%. The more the concentration of D-glucose increased, the more the GSH content depleted.

Effect of high glucose on intracellular reactive oxygen species (ROS)

High glucose significantly increased the intracellular level of ROS in a dose-dependent manner as measured by DCF fluorescence (Fig. 4), and the max increase reached almost 85%.

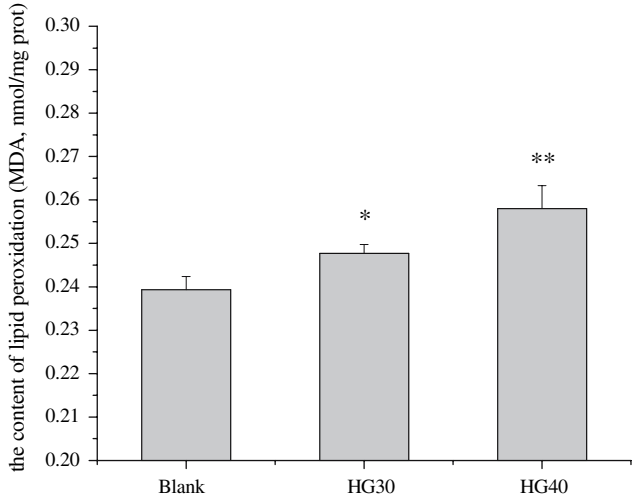

Fig. 2 Lipid peroxidation in ECV304 cells induced by HG30 and HG40. Values are means ± S.D. of four determinations. Blank represents the group of cell with NG; Control (30 mM) and Control (40 mM) represent the group of cell with HG30 and HG40 respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to Blank group

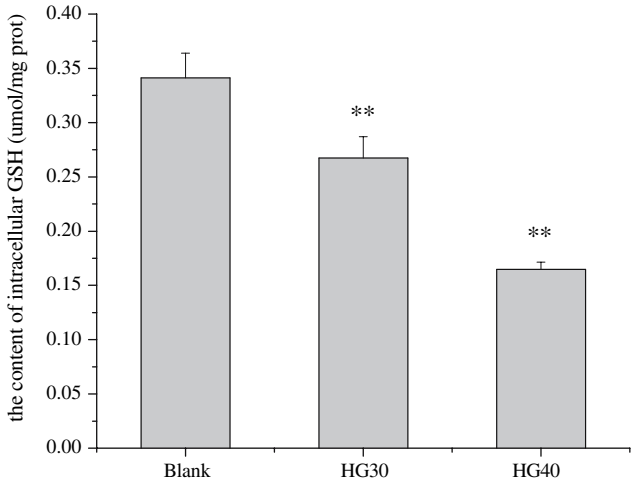

Fig. 3 The content of GSH in ECV304 cells induced by HG30 and HG40. Values are means ± S.D. of four determinations. Blank represents the group of cell with NG, Control (30 mM) and Control (40 mM) represent the group of cell with HG30 and HG40 respectively. $^{**}P < 0.01$ compared to Blank group

Intercellular nitrite/nitrate concentration after high glucose treatment

High glucose-treated endothelial cells would generate reactive nitrogen species, such as NO. To investigate the effect of high glucose on the formation of NO, we determined the levels of nitrite and nitrate, the oxidized product of NO, in cells lysate supernatant and in culture medium respectively by capillary electrophoresis. As shown in Fig. 5a, exposure of ECV304 to HG30 and HG40 significantly increased the content of nitrate in medium (Fig. 5a). The max increase reached almost 25%. Compared with cells under NG, the content of nitrite in supernatant of cells cultured in HG30 and HG40 also significantly increased and the increase reached 40% (Fig. 5b).

Effects of hemin–nitrite–H_2O_2 system on cell viability

In order to distinguish the injury between oxidation and nitration induced by hemin–nitrite–H_2O_2 system, we detected the cell viability induced by hemin–H_2O_2 with or without nitrite. We could find that hemin–H_2O_2 system at the given concentration significantly decreased the cell viability, but nitrite at the concentration of 0.75 mM had no significant influence on cell viability. However, the addition of nitrite significantly augmented the injury in ECV304 cells induced by hemin–H_2O_2 system (Fig. 6).

Effects of high glucose or hemin–nitrite–H_2O_2 system on cellular protein tyrosine nitration

Cells were treated with HG30 and HG40 or hemin–nitrite–H_2O_2 system, then protein tyrosine nitration was detected by western blotting using anti-3-NT antibody. After high glucose treatment, the intensity of those original nitrotyrosine epitopes increased, especially those proteins with molecular masses of ~34, 43, and 50 kDa, which demonstrated that high glucose increased tyrosine nitration in cell protein (Fig. 7).

Tyrosine nitration in cell protein induced by hemin–nitrite–H_2O_2 system was shown in Fig. 8, from which we could find hemin–nitrite–H_2O_2 system selectively increase tyrosine nitration in cell protein. As shown in Fig. 8, in normal cells, nitrotyrosine epitopes were detected in proteins with molecular masses of ~23, 25, 30, 34, 50, 66, 84, 125 and 140 kDa with faint bands at ~43 and 72 kDa (Fig. 8a, lane 1). In hemin–nitrite–H_2O_2 system-treated (Fig. 8a, lane 7) ECV304, roughly three additional proteins displayed evidence of tyrosine nitration (Fig. 8a, ~11, 17, and 20 kDa). Moreover the intensity of those original nitrotyrosine epitopes increased, especially those proteins with molecular masses of ~23, 25, 30, 34, 43, 50, and 66 kDa.

Discussion

The dysfunction and further damage of endothelium play an important role in the development and progression of diabetic microangiopathy and macroangiopathy [37–39]. Diabetic vascular complications have a close association with the increase of glucose in body fluid. Hyperglycemia can produce oxidative stress and ultimate acute endothelial dysfunction in diabetic patients. High-glucose condition reduced cell viability and induced apoptosis in human aorta endothelial cells (HAECs), which would contribute to macrovascular complications associated with diabetes [40]. Thus, we tested whether high glucose condition could reduce cell viability and induce cellular oxidative damage in ECV304. Fig. 1 showed that exposure of cultured ECV304 to high glucose for 72 h could significantly reduce cell viability (Fig. 1), suggesting high glucose could induce cell injury.

Of note, high glucose condition can generate oxidative stress in living body and induce cell injury, including the participation of free radicals [41, 42] and the breakdown of antioxidase system [43]. In agreement with these reports, it was found that high glucose produced oxidative injury, including increasing the content of MDA (Fig. 2), decreasing the content of GSH (Fig. 3), and increasing

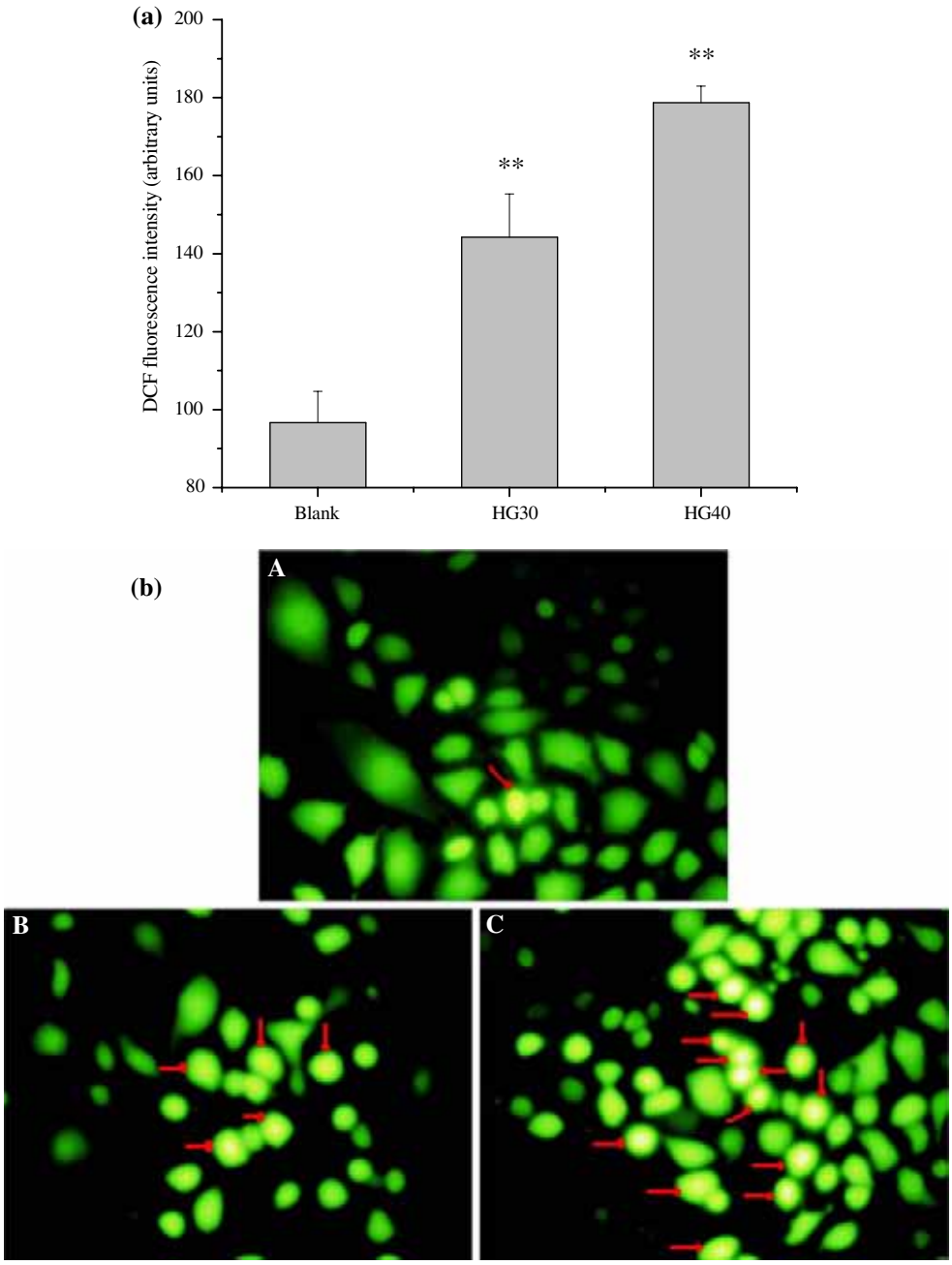

Fig. 4 (**a**) The increase of DCF-fluorescence intensity induced by HG30 and HG40. Values are means ± S.D. of three determinations. Blank represents the group of cell with NG, Control (30 mM) and Control (40 mM) represent the group of cell with HG30 and HG40 respectively. $^{**}P < 0.01$ compared to Blank group. (**b**) The corresponding DCF-fluorescent picture induced by HG30 and HG40. The brightened dot (red arrow) represents the fluorescence of ROS. A represents cells with NG; B represents cells with HG30; C represents cells with HG40

intracellular ROS (Fig. 4). These results indicated that high glucose could induce oxidative injury in ECV304.

NO also participates in diabetic complications as one of the ROS. Myatt et al. [44] found that nitrative stress, which was subsequent to oxidative stress, was seen in diabetic patients. It was well accepted that exposure of endothelial cells to high glucose would produce O_2^-, which can quench NO, reducing the efficacy of the most important vasodilator and generating the strong oxidant species peroxynitrite, a potent nitrating agent which will cause protein tyrosine nitration. We detected the production of NO by determining the content of nitrite/nitrate in ECV304 cultured in high glucose medium. As shown in Fig. 5, a significant increase of the content of nitrite/nitrate in ECV304 was found when the cells were cultured in high glucose medium. In Fig. 4a, it was found that high glucose caused a significant increase of intracellular ROS in ECV304. It is generally accepted that NO will react with O_2^- to generate peroxynitrite. In the present study, we have found the generation of O_2^- and NO under high glucose condition. Thus the generation of tyrosine nitration in ECV304, subsequent to the generation of peroxynitrite, was unavoidable when ECV304 was exposured to high glucose (Fig. 7). As shown in Fig. 7a, by comparison with ECV304 treated under NG conditions,

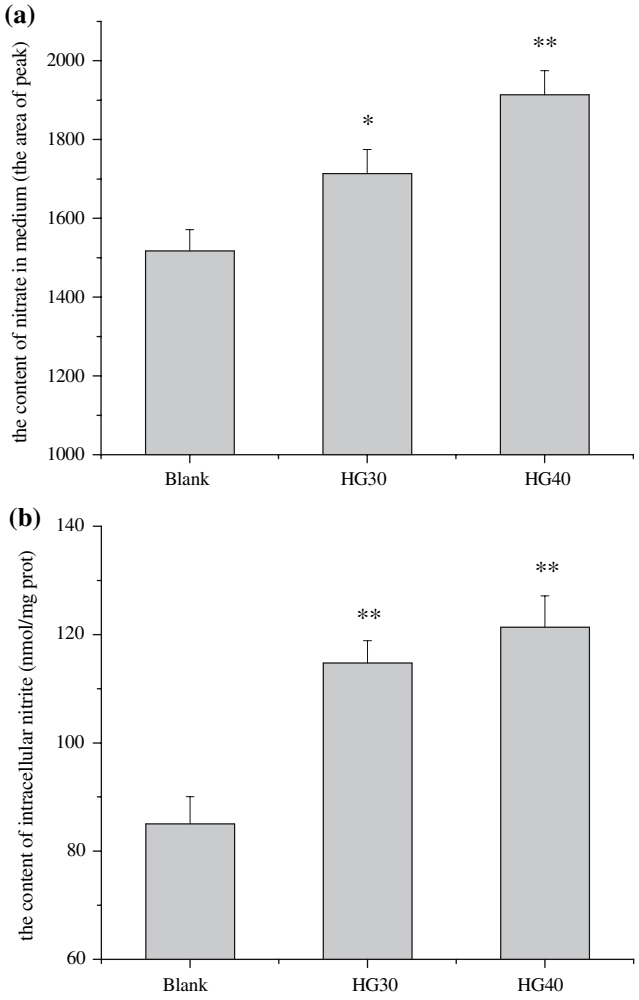

Fig. 5 (**a**) The content of nitrate in medium after cells treated with NG, HG30, and HG40. (**b**) The content of nitrite in cells after cells treated with NG, HG30 and HG40. Values are means ± S.D. of four determinations. Blank represents the group of cell with NG, Control (30 mM), and Control (40 mM) represent the group of cell with HG30 and HG40 respectively. $*P < 0.05$, $**P < 0.01$ compared to Blank group

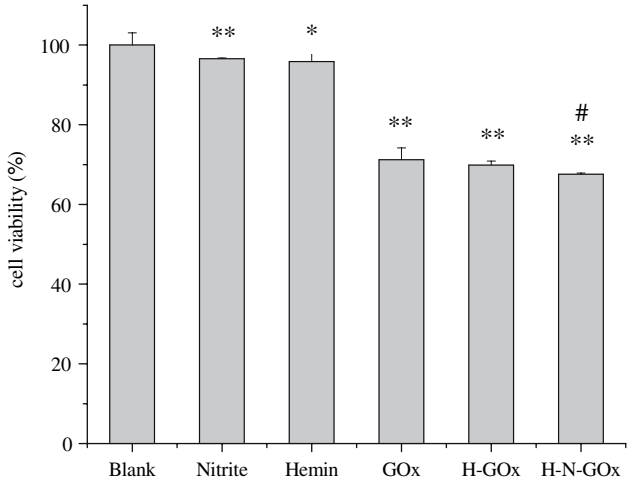

Fig. 6 Effect of hemin–nitrite–H_2O_2 system on cell viability after treated for 72 h. The final concentration was: 2 μM hemin (Hemin), 0.75 mM nitrite (Nitrite), 2 mU glucose oxidase (GOx), Hemin–GOx (H–GOx), and Hemin–nitrite–GOx (H–N–GOx)). Values are means ± S.D. of four determinations. Blank represents the group of cell without hemin, Glucose oxidase (GOx) and NO_2^{-1} $*P < 0.05$, $**P < 0.01$ represent the comparison between control group (Nitrite, Hemin, GOx, H–GOx, H–N–GOx) and blank group; $\#P < 0.05$ compared to H–GOx group

treatment ECV304 with HG30 or HG40 increased the number and intensity of proteins with nitrotyrosine epitopes (molecular mass ranging from ~20 to 140 kDa). In NG cells, nitrotyrosine epitopes were detected in proteins with molecular masses of ~23, 25, 30, 34, 50, 66, 84, 125, and 170 kDa with faint bands at ~43 and 72 kDa (Fig. 7a, lane 1). In HG30 (Fig. 7a, lane 2) or HG40-treated (Fig. 7a, lane 3) ECV304, roughly two additional proteins displayed evidence of tyrosine nitration (Fig. 7a, ~40 and 60 kDa). Moreover, the intensity of those original nitrotyrosine epitopes increased, especially those proteins with molecular masses of ~34, 43, and 50 kDa. These results demonstrated that exposure of ECV304 to high glucose could lead to the generation of tyrosine nitration.

It is well accepted that heme or heme containing proteins could catalyze NO_2^-–H_2O_2 to produce protein tyrosine nitration, which was the more possible mechanism under physiological and pathological conditions to produce tyrosine nitration than peroxynitrite. In order to see if endogenous tyrosine nitration caused by high glucose is similar to exogenous tyrosine nitration by nitrating agents as well as the contribution of tyrosine nitration to cell viability, we used hemin–nitrite–H_2O_2 system as exogenous nitrating agent to treat ECV304. As shown in Fig. 6, the addition of nitrite, although had no effect on cell viability at tested concentration, significantly augmented the cytotoxicity of hemin–H_2O_2 on ECV304. The only explanation was that when nitrite was added, protein nitration happened and caused further injury. At the same time we also found that hemin–nitrite–H_2O_2 system could generate tyrosine nitration in ECV304, which was coincident with our presumption (Fig. 8). As shown in Fig. 8a, by comparison with ECV304 treated under normal condition, treatment with hemin–nitrite–H_2O_2 system increased the number and intensity of proteins with nitrotyrosine epitopes (molecular mass ranging from ~20 to 170 kDa). Compared with cytotoxicity induced by high glucose, using hemin–nitrite–H_2O_2 system could distinguish the contribution of oxidation and tyrosine nitration to cytotoxicity by the increase or decrease of nitrite. We have found that tyrosine nitration could induce further cell injury on the base of oxidation by adding nitrite in ECV304 (Fig. 6). Moreover we also found that the increased intensity of

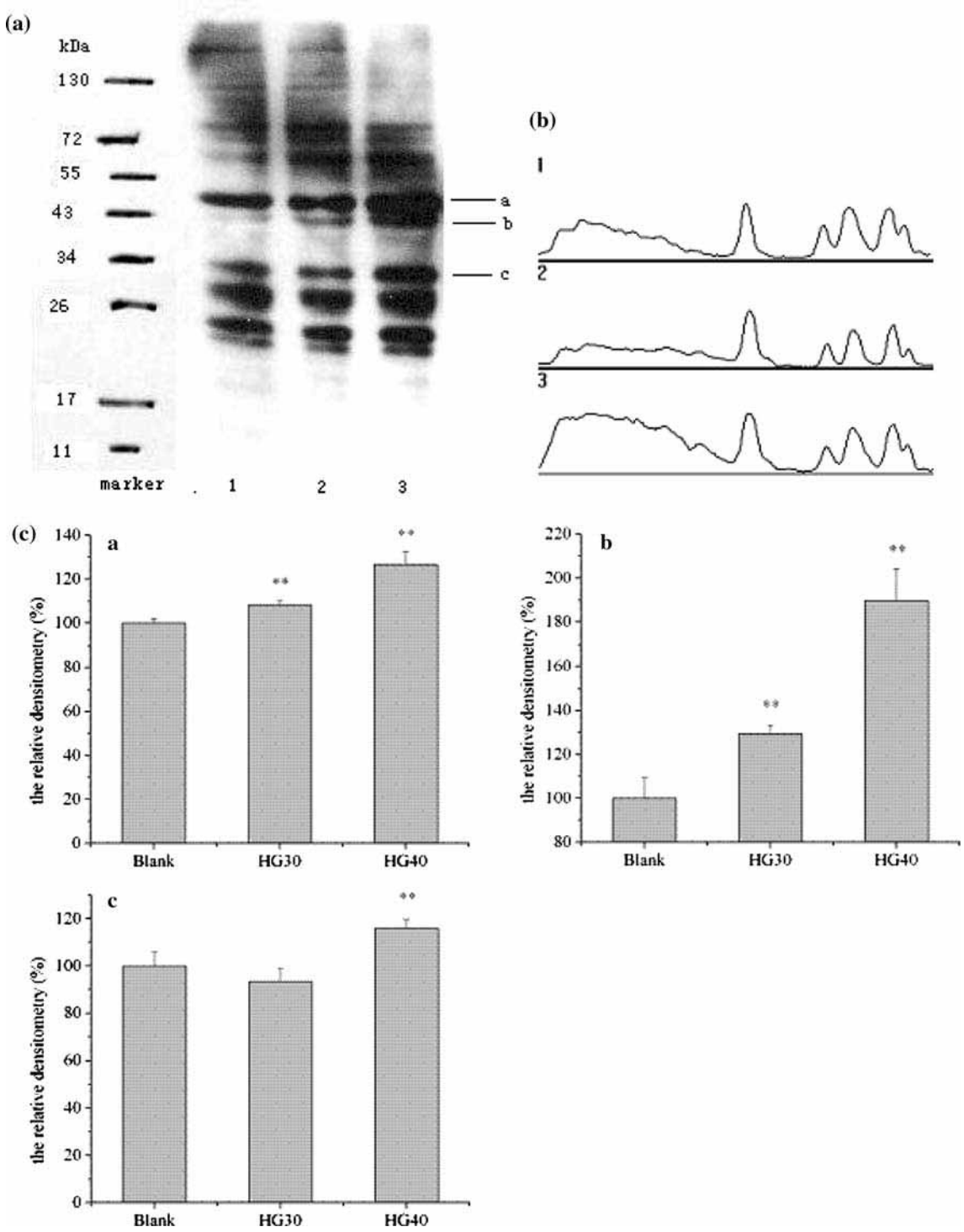

Fig. 7 (**a**) Protein nitration in ECV304 cells treated with HG30 and HG40 for 72 h. Lane 1, cells with NG. Lane 2, cells with HG30. Lane 3, cells with HG40. (**b**) The corresponding densitograms of protein nitration to (**a**) Densitogram 1, cells with NG. Densitogram 2, cells with HG30. Densitogram 3, cells with HG40. (**c**) The changes in protein nitration density at specific molecular weights. Bar graph a, 50 kDa protein. Bar graph b, 43 kDa protein. Bar graph c, 34 kDa protein

nitrotyrosine bands induced by hemin–nitrite–H_2O_2 system at ~11, 20, 66, and 80 kDa compared with hemin–H_2O_2 system, which supported the conclusion that tyrosine nitration induced further cell injury [45].

In Fig. 8a, we found that exogenous hemin–nitrite–H_2O_2 system-induced protein tyrosine nitration mainly were in proteins with molecular masses ranging from ~11 to 80 kDa, while endogenous hyperglycemia-induced

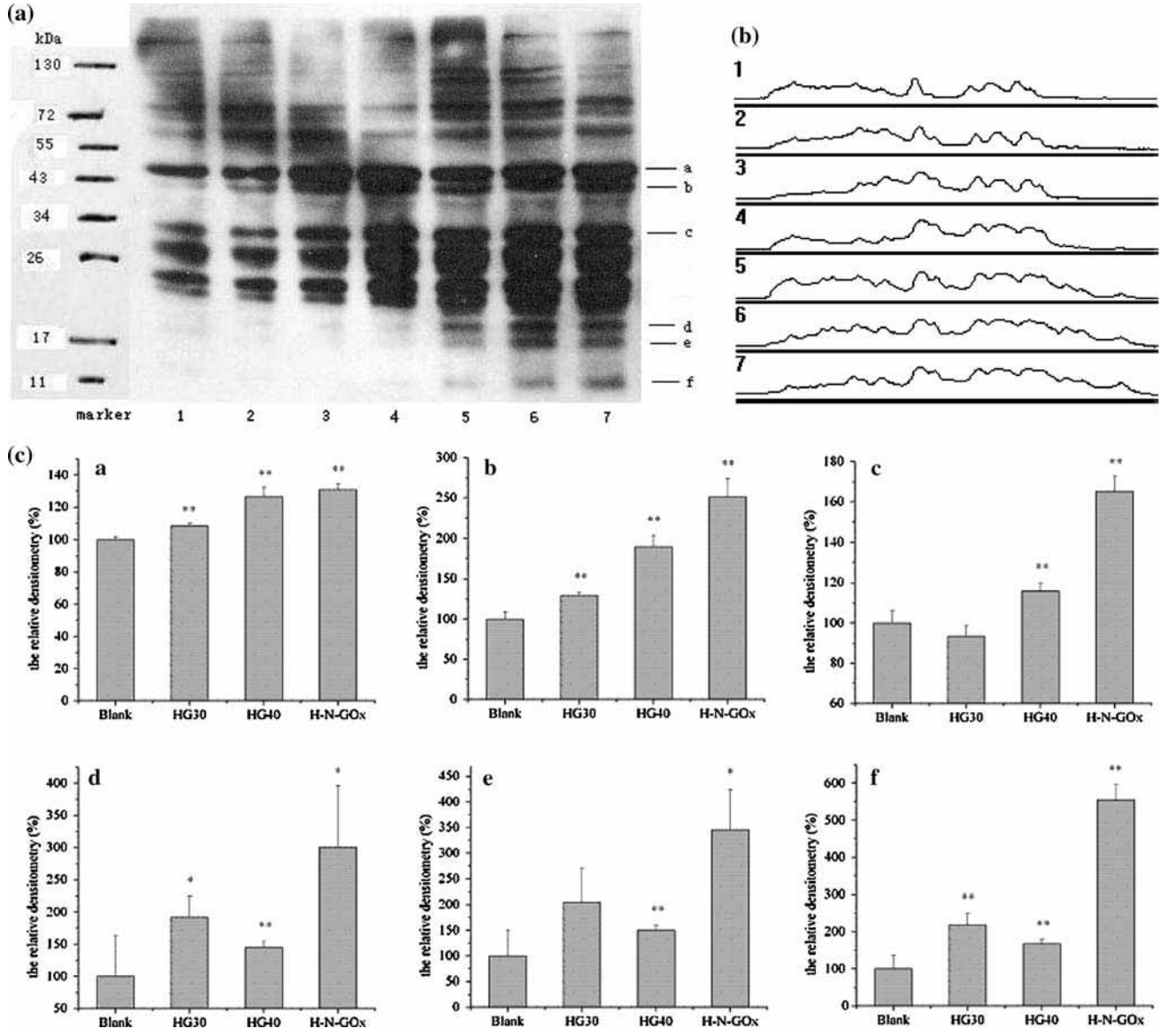

Fig. 8 (**a**) Comparison protein nitration between cells with high glucose and hemin–nitrite–H_2O_2 system. Lane 1, cells without D-glucose and hemin–nitrite–H_2O_2 system. Lane 2, cells with HG30. Lane 3, cells with HG40. Lane 4, cells with hemin. Lane 5, cells with nitrite. Lane 6, cells with hemin–GOx. Lane 7, cells with hemin–nitrite–H_2O_2. (**b**) The corresponding densitograms of protein nitration. Densitogram 1, cells without D-glucose and hemin–nitrite–H_2O_2 system. Densitogram 2, cells with HG30. Densitogram 3, cells with HG40. Densitogram 4, cells with hemin. Densitogram 5, cells with nitrite. Densitogram 6, cells with hemin–GOx. Densitogram 7, cells with hemin–nitrite–H_2O_2. (**c**) The changes in protein nitration density at specific molecular weights. Bar graph a, 50 kDa protein. Bar graph b, 43 kDa protein. Bar graph c, 34 kDa protein. Bar graph d, 20 kDa protein. Bar graph e, 17 kDa protein. Bar graph f, 11 kDa protein

protein nitrotyrosine ranging from ~23 to 80 kDa. Meanwhile the intensity of nitrotyrosine bands at ~90–170 kDa both decreased under the two conditions. In our study the band intensity of protein nitration induced by hemin–nitrite–H_2O_2 system aggravated, especially those proteins with molecular masses of ~20, 25, 30, and 34 kDa. These results indicated that some exogenous nitration species, such as hemin–nitrite–H_2O_2 system, could induce protein nitration to further aggravate cell injury. Our study also indicated that hemin could catalyze nitrating on those proteins with low molecular masses (~11, 17, and 20 kDa). With respect to the decreasing nitration on proteins with molecular weight between 90 and 170 kDa, a further investigation about the mechanism should be studied.

In summary, our current work have studied: (1) high glucose stimulated ECV304 injury in a dose-dependent manner, including reducing cell viability, inducing cell oxidative injury (increased MDA content, decreased GSH content, and increased intracellular ROS), increasing the

production of NO (increased nitrite content in cell and nitrate content in medium), and generating protein tyrosine nitration; (2) Protein tyrosine nitration could induce further cell injury; (3) With regard to some proteins, protein nitration induced by high glucose was similar to those induced by extrinsic factors (hemin–nitrite–H_2O_2 system). The difference between protein tyrosine nitration induced by high glucose condition and extrinsic factors (hemin–nitrite–H_2O_2 system) was that the latter could also generate protein nitration with low molecular masses ($\sim$11, 17, and 20 kDa). Taken together, our results indicated that high glucose could induce human umbilical vein endothelial cell injury, which demonstrated that protein nitration was involved in ECV304 under hyperglycemic condition. Moreover we found that cell injury could be aggravated because of protein nitration. By comparison the protein tyrosine nitration induced by high glucose condition and extrinsic factors (hemin–nitrite–H_2O_2 system), it may be speculated that protein is nitrated selectively to generate nitrotyrosine in diabetic vascular complications.

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References

1. Rubani GM (1993) The role of endothelium in cardiovascular homeostasis and diseases. J Cardiovasc Pharmacol 22:S1–S14
2. Cohen RA (1993) Dysfunction of vascular endothelium in diabetes mellitus. Circulation 87:S67–S76
3. Laight DW, Carrier MJ, Anggard EE (1999) Endothelial cell dysfunction and the pathogenesis of diabetic macroangiopathy. Diabetes Metab Res Rev 15:274–282
4. Cosentino F, Lüscher TF (1998) Endothelial dysfunction in diabetes mellitus. J Cardiovasc Pharmacol 32:S54–61
5. Pănuş C, Moţa M, Vladu D, Vanghelie L, Răducanu CL (2003) The endothelial dysfunction in diabetes mellitus. Rom J Intern Med 41:27–33
6. Lash JM, Nase GP, Bohlen HG (1999) Acute hyperglycemia depresses arteriolar no formation in skeletal muscle. Am J Physiol 277:1513–1520
7. Kawano H, Motoyama T, Hirashima O, Hirai N, Miyao Y, Sakamoto T, Kugiyama K, Ogawa H, Yasue H (1999) Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. J Am Coll Cardiol 34:146–154
8. Title LM, Cummings PM, Giddens K, Nassar BA (2000) Oral glucose loading acutely attenuates endothelium-dependent vasodilation in healthy adults without diabetes: an effect prevented by vitamins C and E. J Am Coll Cardiol 36:2185–2191
9. Shige H, Ishikawa T, Suzukawa M, Ito T, Nakajima K, Higashi K, Ayaori M, Tabata S, Ohsuzu F, Nakamura H (1999) Endothelium-dependent flow-mediated vasodilation in the postprandial state in type 2 diabetes mellitus. Am J Cardiol 84:1272–1274
10. Vehkavaara S, Seppälä-Lindroos A, Westerbacka J, Groop PH, Yki-Järvinen H (1999) In vivo endothelial dysfunction characterizes patients with impaired fasting glucose. Diabetes Care 22:2055–2060
11. Tesfamariam B, Cohen RA (1992) Free radicals mediate endothelial cell dysfunction caused by elevated glucose. Am J Physiol 263:H321–H326
12. Tesfamariam B (1994) Free radicals in diabetic endothelial cell dysfunction. Free Radic Biol Med 16:383–391
13. Giugliano D, Ceriello A, Paolisso G (1996) Oxidative stress and diabetic vascular complications. Diabetes Care 19:257–267
14. Ceriello A (2006) Oxidative stress and diabetes-associated complications. Endocr Pract 12:S60–S62
15. Nishikawa T, Araki E (2007) Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. Antioxid Redox Signal 9:343–353
16. Brezniceanu ML, Liu F, Wei CC, Tran S, Sachetelli S, Zhang SL, Guo DF, Filep JG, Ingelfinger JR, Chan JS (2007) Catalase overexpression attenuates angiotensinogen expression and apoptosis in diabetic mice. Kidney Int 71:912–923
17. Chan NN, Vallance P, Colhoun HM (2000) Nitric oxide and vascular responses in Type I diabetes. Diabetologia 43:137–147
18. Farkas K, Sármán B, Jermendy G, Somogyi A (2000) Endothelial nitric oxide in diabetes mellitus: too much or not enough? Diabetes Nutr Metab 13:287–297
19. Endemann DH, Schiffrin EL (2004) Nitric oxide, oxidative excess, and vascular complications of diabetes mellitus. Curr Hypertens Rep 6:85–89
20. Cosentino F, Eto M, De Paolis P, van der Loo B, Bachschmid M, Ullrich V, Kouroedov A, Gatti CD, Joch H, Volpe M, Luscher TF (2003) High glucose causes upregulation of cyclooxygenase-2 and altes prostanoid profile in human endothelial cells. Circulation 107:1017–1023
21. Reddy S, Bradley J (2004) Immunohistochemical demonstration of nitrotyrosine, a biomarker of oxidative stress, in islet cells of the NOD mouse. Ann N Y Acad Sci 1037:199–202
22. Asghar M, Monjok E, Kouamou G, Ohia SE, Bagchi D, Lokhandwala MF (2007) Super CitriMax (HAC-SX) attenuates increases in oxidative stress, inflammation, insulin resistance, and body weight in developing obese Zucker rats. Mol Cell Biochem 304:93–99
23. Sarchielli P, Galli F, Floridi A, Floridi A, Gallai V (2003) Relevance of protein nitration in brain injury: a key pathophysiological mechanism in neurodegenerative, autoimmune, or inflammatory CNS diseases and stroke. Amino Acids 25:427–436
24. Reynolds MR, Berry RW, Binder LI (2007) Nitration in neurodegeneration: deciphering the "Hows" "nYs". Biochemistry 46:7325–7336
25. Butterfield DA, Reed TT, Perluigi M, De Marco C, Coccia R, Keller JN, Markesbery WR, Sultana R (2007) Elevated levels of 3-nitrotyrosine in brain from subjects with amnestic mild cognitive impairment: implications for the role of nitration in the progression of Alzheimer's disease. Brain Res 1148: 243–248
26. Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA (2003) Proteomic identification of nitrated proteins in Alzheimer's disease brain. J Neurochem 85: 1394–1401
27. Liu D, Bao F, Wen J, Liu J (2007) Mutation of superoxide dismutase elevates reactive species: comparison of nitration and oxidation of proteins in different brain regions of transgenic mice with amyotrophic lateral sclerosis. Neuroscience 146:255–264
28. Casoni F, Basso M, Massignan T, Gianazza E, Cheroni C, Salmona M, Bendotti C, Bonetto V (2005) Protein nitration in a mouse model of familial amyotrophic lateral sclerosis: possible multifunctional role in the pathogenesis. J Biol Chem 280: 16295–16304

29. Zou MH, Shi C, Cohen RA (2002) High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. Diabetes 51:198–203
30. El-Remessy AB, Bartoli M, Platt DH, Fulton D, Caldwell RB (2005) Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration. J Cell Sci 118:243–252
31. van der Vliet A, Eiserich JP, Halliwell B, Cross CE (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. J Biol Chem 272:7617–7625
32. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
33. Rajeswari O, Natarajan R, Nadler JL, Kumar D, Kalra VK (1991) Glucose induces lipid peroxidation and inactivation of membrane-associated ion-transport enzymes in human erythrocytes in vivo and in vitro. J Cell Physiol 149:100–109
34. Hissin PJ, Hilf RA (1976) Fluorimetric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74:214–226
35. Jiao HL, Ye P, Zhao BL (2003) Protective effects of green tea polyphenols on human HepG2 cells against oxidative damage of fenofibrate. Free Radic Biol Med 35:1121–1128
36. Ye J, Wang S, Leonard SS, Sun Y, Butterworth L, Antonini J, Ding M, Rojanasakul Y, Vallyathan V, Castranova V, Shi X (1999) Role of reactive oxygen species and p53 in chromium(VI)-induced apoptosis. J Biol Chem 274:34974–34980
37. Zou MH, Ullrich V, Cohen R (2004) Peroxynitrite and vascular endothelial dysfunction in diabetes mellitus. Endothelium 11: 89–97
38. Ruderman NB, Williamson JR, Brownlee M (1992) Glucose and diabetic vascular disease. FASEB J 6:2905–2914
39. Cohen RA (2005) Role of nitric oxide in diabetic complications. Am J Ther 12:499–502
40. Duffy A, Liew A, O'Sullivan J, Avalos G, Samali A, O'Brien T (2006) Distinct effects of high-glucose conditions on endothelial cells of macrovascular and microvascular origins. Endothelium 13:9–16
41. Camici GG, Schiavoni M, Francia P, Bachschmid M, Martin-Padura I, Hersberger M, Tanner FC, Pelicci P, Volpe M, Anversa P, Lüscher TF, Cosentino F (2007) Genetic deletion of p66(Shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. Proc Natl Acad Sci USA 104:5217–5222
42. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M (2000) Normalising mitochondrial superoxide production blocks three pathways of hyperglycemic damage. Nature 404:787–790
43. Tachi Y, Okuda Y, Bannai C, Okamura N, Bannai S, Yamashita K (1998) High concentration of glucose causes impairment of the function of the glutathione redox cycle in human vascular smooth muscle cells. FEBS Let 421:19–22
44. Myatt L, Cui X (2004) Oxidative stress in the placenta. Histochem Cell Biol 122:369–382
45. Zhao YL, Li HL, Gao ZH, Gong YF, Xu HB (2006) Effects of flavonoids extraced from Scutellaria baicalensis Georgi on Hemin–nitrite–H_2O_2 induces liver injury. Eur J Pharmacol 536:192–199