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Fatty Acid-Binding Protein 4 mediates apoptosis via endoplasmic reticulum stress in mesangial cells of diabetic nephropathy

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ABSTRACT

Type 2 diabetes is characterized by hyperglycemia and deregulated lipid metabolism with increased plasma non-esterified fatty acids (NEFA). Apoptosis of glomerular cells is a hallmark in diabetic glomerulosclerosis. Fatty acid-binding protein 4 (FABP4), a carrier protein for fatty acids, has been linked to diabetes and diabetic nephropathy (DN). Here we aimed to investigate the link between FABP4 and apoptosis in diabetic glomerulosclerosis. We first evaluated the presence of FABP4 and ER stress markers as well as apoptosis-related proteins in renal biopsies of patients with DN. Then we used FABP4 inhibitor BMS309403 or siRNA to further investigate the role of FABP4 in ER stress and apoptosis induced by NEFA or high glucose in cultured human mesangial cells (HMCs).

We found FABP4 was expressed mainly in glomerular mesangial cells of the human renal biopsies and the glomerular FABP4 was increased in renal biopsies of DN. The up-regulation of FABP4 was accompanied with increased glucose-regulated protein 78 (GRP78) and Caspase-12 as well as down-regulated B-cell CLL/lymphoma 2 (Bcl-2) in glomeruli. Along with the induction of FABP4 and apoptosis, GRP78 and its three sensors as well as C/EBP homologous protein (CHOP) and Caspase-12 were induced in HMCs treated with NEFA or high glucose and these responses were attenuated or even abrogated by treating with FABP4 inhibitor or FABP4 siRNA. Ultrastructure observation confirmed the lipotoxicity of oleic acid by showing the morphological damage in HMCs. Our data suggest that FABP4 in glomerular mesangial cells is up-regulated in DN and FABP4 mediates apoptosis via the ER stress in HMCs.

1. Introduction

Diabetic nephropathy (DN) is becoming an increasingly important cause of morbidity and mortality worldwide as a consequence of increasing prevalence of type 2 diabetes (Lizicarova et al., 2014). In addition to hyperglycemia, increased plasma non-esterified fatty acids (NEFA) are detected in type 2 diabetes and further identified as a risk factor of type 2 diabetes (Chehade et al., 2013; Han et al., 2011; Lizicarova et al., 2014). Fatty acid-binding protein 4 (FABP4, also known as AFABP or aP2) is a small cytoplasmic lipid chaperone that plays an important role in the trafficking of fatty acids in subcellular compartments and influencing lipid metabolism, as well as insulin sensitivity (Baar et al., 2005; Elmasri et al., 2009; Kralisch and Fasshauer, 2013). FABP4 is preferentially produced in and released from adipocytes, but is also produced in significant amounts in macrophages and endothelial cells (Coe and Bernlohr, 1998; Elmasri et al., 2009; Kralisch and Fasshauer, 2013; Raclot et al., 1997). Experiments in vitro and in vivo performed on macrophages showed that FABP4 could be induced by saturated fatty acids or advanced glycation end-products and FABP4 is an obligatory intermediate for endoplasmic reticulum (ER) stress responses to lipids (Erbay et al., 2009; Wang et al., 2011).

The endoplasmic reticulum is a central organelle entrusted with lipid synthesis, protein folding and protein maturation. It is involved in the intrinsic pathway of apoptosis (Bravo et al., 2013; Cunard and Sharma, 2011; Eizirik et al., 2013; Laybutt et al., 2007; Liu et al., 2008; Preston et al., 2009; Rasheva and Domingos, 2009; Ron and Walter, 2007). Disruption of ER homeostasis leads to accumulation of unfolded proteins and successively activates the unfolded protein response (UPR), which is also referred to as the ER stress response, and then triggers apoptosis (Rasheva and Domingos, 2009; Ron and Walter, 2007; Scull and Tabas, 2011; Szegezdi et al., 2006). It has also been proved that hyperglycemia and the elevated plasma levels of NEFA initiate ER stress in type 2

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diabetes (Giacca et al., 2011; Karaskov et al., 2006; Preston et al., 2009; Sieber et al., 2010). NEFA, such as palmitic acid, has been identified as a proapoptotic factor in some cell types including pancreatic beta cells, hepatocytes and renal tubular cells (Busch et al., 2002; Giacca et al., 2011; Gu et al., 2010; Ibrahim et al., 2011; Sieber et al., 2010; Soumura et al., 2010; Wrede et al., 2002). Recent studies implicated ER stress, as a potential mediator of NEFA-induced apoptosis, was involved in lipotoxicity in diabetes and its complications including atherosclerosis and DN (Cunard and Sharma, 2011; Giacca et al., 2011; Laybutt et al., 2007; Szegezdi et al., 2006). The mitigation of ER stress with a chemical chaperone of FABP4 not only prevents macrophage FABP4 production, but also leads to marked protection against macrophage ER stress, apoptosis and atherosclerosis (Erbay et al., 2009). The FABP4-deficient mouse model confirms the critical role of FABP4 in mediating macrophage ER stress by showing the protection from hypercholesteremia-induced macrophage ER stress and apoptosis in atherosclerotic lesions (Erbay et al., 2009).

Several evidence demonstrated cell death via apoptosis contributing to diabetic glomerulosclerosis (Liu et al., 2008; Raptis and Viberti, 2001; Sanchez-Niño et al., 2010; Sieber et al., 2010). Considering the role of FABP4 in the ER stress of metabolic syndrome and atherosclerosis (Erbay et al., 2009; Furuhashi et al., 2007; Kralisch and Fasshauer, 2013; Makowski et al., 2001), we assumed that there might be a link between FABP4 and apoptosis in DN. We hypothesized that FABP4 might mediate apoptosis via ER stress in DN. To test this hypothesis, we first evaluated the presence of FABP4 and ER stress markers as well as apoptosis-related proteins in renal biopsies of patients with DN. Then we used FABP4 inhibitor BMS309403 or RNA interference to further investigate the role of FABP4 in ER stress and apoptosis induced by NEFA or glucose in vitro.

2. Materials and methods

2.1. Antibodies and reagents

Unless otherwise noted, all chemicals and reagents were obtained from Sigma (St. Louis, MO, USA). Rabbit monoclonal anti-glucose-regulated protein 78 (GRP78), anti-inositol requiring 1α (IRE1α) (phospho S724) and polyclonal anti-Caspase-12 antibodies were purchased from Abcam (Hong Kong, China). The goat polyclonal antibody against FABP4 was purchased from R&D Systems (Minneapolis, MN, USA). The rabbit monoclonal antibody against Bcl-2-associated X protein (BAX) and polyclonal antibodies against C/EBP homologous protein (CHOP), phosphorylated protein kinase R (PKR)-like ER kinase (p-PERK), B-cell CLL/lymphoma 2 (Bcl-2) and β-actin were purchased from Santa Cruz Biotechnology (CA, USA). Mouse monoclonal antibody against activating transcription factor 6α (ATF6α) was purchased from Novus Biologicals (Littleton, USA). Rabbit polyclonal anti-cleaved Caspase-3 antibody was purchased from Cell Signaling (Boston, USA). Fluorescent secondary antibodies Dylight 488 rabbit anti-goat IgG and Dylight 594 goat anti-mouse IgG were purchased from Earthox (San Francisco, USA). All other immunohistochemical reagents including anti-CD34 antibody were obtained from ZSGB-BIO (Beijing, China).

2.2. Human renal biopsy

We collected renal biopsies from 45 patients (30 males and 15 females) with established DN from 2003 to 2011. All these renal biopsies met the standard histological criteria for DN diagnosis (Japanese Society of Nephrology, 2011). Other concomitant renal diseases were excluded for all the participants with DN. Renal biopsies with the diagnosis of minimal change disease (MCD) (Japanese Society of Nephrology, 2011) (10 cases) served as controls. The mean value of serum creatinine of DN (1.95 mg/dl) was significantly elevated compared to control (0.87 mg/dl) ($P = 0.043$). Serum creatinine levels showed the subjects in DN group suffered significant renal functional damages than control. All formalin-fixed renal biopsy tissues, both control and DN, were obtained from the Department of Pathology, Shinshu University School of Medicine. All human biopsy tissues were obtained with patient's consents and permission of the local ethics committee.

2.3. Immunohistochemistry and double immunofluorescence labeling

Formalin-fixed human renal tissue was embedded in paraffin, cut in 4-μm sections, and immunostained for FABP4, GRP78 (an ER chaperone, also known as BIP or HSPA5), as well as caspase-12 and Bcl-2 basing in line with the instructions of a Polink-2 Plus or Histostain-Plus Kit (ZSGB-BIO). After microwave treatment in 0.01 mol/l sodium citrate (80 °C for 50 min, then 40 °C for 10 min), sections were incubated with the primary antibody against FABP4 (1:200), GRP78 (1:100), Bcl-2 (1:30) and Caspase-12 (1:1000), then stained with the corresponding secondary antibodies and visualized by reaction with DAB before counterstaining with hematoxylin. The negative control by using PBS to replace the primary antibody showed no staining. All the sections were examined by light microscopy (Olympus AX70) and the stainings in both about 100 glomeruli from renal biopsies of DN and about 30 glomeruli from control renal biopsies were quantified by Image-Pro-Plus 6.0 (Media Cybernetic, Silver Spring, MD, USA).

For double immunofluorescence labeling assay, the sections were incubated with anti-FABP4 antibody and anti-CD34 antibody overnight at 4 °C and finally with the appropriate fluorescent secondary antibodies (Dylight 488 rabbit anti-goat IgG or Dylight 594 goat anti-mouse IgG) for 2 hours at room temperature. Fluorescent specimens were examined with a fluorescence microscope (Olympus, BX63).

2.4. Cell cultures

Human mesangial cells (Clonetics Corporation, Clonetics, San Diego, CA) were inoculated for serial passage in 25 cm^2 flasks with 5 ml DMEM-F12 medium (Gibco BRL Life Technologies, Beijing, China) containing 5% fetal bovine serum (FBS), 5.6 mmol/l glucose, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.5. FABP4 inhibitor BMS309403 treatment

Cells were seeded at 1×10^6 in 3 ml medium per well in a six-well plate. Cells were treated serum-free overnight to bring them to about 80% confluence. Then the medium was replaced and the cells were cultured for 24 hours in DMEM-F12 as discussed but containing 200 μmol/l oleic acid, 400 μmol/l palmitic acid or 30 mmol/l glucose respectively with or without the presence of BMS309403 (10 μmol/l, Calbiochem, Darmstadt, Germany). The medium containing albumin-conjugated oleic acid or palmitic acid was prepared by the protocol of Cousin et al. (2001). Briefly, sodium salts of oleic acid or palmitic acid were dissolved in 50% ethanol or 0.1 mol/l NaOH solution and gently warmed to facilitate dissolution without damaging the fatty acids. The solution containing albumin-conjugated oleic acid or palmitic acid was prepared in 10% fatty acid-free BSA in a phosphate-buffered saline (PBS).

2.6. Small interfering RNA (siRNA) transfection

Human mesangial cells (HMCs) were transfected at about 50–60% confluence with siRNAs specific to FABP4 (Yingrun Biotechnology, Changsha, China) or nontargeting siRNAs (Yingrun Biotechnology, Changsha, China) as a negative control using FuGENE

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