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Calcitriol modulates receptor for advanced glycation end products (RAGE) in diabetic hearts☆

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ABSTRACT

Background: Receptor for advanced glycation end products (RAGE) signaling pathway plays a vital role in diabetic cardiovascular complications. Calcitriol has been shown to exert various beneficial cardiovascular effects. The purpose of this study is to determine whether calcitriol can modulate RAGE expression, and study the potential mechanisms in diabetic hearts.

Methods: Streptozotocin (65 mg/kg, intraperitoneal injection once) induced diabetic rats were treated with or without subcutaneous injections of calcitriol at a dose of 150 ng/kg/day for 4 weeks. Western blot was used to evaluate protein expressions of myocardial RAGE, TNF-α, p65 subunit of NF-κB (p65), α subunit of inhibitor of κB (IκBα), subunits of NADPH oxidase (NOX4 and $p22^{phox}$), angiotensin II type 1 receptor (AT1R), TGF-β1, TGF-β receptor I, total and phosphorylated SMAD2/3 and ERK, matrix metalloproteinases 2 (MMP2), tissue inhibitors of metalloproteinases 2 (TIMP2) and procollagen I.

Results: As compared to control, diabetic rats had increased expressions of cardiac RAGE, TNF-α, $p22^{phox}$, AT1R, and TGF-β1, which were significantly attenuated in the diabetic rats treated with calcitriol. Calcitriol-treated diabetic hearts also had lesser expressions of p-SMAD2/3 and p-ERK signaling than those of diabetic hearts. Moreover, diabetic hearts had increased expressions of MMP2 and procollagen I and decreased TIMP2. However, calcitriol reverted the diabetic effects in procollagen I but not in MMP2 or TIMP2.

Conclusions: Calcitriol decreased diabetic effects on RAGE and fibrosis, which may be caused by its modulation on AT1R and the anti-inflammatory and antioxidative potentials. Therefore, calcitriol may attenuate diabetic cardiomyopathy.

1. Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in patients with diabetes mellitus (DM). Advanced glycation end products (AGE) have been implicated in the pathogenesis of diabetic vascular complications and diabetic cardiomyopathy, via AGE-modified proteins associated with cellular dysfunction or interactions with receptor for AGE (RAGE). In addition to hyperglycemia, oxidative stress, inflammation, aging, and renal failure all contribute to the generation of AGE. Not only AGE, a number of other ligands that tend to be released or accumulated in tissues during inflammatory disorders, immune responses, and chronic degenerative diseases also interact with RAGE [1,2]. RAGE engagement with its ligands activates multiple cellular signaling pathways, such as MAP kinases, SAPK/JNK, rho-GTPases, JAK/STAT and nuclear factor-κB (NF-κB), that are associated with increased production of pro-inflammatory, pro-atherogenic mediators and further enhances the expression of RAGE [2–4]. Therefore, ligands and RAGE interaction forms a positive feedback loop that converts acute inflammatory stimulus into sustained cellular dysfunction and further magnifies tissue damage. Animal studies have shown that RAGE blockade stabilizes atherosclerosis and vascular inflammation in established DM [5,6]. Accordingly, regulation of RAGE signaling pathway has emerged as a promising target to treat diabetic cardiomyopathy and vascular complications. However, effective treatment for eliminating RAGE in DM is limited.

Accumulating epidemiological data suggest that the vitamin D status is important for achieving optimal cardiovascular function. Vitamin D

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deficiency increases the risk of cardiovascular diseases and DM [7–9]. $1,25(OH)_2D$ (calcitriol), the hormonally active metabolite of vitamin D has been proved to be beneficial in patients with DM [8,10]. Suppression of renin–angiotensin–aldosterone system [11,12], anti-inflammatory actions [13–15] and antioxidant properties [12,16] are involved in the beneficial effects of calcitriol on cardiovascular system. Moreover, myocardial fibrosis is a major feature of diabetic cardiomyopathy. Up-regulation of transforming growth factor-β1 (TGF-β1), a profibrotic cytokine, and dysregulation of extracellular matrix remodeling contribute to the development of cardiac fibrosis [17]. Vitamin D receptor-knockout mice demonstrated altered gene expressions of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP), suggesting that calcitriol plays a role in heart extracellular matrix metabolism [18]. Since the cellular effects of calcitriol closely link to the pathophysiology of RAGE, it is possible that calcitriol may regulate the genesis and effects of RAGE. Therefore, the present study investigated the expressions, effects and mechanisms of calcitriol on RAGE in diabetic hearts.

2. Methods

2.1. Animal and tissue preparations

This investigation was approved by the Institutional Animal Care and Use Committee of Taipei Medical University and complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Male Wistar-Kyoto (WKY) rats (10 weeks old) received an intraperitoneal injection of streptozotocin (65 mg/kg of body weight, Sigma, St. Louis, MO) to induce DM with a fasting plasma glucose level above 15 mmol/L, measured with a glucometer (Ascensia Elite, Bayer Health Care, Mishawaka, IN) as described previously [19]. The rats were housed in standard environmental conditions and maintained on commercial rat chow and tap water ad libitum. At 12 weeks of age, diabetic WKY rats were administered subcutaneously with calcitriol (150 ng/kg, Sigma) or vehicle (1 mL/kg 1,2-propanediol, the same volume as calcitriol) daily for 4 weeks [20]. The diabetic WKY, calcitriol-treated diabetic WKY or WKY (control) rats were sacrificed at 16 weeks of age. Each heart was rapidly excised and dissected. Cardiac tissues were rinsed in a cold physiological saline solution. Transverse tissue pieces from the atria were snap-frozen in liquid nitrogen for protein isolation.

2.2. Western blot analysis

Equal amounts of proteins were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis as described previously [19]. Blots were probed with antibodies against RAGE (Pierce-Thermo Fisher Scientific Inc., Rockford, IL), tumor necrosis factor-alpha (TNF-α) (eBioscience, San Diego, CA), p65 subunit of NF-κB (p65) and α subunit of inhibitor of κB (IκBα) (Santa Cruz Biotechnology, Santa Cruz, CA), subunits of NADPH oxidase (NOX), including NOX4 and $p22^{phox}$ (Santa Cruz), angiotensin II type 1 receptor (AT1R) (Abcam, Cambridge, UK), TGF-β1 (Cell Signaling, Beverly, MA), TGF-β receptor I (TGF-βRI) (Millipore, Billerica, MA), total and phosphorylated SMAD2/3 (p-SMAD2/3) (Cell Signaling), total and phosphorylated extracellular-signal-regulated kinases (p-ERK) (Cell Signaling), MMP2 (Santa Cruz), TIMP2 (Millipore), and procollagen I (Santa Cruz). Secondary antibodies were conjugated with horseradish peroxidase (Leinco Technology, St. Louis, MO). Bound antibodies were detected with an enhanced chemiluminescence detection system (Millipore) and analyzed with AlphaEaseFC software (Alpha Innotech, San Leandro, CA). Targeted bands were normalized to cardiac α-sarcomeric actin (Sigma) to confirm equal protein loading.

2.3. Statistical analysis

All quantitative data are expressed as the mean ± S.E.M. Statistical significance among different groups was determined by one-way ANOVA with *post-hoc* tests of Fisher's least significant difference. A P value <0.05 was considered to indicate statistically significant differences.

3. Results

3.1. RAGE, inflammation and oxidative stress in control, diabetic and calcitriol-treated diabetic hearts

As shown in Fig. 1, diabetic hearts had larger RAGE expressions than control or calcitriol-treated diabetic hearts. However, the control and calcitriol-treated diabetic hearts had similar RAGE expressions. In addition, control and calcitriol-treated diabetic hearts had a similar TNF-α expression. Therefore, calcitriol attenuated the larger expressions of

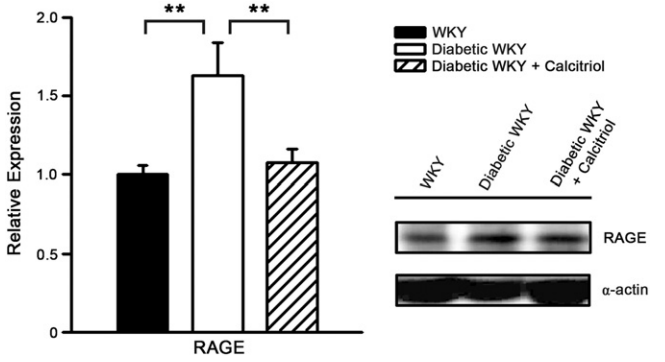

Fig. 1. Expressions of atrial receptor for advanced glycation end products (RAGE) from Wistar-Kyoto (WKY), diabetic WKY, and calcitriol-treated diabetic WKY rats. Representative immunoblots and average data of RAGE from WKY ($n = 9$), diabetic WKY ($n = 9$), and calcitriol-treated diabetic WKY ($n = 9$) rats. $^{**}P<0.01$.

TNF-α in diabetic hearts (Fig. 2A). Diabetic and calcitriol-treated diabetic hearts had lager IκBα and p65 expressions than control, whereas diabetic and calcitriol-treated diabetic hearts had a similar IκBα and p65 (Fig. 2B). In addition, diabetic hearts had increased expressions of $p22^{phox}$ than control or calcitriol-treated diabetic hearts. Nevertheless, control, diabetic and calcitriol-treated diabetic hearts had a similar NOX4 expression (Fig. 3).

3.2. Angiotensin II receptor type 1, TGF-β1, TGF-β receptor type I and signaling in control, diabetic and calcitriol-treated diabetic hearts.

As shown in Fig. 4, diabetic hearts had larger expressions of AT1R or TGF-β1 than control or calcitriol-treated diabetic hearts. The control and calcitriol-treated diabetic hearts had similar expressions of AT1R and TGF-β1. Moreover, control, diabetic and calcitriol-treated diabetic hearts also had similar expressions of TGF-βRI.

Both diabetic and calcitriol-treated diabetic hearts had increased expressions of total SMAD2/3 as compared to control. However, only diabetic hearts had a larger expression of p-SMAD2/3 than control. There was a similar expression of p-SMAD2/3 between control and calcitriol-treated diabetic hearts. Therefore, calcitriol-treated diabetic hearts significantly decreased p-SMAD2/3 expressions compared to diabetic hearts (Fig. 5A). However, the total ERK expressions were not statistically different between diabetic and calcitriol-treated diabetic hearts. In contrast, the expressions of p-ERK were markedly increased in diabetic hearts compared to control, which were significantly attenuated in calcitriol-treated diabetic hearts (Fig. 5B).

3.3. Extracellular matrix in control, diabetic, and calcitriol-treated diabetic hearts

Diabetic or calcitriol-treated diabetic hearts had larger MMP2, but lesser TIMP2 expressions than control hearts. Diabetic and calcitriol-treated diabetic hearts had similar expressions of MMP2 and TIMP2. Moreover, diabetic hearts had enhanced procollagen I expressions compared to control or calcitriol-treated diabetic hearts. The control and calcitriol-treated diabetic hearts had a similar procollagen I expression (Fig. 6).

4. Discussion

In this study, for the first time, we have demonstrated that calcitriol treatment attenuated the up-regulations of RAGE in diabetic hearts. In addition, calcitriol also modulated the up-regulated effects of TNF-α, $p22^{phox}$, AT1R, TGF-β1 on diabetes, which may result in attenuation of p-SMAD2/3 and p-ERK signaling in diabetic hearts. Moreover, we also found that calcitriol treatment may attenuate the diabetes-regulated procollagen I, but not in MMP2 or TIMP2. All of these effects may