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Modulation of AT-1R/MAPK cascade by an olmesartan treatment attenuates diabetic nephropathy in streptozotocin-induced diabetic mice

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ABSTRACT

There is increasing evidence that angiotensin (Ang)-II plays an unprecedented role in diabetic complications. It could also be an important therapeutic target for ameliorating various diseases, especially diabetic nephropathy (DN). We therefore studied the beneficial effects of olmesartan, an Ang-II type 1 receptor (AT-1R) blocker in streptozotocin (150 mg/kg, BW)-induced diabetic kidney disease in mice. The diabetic kidney mice displayed upregulated protein expression levels of AT-1R, AT-2R, ERK-1/2, p-p38 MAPK, p-MAPKAPK-2, ET-1, p-JNK, p-c-Jun, TGF-β1, and gp91-phox, and all of these effects were expectedly downregulated by an olmesartan treatment. Also, immunohistochemical analysis, and Azan-Mallory and HE staining were performed to examine the expression of collagen-III and fibronectin, renal fibrosis, and hypertrophy, respectively. Furthermore, olmesartan treatment significantly abrogated the downregulation of ACE-2 and Ang-(1–7) *mas* R protein expression in diabetic kidney mice. Considering all these findings together, the AT-1R/MAPK pathway might be a potential therapeutic target in diabetes kidney disease, and olmesartan treatment could have beneficial effects on DN by modulating the AT-1R/MAPK pathway.

1. Introduction

Activation of the local renin–angiotensin system (RAS) was demonstrated to be involved in the progression of kidney damage, including diabetic nephropathy (DN). Studies in experimental animal models of DN have demonstrated that RAS component expression was upregulated, and RAS blockade had beneficial effects on these animals (Burns, 2000). A large number of clinical trials have shown that angiotensin converting enzyme inhibitors (ACE) and angiotensin (Ang)-II type 1 receptor (AT-1R) antagonists produced positive outcomes during DN treatment (Lewis et al., 2001; Brenner et al., 2001; Parving et al., 2001). It has been demonstrated that hyperglycemia-mediated stimulation of the RAS eventually leads to renal tissue injury, possibly through the activation of the extracellular signal-regulated kinase (ERK)/p38 mitogen activated protein kinase (MAPK) pathway, in the diabetic state (Fujita et al., 2004). The components of RAS have differential signaling effects on ERK/p38 MAPK, and Ang-II, which is a crucial component of the RAS, was found to stimulate ERK 1/2 as well as p38 MAPK, while the Ang-(1–7) *mas* receptor is believed to play a protective role against hyperglycemia induced ERK/p38 MAPK signaling in the diabetic state (Gava et al., 2009). The MAPK family is made up of serine/threonine kinases from three distinct subfamilies, namely ERK, p38 MAPK, and c-junctional N-terminal kinases (JNK). By regulating transcription factor activation, MAPK play a central role in diverse cellular responses including survival, growth, differentiation, and apoptosis (Geilen et al., 1996). Of these, ERK was shown to be crucial for cell proliferation and differentiation, while p38 MAPK and JNK are believed to mediate cellular stress and apoptosis. Studies have shown that under high glucose conditions, ERK is activated in the glomeruli and mesangial cells (Awazu et al., 1999; Haneda et al., 1997). The other

Abbreviations: ACE-2, angiotensin converting enzyme-2; Ang-(1–7) *mas* R, angiotensin-(1–7) *mas* receptor; Ang-II, angiotensin-II; AT-1R, Ang-II type 1 receptor; DN, diabetic nephropathy; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; p-JNK, phospho-c-Jun N-terminal kinases; RAS, rennin–angiotensin system; STZ, streptozotocin.

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members of the MAPK family, p38 MAPK and JNK, were originally thought to mediate the cellular stress response and apoptosis. However, recent reports have emphasized the roles of p38 MAPK and JNK in mediating renal hypertrophy (Fujita et al., 2004). Ang-II is a potent vasoactive peptide that also plays regulatory roles in inflammation, cell proliferation, apoptosis, and fibrosis (Long et al., 2004; Braam et al., 1993; Seikaly et al., 1990; Donoghue et al., 2000). Numerous studies have demonstrated the potential link between Ang-II and the regulation of extracellular matrix gene expression.

Hyperglycemia-induced stimulation of RAS component expression, especially Ang-II, is well known to cause potential damage to the kidney cells through a variety of mechanisms, such as pressure-induced renal injury, activating renal fibroblasts to become myofibroblasts; stimulating the production of profibrotic cytokine transforming growth factor (TGF)-β; inducing oxidative stress; stimulating the production of chemokines and osteopontin, both of which can cause local inflammation; and stimulating vascular and mesangial cell proliferation and hypertrophy (Long et al., 2004). The levels of Ang-II in the kidneys exceed the circulating levels by 100- to 1000-fold (Braam et al., 1993; Seikaly et al., 1990). The discovery of ACE-2 has provided a significant opportunity for researchers to explore the exact mechanism of action of Ang-II in DN. ACE-2 has been shown to mediate the conversion of Ang-II to the heptapeptide Ang-(1–7) (Donoghue et al., 2000; Tipnis et al., 2000). It has also been reported that Ang-(1–7) inhibits Ang-II mediated MAPK activation by binding to the Mas receptor (*mas* R) (Su et al., 2006). Gava et al. reported that Ang-(1–7) inhibits high glucose-induced p38 MAPK stimulation, which is believed to play a role in nephron injury associated with renal cell hypertrophy, and TGF-β1 production, and Ang-(1–7) provides protection against nephron injury by downregulating p38 MAPK expression (Gava et al., 2009). Recently, we reported that telmisartan, a selective AT-1R antagonist has beneficial effects in streptozotocin (STZ)-induced diabetic mice, mainly by upregulating Ang-(1–7) *mas* R expression and increasing ACE-2 expression. We also reported that anti-fibrotic effects of telmisartan are derived from the attenuation on the phosphorylation of p38 MAPK expression in diabetic mice (Lakshmanan et al., 2011). In addition, it has been proven that Ang-II stimulates the ERK1/2 cellular response in different cell types (Uchiyama-Tanaka et al., 2001), and also Ang-II induces fibronectin expression by stimulating ERK1/2 and p38 MAPK in human peritoneal mesothelial cells (Kiribayashi et al., 2005).

Considering these reports, it appears that Ang-II could be an important therapeutic target for ameliorating hyperglycemia-induced renal complications. Numerous recent studies have provided new insights into the roles of Ang-(1–7) *mas* R and ACE-2 in diabetic complications. Moreover, various reports have stated that AT-1R antagonists play a significant role in ameliorating diabetic-induced kidney diseases. Despite their significant role, the exact mechanism by which AT-1R antagonists attenuate kidney disease is unclear. To the best of our knowledge, this is the first report describing the beneficial effects of olmesartan, a selective AT-1R antagonist, in STZ-induced diabetic kidney mice; i.e., it modulates the AT-1R/MAPK cascade and upregulates Ang-(1–7) *mas* R and ACE-2 protein expressions.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma (Tokyo, Japan). Olmesartan medoxomil was generously donated by Daichi-Sankyo Pharmaceutical (Tokyo, Japan).

2.2. Diabetes induction

Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (Sigma, St. Louis, MO, USA) at a dose of 150 mg/kg body weight (BW) to 8- to 10-week-old male C57BL/6 JAX mice, which were obtained from Charles River Japan Inc. (Kanagawa, Japan). STZ was dissolved in 20 mM sodium citrate saline buffer (pH 4.5) and injected within 5 min of preparation. Age-matched male C57BL/6 JAX mice were injected with 100 μl of citrate buffer and used as non-diabetic normal mice. The mice were maintained with free access to water and chow throughout the study period and were treated in accordance with the guidelines for animal experimentation of our institute.

2.3. Experimental protocol

At 3 days after the STZ injection, the blood glucose (BG) levels of the mice were measured using Medi-safe chips (Terumo Inc., Tokyo, Japan). The mice with blood glucose levels of >300 mg/dL were considered to be diabetic and included in the study. As a result, thirty mice were divided into the following three groups: (1) age-matched vehicle-treated mice (NG; $n = 10$), (2) age-matched vehicle-treated STZ-induced diabetic mice (DG; $n = 10$), and (3) age-matched olmesartan-treated STZ-induced diabetic mice (OG; $n = 10$). Olmesartan medoxomil was dissolved in 0.5% hydroxyethyl cellulose (HEC) and administered orally for 28 days at a dose of 5 mg/kg, BW. The NG and DG mice received 0.5% HEC alone. At 28 days after the STZ injection or olmesartan treatment, the mice were anesthetized with a single i.p. injection of pentobarbital (50 mg/kg, BW), and their kidneys were excised and decapsulated. Half of the kidneys were immediately snap-frozen in liquid nitrogen and stored at −80 °C until the subsequent protein analysis. The remaining excised kidneys were fixed in 10% formalin and used for the histopathological studies.

2.4. Western blotting

Protein lysate was prepared from the kidneys, as described previously. The total protein concentrations of the samples were measured by the bicinchoninic acid (BCA) method (Thandavarayan et al., 2010). To determine the protein levels of AT-1R, Ang-II type 2 receptor (AT-2R), ACE-2, Ang-(1–7) *mas* R, p38 MAPK, phospho-p38 MAPK, phospho-MAPK-activated protein kinase-2 (p-MAPKAPK-2), ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, phospho-c-JUN, ET-1, gp91-phox, and TGF-β1, equal amounts of protein extract (30 μg) were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Bio-Rad, CA, USA) and transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked with 5% non-fat dried milk or 5% bovine serum albumin in Tris buffered saline Tween (20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20). The primary antibodies against AT-1R, AT-2R, ACE-2, ET-1, and gp91phox were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); those against p-p38 MAPK, p-MAPKAPK-2, p-ERK1/2, p-JNK, and p-c-JUN were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA); and those against Ang-(1–7) *mas* R and TGF-β1 were obtained from Alomone Labs Ltd. (Jerusalem, Israel) and Promega Corporation (Madison, USA), respectively. All of the antibodies were used at a dilution of 1:1000. The membrane was incubated overnight at 4 °C with the primary antibody, and the bound antibody was visualized using the respective horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The levels of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (for TGF-β1) were estimated in every sample to ensure the equal loading of the