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

Yanzhang Li, Shuxia Wang *

Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, KY 40536, United States

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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.

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

* Corresponding author. Address: Graduate Center for Nutritional Sciences, University of Kentucky, Wethington Bldg. Rm 583, 900 S. Limestone Street, Lexington, KY 40536, United States. Fax: +1 859 257 3646.
E-mail address: swang7@uky.edu (S. Wang).

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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-β1 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-β1 levels in the conditioned media were measured by TGF-β1 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-β1 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,