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Original Article

Losartan alleviates hyperuricemia-induced atherosclerosis in a rabbit model

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Abstract: Objective: To investigate the mechanisms underlying the therapeutic effects of losartan on hyperuricemia-induced aortic atherosclerosis, in an experimental rabbit model. Methods: Male rabbits (n = 48) were divided into control, hyperuricemia (HU), hypercholesterolemia + hyperuricemia (HC + HU) and high-purine with 30-mg/kg/d losartan (HU + losartan) groups. Serum uric acid (UA) and plasma renin and angiotensin II activities were determined. Aortic tissue specimens were analyzed for histological changes and proliferating cell nuclear antigen (PCNA). Liver tissues were sampled for quantitative analyses of liver low-density lipoprotein receptor (LDLR) mRNA and protein via reverse transcription polymerase chain reaction and western blotting. Results: After 12 weeks, serum UA and plasma renin and plasma angiotensin II activities were enhanced in the HU and HU + HC groups ($P < 0.001$) compared to the control, whereas in the HU + losartan group plasma renin activity was not different and serum UA concentrations as well as plasma angiotensin II activity were moderately enhanced ($P < 0.05$). Smooth muscle cell (SMC) PCNA expression increased strongly in the HU and HU + HC groups ($P < 0.001$), but was less pronounced in the HU + losartan group. In contrast, transcription and expression of LDLR mRNA and protein were significantly higher in the control and HU + losartan groups compared to the HU and HU + HC groups. Both the HU and HU + HC groups had elevated intima thickness and intima areas compared to the control and HU + losartan groups. Conclusions: Losartan can alleviate experimental atherosclerosis induced by hyperuricemia.

Keywords: Atherosclerosis, hyperuricemia, losartan, vascular smooth muscle cell, proliferating cell nuclear antigen

Introduction

Atherosclerosis (AS) is a major public health concern worldwide especially when it affects major blood vessels such as the coronary artery. The etiology of AS is thought to be multifactorial and risk factors include hypercholesterolemia, hypertriglyceridemia, hypertension, smoking, hyperglycemia and obesity [1], but a genetic predisposition has also been proposed [2, 3]. Hyperuricemia (HU), which is associated with metabolic syndrome [4], has been proposed as a potential risk factor for subclinical AS [5-7] and was reported to be a risk factor for left ventricular hypertrophy independent of hypertension [8]. However, up to now, it is not unambiguously clear whether HU is an independent or dependent risk factor for coronary heart disease [9, 10]. Losartan is an angiotensin II type 1 receptor antagonist mainly used for the treatment of hypertension and diabetic nephropathy. Losartan intervention for endpoint reduction in hypertension (LIFE) study demonstrated that losartan was therapeutically superior to atenolol in reducing cardiovascular morbidity and mortality, with a comparable reduction in blood pressure [11]. An additional study found that the therapeutic benefits of losartan in terms of cardiovascular events partially resulted from a lowering of serum uric acid levels [12]. In the present study, we investigated whether HU triggers AS and the effects of losartan in a rabbit HU model.

Materials and methods

Laboratory animals

The study protocol was approved by the Animal Research Committee at Shanghai Jiaotong University College of Medicine in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Forty-eight male, specific-pathogen-free New

Zealand white rabbits, weighing approximately 3.0 kg, were bred at the Laboratory Animal Center, Shanghai Jiaotong University College of Medicine (License No. WYDW 2014-0015). The animals were maintained in a temperature-regulated environment (24°C) on a 12-h light/dark cycle, housed in individual cages with soft bedding and given free access to 120-gram of standard rodent chow and tap water.

Experimental protocol

All animals were quarantined and acclimated for 1 week and randomly divided into 3 equal groups (n = 12): A control group fed with a 12-week routine rodent chow, a hyperuricemia (HU) group fed on a 12-week high-purine chow containing 2% potassium oxonate and 3% adenosine [13]; a hypercholesterolemia + hyperuricemia (HC + HU) group fed on a 12-week high-lipid and purine chow containing 2% cholesterol, 0.5% sodium cholate, 0.2% propylthiouracil and 3% porcine fat; a hyperuricemia + losartan (HU + losartan) group fed initially for 4-weeks solely on high-purine chow and subsequently on an 8-week high purine chow containing 30-mg/kg/d grounded losartan.

Clinical biochemistry assays

Fasting (> 14 h) blood samples were harvested from the central auricular artery before randomization, at 8 weeks and 12 weeks, respectively. The blood samples were centrifuged to collect serum for assaying serum UA and plasma activities of renin and angiotensin II, using an automatic biochemistry analyzer (7600-020; Hitachi Medical Corporation, Tokyo, Japan). All experiments were performed on independent duplicates.

Histological and immunohistochemical examinations

All animals were sacrificed at 12 weeks, and aortic specimens were harvested for histological and immunohistochemical examination of AS plaques. The specimens were fixed in 10% formaldehyde, embedded in paraffin, sectioned between 0.5 and 0.9 μm, and stained with hematoxylin and eosin. An automatic imaging analysis system (Leica Microsystems, Wetzlar, Germany) was used for measurements of the thickness and area of the tunica intima and media. Proliferating cell nuclear antigen (PCNA; Neomarkers, Inc., Waltham, MA, United States) was immunohistochemically stained using a streptavidin peroxidase conjugate kit (Roche Diagnostics, Basel, Switzerland), with phosphate balanced solution as the negative control. PCNA-positive cells were nucleus-stained brownish, and 5 high-power fields were examined using the imaging analysis system for the calculation of the PCNA positivity percentage, equal to the number of PCNA-positive cells divided by the total number of counted cells × 100%. All experiments were performed as independent duplicates.

Transmission electron microscopy

Aortic AS specimens were fashioned into 2 mm × 2 mm × 10 mm strips and fixed in 2% glutaraldehyde at 4°C. The specimens were rinsed and re-fixed in osmium tetroxide, dehydrated in an ethanol gradient and post-fixed in 3 washes of acetone for 30 min per wash. The post-fixed specimens were embedded for 48 h at 40°C and 60°C, respectively. The embedded specimens were fashioned in a trapezium, ultrathin sectioned at 50-90 nm, and mounted on a copper-coated fine mesh. The sections were sequentially stained with lead citrate for 5-15 min and uranyl acetate for 5-15 min for examination using a Hitachi-7650 transmission electron microscope (TEM, Japan) at 120 kV. All experiments were performed in independent duplicates.

LDL receptor reverse transcription polymerase chain reaction

Liver specimens were harvested and pre-processed at -80°C for LDL receptor (LDLR) semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) RNA extraction. The total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, US) and processed into cDNA (Invitrogen, Carlsbad, CA, US) according to the manufacturers' instructions. PCR primers for LDLR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; internal control) were designed using BLAST: Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and synthesized by Sangon Biotech, Shanghai, China. PCR products (5 μL for each sample) were stained with ethidium bromide and electrophoresed in 15 g/L agarose gels at 75 V. The level of LDLR mRNA is expressed as the intensity ratio of the target

band to the internal control band (GAPDH) on a gel imaging analysis system (Bio-Rad, Philadelphia, PA, US).

Western blot assay for LDL receptor

Total liver protein samples were extracted using RAPI lysis buffer (Beyotime Institute of Biotechnology, Nantong, China) and quantitated using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Nantong, China). Denatured protein samples were electrophoresed in sodium dodecyl sulfate polyacrylamide gel (Beyotime Institute of Biotechnology, Nantong, China), at 100 V and a current intensity of 120 mA, and then transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). LDLR was immunoblotted by incubating with LDLR antibody (1:1,500) (BioVision, Inc., Milpitas, CA, US) at 4°C overnight and with the secondary antibody (1:2,000) (BioVision, Inc., Milpitas, CA, US) at room temperature for 2 h. The binding immunoblots were chemiluminated using an enhanced chemiluminescent detection kit (Pierce Biotechnology, Inc., Rockford, IL, US). Tubulin was immunoblotted by using the same experimental protocol as the internal control.

Statistical analysis

The statistical software package SAS 6.12 (SAS Institute, Cary, NC, United States) was used for statistical analyses. All continuous data are expressed as the mean ± standard deviation and means compared using the one-way or repeated measures analysis of variance. All categorical data were expressed as *n* (%) and compared using the Fisher exact probability test. *P* values less than 0.05 were considered to be statistically significant.

Results

Losartan reverses hyperuricemia-induced increases in serum UA and plasma vasoactive substances

The 4 groups had similar serum UA levels and plasma renin/angiotensin activities at the baseline ($P > 0.05$).

Levels of serum UA and plasma renin/angiotensin activities remained unchanged in the control group 12 weeks later (all *P* values > 0.05); however, these biochemical measures significantly increased in both the HU group (serum UA, 50.4 ± 6.0 μM *vs.* 85.2 ± 7.0 μM, $P < 0.001$, plasma renin, 6.4 ± 0.4 μmol/L *vs.* 8.1 ± 0.4 μmol/L, $P < 0.001$, plasma angiotensin II, 10.6 ± 0.9 μmol/L *vs.* 14.6 ± 1.1 μmol/L, $P < 0.001$) and the HU + HC group (serum uric acid 49.8 ± 5.3 μM *vs.* 88.8 ± 7.5 μM, $P < 0.001$, plasma renin, 6.4 ± 0.4 μmol/L *vs.* 8.3 ± 0.5 μmol/L, $P < 0.001$; plasma angiotensin II, 10.6 ± 0.9 μmol/L *vs.* 14.1 ± 1.1 μmol/L, $P < 0.001$), to a similar extent.

In contrast, plasma renin activity did not change in the HU + losartan group, but serum UA concentrations (50.82 ± 4.89 *vs.* 56.83 ± 5.34, $P < 0.05$) and angiotensin II activity (10.6 ± 1.0 *vs.* 11.9 ± 1.0, $P < 0.05$) also significantly increased in the HU + losartan group after 12 weeks of the diet compared to the baseline (**Figure 1**).

Losartan alleviates hyperuricemia-induced aortic atherosclerosis

Microscopic measurements showed that the HU and HC + HC groups had a significantly similar increase in intima thickness (control *vs.* HU *vs.* HC + HU, 210.2 ± 8.2 μm *vs.* 486.7 ± 11.8 μm *vs.* 493.7 ± 10.2 μm, $P < 0.001$) (**Figure 2A**) and area (19.9 ± 29.2 × 10^3 μm^2 *vs.* 280.7 ± 45.1 × 10^3 μm^2 *vs.* 292.5 ± 42.1 × 10^3 μm^2, $P < 0.001$) (**Figure 2C**) compared to the control group, while the HU + losartan group exhibited a tunica intima similar to that in the control group in terms of intima thickness (211.3 ± 16.5 μm, $P > 0.05$) (**Figure 2A**) and area (93.2 ± 12.2 × 10^3 μm^2, $P > 0.05$) (**Figure 2C**). However, the groups were comparable with respect to media thickness, except for the HU + losartan group, which showed significantly less thickness ($P < 0.001$) (**Figure 2B**) but an area without significant difference (**Figure 2D**).

Losartan inhibits hyperuricemia-induced PCNA overexpression in aortic tunica intima

Immunohistochemistry revealed PCNA as brownish particles present in the tunica intima. Compared to the control group (**Figure 3A**), HU (**Figure 3B**) and HC + HU (**Figure 3C**) groups had a significant and similar increase in PCNA immunopositivity (control *vs.* HU *vs.* HC + HU, 9.6% ± 1.6% *vs.* 28.4% ± 1.6% *vs.* 31.6% ±

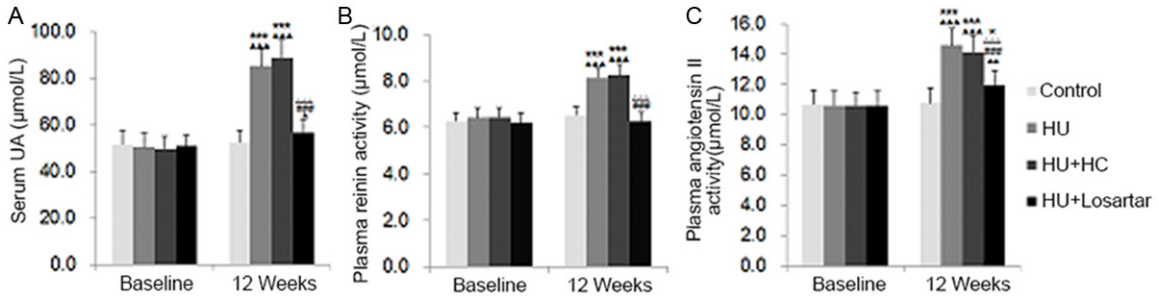

Figure 1. Changes in (A) serum UA, plasma activities of (B) renin and (C) angiotensin II at baseline and 12 weeks from baseline. All data are expressed as the mean ± SD (n = 12). Compared to the control group, $^{*}P < 0.05$, $^{***}P < 0.001$; compared to the model group, $^{\Delta}P < 0.05$, $^{\Delta\Delta\Delta}P < 0.001$; compared to the hypercholesterolemia group, $^{\#\#\#}P < 0.001$; compared to baseline in the same group, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$.

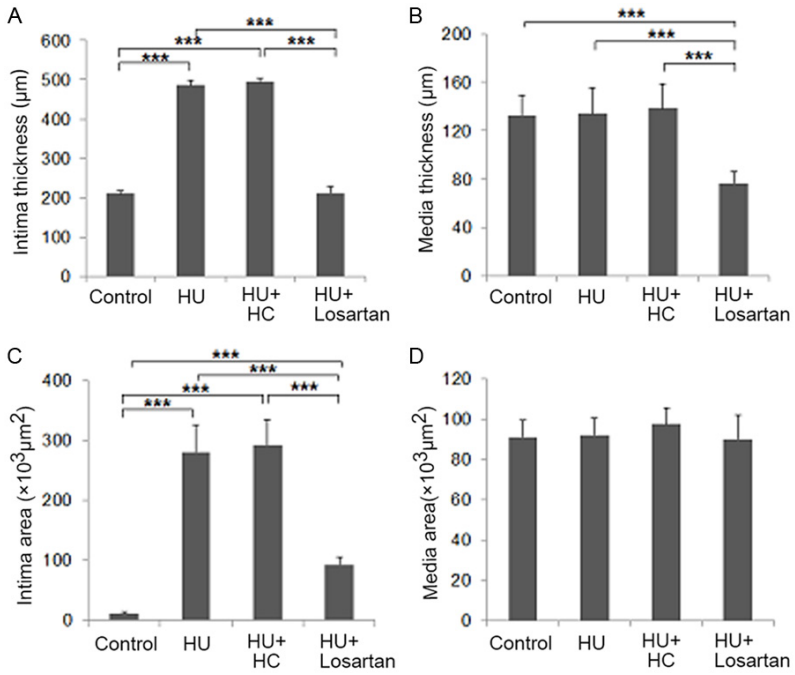

Figure 2. Macroscopy, histology and microscopic measurements of the inner aortic wall at 12 weeks: A: Intima thickness. B: Media thickness. C: Intima area. D: Media area. All data are expressed as the mean ± SD (n = 12) $^{***}P < 0.001$.

4.6%, $P < 0.01$), while the HU + losartan group (**Figure 3D**) exhibited a significantly smaller positivity percentage than the HU and HC + HU groups (16.5% ± 3.2%, $P < 0.001$; **Figure 3E**).

Losartan regresses hyperuricemia-induced mitochondrial injury of intima endothelial cells

On transmission electronic microscopy examination, the control group exhibited a smooth endothelial lining, and well aligned SMCs in which the mitochondria were well preserved in terms of their numbers and integrity (**Figure 4A**). HU (**Figure 4B**) and HC + HU (**Figure 4C**) groups showed a significant hyperplasia of endothelial cells and disorganized SMCs containing a large number of vacuoles and lytic mitochondria, with rupture and lysis of mitochondrial crests. In contrast, the HU + losartan group specimens had an ultramicroscopic morphology similar to the control group (**Figure 4D**).

Losartan antagonizes hyperuricemia-induced downregulation of liver LDLR mRNA and protein expression

Compared to the control group, the HU group had a significantly lower relative expression level of liver LDLR mRNA ($P < 0.001$), while the HC + HU group had a significantly greater downregulation of liver LDLR mRNA transcription than the HU group ($P < 0.001$). In contrast, the HU + losartan group exhibited significantly upregulated liver LDLR mRNA transcription compared to the other 3 groups ($P < 0.001$). For liver LDLR protein expression, the HU and HC + HU groups had significantly and similarly reduced expressions ($P < 0.001$) compared to the control group; however, the HU + losartan group showed a significantly less reduction compared to the HU and HC + HU groups, although still

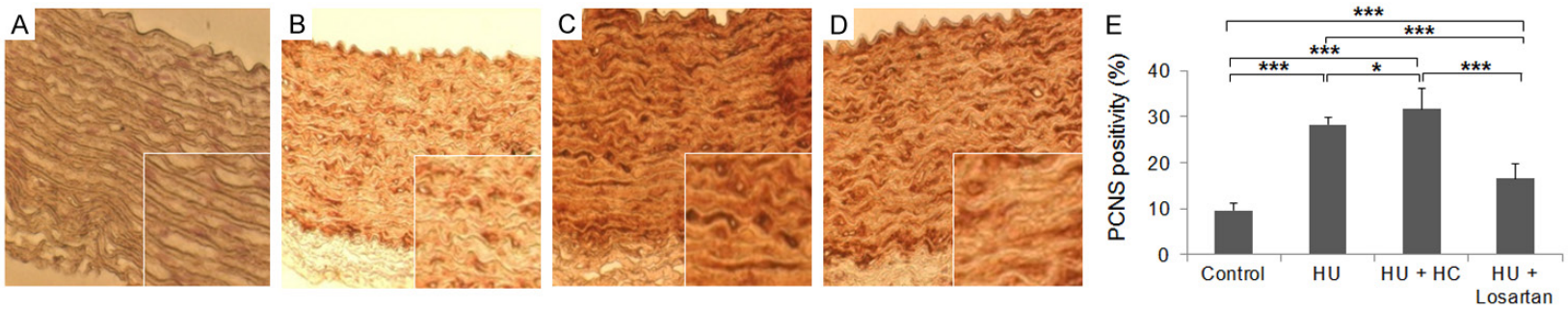

Figure 3. Representative microphotographs of the (A) control group, (B) HU group, (C) HU + HC group and (D) HU + losartan group (× 100; inserted images × 200). (E) The bar chart represent the PCNA positivity percentage expressed as the mean ± SD (n = 12).*P < 0.05, ***P < 0.001.

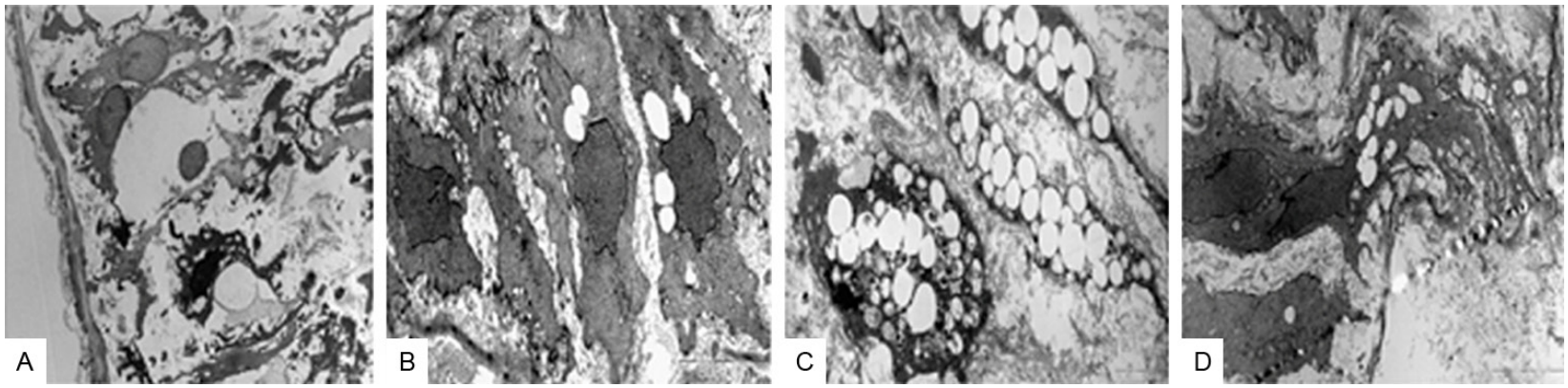

Figure 4. Transmission electronic micrographs of the tunica intima and media at 12 weeks. A: Control group. B: HU group. C: HU + HC group. D: HU + losartan group.

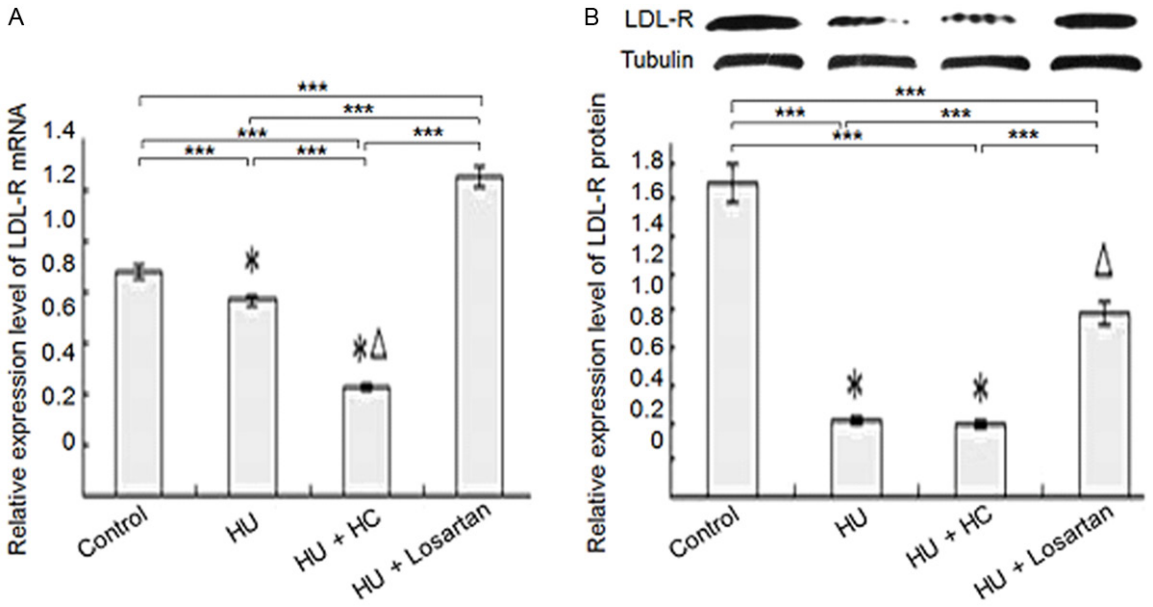

Figure 5. Relative expression levels of liver LDL receptor (A) mRNA and (B) protein. The upper panel shows representative PCR product bands or immunoblots and the lower panel shows the bar charts detailing the relative expression levels of liver LDL receptor mRNA and protein. All data are expressed as the mean ± SD (n = 12) $^{***}P < 0.001$.

below the level in the control group ($P < 0.001$); **Figure 5B**).

Discussion

As is characterized by endothelial injury, accumulation of cholesterol, infiltration of macrophages and proliferation of SMCs [14] and classical risk factors are hyperlipidemic states, diabetes mellitus, smoking and hypertension [1]. A major contributor to endothelial injuries is reactive oxygen species (ROS) with intracellular ROS production in vascular SMCs being mainly mediated by membrane-bound NAD (P) H oxidases. Angiotensin II is a powerful inducer of NAD (P) H oxidase and can stimulate the production of ROS eightfold in vascular SMCs [15]. In addition, angiotensin II elicits inflammatory responses in human vascular SMCs by stimulating cytokine production and activating nuclear factor-kappaB (NFkB) [16]. Increased ROS production leads to oxidation of LDL-cholesterol (oxLDL), which in turn induces fibroblast growth factor 1 (FGF-1) release believed to be a mediator of endothelial and SMC proliferation [17].

The oxidized lipoproteins are engulfed by migrating macrophages resulting in the formation of foam cells. If cholesterol derived from the uptake of oxidized LDL particles cannot be mobilized from the cell to a sufficient extent, it accumulates as cytosolic droplets, building the fatty streaks of the plaques in the tunica intima of arteries [18]. Our data has demonstrated that in the HU and HU + HC groups, considerable damage occurred to SMCs with a hypertrophic intima, accompanied by overexpression of PCNA, a marker indicative of cellular DNA replication. Importantly, it is noteworthy that UA alone can induce over proliferation of vascular SMCs.

Though the role of HU as an independent or dependent risk factor for coronary heart disease is not unequivocally known [9, 10], several groups have reported that there is a direct causal role for UA in the pathogenesis of hypertension and atherosclerosis [19-22], which is in agreement with our results, which showed that HU alone could induce experimental AS in rabbits to an extent similar to HU, with complicating HC. In contrast to AS risk factors like hypertension and hyperlipidemic states, less data has been published about the mechanisms related to UA and AS. Corry et al. reported that UA stimulates proliferation, angiotensin II production and oxidative stress in vascular SMCs through tissue renin-angiotensin system (RAS). Corry et al. and Kanellis et al. noted that UA also can react to form radicals, increase lipid oxidation and induce various pro-oxidant effects in vascular cells [22].

It has been shown, that treatment with losartan can effectively reduce angiotensin induced ROS production [15]. Our results also demonstrated that losartan could alleviate HU-induced experimental AS by downregulating angiotensin II activity. Our data also showed that HU alone could significantly increase plasma renin/angiotensin activity in a way similar to HC with complicating HU, while medication with losartan could inhibit the RAS and decrease serum UA levels, which is in agreement with previous studies, which suggested that RAS regulates UA metabolism by controlling excretion and reabsorption of UA in the proximal convoluted tubule [23]. Plasma renin activity was not enhanced by losartan treatment in the present study.

LDL receptor, a glycoprotein expressed on hepatocytes, endothelial cells, SMCs and monocytes/macrophages function to transport serum LDL and very low-density lipoprotein into hepatocytes, actions mediated by apolipoproteins B and E [24]. In both the HU and HU + HC groups, LDLR were significantly downregulated, an effect reversed by losartan. Up until now, only downregulation of the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) by losartan medication have been reported [25, 26], but since blood lipid patterns are strongly associated with serum UA levels [27], losartan medication also changed LDL serum concentrations via UA elimination, and LDLR expression returned to normal levels.

In conclusion, HU alone can induce experimental AS by causing endothelial dysfunction and mitochondrial injury, SMCs over proliferation and liver LDL dysmetabolism. The reversal effects of losartan suggest that RAS is pivotal for the pathogenesis of HU-induced AS. The bench-to-bed translation of the present work is that the addition of losartan or other RAS inhibitor may be therapeutically beneficial in hyperuricemic patients at high risk of AS. It remains to be investigated whether high serum UA levels contribute to the occurrence and progression of AS through pathogenic mechanisms others than RAS.

Disclosure of conflict of interest

None.

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