Dynamic Changes in Plasma Urotensin II and Its Correlation With Plaque Stability

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Abstract: Urotensin II (UII) is involved in the formation of atherosclerosis, but its role in the stability of atherosclerotic plaques is unknown. The purpose of this study was to observe the dynamic changes in plasma UII and analyze its relationship to the stability of atherosclerotic plaques. One hundred thirty-five consecutive patients with acute coronary syndrome (ACS) were enrolled. The plasma UII levels were measured immediately after admission and during three-month follow-up. A vulnerable plaque model was established using local transfection of a recombinant P53 adenovirus into plaques in rabbits fed with a high-cholesterol diet and subjected to balloon arterial injury. The levels of plasma UII were measured weekly. The changes in plasma UII during the formation of atherosclerotic plaques and before and after plaque transfection were observed. The morphology of the plaques and the expression, distribution, and quantitative expression of UII in the plaques also were observed. Our results showed that the levels of plasma UII in patients with ACS at admission were lower than levels observed at the three-month follow-up. UII dynamic changes and its correlation with plaque stabilities were further verified in rabbits with atherosclerotic vulnerable plaques. The UII levels in rabbits were significantly decreased immediately after the P53 gene transfection, which led to plaque instability and rupture. These results suggested that UII expression was down-regulated in ACS, which may be related to its ability to modulate mechanisms involved in plaque stability and instability.

Key Words: biomarker, acute coronary syndrome, atherosclerosis, cardiovascular disease, coronary heart disease

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INTRODUCTION

The primary pathology of coronary heart disease (CHD) is coronary atherosclerosis, characterized by dynamic changes in the stability of atherosclerotic plaques.1 Unstable plaques are easily eroded or ruptured, leading to thrombosis that results in complete or partial occlusion of coronary arteries.2 This condition is clinically named acute coronary syndrome (ACS), which is the primary cause of death in patients with CHD.2 Therefore, the early detection of vulnerable individuals is crucial for preventing severe adverse clinical events in CHD patients. However, biomarkers that could be used in clinical settings and help predict the future course of plaque formation and stability have not been identified.

Urotensin II (UII) is an extremely potent vasoactive substance.3 When UII binds to the G protein-coupled receptor, GPR14 (also named as UT), it produces a range of biological effects and is involved in many cardiovascular-related diseases, including essential hypertension, coronary heart disease, congestive heart failure, diabetes mellitus, and renal failure.3–5 Also, recent evidence has suggested that UII contributes to the development of atherosclerotic cardiovascular diseases.6–9 For example, UII induces monocyte chemotaxis and contributes to the recruitment of monocytes expressing the UT receptor to atherosclerotic lesions.10 Subsequently, this event promotes the accumulation of inflammatory cells in the plaque and accelerates foam cell formation,11,12 which indicates that UII is involved in the development of athero-sclerotic plaques. However, the change of UII in atherosclerotic plaques and its correlation with the stability are not clear.

At present, the results of several comparative studies on plasma UII levels in patients with ACS or with stable coronary heart disease (CHD) were inconsistent and ambiguous. Khan et al13 and AI Kindi et al14 found that the UII plasma levels were elevated in ACS patients immediately after clinical presentation. On the other hand, Babińska et al and Joyal et al reported that the UII plasma levels were significantly decreased in ACS.15,16 In these studies, the comparison of plasma UII levels between ACS and CHD patients were comparisons among different individuals with confounding factors, including differences in plaque loads and percutaneous coronary interventions (PCIs) among the groups that biased the analysis of the results.

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Ethics approval and consent to participate: This study complied with the Declaration of Helsinki and was approved by the ethics committee of the Xuanwu Hospital Capital Medical University (approval number LYS [2016] 010). Written informed consent was obtained from patients and healthy control subjects. This study conformed to the Guide for the Care and Use of Laboratory Animals and was approved by the Laboratory Animal Administration Committee of Capital Medical University (Ethics protocol number AEEI-2015-001). The methods were carried out in the experiments in accordance with the approved guidelines.

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To investigate the relationship between plasma UII levels and the stability of atherosclerotic plaques, we observed the dynamic changes in plasma UII levels in the same patients during acute events and stable periods to avoid the effects of the confounding factors mentioned above. Furthermore, we used a rabbit atherosclerotic vulnerable plaque model to explore alterations in UII expression during changes in atherosclerotic plaque stability.

MATERIALS AND METHODS

Study Subjects

One hundred thirty-five consecutive ACS patients, without age limit, were admitted to the cardiology department and enrolled in the study. Patients with chronic stable coronary heart disease, other cardiovascular diseases, including chronic heart failure, congenital heart disease, valvular disease, and cardiomyopathy, other systemic diseases, including chronic liver, and kidney insufficiency, hematological diseases, digestive system diseases, connective tissue diseases, neurological diseases, neoplastic diseases, severe infections, trauma, and pregnant or lactating patients were excluded. For the follow-up assessment, ACS patients who received standard secondary prophylactic treatment for coronary heart disease were discharged after 3 months if they had not experienced an acute ischemic attack or were clinically stable, which represented plaque stability. All other patients received standard secondary prophylaxis for coronary heart disease, including statins, after discharge. Forty-eight healthy nursing and care worker volunteers working in the cardiology department served as healthy control subjects. The study complied with the Declaration of Helsinki, a statement of ethical principles for medical research involving human subjects, and was approved by the ethics committee of Xuanwu Hospital Capital Medical University [LYS (2016) 010]. Written informed consent was obtained from the patients and healthy control subjects.

UII Measurement

The baseline data from the patients were recorded, and the UII levels were measured immediately upon admission and at the three-month follow-up time using radioimmunoassay as previously described. Briefly, venous blood was collected into tubes containing ethylene diamine tetraacetic acid and aprotinin. The blood samples were centrifuged immediately for 15 minutes (2000 g at 4°C), and the supernatants were stored at −70°C until measurement. For the UII immunoreactivity assay, the samples displaced the traces parallel to the standard curves and the cross-reactivity between human and rabbit UII was 100%. No cross-reactivity was found among human and rabbit angiotensin II, brain natriuretic peptide, or endothelin. The UII intra-assay and interassay coefficients of variation for the blood samples were <10%. The high-sensitivity C-reactive protein (hs-CRP) content was determined using a highly sensitive enzyme immunoassay kit for C-reactive protein (hs-CRP ELA Kit; Beijing Yonghan Xinggang Biotechnology Co, Ltd, Beijing, China).

Animal Study Using the Rabbit Atherosclerotic Vulnerable Plaque Model

Thirty-two male Japanese white rabbits (conventional, 8-week-old and weighing 1.5–2.0 kg) were provided by the Laboratory Animal Center of Capital Medical University. The rabbits were randomly divided into 4 groups: control, normal diet; high-fat diet (HFD) (containing 5% lard, 1.5% cholesterol, and 5% egg yolk powder) alone; HFD plus BI, high-fat diet plus balloon injury (BI); and HFD plus BI plus P53, high-fat diet plus BI plus P53 transfection. The rabbits were individually housed in metal cages in an air-conditioned room under a 12-hour light/dark cycle. Water was allowed ad libitum, and 100 g/d food was provided to each rabbit. No adverse events were observed. The rabbits were anesthetized with a 1:1 Zoletil 50 and ketamine mixture (1 mL/kg) given intramuscularly, and the right femoral artery was exposed. A 3F Fogarty balloon catheter (American Baxter Healthcare Corporation) was introduced through the right femoral artery and advanced 25 cm proximally into the iliac artery then into the abdominal aorta. The balloon was inflated with saline to distend the abdominal artery and pulled back to the femoral artery. This step was repeated 3 times. Eight weeks after arterial injury, the rabbits in the HFD plus BI group were transsected with $0.5 \times 10^{13}$ pfu/L of a recombinant human P53 adenovirus gene (Shenzhen Sebano Gene Technology Co, Ltd, Shenzhen, China). The atherosclerotic vulnerable plaque model was replicated, as previously described. In brief, after the rabbits were intravenously heparinized (1000 IU) and anesthetized as mentioned above, 30 μL of recombinant human p53 adenovirus gene suspension ($0.5 \times 10^{13}$ pfu/L) were injected into the abdominal aortic segments rich in atheromatic plaques, which were mainly located between the right renal and the common iliac arteries. The suspension was left in situ for 10 minutes after the temporary occlusion of the aortic segment with artery clamps. The site of injection was marked with a nesis, and the abdominal cavity was closed. Blood samples were collected weekly from the auricular vein, and the UII plasma levels were determined using radioimmunoassay.

The animal experiments were approved by the Laboratory Animal Administration Committee of Capital Medical University (Ethics protocol number AEEI-2015-001) and conducted according to the Guidelines for Animal Experimentation of Capital Medical University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011). All sections of this report adhered to the ARRIVE Guidelines for reporting animal research.

Histopathology, Immunohistochemistry, and Plaque Stability Evaluation

In the ninth week, the rabbits were injected intramuscularly with a 1:1 mixture of Zoletil 50 and Ketamine (1 mL/kg). The arteries were quickly dissected, rinsed with cool PBS, fixed in 4% paraformaldehyde, and embedded in paraffin. Once the arterial samples were collected, the rabbits were euthanized by injecting 20–50 mL of air into the ear vein. The paraffin-embedded arteries were sectioned at 5 μm and the
sections were placed on microscope slides, deparaffinized, and stained for histological analysis with hematoxylin and eosin, Verhoeff-van Gieson, Sirius red, and Oil-red O. Additional sections were stained for immunohistochemistry using Mac-2, alpha-smooth muscle actin, and UII antibodies as previously described.20 The areas with positive staining for lipids, collagen, vascular smooth muscle cells (VSMCs), and macrophages were expressed as the percentage of the stained area divided by the plaque area, with least at 10 high power fields (×400) that were sampled. The plaque vulnerability index was calculated as (macrophage staining% plus lipid staining%)/(SMCs% plus collagen fiber%).21 All quantification of image data was performed using ImageJ software (NIH, Bethesda, MD).

Quantitative Real-time Polymerase Chain Reaction Analysis

Total RNA was extracted using the Trizol reagent method (Invitrogen), and total RNA (1 μg) was reverse-transcribed to generate cDNA using the GoScript Reverse Transcription System (Promega, Madison, WI). The quantitative real-time polymerase chain reaction was performed using the iCyclerIQsystem (Bio-Rad, Hercules, CA) as previously described.22 The following primers were used: UII-Forward primer: 5'-CTTCAGCTTCCCCGCC-3', UII-Reverse primer: 5'-GACCTCGCACCCAAAAC-3'. UII expression was assessed as the relative expression of the gene of interest to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Statistical Analysis

All values were expressed as means ± SD. The statistical analyses were performed using the two-tailed unpaired Student’s t test when comparing 2 groups and with one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test for multiple comparisons. To analyze dynamic changes in plasma UII levels among the different groups of rabbits, 2-way repeated measures ANOVA followed by Tukey’s post-hoc tests were performed. All analyses were conducted using the GraphPad Prism version 7 Software. P-values < 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

The characteristics of the ACS subjects are summarized in Table 1. The healthy control group included primarily young women with a low incidence of atherosclerosis. Low-density lipoprotein levels in ACS patients at the follow-up were significantly decreased than at admission (1.75 ± 0.48 mmol/L, 2.66 ± 0.81 mmol/L, P < 0.05). There were no other differences between the individual patient cohorts that may contribute to the observed results.

UII Content in Plasma from ACS Patients

The UII plasma levels in ACS patients were higher than the healthy control subjects (Fig. 1A). We divided the ACS patients into 3 groups [unstable angina pectoris (UAP), non-ST-segment elevation myocardial infarction (NSTEMI), and...
STEMI] and observed that UII was significantly lower in patients with STEMI compared with the UAP and NSTEMI groups (Fig. 1B). Interestingly, UII was higher again in ACS patients 3 months after discharge (Fig. 1C).

UII and hs-CRP Levels in the Rabbit Vulnerable Plaque Model

To observe dynamic UII changes, a vulnerable plaque model was created using rabbits fed with a HFD, BI, and P53 transfection. The results showed that HFD plus BI significantly increased the UII levels (Fig. 2A). However, we observed that the UII levels decreased immediately after P53 transfection, then increased significantly one week after P53 transfection (Fig. 2A). Because serum hs-CRP levels are a sensitive indicator of inflammation, which is closely related to the progression of plaque formation, we measured the hs-CRP levels in the rabbits. The HFD plus BI treatment resulted in a significant increase in hs-CRP content, which did not decrease after P53 transfection (Fig. 2B).

Histological Changes in the Plaques

To verify the success of the atherosclerotic vulnerable plaque model, HE and elastin staining were assessed in the iliac and abdominal aortic arteries of the rabbits. Compared with the control group, HFD induced increased arterial thickness and disordered elastic fibers. The BI or BI plus P53 transfection further aggravated these changes, and numerous inflammatory cells infiltrated the vessels, which was accompanied by thinning of the fibrous cap and intraplaque bleeding (Fig. 3). We also measured plaque stability using the vulnerability index. As shown in Figure 4, the vulnerability index was significantly higher in the HFD plus BI plus P53 group (Fig. 4). These results demonstrated that localized P53 transfection of the plaques altered the composition and vulnerability of the abdominal artery plaques.

UII Expression and Distribution

Compared with the control group, the HFD plus BI and the HFD plus BI plus P53 treatment groups exhibited increased UII mRNA expression (Fig. 5A). In the HFD alone
group, UII expression was not induced in the plaques (Fig. 5A). As seen in Figure 5B, immunohistochemical staining revealed that UII expression (green) was localized primarily in endothelial and foam cells, and in the smooth muscle cell–rich compartment of the plaques and the arterial tissues of the HFD plus BI group. The HFD plus BI plus P53 transfection group exhibited strong UII protein expression, which was mainly concentrated in the cytoplasm (Fig. 5B). However, minimal UII expression was observed in the control and HFD groups (Fig. 5B).

Previous studies13,15 have found that low UII levels after acute myocardial infarction are associated with negative adverse events, including death, heart failure, emergency revascularization, or more severe myocardial injury.

**DISCUSSION**

In this study, the levels of plasma UII in patients with ACS were significantly higher than in healthy controls ($P < 0.05$). Previous studies have demonstrated a positive correlation between plasma UII levels and the severity of atherosclerosis.23,24 It is noted that age and sex are uncontrollable risk factors in the development of atherosclerosis.25 The degree of atherosclerosis increases with age. Compared with men, the incidence of atherosclerosis in premenopausal women is significantly lower. Therefore, primarily premenopausal women were selected as healthy control subjects in this study, and the degree of atherosclerosis was significantly lower in that group.

The changes in plasma UII levels in patients with different types of ACS revealed a decreasing trend from UAP to NSTEMI to STEMI. However, there was no statistical difference between the UAP and acute myocardial infarction groups because of the small number of cases of UAP. One limitation of this study was that the UAP sample size was small, so it may not accurately represent the actual range of UII levels of the patients in the UAP subgroup. Most cases of ACS result from the loss of integrity of the protective
FIGURE 4. Plaque vulnerability index determination. 

A, Macrophages were detected using Mac-2 staining. B, SMCs actin staining was detected using α-SMA. C, Collagen fibers were detected using Sirius red staining. D, Lipids were detected using oil red O staining. 

E, Determination of plaque vulnerability index in each group. The data are expressed as means ± SD. Statistical comparisons were conducted using one-way ANOVA followed by Tukey’s multiple comparison tests; *P < 0.05 versus control or HFD, #P < 0.05 versus control or HFD. α-SMA, alpha-smooth muscle actin.
covering of the atherosclerotic plaque. This occurs with plaque rupture that results from disruption of the fibrous cap overlying the plaque or erosion that disturbs the endothelial lining of the plaque. Compared with non–ST-elevation ACS, a higher percentage of cases in acute STEMI result from plaque rupture.26 This may indicate that plaques in STEMI are more unstable and, therefore, UII levels are lower.

Most of the previous studies that compared plasma UII levels in patients with different types of coronary heart disease, such as ACS and stable coronary heart disease, were not included based on strict definitions of ACS or stable CHD. Also, the effects of PCI treatment on UII were not considered, so the results were not consistent. To avoid the influence in this study of the intergroup confounding factors mentioned above, we followed patients with acute myocardial infarction and compared the changes in plasma UII levels at admission and 3 months after treatment when the plaque conditions were stable. The results indicated that the UII levels in the stable period (e.g., plaque stability) were significantly higher than during the acute stage (plaque instability). These observations suggested that the level of UII may reflect the dynamic changes in the plaque condition and the plaque stability or instability.

Studies have shown that UII plays a role in the development and progression of atherosclerosis.8,27 UII stimulates endothelial and smooth muscle cell proliferation, inhibits endothelial cell apoptosis, increases collagen 1, and decreases MMP-1 expression.28–32 Taken together, these effects promote cell- and matrix-enriched plaques, suggesting that UII might be protective against plaque rupture. Several studies have shown that ACS patients have significantly lower UII plasma levels than patients with stable coronary heart disease.16,33 A low UII level after acute myocardial infarction is associated with negative adverse events, including death, heart failure, myocardial infarction, or emergency revascularization.13 Based on the above basic and clinical studies, UII may play an important role in plaque stability,
and low UII expression may be used to predict plaque instability and the occurrence of adverse clinical events.

We established a rabbit atherosclerotic vulnerable plaque model to verify the relationship between the dynamic changes in plasma UII and plaque stability. Rabbits are a suitable model for atherosclerosis research due to their rapid development of hyperlipidemia and atherosclerosis resulting from their sensitivity to dietary fat and cholesterol. Also, rabbit atherosclerotic lesions resemble those observed in humans, ranging from early-stage lesions (fatty streaks) to complicated lesions (fibrous plaques). Finally, rabbits exhibit lipoprotein profiles that are more similar to humans than mice.34 The atherosclerotic plaques produced in this study were incubated transmurally with recombinant adenovirus carrying a P53 transgene, which has been demonstrated to induce remodeling in atherosclerotic plaque destabilization.35

The destabilization of local preexisting atherosclerotic plaques was induced using an intra-arterial injection of the vector, which was characterized by infiltration of numerous inflammatory cells, thinning of the fibrous caps, and intraplaque bleeding (Fig. 3). Interestingly, one day after transfection, the plasma UII levels decreased significantly. However, the plasma UII levels increased one week later, although the morphological manifestations of the plaques were still unstable. Moreover, the UII plasma levels were positively correlated with the expression of UII mRNA in the plaques (Figs. 2 and 5).

Apoptosis of plaque cells induced by the P53 gene, which is believed to be primarily responsible for the development of the destabilized cap phenotype through a proportional decrease in collagen production by the cap VSMCs, is reported to be extremely rapid, occurring within several hours after transfection.36 Thus, the concomitant decrease of the plasma UII levels 24 hours after transfection suggested that UII may be an essential mediator of plaque destabilization. The upregulation of UII expression in the plaques and increased UII levels in plasma one week after transfection may be related to the inherent resistance of the plaques to injury and the activation of repair potential in the plaques induced by remodeling after transfection. A similar phenomenon has been reported in clinical studies, in which plasma UII levels increased after acute myocardial infarction (plaque instability or rupture) or percutaneous coronary intervention procedures (plaque injury), and the clinical prognosis was positively correlated with the increased plasma UII levels.14,17 Therefore, this study demonstrated that the changes in plasma UII levels were positively correlated with the expression of UII in the atherosclerotic plaques, and UII was involved in modulating plaque stability and instability. The transformation of a stable plaque to an unstable one involves complex mechanisms, including cell apoptosis and extracellular matrix degradation that promote plaque destabilization and rupture. Therefore, the effect of UII was related to its ability to modulate mechanisms involved in plaque stability and instability, which needs additional exploration. We provided evidence that supported a potential role for UII in the destabilization and rupture of atherosclerotic plaques. Using a rabbit animal model, we confirmed that HFD plus BI increased UII levels in the experimental rabbits, and more importantly, UII levels were significantly decreased after P53 transfection, which led to plaque instability.

Our results demonstrated that UII was significantly decreased in ACS patients, especially in patients with STEMI, which indicated adverse clinical events. This result was further verified by the rabbit vulnerable plaque model. In conclusion, the level of plasma UII was significantly decreased in ACS and may serve as a reliable biological marker to reflect the progression and stability of atherosclerotic plaques and could prove useful in predicting adverse events.

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