Changes of Gene Expressions in Spontaneously Hypertensive Rat Model After Losartan Treatment

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Background and Objectives: The renin angiotensin system seems to play an important role in the development of cardiac and vascular hypertrophy in hypertension. The changes in pathology, and gene expressions of the angiotensin II receptor type 1A (ATIA) and angiotensin converting enzyme (ACE) were investigated in order to explore the effects of losartan in spontaneously hypertensive rat (SHR) models.

Materials and Methods: Twelve week-old male Wistar rats were grouped as follows: control (C) group, hypertension (H) group, and losartan (L) group in which SHR was treated with losartan (10 mg/kg/day). Western blot and reverse transcription-polymerase chain reaction analysis regarding seven genes such as endothelin-1, ACE, ATIA, neutrophil cytosolic factor, brain natriuretic peptide, troponin I, endothelial nitric oxide synthase were performed.

Results: Systolic blood pressure was significantly decreased in the L group compared with the H group in weeks 3 and 5. ACE and ATIA proteins in the L group were lower than H group in week 5.

Conclusion: Losartan reduced blood pressure, cardiac hypertrophy and protein expressions of ACE and ATIA. Changes of protein expressions were more sensitive than changes in pathology. Further study is needed for the differing doses of losartan in SHR models. (Korean Circ J 2012;42:761–768)

KEY WORDS: Hypertension; Receptors, angiotensin; Angiotensin converting enzyme; Gene expression.

Introduction

Hypertension or elevated arterial blood pressure is a substantial public health issue in industrialized societies. It is a major risk factor in regards to many common causes of morbidity and mortality including stroke, myocardial infarction, congestive heart failure, and end-stage renal disease. Although hypertension is linked to many diseases, it’s definite causes are still poorly understood. Various factors mediate hypertension. The renin-angiotensin-aldosterone system (RAAS) seems to play a key role in the development of hypertension. The principle mediator regarding the physiological actions of the RAAS is the octapeptide angiotensin (QAT). There is considerable evidence supporting the involvement for the inappropriately elevated levels of angiotensin (AT) II in many forms of hypertension.

Angiotensin II is intimately involved in many regulatory mechanisms in the cardiovascular system besides blood pressure. It stimulates the proliferation of smooth muscle cells (SMCs) in the arterial wall, enhances collagen deposition, influences matrix components, alters structure and thickness of the arterial wall in both resistant as well as conduit arteries, modulates sympathetic activity, stimulates endothelin (ET)-1 release from vascular endothelium and tunica adventitia, and influences bioavailability of nitric oxide and endothelial functions.

Pathological alterations in the majority of these regulatory systems were observed in hypertension. Most of the known AT II effects are mediated via the AT II receptor type 1A (ATIA) and therefore administration of an AT receptor blocker offers the possibility to modify numerous AT II actions within the cardiovascular system.

The spontaneously hypertensive rats (SHR) of the Okamoto-Aoki...
strain were developed by the selective breeding of Wistar-Kyoto stock for higher blood pressure.10 SHR s spontaneously and consistently develop moderate-to-severe hypertension between 7 and 15 weeks of age and have served as models of genetic hypertension in humans. Several studies were reported in SHR models.12-19

Losartan is a highly specific, non-peptide antagonist of the AT II receptor type 1.10 AT II receptor antagonists are a group of drugs that are used in the treatment of hypertension. Their mechanism of action involves the specific blockade of AT II receptors, which inhibits vasoconstriction and prevents vascular and cardiac hypertrophy. There have been several articles concerning the effect of losartan in the SHR model.12-19

The effects were different depending on the dose of losartan. However, there were rare reports with regards to gene changes.

The purpose of this study was to investigate the changes in pathology, and gene expressions of ATIA, and the angiotensin converting enzyme (ACE) in SHR models after losartan treatment.

Materials and Methods

Materials

Twelve-week-old male Wister-Kyoto rats, weighing approximately were used for this study. All rats were housed in climate-controlled conditions with a 12 hours light: 12 hours dark cycle, and had free access to chow and water.

The rats were grouped as follows: control (C) group (n=18), hypertension (H) group (n=18), losartan (L) group (n=18) in which losartan was administrated daily by gavage in a dose of 10 mg/kg/day.

The rats were sacrificed in weeks 3 and 5. The heart and kidney tissues were removed and immediately frozen at -70°C for protein and gene analysis, post-fixed in 10% formalin, and processed routinely for paraffin embedding. All protocols were approved by the Institutional Animal Care and Use Committees of the School of Medicine of Ewha Womans University (approval No. 11-0169).

Methods

Organ weights

The rats were weighed and their general appearance was observed during the study period. The animals were sacrificed at the scheduled time and the hearts and kidneys were rapidly removed. The wet weights of the left ventricle (LV) and kidney were measured, and the ratio of organ weight to body weight (BW) was calculated.

Estimation of systemic blood pressure

The animals were placed in the supine position and instrumented with an arterial pressure line (Physiological Pressure Transducer, MLT 1199; AD Instruments, Oxfordshire, UK). Arterial pressures were estimated using the input from an ambient-pressure reference (APR-1; Data Sciences) on day 1 in weeks 3 and 5.

Morphometric analysis of the heart tissues

LV including the interventricular septum was weighed after the right and left atria and the right ventricular free wall was dissected. The LV mass index was calculated by dividing the LV weight by the BW of each animal. The LV heart tissue was fixed in formaldehyde and embedded as a paraffin section in all three groups. Masson’s Trichrome staining was used in order to observe the degree of fibrosis (i.e., degree of collagen penetrating the heart tissue). The area with the most and least degree of fibrosis within each group was selected and photographed under 200 HPF light microscopy. The photographs were processed through an image analysis program (analySIS) and the area of collagen penetration, i.e., the area stained blue with Masson’s Trichrome staining was measured.

Ribonucleic acid extraction and complementary deoxyribonucleic acid synthesis

Total ribonucleic acid (RNA) was extracted by using TRIzol Reagent™ (Invitrogen, Carlsbad, CA, USA), according to the Trizol method protocol and resuspended in diethyl pyrocarbonate water. The final RNA amount was spectrophotometrically determined at 260/280 nm. Quality was assessed as the absence of smear for 18S and 28S bands analyzed by means of Bio analyzer 2100 (Agilent technologies, Palo Alto, CA, USA). RNA samples were stored at -70°C prior to usage. complementary deoxyribonucleic acid (cDNA) synthesis were synthesized by 1 μg of total RNA, according to the manufacture’s protocol (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA, USA).20

Gene expression analysis by a real time reverse transcription–polymerase chain reaction

Real-time quantitative polymerase chain reaction (PCR) was performed in triplicate in 384-well plates. A 384-well high-throughput analysis was performed by using the ABI Prism 7900 Sequence Detection System Software (Applied Biosystems, Foster City, CA, USA) and white colored 384-well plates (ABgene, Hamburg, Germany) for intensification of the fluorescent signals by a factor of three. The system operated using a thermal cycler and a laser that is directed via fiber optics to each of the 384 sample wells. Fluorescent emissions from each sample were collected by a charge-coupled device-camera and the quantitative data were analyzed using the Sequence Detection System Software (SDS version 2.0, Applied Biosystems, Foster City, CA, USA). Reaction mixtures contained 10 pmol/μL of each primer and 2X SYBR Green PCR Master Mix (Applied
The resulting first-strand of cDNA normalized by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The normalized cDNA was used as a template for the PCR process. The specific primers for rat ET-1 were 5'-TCTCGGAGAGCAGACAC-3' (forward) and 5'-TGGACACTGGGATTTTCCT-3' (reverse).

The specific primers for ACE were 5'-GCCAACCTATACACATGATG-3' (forward) and 5'-ATGGAGAAGAGATCAATGGA-3' (reverse).

The specific primers for AT1A were 5'-CTGCGGAGTGTACATGACAT-3' (forward) and 5'-AAATGCTCCTGTTAGGT-3' (reverse).

The specific primers for neuropil cytosolic factor (NCF) 1 were 5'-GGCAAAGTTGAACAAATC-3' (forward) and 5'-ACCCTTGGTCTGGTACT-3' (reverse).

The specific primers for ACE were 5'-GCCAACCTATACACATGATG-3' (forward) and 5'-ATGGAGAAGAGATCAATGGA-3' (reverse).

The tissue was homogenized in a 10 mM Tris HCl buffer, pH 7.4 containing 0.5 mM ethylenediaminetetraacetic acid, pH 8.0, 0.25 M sucrose, 1 mM PMSF, 1 mM Na,VO₃, and a protease inhibitor cocktail (Roche-Boehringer-Mannheim). After centrifugation, the supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples equivalent to 25 μg of protein content were loaded and separated by size in 10% SDS-PAGE. The proteins on the acrylamide gel were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) at 400 mA in a transfer buffer containing 25 mM Tris and 192 mM glycine, pH 8.4. The peripheral vascular disease membranes were blocked in TBS with 5% non-fat dry milk at room temperature for 1 hour in 0.1% Tween 20 and incubated with the appropriate primary antibodies, including ET-1, ACE, AT1A, NCF, endothelial nitric oxide synthase (eNOS), brain natriuretic peptide (BNP), troponin I (Cell Signaling Technology Inc., Danvers, MA, USA) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc., Danvers, MA, USA) for 1 hour at room temperature. After undergoing washing, the membrane was visualized by a chemiluminescent reaction using an enhanced chemiluminescence-detection kit system (Amersham Biosciences, Buckinghamshire, UK).

The cell lysate from the solubilized tissue was mixed with Pierce BCA reagents (Pierce, Rockford, IL, USA) and incubated for 30 minutes. Moreover, the protein content was quantified with a Molecular Devices enzyme linked immuno sorbent assay reader (Amersham Pharmacia Biotechnology, Sunnyvale, CA, USA) at 562 nm based on the bovine serum albumin standard curve.

**Statistical analysis**

Results were expressed as the mean±standard deviation. An unpaired two-tailed t-test and Mann-Whitney test were used, and a p value of less than 0.05 was considered statistically significant. Statistical Package for the Social Sciences (SPSS) 12.0 for Windows (SPSS, Chicago, IL, USA) was used for all statistical analyses.

**Results**

**Systemic blood pressure after losartan treatment**

Blood pressure was significantly higher in the H group compared with the C group on day 1 (219.8±10.5 mm Hg vs. 130.7±5.8 mm Hg, p<0.05), in week 3 (211.5±16.1 mm Hg vs. 131.3±7.4 mm Hg, p<0.05), and week 5 (221.2±14.2 mm Hg vs. 175±6.2 mm Hg, p<0.05).

Blood pressure was significantly lower in the L group compared with the H group in week 3 (147.7±7.5 mm Hg, p<0.05) and week 5 (139.0±16.9 mm Hg, p<0.05) (Fig. 1).

**Body weight, left heart, left heart/body weight ratio, kidney weight, kidney/body weight ratio after losartan treatment**

Body weight was statistically lower in the H group compared with the C group in week 3 (327.2±24.7 g vs. 474.8±12.9 g, p<0.05) and week 5 (320±10.2 g vs. 480±27.2 g, p<0.05).

Left heart (LH) weight was significantly higher in the H group compared with the C group (0.88±0.01 g vs. 0.98±0.01 g, p<0.05) in week 3 and significantly decreased in the L group compared with the H group in week 3 (0.88±0.01 g vs. 0.98±0.01 g, p<0.05).

Kidney/BW ratio was significantly higher in the H group compared with the C group in week 5 (0.40±0.02 g vs. 0.32±0.01 g, p<0.05) and significantly decreased in the L group compared with H group in week 5 (0.37±0.01 vs. 0.40±0.02, p<0.05) (Table 1).
Pathologic finding in heart tissues

Under light microscopy, collagen was observed to penetrate between the LV myocardiocytes, staining blue as noted in Fig. 2. The degree of penetration was not equally dispersed and differed in each area. Most areas presented little collagen infiltration and these areas were similar in each group. By quantitative analysis, collagen penetration in the H group was significantly higher than that in the C group (1280.8 ± 863.1 vs. 457.1 ± 270.2, p < 0.05).

There was no statistically significant difference between the H and the L group but the L group showed a slight decrease in collagen (Table 2).

Serum renin levels after losartan treatment

Serum renin levels were significantly higher in the H group compared with the C group in week 3 (9.6 ± 2.3 ng/mL/hr vs. 5.4 ± 1.5 ng/mL/hr, p < 0.05) and week 5 (8.0 ± 2.5 ng/mL/hr vs. 4.0 ± 0.9 ng/mL/hr, p < 0.05). Also, there was no significant difference between the L and H group (Fig. 3A).

Serum aldosterone levels after losartan treatment

Serum aldosterone levels were significantly higher in the H group compared with the C group in week 5 (78.5 ± 5.3 pg/mL vs. 36.5 ± 9.0 pg/mL, p < 0.05) and significantly lower in the L group (65.2 ± 2.8 pg/mL, p < 0.05) compared with the H group in week 5 (Fig. 3B).

Table 1. Body, weight LH, LH/BW, KD and KD/BW after losartan treatment

| Group | BW (g) | LH (g) | LH/BW | KD (g) | KD/BW |
|-------|--------|--------|-------|--------|-------|
| C     | 474.8 ± 12.9 | 0.84 ± 0.01 | 0.18 ± 0.02 | 1.53 ± 0.1 | 0.33 ± 0.07 |
| H     | 327.7 ± 24.7* | 0.98 ± 0.08* | 0.30 ± 0.06* | 1.14 ± 0.07 | 0.35 ± 0.02 |
| L     | 340.1 ± 31.6* | 0.88 ± 0.01* | 0.27 ± 0.07 | 1.33 ± 0.07 | 0.36 ± 0.01 |
| C     | 480 ± 27.2 | 0.86 ± 0.06 | 0.18 ± 0.01 | 1.49 ± 0.06 | 0.32 ± 0.01 |
| H     | 320 ± 10.2* | 0.93 ± 0.08 | 0.29 ± 0.08* | 1.25 ± 0.01 | 0.40 ± 0.02* |
| L     | 322.8 ± 6.8* | 0.82 ± 0.04 | 0.26 ± 0.07 | 1.22 ± 0.08 | 0.37 ± 0.018* |

*Significantly different from group C group p < 0.05, †Significantly different from group H group p < 0.05. BW: body weight, LH: left heart, KD: kidney, C: control, H: hypertension, L: losartan

Table 2. Change of collagen by Masson’s Trichrome staining in the heart tissues

| Group | C group | H group | L group |
|-------|---------|---------|---------|
| BW (g) | 1280.8 ± 863.1* | 457.1 ± 270.2 | 880.1 ± 370.3 |

*Significantly different from group C group p < 0.05. C: control, H: hypertension, L: losartan

Fig. 1. Systemic blood pressures after losartan treatment. Blood pressure was significantly higher in the H group compared with the C group on day 1, week 3, and week 5. Blood pressure was significantly lower in the L group compared with the H group in weeks 3 and 5. *Significantly different from group C group p < 0.05, †Significantly different from group H group p < 0.05. C: control, H: hypertension, L: losartan.

Fig. 2. Change of collagen by Masson’s Trichrome staining in the heart tissues (×200). Collagen penetration in the H group was significantly higher than that in the C group. There was no statistically significant difference between the H and the L group but the L group showed a slight decrease in collagen. C: control, H: hypertension, L: losartan.
Serum angiotensin converting enzyme levels after losartan treatment

Serum ACE level did not significantly differ among the three groups (Fig. 3C).

Serum angiotensin levels after losartan treatment

Serum AT levels were significantly higher in the H group compared with the C group on day 1 (1.18±0.23 pg/mL vs. 0.57±0.34 pg/mL, p<0.05), in week 3 (3.66±2.2 pg/mL vs. 0.59±0.18 pg/mL, p<0.05) and week 5 (3.1±1.78 pg/mL vs. 0.52±0.16 pg/mL, p<0.05). In addition, the serum AT level in the L group was significantly lower than that in the H group on day 1 (0.75±0.08 pg/mL in week 3 (0.60±0.2 pg/mL, p<0.05) and week 5 (0.9±0.51 pg/mL, p<0.05) (Fig. 3D).

Serum high sensitive C-reactive protein

Serum high sensitive-C reactive protein (hs-CRP) levels were significantly decreased in week 3 in the L group compared with the H group (p<0.05) (Table 3).

Gene expressions levels of angiotensin converting enzyme, angiotensin II receptor type 1A and the neutrophil cytosolic factor in week 5

Angiotensin II receptor type 1A and NCF gene expressions were significantly higher in the H group (p<0.05) (Table 4). NCF gene expressions were significantly decreased in the L group compared with the H group (p<0.05) (Table 4).

Endothelin-1, angiotensin converting enzyme, angiotensin II receptor type 1A and neutrophil cytosolic factor 1 protein contents by western blot analysis in the heart tissues

Endothelin-1 protein contents were not different among the three groups (Fig. 4A).
Angiotensin converting enzyme protein content was significantly higher in the H group compared with that in the C group in weeks 3 and 5 (p<0.05) and significantly lower in the L group compared with the H group in week 5 (p<0.05) (Fig. 4B).

The ATIA protein level was significantly higher in the H group compared with the C group in weeks 3 and 5 (p<0.05) and significantly lower in the L group compared with the H group in week 5 (p<0.05) (Fig. 4C). NCF 1 protein contents were significantly higher in the H group compared with the C group in weeks 3 and 5 (p<0.05) and significantly lower in the L group compared with the H group in week 5 (p<0.05) (Fig. 4D).

**Brain natriuretic peptide, troponin I and endothelial nitric oxide synthase protein contents by western blot analysis in the heart tissues**

Brain natriuretic peptide and troponin I protein contents were significantly higher in the H group compared with the C group in week 5 (p<0.05) but there was no difference between the L group and the C group. eNOS protein contents were significantly higher in the H group compared with the C group in weeks 3 and 5 (p<0.05), but there was no difference between the H group and the L group (data was not shown).

**Discussion**

We demonstrated hypertension-related cardiac changes in the heart tissues.

**Table 3. Serum hs-CRP level after losartan treatment (mg/L)**

| Week | C group | H group | L group |
|------|---------|---------|---------|
| 3    | 0.60±0.05 | 0.6±0.02 | 0.51±0.22† |
| 5    | 0.66±0.05 | 0.62±0.03 | 0.56±0.02 |

†Significantly different from group H group p<0.05. C: control; H: hypertension; L: losartan, hs-CRP: high sensitive-C reactive protein

**Table 4. Gene expression levels of ACE, ATIA and NCF1 in the heart tissues in week 5**

| Gene   | C group | H group | L group |
|--------|---------|---------|---------|
| ACE    | 1.34±0.24 | 0.77±0.08 | 0.60±0.15 |
| AT 1A  | 1.20±0.20 | 1.74±0.16* | 1.49±0.24 |
| NCF1   | 0.97±0.11 | 1.89±0.05* | 1.30±0.24† |

*Significantly different from group C group p<0.05, †Significantly different from group H group p<0.05. ACE: angiotensin converting enzyme, AT: angiotensin, NCF: neutrophil cytosolic factor, C: control, H: hypertension, L: losartan

**Fig. 4.** Endothelin-1 (A), ACE (B), angiotensin II receptor type 1A (C), neutrophil cytosolic factor 1 (D) protein contents by western blot analysis in the heart tissues. *Significantly different from group C group p<0.05, †Significantly different from group H group p<0.05. ET: endothelin, ACE: angiotensin converting enzyme, AT: angiotensin, NCF: neutrophil cytosolic factor; C: control; H: hypertension, L: losartan.
SHR model including left ventricular hypertrophy and LH/BW ratio in week 3, increased BNP and troponin I protein content by western blot analysis in week 5, and increased collagen content by histology.

We initiated this study because the RAAS has an important role in systemic hypertension. Specifically, AT II is a major effector of vasoconstriction, cell growth, sodium and water retention, and sympathetic activation. It appears to promote endothelial dysfunction, inflammation, oxidative stress, insulin resistance, and reduced β-cell responsiveness. The close relationship between RAAS and hypertension has led to a compelling indication to block the formation or activity regarding AT II through the use of ACE inhibitors and AT receptor blockers. The mechanism by which the AT II receptor type 1 blocker, one of the RAAS metabolites, mediates hypertension seems to be important.

In our SHR model, increased serum renin and aldosterone levels as well as increased protein expression levels for ACE and ATIA were observed, confirming that RAAS has an important role in the SHR. Increased eNOS protein content was also noted in our SHR model. Hypertension is associated with impaired endothelial function, which can be explained by a decrease in nitric oxide generation or by an enhanced inactivation of NO after its release from the endothelial function. AT II stimulates ET-1 release from the vascular endothelium and tunica adventitia and influences the bioavailability of NO and endothelial functions.

Our study has shown the structural and functional changes of the LV with losartan, an AT II receptor type 1 blocker.

The findings were as follows: In week 1, losartan lowered serum AT levels. In week 3, blood pressure was significantly lowered and LH weight, and kidney/hematocrit ratio also significantly decreased. In week 5, kidney/BW ratio and serum aldosterone levels were significantly decreased. Furthermore, in week 5, NCF gene expressions and protein contents (ACE, ATIA, and NCF) were significantly decreased.

The preventive effect of losartan administration on the enlargement of arterial wall mass may have been reasonably explained by the protective effect of losartan on endothelial cells, and inhibition for the proliferative action of AT II on SMCs. Tea et al. found that the AT2 receptor-mediated vascular mass regression by stimulating SMC apoptosis in vivo, an effect seen during the AT1 receptor blockade. Decreased mass of SMC in the vessel wall may participate in the antihypertensive effect of losartan. Our results revealed several losartan effects in the SHR models.

First, the effect of losartan on decreasing blood pressure appears in week 3 following the onset of losartan treatment. Blood pressure lowering treatments can prevent cardiovascular disease. So, RAAS is targeted by antihypertensive medication such as losartan. The antihypertensive effect differs according to the dose of losartan.

Second, losartan has decreasing effects on LH weight, and kidney/BW ratio in our study. Collagen content was decreased after losartan treatment. Cardiac fibrosis is a detrimental process that causes an imbalance between the extracellular matrix (ECM) deposition and the degradation within the heart resulting in excessive fibroblast proliferation as well as a buildup of ECM proteins within the cardiac interstitial space. As a result, it causes progressive stiffening of the ventricular wall. Fibrosis is associated with hypertension, in addition to congestive heart failure, valvular defects and aging. Losartan has beneficial effects on the heart, which affects not only the myocytes but also the production of collagen and other matrix tissues.

Third, serum AT levels were significantly decreased following the treatment of losartan from day 1. The aldosterone level decreased as well, leading to decreased water and sodium retention. Actual measurements of serum aldosterone levels showed lower levels in the L group compared to the H group, which is significant in regards to the effects of losartan on aldosterone in the RAAS.

Fourth, down regulated ATIA signifies the inhibition of vasoconstriction, sodium retention, somatic nervous system activation, inflammation, and growth-promoting effects. Protein levels of ACE and ATIA were significantly decreased in the L group compared with the H group. We identified decreased messenger ribonucleic acid (mRNA) levels of NCF1 and serum hs-CRP levels. This means that hypertension is speculated to be a response to inflammation. It also suggests that losartan provides an anti inflammatory action. A low dose of losartan did not have an effect on ET-1 and eNOS protein content in this study.

In conclusion, losartan has an effect on blood pressure, cardiac hypertrophy, the serum aldosterone level, the mRNA level of ATIA, and the protein level of ACE, ATIA, and NCF.

The most important limitation is that these are data from rats. Also, the physiology and pathology of rats differ from humans. Moreover, the sample size is relatively small. Thus, we plan to re-search the gene expression levels affected by high doses of losartan treatment.

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