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## EPLERENONE ATTENUATES MYOCARDIAL FIBROSIS IN THE ANGIOTENSIN II-INDUCED HYPERTENSIVE MOUSE: INVOLVEMENT OF OSTEOPONTIN INDUCED BY NADPH OXIDASE

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**Objectives** Oxidative stress and fibrosis are implicated in cardiac remodelling and failure. We tested whether apocynin and eplerenone could decrease myocardial oxidative stress and attenuate cardiac fibrosis and left ventricular diastolic dysfunction in angiotensin II (Ang II, AII)-induced hypertensive mice. The aim of the present study was to clarify the involvement of OPN which involved in the process of Ang II induced fibrosis and heart failure. First, we examined histological changes and gene expression of NADPH oxidase and OPN in the mouse myocardium with immunohistochemistry, western-blot and RT-PCR. Next, to study the involvement of aldosterone and ROS in regulation of OPN synthesis, the effect of aldosterone receptor blocker (eplerenone), and NADPH oxidase inhibitor (apocynin) on expression of NADPH and OPN in the model mouse were examined. Furthermore, the direct effects of these factors on OPN synthesis were studied using cultured cells to examine the NADPH-OPN signalling pathway.

**Methods Animals** In our study, all animal protocols were approved by our Institutional Animal Care and Use Committee. 30 8-week-old male C57BL/6 mice were randomised into 5 groups: (1) control group (n=5); (2) Ang II infused mice group (n=5); (3) apocynin treatment group (n=5); (4) eplerenone treatment group (n=5); (5) apocynin and eplerenone cotreatment group (n=5). Ang II infused mice were imbedded the osmotic minipump (model 2004, Alza) which is full of angiotensin II, its administrating speed designed about 2 µg/kg/min, keep on 4 weeks. At the same time the mice were accepted the gastric infusion with apocynin (100 mg/kg/day) and eplerenone (200 mg/kg/day) and cotreatment, designed the course of treatment as 5 days irrigation and 2 days interval, carried out four courses.

**Blood Pressure and Heart Rate** Systolic blood pressure (SBP) and heart rate (HR) measurements were done using a tail cuff system (Visitech Systems, NC, USA) at the end of the study. A minimum of 5 preliminary cycles was performed before collecting 10 measurements for each mouse.

**Echocardiographic Analysis** Transthoracic echocardiography was performed at the end of the study. End-diastolic left ventricular internal diameter (LVDd), end-systolic left ventricular internal diameter (LVDs) and left ventricular posterior wall thickness (PW) were measured. Percentage fractional shortening (FS%) and ejection fraction (EF%) was calculated to estimate the cardiac systolic function. The left lateral position was used to obtain an optimal Doppler image quality. The LV inflow tract was interrogated from the apical four-chamber view with the sample volume at the tips of the mitral leaflets. The E wave velocity (E/A) ratio and isovolumic relaxation time (IVRT) were measured as estimates of the cardiac diastolic function.

**Histological Analysis** For histological analysis, hearts were fixed with 10% formalin by perfusion fixation. Fixed hearts were embedded in paraffin, sectioned at 5 µm and stained with Masson's trichrome (MT) to enable investigation of the overall morphology and fibrosis. Myocyte breadth was measured from sections stained with hematoxylin-eosin, and suitable cross sections were defined as having nearly circular capillary profiles and nuclei. For measurement of the myocyte breadth, 100 cells (per animal) from the left ventricular lateral-mid free wall were randomly chosen and

analysed. The collagen fraction was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the myocyte area in the entire visual field of the section. Approximately 40 arterial cross sections were examined in each heart.

**Assay of Oxidative Stress** Myocardial tissues were homogenised in RIPA lysis buffer and the homogenates were used for assay. The tissue level of total glutathione (reduced glutathione/oxidised glutathione (GSH/GSSG)) in the LV was determined by the glutathione reductase and 5, 5'-dithiobis-(2-nitrobenzoic acid) recycling assay. The activity of glutathione peroxidase (GPx) was determined by using hydrogen peroxide assay, and the rate of disappearance of NADPH was recorded spectrophotometrically (412 nm) at 37°C. The lipid peroxide content of the LV was determined by estimation of malondialdehyde (MDA) contents by using the lipid peroxidation MDA assay kit.

**Reverse Transcription-PCR** RNA was isolated according to the TRIZOL protocol. The RNA was dissolved in diethylpyrocarbonate-treated water, quantified spectrophotometrically at 260 nm and stored at -80°C. Reverse transcription-PCR (RT-PCR) of myocardial tissues of mice were performed according to the Omniscript Reverse Transcription Handbook. Message expression was quantified with the use of the Lightcycler instrument (Roche) with SYBR green dye. The mouse primers used for amplification of NOX1, NOX2, NOX4, OPN and GAPDH, as an internal control, were designed according to the manufacturer's instructions. RT-PCR was performed with an ABI PRISM7700 Sequence Detection System by the relative standard curve method. The target amount was determined from the relative standard curves constructed with serial dilutions of control total RNA.

**Western Blotting** Previously frozen heart tissues were extracted with RIPA lysis buffer containing protease inhibitor, minced with scissors and sonicated for 10 s on ice. Homogenates were pelleted at 12000\*g for 15 min at 4°C, and supernatants were collected for western blotting. Protein concentrations were determined using the Bradford protein assay. Tissue lysates (30 µg) were separated by 10% Bis-Tris gels and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated overnight at 4°C with specific antibodies to NOX1, NOX2, NOX4, OPN and β-actin. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, immune complexes were detected using the enhanced chemiluminescence method. Proteins levels from western blots were evaluated by quantifying the band intensities using ImageJ software (National Institutes of Health).

**Immunohistochemistry** Hearts were embedded in paraffin, and 5 µm cross sections were cut. After 10 mmol/l citrate buffer antigen retrieval, the sections were incubated with NOX1, NOX2, NOX4 and OPN polyclonal antibody, followed by an incubation for 30 min at room temperature with fluorescence-conjugated secondary antibody or visualised with HRP/diaminobenzidine. Three independent fields in perivascular regions of myocardium from each mouse were examined under a 20\*10 objective lens, and the positive cells were counted.

**Cell Cultures** Cardiac fibroblasts were from ventricles of Balb/c mice and grown in DMEM with 10% fetal bovine serum as previously described. Experiments were performed on secondary cultures. Cells were plated in MULTI-WELL 6-well plates for 12 h in serum-free DMEM media, then treated with Ang II (10<sup>-7</sup> mol/l) for 4 h. Some cells were pretreated with eplerenone (10<sup>-5</sup> mol/l), apocynin (10<sup>-5</sup> mol/l) for 30 min and then stimulated with Ang II (10<sup>-7</sup> mol/l). The relative NOX1, NOX2, NOX4, OPN levels were determined by western blots.

**Statistical Analyses** Data are presented as mean±SEM and were analysed using one-way analysis of variance (ANOVA) followed by

Tukey or Bonferroni methods for post hoc analysis and two-tailed t-test when appropriate. A value of  $p < 0.05$  was considered statistically significant.

**Results** Blood Pressure and Heart Rate. Ang II treatment elevated blood pressure in mice and apocynin or eplerenone treatment didn't significantly reduce the Ang II-induced elevation of blood pressure. Apocynin and eplerenone co-treatment significantly lowered blood pressure of the Ang II infused mice. (The blood pressure of each group was  $116.4 \pm 5.0$ ,  $166.7 \pm 13.6$ ,  $150.2 \pm 16.8$ ,  $153.2 \pm 21.9$ ,  $133.8 \pm 8.9$  mm Hg, respectively.) The co-treatment group significantly decreased heart rate compare with the groups through the treatment of Ang II.

**Cardiac Hypertrophy** Ang II-induced increase of HW/BW (ratio of heart weight to bodyweight,  $4.4 \pm 0.1$  in sham vs  $6.6 \pm 0.6$  in AII,  $p < 0.05$ ) was partly inhibited by apocynin or eplerenone treatment and significantly inhibited by combination treatment ( $5.6 \pm 0.5$  in cotreatment vs  $6.6 \pm 0.6$  in AII,  $p < 0.05$ ). Ang II-induced increase of LVW/BW (ratio of left ventricular weight to bodyweight) didn't abolish by apocynin or eplerenone or combination treatment. As the same, Ang II treatment significantly increased myocyte breadth of the mice, however apocynin or eplerenone or combination treatment almost didn't work.

**Cardiac Fibrosis** Quantitative analysis of percentage fibrotic areas confirmed a significant increase in AngII-treated ( $47.1\% \pm 3.1\%$ ,  $p < 0.05$ ) compared with sham mice ( $13.2\% \pm 0.2\%$ ). Fibrotic areas in apocynin, eplerenone and combination treated mice were significantly attenuated ( $32.4\% \pm 1.8\%$ ,  $29.8\% \pm 1.4\%$  and  $29.0\% \pm 0.8\%$ , respectively,  $p < 0.05$ ) as compared with AngII alone.

**Cardiac Function and Remodelling** To investigate cardiac function, we performed echocardiographic examination. Posterior wall thickness (PW), end-diastolic left ventricular internal diameter (LVDd), end-systolic left ventricular internal diameter (LVDs) did not differ between any of the groups. LV systolic function was measured by ejection fraction (%EF). There were no significant differences in EF % between the five groups, while with the slightly impaired LV systolic function in Ang II-treated mice. Moreover, LV diastolic function was evaluated by isovolumic relaxation time (IVRT). This parameter was increased in WT mice after Ang II treatment, reflecting the impaired LV diastolic function. Apocynin or eplerenone treatment decreased IVRT but not showed significance compared with AII-treated mice. LV diastolic function was significantly improved by combination treatment.

**Myocardial Oxidative Stress** The GSH/GSSG ratio was decreased and MDA levels were increased after Ang II treatment in mice, indicative of increased myocardial oxidative stress. However, both the GSH content and the activity of GPx remained unchanged in these hearts. Treatment of mice with apocynin and eplerenone and combination inhibited the decrease in the GSH/GSSG ratio and the increase in MDA levels in the heart, respectively. Treatment with apocynin and eplerenone and combination didn't affect myocardial GSH content or GPx activity.

**Expression of OPN, NADPH oxidase Protein** Immunohistochemical staining for OPN, NOX1, NOX2, NOX4 and PKC $\xi$  was performed. There was no OPN staining in hearts from WT mice. Hearts from Ang II-treated mice exhibited OPN staining that was mainly present in the interstitium. Apocynin or eplerenone or combination treatment markedly blunted the Ang II-induced OPN staining. There was little NOX1, NOX2, NOX4 and PKC $\xi$  staining in hearts from WT mice and clearly abrogated in apocynin or eplerenone or combination treatment group. With apocynin and eplerenone treatment, OPN protein level was 28% and 39% lower, respectively, than with Ang II treatment alone ( $p < 0.05$ ). Western blot and RT-PCR showed the consistent result with the immunohistochemical staining. Effects of Ang II, apocynin, eplerenone, and

Inflammatory/Fibrotic Cytokines on OPN Synthesis by Cardiac Fibroblasts in Culture. The direct effects of Ang II, apocynin and eplerenone on OPN protein expression of cardiac fibroblasts in culture were examined by western blot. Ang II ( $10^{-7}$  mol/l) significantly increased OPN protein levels. Cells which treated with apocynin caused a decrease of OPN expression in a dose-dependent manner. Eplerenone significantly downregulated the OPN and PKC $\xi$  expression in Ang II treated cardiac fibroblasts.

**Conclusions** The results of the present study add the available evidences that apocynin and eplerenone partly prevent pathological remodelling of the heart in Ang II-induced hypertensive mice and also explain the mechanisms underlying the benefits of their coadministration. These findings suggest the potential involvement of NADPH-OPN pathway in regulation of the following process in Ang II-induced diastolic heart failure mice. Although further studies are necessary to elucidate the complex multistep molecular pathways involved, induction of OPN by eplerenone in the hypertensive heart might be a key step in perivascular fibrosis and thus a prime target for therapy.