Expression of NADPH Oxidase and Production of Reactive Oxygen Species in Aorta in an Active Immunization Mouse Model with AT1-EC2 Peptide

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Summary: The antibody against AT1-EC2 plays a role in some kinds of inflammatory vascular diseases including malignant hypertension, preeclampsia, and renal-allograft rejection, but the detailed mechanisms remain unclear. In order to investigate the changes of NADPH oxidase and reactive oxygen species in the aorta in a mouse model which can produce AT1-EC2 antibody by active immunization with AT1-EC2 peptide, 15 mice were divided into three groups: control group, AT1-EC2-immunized group, and AT1-EC2-immunized and valsartan-treated group. In AT1-EC2-immunized group and AT1-EC2-immunized and valsartan-treated group, the mice were immunized by 50 μg peptide subcutaneously at multiple points for 4 times: 0, 5, 10, and 15 days after the experiment. In AT1-EC2-immunized and valsartan-treated group, valsartan was given at a dose of 100 mg/kg every day for 20 days. After the experiment, the mice were sacrificed under anesthesia and the aortas were obtained and frozen in liquid nitrogen for the preparation of frozen section slides and other experiments. The titer of AT1-EC2 was assayed by using ELISA. The level of NOX1 mRNA in the aorta was determined by using RT-PCR. The expression of NOX1 was detected by using Western blotting. Confocal scanning microscopy was used to assay the α-actin and NOX1 expression in the aortic tissue. The O2·− production was detected in situ after DHE staining. The mice produced high level antibody against AT1-EC2 in AT1-EC2-immunized group and AT1-EC2-immunized and valsartan-treated group, and the level of NOX1 mRNA in the aortic tissues was 1.6±0.4 times higher and the NOX1 protein expression was higher in AT1-EC2-immunized group than in control group. There were no significant differences in the level of NOX1 mRNA and protein expression between control group and AT1-EC2-immunized and valsartan-treated group. The expression and co-localization of α-actin and NOX1 in AT1-EC2-immunized group increased significantly as compared with those in control group, and the O2·− production increased about 2.7 times as compared with control group. There were no significant differences between control group and AT1-EC2-immunized and valsartan-treated group. It is concluded that active immunization with AT1-EC2 can activate NOX1-ROS, and increase vascular inflammation, which can be inhibited by AT1 receptor blocker valsartan. This may partially explain the mechanism of the pathogenesis of inflammatory vascular diseases related to antibody against AT1-EC2.

Key words: AT1-EC2 peptide; NADPH oxidase; reactive oxygen species; vascular inflammation

The renin-angiotensin system plays a pivotal role in the pathogenesis of hypertension, atherosclerosis and other cardiovascular diseases, which is mediated by angiotensin II and angiotensin II type 1 (AT1) receptor. The AT1 receptor belongs to G-protein-coupled receptor superfamily, which includes four extracellular peptide regions and three intracellular peptide regions, and the extracellular peptide regions can receive the stimulating signals. Now many studies[1-3] reported that there are autoantibodies against the AT1 receptor in patients with malignant and refractory hypertension, preeclampsia, and renal-allograft rejection. The autoantibody against the second extracellular loop peptide (EC2) of AT1 receptor can activate AT1 receptor, which may greatly contribute to the pathogenesis of hypertension and inflammatory responses via the agonistic effects[1, 5, 6], but the detailed mechanism remains unclear.

The reactive oxygen species (ROS), including superoxide anions (O2·−), hydrogen and hydroxyl radicals, play a vital role in the inflammatory reaction and pathogenesis of vascular diseases, which are mainly produced from a serials redox enzymes such as NADPH oxidase (NOX), xanthine oxidase, and cytochrome P450[7]. NOX was first found in neutrophils and is composed of...
gp91phox, p47phox and other subunits which now are termed NOX2, and subsequently a serial of NOXs were found in non-phagocytes, such as NOX1, NOX3 and NOX4 which has a homologs of gp91phox[8]. NOX1 is expressed mainly in vascular system[9].

In order to investigate the pro-inflammatory mechanisms of antibody against AT1-EC2 peptide, in present study, we studied the effect of AT1-EC2 antibody on the expression of NOX1 and production of ROS in a C57BL/6 mouse model carrying AT1-EC2 antibody for a few weeks produced by active immunization with AT1-EC2 peptide.

1 MATERIALS AND METHODS

1.1 Preparation of AT1 Peptide

According to the methods established in our lab[3], a peptide corresponding to amino acids 165-191 of the EC2 peptide of the human AT1 receptor was produced using an automated multiple solid-phase peptide synthesizer (Shimadzu, Japan). The peptide was judged to be pure by high performance liquid chromatography (HPLC) and at least 95% purity was achieved. The sequence of the peptide was as follows: Ile-His-Arg-Asn-Val-Phe-Phe-Ile-Glu-Asn-Thr-Asn-Ile-Thr-Val-Cys-Ala-Phe-His-Tyr-Glu-Ser-Gln-Asn-Ser-Thr-Leu. The peptide was conjugated with glutaraldehyde used for the active immunization protocol.

1.2 Animal Active Immunization and Other Procedures

Fifteen C57BL/6 mice (8 weeks old) were purchased from Hubei Research Center of Laboratory Animal (Wuhan, China) and divided into 3 groups randomly: control group, AT1-EC2-immunized group, and valsartan-treated group. The mice in AT1-EC2-immunized and valsartan-treated group were subcutaneously injected with 50 μg AT1-EC2 peptide conjugated with glutaraldehyde in Freund’s complete adjuvant at multiple sites, and at least 95% purity was achieved. The sequence of the peptide was as follows: Ile-His-Arg-Asn-Val-Phe-Phe-Ile-Glu-Asn-Thr-Asn-Ile-Thr-Val-Cys-Ala-Phe-His-Tyr-Glu-Ser-Gln-Asn-Ser-Thr-Leu. The peptide was conjugated with glutaraldehyde used for the active immunization.

1.3 Detection of AT1-EC2 Antibody by ELISA

Antibodies against AT1-EC2 were detected by using ELISA as described previously in our lab[10]. Briefly, AT1-EC2 peptide (10 μg/mL) in buffer was coated on microtiter plates. The wells were saturated with phosphate-buffer saline supplemented with 3% skimmed milk, 0.1% Tween 20, and 0.01% merthiolate. The diluted sera were added to the coated plates overnight at 4°C. After additional washes, horseradish peroxidase-conjugated anti-mouse IgG antibodies were added for 1 h at 37°C. The plates were then washed and the substrate was added for color display. The absorbance (A) was measured at 450 nm and the titer of antibody was calculated.

1.4 Detection of NOX1 mRNA Expression by RT-PCR

The aortas of mice were dissected and snap-frozen in liquid nitrogen. The total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method. One microgram of total RNA was reversely transcribed using RNA PCR Kit (Takara, Japan) and the resulting cDNA was used as a PCR template. The mRNA levels were determined by real-time PCR with ABI PRISM 7900 Sequence Detector system (Applied Biosystem, USA) according to the manufacturer’s instructions. SYBR green I (Takara, Japan) was used as the fluorescence indicator. According to reference[11], the primer of NOX1 was as follows: forward, TTCACCAATTCCCAGGATTGAAGTTGAGTTGGTC; reverse, GACCTGTCACGATGTCAGTGCCCTTGTCAA.

1.5 Detection of NOX1 Expression by Western Blotting

The protein of the aorta was extracted and the concentration was assayed by BCA. 40 μg protein sample was loaded on SDS-PAGE gel and transferred to nitrocellulose membranes (Pierce, USA). After blocking for 2 h with 5% non-fat milk in TBS-T, the membranes were incubated with NOX1 primary antibodies (Rabbit NOX1 antibody, Sigma-Aldrich, USA) at 4°C overnight. After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. After washes, the membranes were reacted with the enhanced chemiluminescence system (Pierce, USA) and exposed to film. Band intensities were quantified with NIH Image Version 1.61.

1.6 NOX1 and α-actin Co-localization in Aorta under Confocal Microscopy

The NOX1 and α-actin co-localization was observed under a confocal microscope as described previously[12]. Briefly, the aortas were frozen in Tissue-Tek OCT and cut by cryostat into 10 μm-thick sections and mounted on Superfrost/Plus slides. After fixation and blockade, the slides were incubated with rabbit NOX1 antibody (Sigma-Aldrich, USA) and goat α-actin antibody (Santa Cruz, USA) overnight at 4°C. Next morning, after washing with PBS-T, the Alexa Fluor-488- and Alexa Fluor-555-conjugated secondary antibodies (Invitrogen, USA) were incubated for 1 h at room temperature. Then, the slides were washed, mounted, and subjected to confocal microscopic analysis (Nikon A1Si, Japan). And the Image-Pro Plus software was used to analyze the results.

1.7 Detection of O2⁻ Production in Aorta

As described previously[13], dihydroethidium (DHE) was used to assay the production of ROS O2⁻ in situ. Briefly, the unfixed tissue slides from different groups were incubated with DHE (10 μmol/L) in PBS at room temperature for 30 min. Then, the slides were washed, fixed, mounted, and subjected to fluorescence microscopic analysis (OLYMPUS IX71, Japan).

1.8 Statistical Analysis

The data were expressed as ̅±s. The significance of differences was estimated by ANOVA followed by Student-Newmann-Keuls multiple comparison tests. P<0.05 was considered significant. All statistical analyses were performed with SPSS 11.0.