



Glycated albumin activates NADPH oxidase in rat mesangial cells through up-regulation of p47phox

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ABSTRACT

Glycated albumin, an early-glycation Amadori-modified protein, stimulates transforming growth factor- β (TGF- β) expression and increases the production of the extracellular matrix proteins in mesangial cells, contributing to the pathogenesis of diabetic nephropathy. Glycated albumin has been shown to increase NADPH oxidase-dependent superoxide formation in mesangial cells. However, the mechanisms are not well understood. Therefore, in the present studies, we determined the mechanisms by which glycated albumin activates NADPH oxidase in primary rat mesangial cells and its contribution to glycated albumin-induced TGF- β expression and extracellular matrix protein production. Our data showed that glycated albumin treatment stimulated NADPH oxidase activity and increased the formation of superoxide formation in rat mesangial cells. Moreover, glycated albumin treatment stimulated the expression and phosphorylation of p47phox, one of the cytosolic regulatory subunits of the NADPH oxidase. However, the levels of other NADPH oxidase subunits including Nox1, Nox2, Nox4, p22phox, and p67phox were not altered by glycated albumin. Moreover, siRNA-mediated knockdown of p47phox inhibited glycated albumin-induced NADPH oxidase activity and superoxide formation. Glycated albumin-induced TGF- β expression and extracellular matrix production (fibronectin) was also inhibited by p47phox knock down. Taken together, these data suggest that up-regulation of p47phox is involved in glycated albumin-mediated activation of NADPH oxidase, leading to glycated albumin-induced expression of TGF- β and extracellular matrix proteins in mesangial cells and contributing to the development of diabetic nephropathy.

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1. Introduction

Diabetic nephropathy (DN) is the most common cause of end stage renal failure. About 20–30% of people with type 1 and type 2 diabetes develop DN. DN is characterized by both glomerulosclerosis with thickening of the glomerular basement membrane and mesangial matrix expansion, and tubulointerstitial fibrosis. Mesangial cells produce excessive amount of extracellular matrix proteins, contributing to the glomerulosclerosis.

Diabetes is associated with increased modification of proteins. Typically, glucose reacts nonenzymatically with amino groups of proteins to produce intermediate Amadori products (such as glycated albumin) and finally form a class of irreversibly cross-linked, fluorescent moieties termed advanced glycation end products (AGE) [1]. Glycated albumin is the major form of circulating glycated proteins in vivo and its levels are increased in diabetes [2,3]. Accumulating evidence suggests that elevated concentrations of

glycated albumin or AGE play a role in DN [2,4,5], possibly through up-regulation of TGF- β and stimulation of collagen and fibronectin expression in mesangial cells [6–9].

Studies have shown that glycated albumin or AGE stimulates reactive oxygen species (ROS) generation in mesangial cells [9,10]. It is known that an important source of ROS generation in kidneys of diabetes is NADPH oxidase [11]. NADPH oxidase is composed of two membrane-associated components, p22phox and gp91phox (Nox-2), and four cytosolic components, p47phox, p67phox, p40phox and rac-1/2 [12]. There are seven homologues of phagocytic Nox-2 proteins that have been detected in nonphagocytic cells (Nox1–5; Duox1–2). In the kidney, Nox-1, Nox-2, Nox-4, p22phox, p47phox, and p67phox have been found to be expressed in renal cortex as well as in mesangial cells [13,14]. Enhanced expression of renal NADPH oxidase including p47phox, p91phox or p22phox has been observed in the kidneys from diabetic animals or patients [11,15,16]. Moreover, inhibition of NADPH oxidase by apocynin prevented the development of early DN [13] or AGEs-mediated kidney damage in a type 1 diabetes animal model [17], suggesting that activation of NADPH oxidase contributes to DN. Glycated albumin has been shown to increase NADPH oxidase-dependent superoxide

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formation in mesangial cells [10]. However, the mechanisms by which glycated albumin-induced activation of NADPH oxidase in mesangial cells are not well understood.

In the present studies, we determined the mechanisms of glycated albumin-mediated activation of NADPH oxidase in primary rat mesangial cells. The results showed that glycated albumin up-regulates p47phox expression/phosphorylation, resulting in increased NADPH oxidase activity and extracellular matrix production in mesangial cells. These studies establish a molecular link between glycated albumin, altered NADPH oxidase activity, and extracellular matrix production in mesangial cells key to diabetic nephropathy through control of p47phox expression.

2. Methods and materials

2.1. Materials

Lucigenin, NADH, NADPH, Tiron, diphenylene iodonium (DPI), and anti-phospho-serine antibody were purchased from Sigma (St. Louis, Missouri, USA). Apocynin was purchased from Acros Organics (Morris plains, NJ). MnTBAP was purchased from Calbiochem (La Jolla, CA, USA). Dihydroethidium was purchased from Molecular Probes (Eugene, OR, USA). All drug solutions were prepared fresh before each experiment. Antibodies against Nox1, Nox2, p22phox, p47phox, Nox4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-fibronectin antibody was purchased from Life technologies (Gaithersburg, MD).

Glycated and nonglycated bovine serum albumin were purchased from Sigma (St. Louis, Missouri). Glycated albumin contained 3 mol of fructoselysine per mol albumin. The absence of AGE in the glycated albumin was assessed by measuring of AGE-related fluorescence at excitation maximum of 370 nm and emission maximum of 440 nm as described previously [18]. Endotoxin (LPS) was not detectable in the glycated or nonglycated albumin by the use of Limulus Amebocyte Lysate test kit (Sigma).

2.2. Cell culture

Primary rat mesangial cells (RMCs) were the generous gift from Dr. Anne Woods, University of Alabama at Birmingham as described previously [19]. Cells were cultured in RPMI 1640 medium supplemented with 20% heat-activated fetal bovine serum, 5 mM D-glucose, 2 mM L-glutamine, 1% (v/v) nonessential amino acids, 2 mM sodium pyruvate, 10 µg/ml transferrin, 5 ng/ml sodium selenite, and 0.6 international units/ml insulin. Experiments in this study were performed on cells between the 5th and 10th passages.

2.3. Measurement of NAD(P)H-dependent oxidase activity

NADPH oxidase activity in cell homogenates was measured as described previously [20]. Briefly, RMCs were cultured and made quiescent by culturing in serum and insulin-free RPMI 1640 media for 48 h. Cells were then treated with serum-free RPMI 1640 media (with 5 mM glucose) containing control or different concentrations of glycated albumin in the presence or absence of DPI (10 µM) or apocynin (20 µM) for 24 h. After treatment, cells were homogenized and used immediately to measure NADPH dependent superoxide generation. To start the assay, 100 µl of homogenates were added to 900 µl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin, 100 µM NADPH and 100 µM NADH. Photo emission was measured every 15 s for 10 min in a luminometer (Centro LB 960 microplate reader, Berthold technologies). A buffer blank was subtracted from each reading. The superoxide generation was expressed as relative chemiluminescence (light) units (RLU) $\times 10^4$ /per µg protein.

2.4. Dihydroethidium (DHE) staining

RMCs were cultured and treated with control or glycated BSA (200 µg/ml) in the absence or presence of DPI (10 µM) or apocynin (20 µM) for 24 h. This concentration of glycated albumin (200 µg/ml) is similar to that found in clinical specimens (200–600 µg/ml) and has been observed to stimulate extracellular matrix production in mesangial cells [7]. After treatment, cells were treated with the superoxide-sensitive fluorescent dye dihydroethidium (DHE) (5 µM in PBS) and were incubated in the dark for 15 min at 37 °C. Fluorescence was visualized under a fluorescence microscope.

2.5. Real-time PCR

After treatments, total RNA from RMCs was extracted. Total RNA (2 µg) was converted to cDNA using MLV reverse transcriptase (Promega). Real-time quantitative RT-PCR analyses were performed using SYBR Green PCR Master Mix kit with a MyiQ real-time PCR thermal cycler (Bio-Rad). Standard curves were generated using 18S RNA primers. The mRNA expression levels of test genes were normalized to 18S levels. Primers were synthesized by Integrated DNA Technologies. The primers for the components of NADPH oxidase including p22phox, Nox1, Nox4, Nox2(gp91), p47phox and p67phox were synthesized as described previously [21].

2.6. Immunoblotting analysis

RMCs were treated with control or glycated albumin (200 µg/ml) for 24 h. After treatment, conditioned media were collected. Fibronectin levels in the conditioned media were determined by immunoblotting. In addition, cells were harvested and cell homogenates were prepared. Equal amounts of proteins in cell homogenates were subjected to SDS-PAGE and transferred to nitrocellulose membranes to detect p47phox levels with anti-p47phox antibody. The enhanced chemiluminescence detection system (Pierce) was used for visualization of immunoreactive bands. β -actin was used as a loading control. Immunoblots were analyzed by scanning densitometry and quantified by Quantity One Gel analysis (Bio-Rad).

For detection of phosphorylation of p47phox, after treatment, cell lysates were immunoprecipitated (IP) using anti-p47phox antibody and immunoblotted with anti-phospho-serine antibody. The protein levels of p47phox in the immunoprecipitates were also determined using anti-p47phox antibody. The phosphorylated p47phox levels were normalized to the total p47phox protein levels.

2.7. TGF- β 1 measurement

RMCs were treated with control or glycated albumin (200 µg/ml) in the absence or presence of DPI (10 µM), apocynin (20 µM), Tiron (5 mM), or MnTBAP (50 µM) for 24 h. After treatment, conditioned media were collected. Total TGF- β 1 levels in the conditioned media were measured by TGF- β 1 ELISA kit from R&D system. The mean values of triplicate samples were converted into concentrations of TGF- β (pM) using a standard curve obtained with standard TGF- β 1 provided from the kit.

2.8. Transfection of cells with siRNA-p47phox

RMCs were transiently transfected with siRNA targeting to p47phox (sc-45918 from Santa Cruz) by using the siRNA transfection reagent (sc-29528). For a negative control, cells were transfected with a control siRNA duplex (sc-37007). After 48 h of transfection,

cells were treated with control or glycated BSA (200 $\mu\text{g}/\text{ml}$) for 24 h. After treatment, conditioned media were collected. Fibronectin and total TGF- β levels in the conditioned media were determined by immunoblotting and ELISA, respectively. The cells were harvested and cell homogenates were prepared. The NADPH oxidase activity was measured by lucigenin assay as described above. The efficient knock down of p47phox was determined by immunoblotting.

2.9. Statistical analysis

Data are expressed as the mean \pm S.E. Statistical evaluation of the data was performed using ANOVA or student *t* test as appropriate, considering the *p* value of <0.05 as significant.

3. Results

3.1. Glycated albumin activated NADPH oxidase and resulted in increased superoxide formation in rat mesangial cells (RMCs)

It has been shown that glycated albumin induced NADPH oxidase-dependent superoxide production in human mesangial cells [10]. However, the mechanisms are not well understood. Therefore, in the following studies, we first confirmed the effect of glycated albumin on NADPH oxidase activation and superoxide production in RMCs and further determined the involved mechanisms.

Using lucigenin (5 μM)-enhanced chemiluminescence assay with NADH/NADPH (100 μM) as substrates, we measured NADPH oxidase activity in the homogenates of RMCs after glycated albumin treatment. As shown in Fig. 1A and B, glycated albumin increased NADPH oxidase activity in mesangial cells in a dose and time-dependent manner. The maximum effect was achieved after 24 h of glycated albumin treatment at the concentration of 200 $\mu\text{g}/\text{ml}$. Control albumin treatment has no effect on NADPH oxidase activity. Moreover, intracellular superoxide levels were measured using the superoxide-sensitive dye dihydroethidium (DHE) staining and fluorescence microscopy. As shown in Fig. 1C, glycated albumin treatment (24 h) increased DHE staining as compared to control albumin, which was inhibited by NADPH oxidase inhibitors, DPI (10 μM) and apocynin (20 μM). The concentration of apocynin used for the current study is far below that needs for its antioxidant effect [22]. Therefore, our data suggest that glycated albumin increases the formation of NADPH oxidase-driven superoxide in mesangial cells.

3.2. Effect of glycated albumin on the expression of NADPH oxidase subunits in RMCs

To further determine the mechanisms of glycated albumin-mediated activation of NADPH oxidase in RMCs, the effect of glycated albumin on mRNA levels of the components of NADPH oxi-

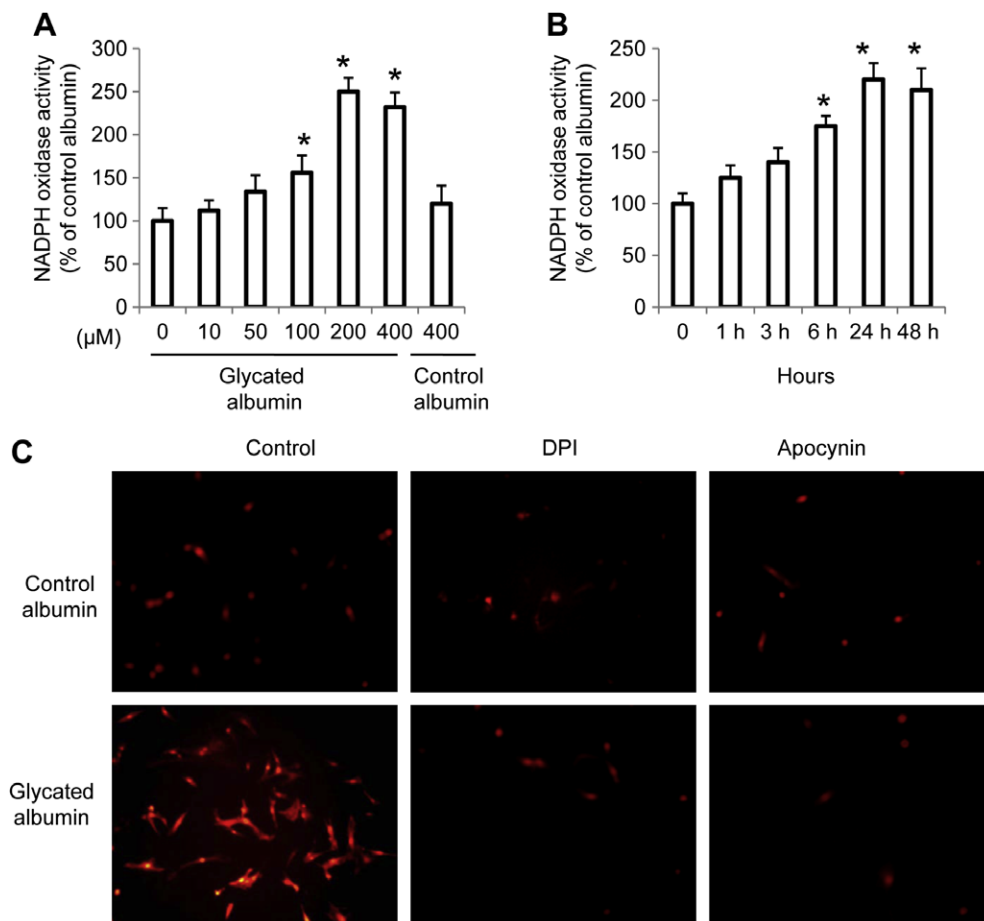


Fig. 1. NADPH oxidase activity and superoxide levels were increased in primary rat mesangial cells (RMCs) after glycated albumin treatment. (A) RMCs were treated with control albumin or glycated albumin at different concentrations for 24 h. (B) RMCs were treated with glycated albumin or control albumin (200 $\mu\text{g}/\text{ml}$) for different time periods. After treatment, RMCs were harvested and NADPH oxidase activity in cell homogenates was measured as described in Section 2. The experiments were repeated three times. The results shown are means \pm SE. **p* < 0.05 vs. control (0). (C) RMCs were treated with glycated albumin or control albumin (200 $\mu\text{g}/\text{ml}$) in the presence or absence of DPI (10 μM) or apocynin (20 μM) for 24 h. Cells were stained with the superoxide-sensitive dye dihydroethidium (DHE) and observed under a fluorescence microscope. The experiments were repeated three times. The images were acquired with identical acquisition parameters and representative images are shown.

dase was determined. The results showed that treatment with glycated albumin did not significantly alter the mRNA levels of p22phox, Nox1, Nox2, Nox4, or p67phox in mesangial cells (Supplemental S1). However, p47phox mRNA levels were significantly increased after 6 h and 24 h of glycated albumin treatment (Fig. 2A). p47phox protein levels in the cell homogenates were also significantly increased after glycated albumin treatment (Fig. 2B). In addition, the ratio of phosphorylated p47phox to total p47phox protein levels was significantly increased in RMCs after glycated albumin treatment (Fig. 2C). Together, these studies indicate that glycated albumin up-regulated p47phox expression and stimulated p47phox phosphorylation in mesangial cells, which may contribute to glycated albumin-mediated activation of NADPH oxidase.

3.3. Glycated albumin stimulated TGF- β and extracellular matrix (fibronectin) production in RMCs, which was inhibited by NADPH oxidase inhibitors and superoxide scavengers.

Glycated albumin has been shown to stimulate TGF- β and fibronectin expression in mesangial cells [7]. In the present studies, we determined whether NADPH oxidase induced superoxide

formation involves in glycated albumin-induced TGF- β and fibronectin expression. Our results showed that glycated albumin treatment stimulated TGF- β and fibronectin expression in RMCs. Control albumin had no significant effect on TGF- β and fibronectin levels in RMCs. Furthermore, administration of NADPH oxidase inhibitors-DPI and apocynin (Fig. 3A and B) or superoxide scavenger-Tiron (5 mM) or SOD mimic-MnTBAP (50 μ M) (Fig. 3C and D) prevented glycated albumin-mediated increases in TGF- β and fibronectin levels in mesangial cells, suggesting that NADPH oxidase-dependent superoxide generation involves in glycated albumin-mediated increases in TGF- β levels and extracellular matrix protein production in mesangial cells. siRNA-mediated p47phox knock down prevented glycated albumin stimulated NADPH oxidase activity and TGF- β and fibronectin levels in RMCs.

To further investigate the potential importance of glycated albumin-mediated increase in p47phox levels in superoxide production in RMCs, cells were transiently transfected with siRNA-p47phox to knock down p47phox. The results showed that inhibition of p47phox abolished glycated albumin-mediated increase in superoxide production in mesangial cells (Fig. 4A). In addition, glycated albumin-induced increase in TGF- β levels (Fig. 4C) and fibronectin expression (Fig. 4B) was also inhibited by p47phox knock

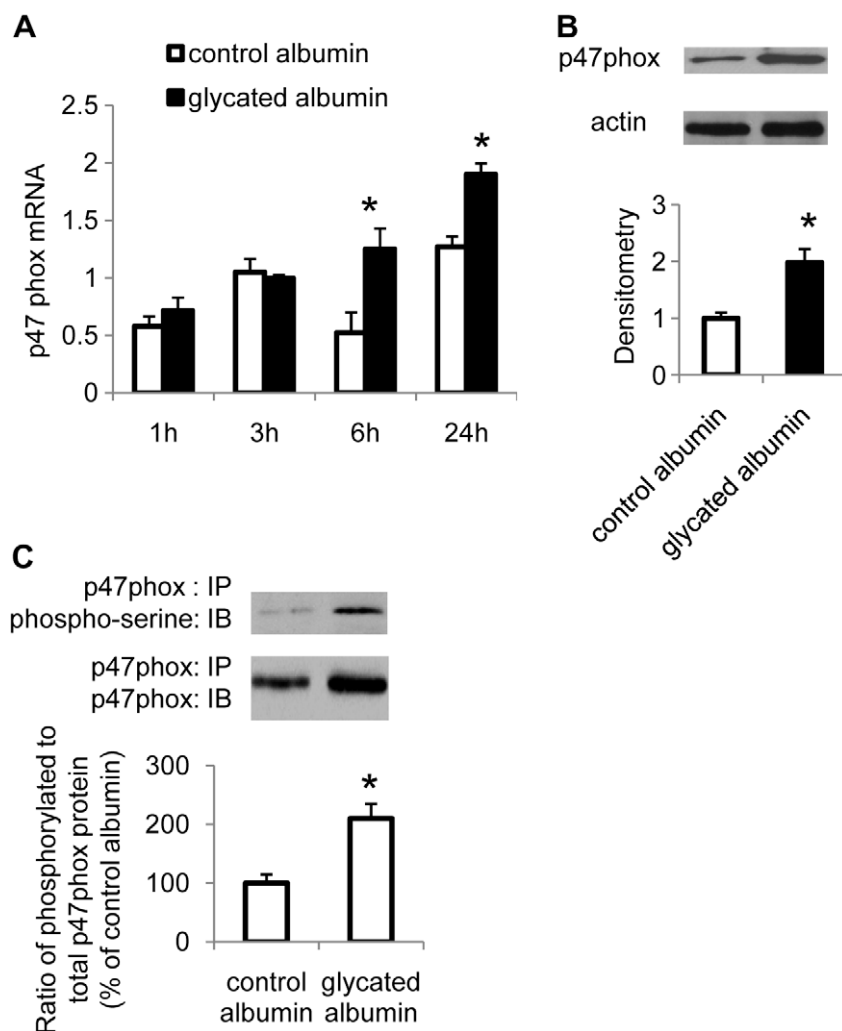


Fig. 2. Glycated albumin stimulated p47phox expression and phosphorylation in mesangial cells. RMCs were treated with glycated albumin or control albumin (200 μ g/ml) for different time periods. After treatment, cells were harvested. (A) The mRNA levels of p47phox were determined by real-time PCR and normalized to 18S RNA. (B) The protein levels of p47phox after 24 h of glycated albumin treatment were determined by immunoblotting. β -actin was used as an internal control. (C) Cell lysates were immunoprecipitated (IP) using anti-p47phox antibody and immunoblotted (IB) using anti-phospho-serine antibody to detect phosphorylated p47phox at serine residues or using anti-p47phox antibody to determine total p47phox protein levels. The immunoblots were analyzed by densitometry. Results were normalized to total p47phox levels. Results are the mean \pm SE ($n = 3$). * $p < 0.05$ vs. control albumin.

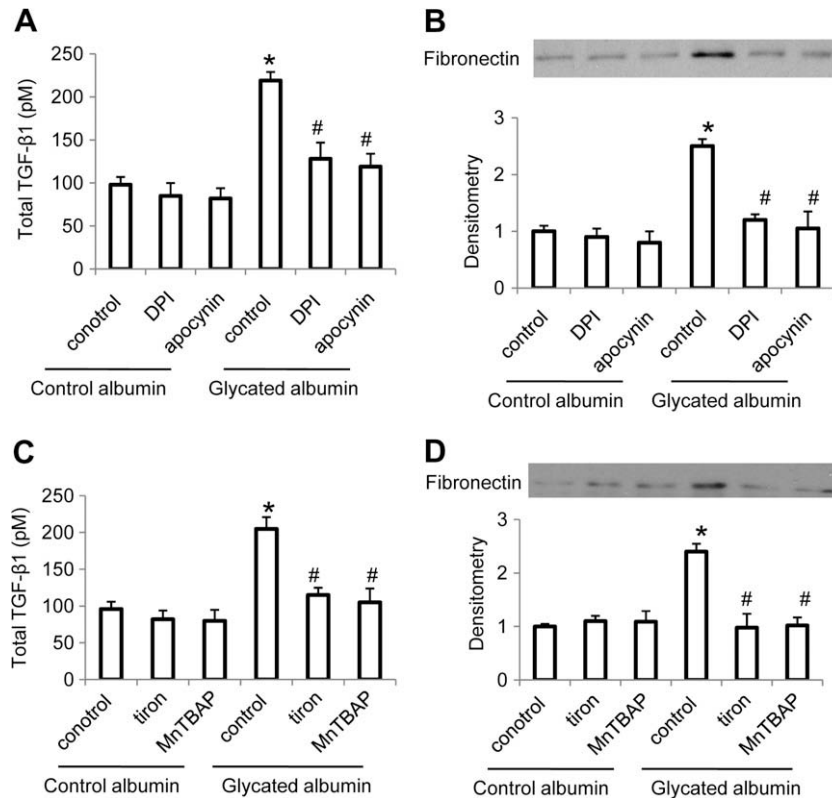


Fig. 3. Glycated albumin-induced increases in TGF- β and fibronectin levels were inhibited by NADPH oxidase inhibitors and superoxide scavengers. RMCs were treated with glycated albumin or control albumin (200 μ g/ml) for 24 h in the presence or absence of NADPH inhibitors-DPI (10 μ M) or apocynin (20 μ M) (A and B) or superoxide scavenger-Tiron (5 mM) or SOD mimic-MnTBAP (50 μ M) (C and D). After treatment, cell conditioned media were collected. Total TGF- β levels and fibronectin levels in the conditioned media were determined by ELISA and immunoblotting, respectively. Results are the mean \pm SE ($n = 4$). * $p < 0.05$ vs. control in control albumin group; # $p < 0.05$ vs. control in glycated albumin group.

down. The efficient knock down of p47phox in mesangial cells was confirmed by immunoblotting (Fig. 4D). Taken together, these data suggest that up-regulation of p47phox is involved in glycated albumin-induced NADPH oxidase activation and increased extracellular matrix production in mesangial cells.

4. Discussion

Our studies showed that glycated albumin stimulated p47phox expression and phosphorylation, leading to activation of NADPH oxidase and increased formation of superoxide in rat mesangial cells. Moreover, siRNA-mediated knockdown of p47phox prevented glycated albumin-induced superoxide production and extracellular matrix production in mesangial cells. Together, our studies provide first evidence that glycated albumin stimulates TGF- β and resultant extracellular matrix production in rat mesangial cells through p47phox-dependent NADPH oxidase activation and superoxide formation.

Increased modification of proteins has been observed under diabetic conditions. Typically, glucose reacts nonenzymatically with amino groups of proteins to produce intermediate Amadori products (such as glycated albumin) and finally form a class of irreversibly cross-linked, fluorescent moieties termed advanced glycation end products [1]. Accumulating evidence suggests that elevated concentrations of glycated albumin play a role in the development of diabetic nephropathy [23–26]. One mechanism is through glycated albumin-mediated generation of reactive oxygen species (ROS) in mesangial cells [10]. It is known that NADPH oxidase is one of the major sources of ROS generation in diabetic kidneys [11]. Glycated albumin has been shown to induce NADPH oxidase-dependent superoxide production in human mesangial

cells [10]. Consistently, our studies demonstrated that glycated albumin stimulated NADPH oxidase activity and increased the formation of superoxide in rat mesangial cells. Moreover, we found that the mRNA levels of NADPH oxidase subunits including Nox1, Nox2, Nox4, p22phox or p67phox were not significantly altered by glycated albumin treatment. However, p47phox mRNA levels as well as protein levels in the cell homogenates were significantly increased by glycated albumin. In addition, glycated albumin also stimulated phosphorylation of p47phox in mesangial cells. Together, these studies suggest that increased p47phox expression and phosphorylation may be a mechanism involving in glycated albumin-mediated activation of NADPH oxidase in mesangial cells. It appears that the effect of glycated albumin on p47phox expression is cell type specific, since no changes in p47phox levels were revealed in glycated albumin treated cardiomyocytes [27]. In addition, glycated albumin has been shown to stimulate PKC activity in mesangial cells [6,10]. Increased PKC activity may up-regulate p47phox expression and contributes to glycated albumin-induced NADPH oxidase activity. At this time, the relevant receptor and down-stream signaling pathways that mediate the glycated albumin response in mesangial cells is not clear and needs to be further investigated.

Our studies showed that glycated albumin treatment stimulated TGF- β levels and increased the expression of extracellular matrix protein (fibronectin) in mesangial cells, which is consistent with the studies from Cohen et al. [23–26]. Furthermore, we demonstrated that p47phox-dependent NADPH oxidase activity mediates glycated albumin-induced stimulation of TGF- β and resultant extracellular matrix protein production in mesangial cells. It is known that glycated albumin activated NF- κ B in a variety of cell types [28–33]. NF- κ B is an important redox-sensitive

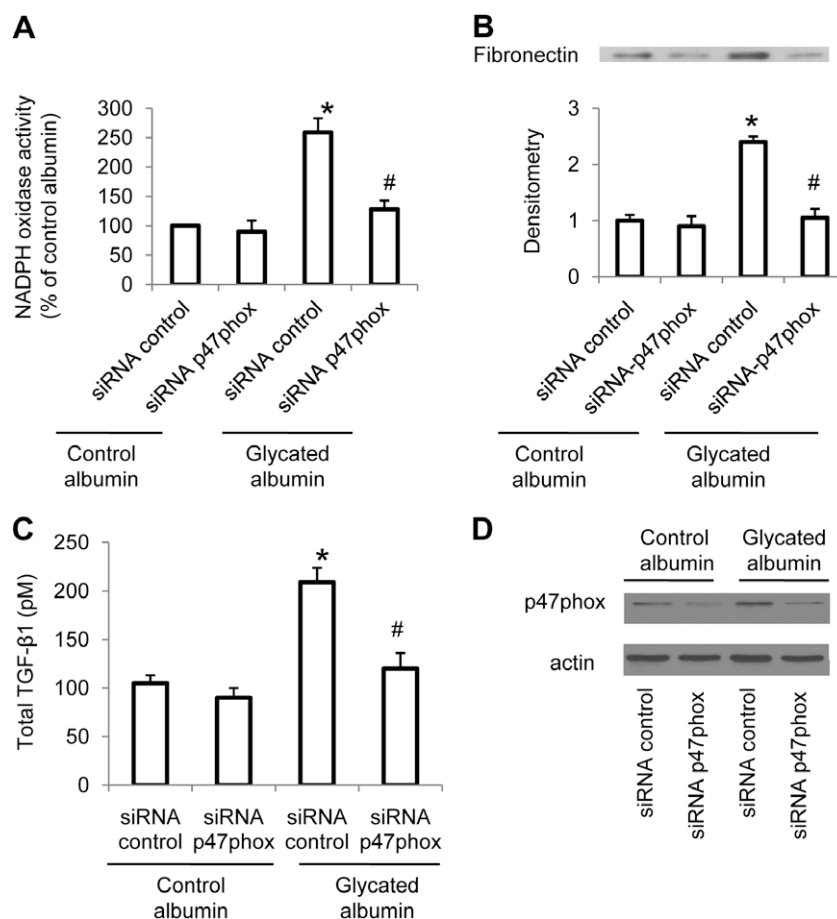


Fig. 4. siRNA-mediated p47phox knock down inhibited NADPH oxidase activity and TGF- β and fibronectin levels in mesangial cells after glycated albumin treatment. RMCs were transiently transfected with siRNA-p47phox or control siRNA for 48 h. Then cells were treated with glycated albumin or control albumin for 24 h. The NADPH oxidase activity in cell homogenates was measured by lucigenin assay (A). Fibronectin protein levels in cell lysates were determined by immunoblotting (B). Total TGF- β levels were determined by ELISA (C). The efficient knockdown of p47phox was confirmed by immunoblotting (D). Results are the mean \pm SE ($n = 4$). * $P < 0.05$ vs. siRNA control in control albumin group; # $p < 0.05$ vs. siRNA control in glycated albumin group.

transcription factor and has been implicated in the development of renal diseases [34]. Therefore, it is possible that glycated albumin-mediated increase in NADPH-oxidase-dependent superoxide formation activates NF- κ B, resulting in increased expression of TGF- β in mesangial cells. Further studies need to be performed to investigate the above possibility.

In summary, our data indicated that glycated albumin up-regulated p47phox expression/phosphorylation and resulted in activation of NADPH oxidase in rat mesangial cells. In addition, NADPH oxidase-driven superoxide formation mediates glycated albumin-induced increase in TGF- β levels and extracellular matrix production, contributing to the development of diabetic nephropathy.

Disclosures

No conflicts of interest are declared by authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.04.084.

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