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Knockout of immunoproteasome subunit β2i ameliorates cardiac fibrosis and inflammation in DOCA/Salt hypertensive mice

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ABSTRACT

The immunoproteasome is a multicatalytic protease complex in all eukaryotic cells, which plays a key role in regulating essential cellular processes. However, the role of immunoproteasome subunit β2i in regulation of cardiac fibrosis and inflammation in deoxycorticosterone-acetate (DOCA)/salt mice remains unknown. Wild-type (WT) and β2i knockout (KO) mice were subjected to uninephrectomy and DOCA/salt treatment for 21 days. Blood pressure was measured by the tail-cuff system. Cardiac function and remodeling were examined by echocardiography, hematoxylin-eosin (H&E) and Masson's trichrome staining. The gene and protein expressions were detected by quantitative real-time PCR, and Western blot analysis. After 21 days, DOCA/salt treatment significantly up-regulated the expression of β2i mRNA and protein in the hearts. Moreover, systolic blood pressure and heart weight/body weight (HW/BW) ratio were significantly higher in DOCA/salt mice than in sham groups, and these effects were markedly reversed in β2i knockout mice. Importantly, DOCA/salt-induced cardiac fibrosis, inflammation and the expression of collagen I, collagen III, α-SMA, IL-1β, IL-6 and TNF-α in the wild-type hearts, which were markedly attenuated by β2i knockout. These beneficial effects were due, at least in part, to the inhibition of IκBα/NF-κB and TGF-β1/Smad2/3 signaling pathways. Collectively, these findings indicate that knockout of β2i ameliorates DOCA/salt-induced cardiac fibrosis and inflammation, and may be a novel potential therapeutic target for hypertensive heart diseases.

1. Introduction

Hypertension is a multi-factorial chronic inflammatory disease. Cardiac remodeling is associated with hypertensive heart diseases, which is characterized by cardiac hypertrophy, fibrosis and inflammation [1]. In the progression of hypertension, myocardial fibrosis and inflammation are the critical mechanism which contributes to the pathogenesis and development of hypertensive cardiovascular diseases [2]. In addition to the rennin-angiotensin system (RAS) [3], administration of deoxycorticosterone acetate (DOCA) in combination with a high salt intake to animals can also induce hypertension and cardiac remodeling, and DOCA/salt animals mimic most of the changes observed in chronic cardiovascular remodeling in humans [4,5]. However, the DOCA/salt hypertensive rat model shows a significantly decreased circulating renin concentration and thus has been regarded as an angiotensin-independent model [6].

Ubiquitin–proteasome system (UPS) plays a critical role in degrading misfolded, damaged, or oxidized cellular proteins [7]. The 26S proteasome complexes are comprised of a 20S catalytic barrel and two 19S regulatory "lids". The 20S catalytic core consists of three standard subunits β1, β1 and β5, which perform distinct proteolytic activities, including caspase-like, trypsin-like, and chymotrypsin-like. Interestingly, in response to inflammatory cytokines such as interferon-γ (IFN-γ), the standard subunits can be replaced by the inducible immunoproteasome subunits such as β1i (low molecular mass polypeptide-2, LMP2), β2i (multicatalytic endopeptidase complex-like-1, MECL1) and β5i (LMP-7), which

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possess broader biological functions, including cell growth, proliferation, oxidative stress, inflammatory response, signal transduction and muscle mass [7,8]. It has been reported that immunoproteasome subunits were lowly expressed in basal condition, and were significantly increased in different cell types such as human blood mononuclear cells and the mouse heart tissue [9,10]. Moreover, our recent data also showed that both expression level and activity of immunoproteasome catalytic subunits (β2i, β2i and β5i) were significantly increased in angiotension II-treated heart, inhibition of proteasome activity by bortezomib markedly attenuated Ang II-induced cardiac remodeling, demonstrating a critical role of immunoproteasome in the heart [11]. However, the effect of β2i on DOCA/salt-induced cardiac fibrosis and inflammation remains unclear.

In the present study, we investigated the effect of β2i on myocardial fibrosis and inflammation in a DOCA/salt hypertensive mouse model. Our results showed that β2i deficiency markedly reduced cardiac fibrosis and inflammation induced by DOCA/salt stress. This effect was possibly associated with inhibition of IκBα/NF-κB and TGF-β1/Smad2/3 signaling pathways.

2. Materials and methods

2.1. Animal models

Male wild type (WT) and β2i knockout (β2i KO) mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). All experiments were approved by the Animal Care and Use Committee of Capital Medical University and performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85–23, 1996). WT and β2i KO mice at the age of 10–12 weeks were subjected to uninephrectomy and DOCA/salt treatment for 21 days as previously reported [5]. Briefly, one 1-cm incision was made between the shoulder blades, and a 50 mg DOCA pellet (Innovative Research of America, Saratosa, Florida, USA) was implanted subcutaneously. DOCA/treated mice were given water containing 1.0% NaCl. Sham mice were uni-nephrectomized but received no DOCA implant and were given tap water.

2.2. Blood pressure measurement

Mice were trained and systolic blood pressure (SBP) was measured by the tail-cuff system (Softron BP98A; Softron Tokyo, Japan) as described previously [11–13].

2.3. Echocardiography

Transthoracic echocardiography was performed on mice at day 21 after DOCA/salt stress. Animals were anesthetized with isoflurane (3%) and then moved to a thermally controlled surgical table that maintained core body temperature, and had nose cone anesthesia (3% isoflurane). M-mode echocardiography was acquired by using a 30 MHz probe (Vevo 770 system; VisualSonics, Toronto, Ontario, Canada) as described previously [11–13]. The LV ejection fraction (EF%) and LV fractional shortening (FS%) were calculated. The LVFS was calculated as: $FS\% = [(LVIDd - LVIDs)/LVIDd] \times 100(\%)$.

2.4. Histopathology and immunohistochemistry

All animals were anesthetized by an overdose of pentobarbital (100 mg/kg, ip). Hearts were quickly dissected out and rinsed with cool sterile saline. Heart weight (HW) and body weight (BW) was calculated as an index of LV hypertrophy [12,14]. For histological analysis, hearts from WT and β2i KO mice were subsequently fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were then stained with hematoxylin and eosin (H&E) and Masson's trichrome reagent. Immunohistochemistry staining was performed with anti-rabbit antibodies against Mac-2 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). Digital photographs were taken at × 200 magnification of over 20 random fields from each heart, and the positive areas were analyzed by Image Pro Plus 3.0 (Nikon, Tokyo, Japan).

2.5. Quantitative real-time PCR analysis

Total ventricular RNA was extracted by using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Two μg of total RNA was reversed transcribed by oligo (dT)-primed RT (iScript cDNA synthesis kit; Bio-Rad Laboratories). Real-time quantitative PCR reactions were performed using SYBR Green kit (Takara, Otsu, Shiga, Japan) and iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The relative expression of TGF-β1, collagen I, collagen III, IL-6, IL-1β and TNF-α were expressed as a ratio to that of GAPDH. The following primers were used: collagen I-F 5′- GAGTACTGGATCGACCCTAACCA -3'; collagen I-R 5′-GACGGCTGAGTAGGGAACACA-3'; collagen III –F 5′-TCCCCTGGAATCTGTGAATC -3'; collagen III –R 5′-GACGGCTGAGTAGGGAACACA-3'; IL-1β–F 5′-TTGGGCCTCAAAGGAAAGAAT-3'; IL-1β–R 5′-TGGGTATTGCTTGGGATCCA -3'; IL-6–F 5′- GCTACCAAACTGGATATAATCAGGA -3'; IL-6–R 5′-CCAGGTAGCTATGGTACTCCAGAA -3'; TNF-α–F 5′- GCCAACGGCATGGATCTC -3'; TNF-α–R 5′- GCAGCCTTGTCCCTTGAAGAG -3'; GAPDH–F 5′-GGTTGTCTCCTGCGACTTCA -3'; GAPDH–R 5-GGTGGTCCAGGGTTTCTTACTC -3'.

2.6. Western blot analysis

Western blot analysis was performed as described [12,14]. Protein levels of β2i, p65, phosphorylation-p65 (p-p65), IκBα, *p*-IκBα, TGF-β1, α-SMA, smad2/3, *p*-smad2/3, H3 and GAPDH were measured.

Fresh heart tissues were homogenized with RIPA lysis buffer (Santa Cruz Biotechnology). Nuclear extracts from the heart tissues were obtained using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China). Equal amounts of protein (50 μg) were separated by SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore, Temecula, CA). The membranes were incubated with appreciate primary antibodies, and then incubated with IR Dye-conjugated secondary antibodies (1:3000, Rockland Immunochemical, Gilbertsville, PA) for 1 h at room temperature. All images were analyzed with a Gel-pro 4.5 Analyzer (Media Cybernetics, USA). GAPDH or H3 was used as loading control. The intensity of blot bands was analyzed using Image J software [13,14].

2.7. Statistical analyses

Data were expressed as mean ± standard deviation (SD) of three independent experiments, each performed in duplicate or triplicate. Significant differences between groups were statistically analyzed using multiple mean comparisons via one-way analysis of variance. Values of $P < 0.05$ were considered statistically significant difference.