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Advanced oxidation protein products induce endothelial-to-mesenchymal transition in human renal glomerular endothelial cells through induction of endoplasmic reticulum stress

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ABSTRACT

Endothelial-to-mesenchymal transition (EndMT) in renal glomerular endothelial cells plays a critical role in the pathogenesis of diabetic nephropathy (DN). Furthermore, advanced oxidation protein products (AOPPs) have been shown to contribute to the progression of DN. However, whether AOPPs induce EndMT in renal glomerular endothelial cells remains unclear. Thus, we investigated the effect of AOPPs on human renal glomerular endothelial cells (HRGECs) and the mechanisms underlying the effects. Our results showed that AOPP treatment lowered the expression of vascular endothelial cadherin, CD31, and claudin 5 and induced the overexpression of α-smooth muscle actin, vimentin, and fibroblast-specific protein 1, which indicated that AOPPs induced EndMT in HRGECs. Furthermore, AOPP stimulation increased the expression of glucose-regulated protein 78 and CCAAT/enhancer-binding protein-homologous protein, which suggested that AOPPs triggered endoplasmic reticulum (ER) stress in HRGECs. Notably, the aforementioned AOPP effects were reversed following the treatment of cells with salubrinal, an inhibitor of ER stress, whereas the effects were reproduced after exposure to thapsigargin, an inducer of ER stress. Collectively, our results indicate that AOPPs trigger EndMT in HRGECs through the induction of ER stress. These findings suggest novel therapeutic strategies for inhibiting renal fibrosis by targeting ER stress.

1. Introduction

Diabetic nephropathy (DN) is a most common cause of end-stage renal disease worldwide, and it is a growing problem across the world. A prominent clinical characteristic of DN and an independent risk factor for the progression of DN is proteinuria, which results from the disruption of the glomerular filtration barrier (Jefferson, Shankland, & Pichler, 2008). Glomerular endothelial cells are an essential component of glomerular filtration barrier and play a crucial role in regulating glomerular permeability. Loss or a reduced number of glomerular endothelial cells will cause dysfunction of glomerular filtration, and then lead to proteinuria (Satchell & Braet, 2009). Furthermore, experimental and clinical studies have indicated that endothelial dysfunction plays a critical role in the pathogenesis of DN (Grutzmacher et al., 2013; Nakagawa et al., 2007; Weil et al., 2012). Moreover, endothelial-to-mesenchymal transition (EndMT), a specific form of epithelial–mesenchymal transition (EMT), has been suggested to play an essential role in the development of endothelial dysfunction and contributes to the development and progression of glomerulosclerosis, which is a critical morphological feature of DN (Li, Qu, & Bertram, 2009; Li et al., 2015). During EndMT, endothelial cells lose their endothelial cell markers, including VE-cadherin, CD31, and claudin 5, and express increased levels of mesenchymal markers such as α-SMA, vimentin, and FSP-1. EndMT was first reported in the embryonic development of the heart (Eisenberg and Markwald, 1995) and Zeisberg, Potenta, Sugimoto, Zeisberg, and Kalluri (2008) reported for the first time that EndMT contributes to the accumulation of myofibroblasts, which is considered to be the key mediators of renal fibrosis, using 3 distinct mouse models of renal disease, including the streptozocin-induced diabetic nephrology. Subsequent studies conducted by Li et al. (2009) demonstrated that EndMT occurs and contributes to the early development and progression of diabetic kidney fibrosis. Thus, understanding the mechanisms of the EndMT program and targeting them might yield novel therapeutic strategies for DN. However, the mechanisms underlying EndMT program remain poorly understood.

Numerous pathologic processes mediated by inflammatory and oxidative stress-associated factors and mechanical factors can cause glomerular endothelial alterations (Bevan et al., 2011; Izawa-Ishizawa et al., 2012; Jaimes, Hua, Tian, & Raij, 2010). Wu et al. (2014)

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suggested that parathyroid hormone induces EndMT in human renal glomerular endothelial cells (HRGECs). Furthermore, AGEs have been reported to trigger EndMT in mouse pancreatic microvascular endothelial cells (Li et al., 2010), and advanced oxidation protein products (AOPPs) have been shown to activate vascular endothelial cells through a receptor of advanced glycation end products (RAGE)-mediated signaling pathway (Guo et al., 2008). AOPPs are dityrosine-containing, crosslinking products that are formed as a result of the reaction between plasma albumin and chlorinated oxidants during oxidative stress (Witko-Sarsat et al., 1996, 1998). AOPPs are first reported to be elevated in patients with chronic kidney disease, and, subsequently, AOPP accumulation was found in patients with metabolic syndrome (Atabek et al., 2006) and diabetes with or without microvascular complications (Kalousova, Skrha, & Zima, 2002; Martin-Gallan, Carrascosa, Gussinye, & Dominguez, 2003). It is worth noting that the levels of AOPPs increase with the deterioration of renal function in chronic kidney diseases, including DN (Liu et al., 2011; Shi et al., 2008; Witko-Sarsat et al., 1996). Previous studies have also indicated that AOPPs induce podocyte apoptosis (Zhou et al., 2009), mesangial cell perturbation (Wei, Zhou, Hou, Liu, & Liang, 2009), and hypertrophy and EMT in proximal renal tubular epithelial cells (Tang, Liang et al., 2015; Tang, Rong et al., 2015). Moreover, Li et al. (2007) demonstrated that chronic accumulation of AOPPs promotes the progression of renal fibrosis. Notably, clinical studies have suggested that the AOPP level is a strong predictor for the progression of IgA nephropathy (Descamps-Latscha et al., 2004). Collectively, these findings strongly suggest that AOPPs play a pathogenic role in the progression of chronic kidney disease, including DN. However, the biological effects of AOPPs on renal glomerular endothelial cells and the mechanisms underlying the aforementioned processes remain unclear.

Endoplasmic reticulum (ER) stress has been widely demonstrated to play a crucial role in renal pathophysiology (Cybulsky, 2010; Inagi, 2010; Kitamura, 2008). ER stress is a physiological or pathological state that results from a variety of disturbances such as glucose deprivation, hypoxia, and viral infection. The accumulation of unfolded proteins in the ER leads to ER stress and, subsequently, to the unfolded protein response (UPR), which is recognized as an adaptive response. However, when ER stress is prolonged or severe, the UPR might also trigger apoptotic pathways. When ER stress occurs, the ER chaperone GRP78 separates from 3 transmembrane transducers of UPR (protein kinase RNA-like ER kinase, activating transcription factor 6, and inositol-requiring enzyme 1) and binds to unfolded proteins within the ER lumen, after which the UPR is initiated to transduce the survival signals. Whereas GRP78 is involved in the protection mechanism that functions during ER stress, CHOP is a critical molecule that has been shown to regulate ER-stress-mediated apoptosis (Tabas & Ron, 2011). Thus, GRP78 and CHOP are regarded as markers of ER stress. ER stress has been shown to induce EMT in lung epithelial cells (Tanjore et al., 2011), PC Cl3 thyroid cells (Ulianich et al., 2008), and proximal renal tubular epithelial cells (Tang, Liang et al., 2015; Tang, Rong et al., 2015). More importantly, ER stress has also been shown to contribute to the damage of renal glomerular endothelial cells (Bouvier et al., 2009; Csordas et al., 2011; Sheikh-Ali, Sultan, Alamir, Haas, & Mooradian, 2010). However, the exact role of ER stress in AOPP-induced damage of renal glomerular endothelial cells has not been previously reported.

In this study, we investigated whether AOPPs induce EndMT and ER stress in HRGECs. Furthermore, we examined whether AOPP-induced EndMT in HRGECs is mediated by ER stress.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Anti- glucose-regulated protein 78 (GRP78), CCAAT/enhancer-binding protein-homologous protein (CHOP), vascular endothelial (VE)-cadherin, claudin 5, vimentin, and fibroblast-specific protein 1 (FSP-1) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CD31 and α-smooth muscle actin (α-SMA) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

To investigate the role of ER stress in AOPP-triggered EndMT in HRGECs, we treated the cells for 24 hours with albumin or AOPPs in the presence or absence of an ER stress inhibitor (salubrinal), or treated the cells with an ER stress inducer (thapsigargin), and then measured the expression of the markers of ER stress and EndMT. Salubrinal and thapsigargin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. AOPPs preparation and content determination

AOPPs-BSA was prepared as previously described (Li et al., 2007; Witko-Sarsat et al., 1998). Briefly, a 100 mg/mL BSA solution was exposed to 200 mmol/L of HOCl for 30 min at room temperature and then dialyzed overnight against phosphate-buffered saline (PBS) to remove free HOCl. The AOPPs preparation was passed through a Detoxi-Gel column (Pierce, Rockford, IL, USA) in order to remove contaminating endotoxins. The levels of endotoxin in the preparations were detected using the Amebocyte lysate assay kit (Sigma, St. Louis, MO, USA) and were examined to be <0.025 EU/mL. The content of AOPPs was measured as described previously (Li et al., 2007; Witko-Sarsat et al., 1996). The AOPPs content in AOPPs-BSA and unmodified BSA were 65.2 ± 2.12 and 0.2 ± 0.04 nmol/mg, respectively.

2.3. Cell culture

The HRGECs were purchased from ScienCell Research Laboratories (San Diego, CA, USA) and cultured (at 37 °C, in a 5% CO_2 atmosphere) in an endothelial cell medium (ScienCell) containing 5% fetal bovine serum, 1% endothelial cell growth supplement, and 1% penicillin/streptomycin solution. In the experiments, we used approximately 80%-confluent cells from passages 2 to 5; these cells were seeded at a density of 2.5×10^5 cells/25 cm^2 in flasks or 1×10^5 cells/well in 6-well dishes and were transferred to serum-free medium for 24 hours before use in all experiments.

2.4. Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocols. The cDNA was synthesized using total RNA (1 μg) with random primers using MMLV reverse transcriptase First Strand Kit (Invitrogen, USA). The qPCR assay was performed using the SYBR® Premix Ex Taq™ kit (TaKaRa, Kyoto, Japan). The following sets of primers were used: β-actin: 5′-TGGCACCCAGCACAATGAA-3′ (forward) and 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′ (reverse); GRP78: 5′-ACCTACTCCTGCGTCGGCGTGTT-3′ (forward) and 5′-CGGCATCGCCAATCAGACGTTCC-3′ (reverse); CHOP: 5′-CCTGGAAATGAAGAGGAAGAATCAA-3′ (forward) and 5′-GGAGGTGCTTGTGACCTCTG-3′ (reverse); VE-cadherin: 5′-TTCACCCAGACCAAGTACAC-3′ (forward) and 5′-CTCGACGATGAAGCTGTATTGC-3′ (reverse); CD31: 5′-GGATGTCAGCACCACCTCTC-3′ (forward) and 5′-TGGGCACTCCTTCCACCAAC-3′ (reverse); claudin 5: 5′- CGTTCGTTGCGCTCTTCGTG-3′ (forward) and 5′-GTTGGCGAACCAGCAGAGTG-3′ (reverse); α-SMA: 5′-TACTACTGCTGAGCGTGAGA-3′ (forward) and 5′-CATCAGGCAACTCGTAACTC-3′ (reverse); vimentin: 5′- TTCAGACAGGATGTTGACAATG-3′ (forward) and 5′-CCTCTTCGTGGAGTTTCTTC-3′ (reverse); and FSP-1: 5′-CTTCTTGGGGAAAAGGACAG-3′ (forward) and 5′- GCGATGCAGGACAGGAAGAC-3′ (reverse). All data were normalized using the internal control β-actin.