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Immobilization stress induces endothelial dysfunction by oxidative stress via the activation of the angiotensin II/its type I receptor pathway

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ABSTRACT

Objective: Psychological stress has been shown to contribute to the development of atherosclerosis; however its underlying mechanism has not been clearly elucidated. We here studied the mechanism by which immobilization stress causes endothelial dysfunction with specific aim of identifying the role of angiotensin II and its type I (AT_1) receptor signaling pathway.
Methods and results: Rats ($n = 30$) were subjected to immobilization stress (120 min/day) for 14 days using a restrainer. During immobilized period, rats were orally administrated with or without the angiotensin converting enzyme (ACE) inhibitor ramipril (3 mg/kg/day, $n = 10$) or AT_1 receptor inhibitor losartan (9 mg/kg/day, $n = 10$). Immobilization significantly increased systolic blood pressure and decreased acetylcholine-induced *ex vivo* relaxation of arteries compared with those of control animals ($n = 10$). Immobilization increased the plasma levels of angiotensin II and ACE activity that were inhibited by treatment with ramipril, but not losartan. Furthermore, immobilization increased the plasma level of malondialdehyde and expression of $gp91^{phox}$ and Rho-associated kinase-1 in arteries, and decreased the arterial eNOS mRNA and oxidized products of NO (nitrite plus nitrate). These functional and biochemical alterations induced by immobilization were significantly reversed by administration of ramipril or losartan.
Conclusions: Immobilization stress induces vascular oxidative stress by activating the angiotensin II/AT_1 receptor signaling pathway, thereby provoking endothelial dysfunction which can contribute to the development of atherosclerosis and hypertension.

1. Introduction

An increasing body of evidence supports that physical and psychological stresses contribute significantly to the development of atherosclerosis [1,2]. Psychosocial stress can provoke atherosclerosis in non-human primates without any traditional risk factors, suggesting the causative role of stress in the development of atherosclerosis [1]. Proneness to stress is estimated as a significant risk factor for coronary artery disease in normotensive humans [2]. Chronic negative life stress may enhance cardiovascular reactivity to stressor in adolescents, thereby increasing the risk for subclinical atherosclerosis [3]. However, the mechanism by which stress causes atherosclerosis has not been clearly elucidated.

The most common allostatic responses to stress involve the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis [4]. In the central nervous system, stress may activate angiotensin II (AngII), thereby increasing the secretion of corticotropin-releasing hormone and central sympathetic activity, leading to activation of HPA axis and adrenomedullary sympathetic nervous system [5,6]. Genetic modifications of renin-angiotensin-aldosteron system (RAAS) has been known to contribute more to the dynamic blood pressure (BP) regulation in response to behavioral stress compared with the static BP, suggesting the important role of RAAS in stress-induced cardiovascular reactivity [7]. Intriguingly, stress can provoke inflammation via activating nuclear factor-κB [8]. Given the key role of inflammation in the pathogenesis of atherosclerosis [9], inflammation may play a pivotal role in the stress-induced atherosclerosis [10]. Importantly, considerable evidence supports the role for Ang II as a proinflammatory mediator [9]. Our goal was to study how stress causes endothelial dysfunction with the specific aim of identifying the role of Ang II and its type I (AT_1) receptor signaling pathway in stress-induced endothelial dysfunction.

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2. Materials and methods

2.1. Immobilization stress animal model

Male Sprague–Dawley rats (8 weeks old, 250–300 g) were acclimatized for 3–5 days to standard laboratory conditions. One group of rats ($n = 10$) was subjected to immobilization stress (120 min/day) for 14 days using an adjustable restraining cage, according to the established protocol [11]. The other two groups ($n = 10$ for each) were subjected to the same immobilization stress for 14 days with oral administration of either the angiotensin converting enzyme (ACE) inhibitor ramipril 3 mg/kg/day (Aventis, Bridgewater, NJ) or the AT_1 receptor inhibitor losartan 9 mg/kg/day (Merck & Co., Inc., Whitehouse Station, NJ) from one day before immobilization until completion of experiment. Systolic BP was measured using a tail cuff and pulse transducer (ADInstruments, Sydney, Australia), and mean of five measurements was used. On the next day after completion of animal treatment, blood sampling was performed after intraperitoneal anesthesia with Ketamine (250 mg/kg), and rats were killed by decapitation. Thoracic and abdominal aortas were retrieved, and samples were kept −70 °C.

2.2. Reverse transcription real-time quantitative polymerase chain reaction analysis

Total RNAs from tissue specimens were isolated using the TRI reagent (Sigma, Saint Louis, MO) according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNAs in a 40 μl reaction volume with use of High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a Perkin-Elmer DNA thermal cycler. Real-time RT-PCR analysis was carried out using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems) and the SYBR green master mixes (2×; Applied Biosystems) as recommended by the manufacturer, and the method was described previously [12]. The primers used were 5′-TGT CTC AGC ATC GAC CGC T-3′ (forward) and 5′-CATCGTGCGGCGAAGG-3′ (reverse) for the AT_1a, 5′-TCTGTTAGT-GGGATGCATGTAATCA-3′ (forward) and 5′-TGTGGGCCTCCAAACC-ATT-3′ (reverse) for the AT_2, 5′-AGCCCGGGACTTCATCAATCAG-3′ (forward) and 5′-GCCCCAAACACCAGCTCACTCTC-3′ (reverse) for the eNOS, and 5′-AAGTCCCTCACCCTCCCAAAAG-3′ (forward) and 5′-AAGCAATGCTGTCACCTTCCC-3′ (reverse) for the β-actin. Thermal cycling for PCR was as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. Samples were amplified simultaneously in triplicate in one assay run. For each sample, the amounts of each target gene and of β-actin (endogenous control) were determined using the relative standard curve method. Standard curves were computed for all genes from a series of five-fold serial template dilutions (3.125–100 ng). The amount of each target gene normalized to β-actin in each sample was compared with that of control group.

2.3. Measurement of endothelium-dependent relaxation

Vascular relaxation was determined using an arterial ring from the superior mesenteric artery (SMA) trunk as described previously [12]. In brief, each aortic ring was threaded with two strands of tungsten wire (120 μm diameter): one anchored in the organ bath chamber and the other connected to a mechanotransducer (model FT-03, Grass). The chamber was perfused at a flow rate of 2.5 ml/min with oxygenated (95% O_2–5% CO_2) Krebs-Ringer bicarbonate solution using a peristaltic pump. Optimal resting tension (0.8–1.0 g) was applied. Rings were pre-contracted with 10^{-6} M norepinephrine, and vascular relaxation was measured in response to either acetylcholine (Ach) or sodium nitroprusside (SNP). Each experimental procedure was performed three times using two SMA aortic rings from each animal with intervening washout with Krebs buffer. Each relaxation response is expressed as a percentage of the contraction induced by norepinephrine.

2.4. Measurement of Ang II and ACE activity assay

The levels of Ang II and ACE activity in plasma were determined using an ELISA kit (Cayman Chemical, Ann Arbor, MI) and an ACE colorimetric assay kit (Bühlmann Laboratories AG, Switzerland), respectively, according to the manufacturers' guide. One unit of ACE activity is defined as the amount of enzyme required to release 1 μmole of hippuric acid per minute and per liter of serum at 37 °C as determined by the colorimetric assay.

2.5. Determination of NO metabolites and lipid peroxidation

The plasma level of nitrite plus nitrate (NO_x), stable metabolites of NO, was measured by using a NO analyzer (Antek, Houston, TX) and quantified with sodium nitrate as a standard [13]. Plasma was mixed with an equal volume of cold ethanol and incubated for 10 min on ice. Mixed samples were centrifuged at 12,000 × *g* for 15 min at 4 °C, and supernatants were transferred to a new tube. Fifty microliters of sample was loaded in a reactor, and NO was quantified as previously described [13]. Lipid peroxidation was determined by measuring thiobarbituric acid-reacting substances using malondialdehyde (MDA) standards as previously described [14]. MDA concentrations were calculated based on its molecular coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.6. Western blot analysis

Thoracic aorta were homogenized in ice cold RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma-Aldrich). Homogenized samples were sonicated and then centrifuged at 12,000 × *g* for 15 min at 4 °C. Western blot analyses were performed to determine the levels of Rho-associated kinase-1 (ROCK1) and gp91^{phox} using their specific antibodies (BD Biosciences, San Jose, CA) as previously described [13].

2.7. Statistical analysis

The data are presented as the mean ± standard deviation (SD). All data were analyzed by means of 1-way ANOVA followed by post hoc analyses for multiple comparisons. Comparisons of non-parametric variables between groups were analyzed using the Mann-Whitney test, and the Wilcoxon signed-rank test was used for comparisons between two related non-parametric variables. Significance was established at a P value <0.05.

3. Results

3.1. Immobilization stress increases systolic BP

The systolic BP was significantly increased in the stress group (129.1 ± 14.3 mm Hg vs 111.5 ± 12.3 mm Hg, $P < 0.05$) after completion of 2-week immobilization compared with baseline (Fig. 1). Systolic BP at endpoint was significantly lower in the stress plus ramipril group (87.7 ± 7.8 mm Hg, $P < 0.01$) and the stress plus losartan group (106.1 ± 15.1, $P < 0.05$) than the stress group (Fig. 1). The weight gain (endpoint − baseline) was significantly decreased in the stress group and the stress plus ramipril group (21.1 ± 11.5 g and 30.4 ± 14.1 g, respectively, both $P < 0.05$), but not in the stress plus losartan group (33.9 ± 9.4 g), compared with the control group (50.5 ± 20.4 g).