Original Article

Allopurinol Reduces Neointimal Hyperplasia in the Carotid Artery Ligation Model in Spontaneously Hypertensive Rats

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Uric acid and oxidative stress promote cardiovascular diseases, including atherosclerosis and hypertension. Xanthine oxidase, through which uric acid is generated, is a free-radical generating enzyme. The aim of the current study was to investigate whether allopurinol, an inhibitor of xanthine oxidase activity, affects vascular remodeling and vascular smooth muscle cell (VSMC) proliferation. In the carotid artery ligation model using spontaneously hypertensive rats (SHR), treatment with allopurinol induced a reduction in the neointima/media ratio by 27% (38.5±34.3% in the control group and 28.1±20.8% in the allopurinol-treated group, respectively, $p<0.01$) without alterations in vascular circumference at 3 weeks after ligation when compared to the control. Allopurinol lowered the serum uric acid concentration (147.0±3.6 μmol/l in the control group and 16.1±3.6 μmol/l in the allopurinol-treated group, respectively $p<0.01$) and xanthine oxidase activity, but not the blood pressure. In an *in vitro* study, high concentrations of uric acid (100 and 200 μmol/l) stimulated VSMC growth, but there was no stimulation of these cells by a low concentration of uric acid (50 μmol/l) or by any of three concentrations of xanthine (50, 100 and 200 μmol/l). In addition, allopurinol (5 μmol/l) had no effect on the cell growth. In conclusion, uric acid is a potent stimulator of VSMC proliferation, and allopurinol prevented vascular remodeling in SHR at least in part by inhibiting uric acid concentration. (*Hypertens Res* 2006; 29: 915–921)

***Key Words*: uric acid, xanthine oxidase, allopurinol, spontaneously hypertensive rat, vascular smooth muscle cell**

Introduction

Several clinical epidemiological studies have suggested that hyperuricemia is as an independent risk factor in the development of cardiovascular diseases (*1–5*). Although the detailed mechanisms of these actions remain unknown, uric acid has been reported to have a proliferative effect on vascular smooth muscle cells (VSMC) (*6*) and is known to be involved in the pathogenesis of vascular endothelial dysfunction and

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hypertension (*7–11*). On the other hand, xanthine oxidase (XO) catalyzes the formation of uric acid from precursors, *i.e.*, xanthine (XA) and hypoxanthine. Several clinical and basic experiments have indicated that XO is one of the principle causes of cardiovascular diseases, including myocardial ischemic reperfusion injury, hypertension and heart failure, and that inhibition of XO improves these pathophysiological conditions (*12–17*). Although XO and uric acid are thought to be intimately involved in the pathogenesis of hypertension (*18–20*), to date, the effect of XO inhibitors on vascular remodeling in hypertensives has not been tested. Because spontaneously hypertensive rats (SHR), an animal model of hypertension, have higher vascular XO activity and serum uric acid levels than normotensive rats (*19*), we used SHR to study the effects of allopurinol on vascular remodeling *in vivo* and VSMC growth *in vitro*.

Methods

Animals

Eight-week-old male SHR (SLC, Shizuoka, Japan) were maintained in a room in an animal facility with a 12-h light/dark cycle and free access to normal rat chow and water. The allopurinol group (n=10) received water containing allopurinol (30 mg/kg body weight [BW]/day, provided by Glaxo SmithKline [Tokyo, Japan]), and the control group was given normal water (n=10). Allopurinol treatments were initiated on the day of the surgical procedure and continued for 21 days. Investigators performing the operative procedures and investigational analysis were blind with respect to the treatment and control groups. Experimental protocols were approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Tottori University.

Carotid Artery Ligation Model

Rats were anesthetized by intraperitoneal administration of ketamine (80 mg/kg BW; Sankyo, Tokyo, Japan) and xylazine (5 mg/kg BW; Bayer, Tokyo, Japan). Following a midline incision in the neck, both common carotid arteries (CCA) were exposed and the left CCA was ligated with 8-0 nylon just proximal to the carotid bifurcation (*21*, *22*). A carotid artery ligation model was employed as XO/XA dehydrogenase is mainly expressed in the endothelium (the carotid artery ligation model differs from the commonly used balloon-injury model in that the endothelium remains intact).

Histomorphometric Analysis

After 3 weeks, rats were anesthetized and blood was obtained from the inferior vena cava, followed by perfusion with 10% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min at 100 mmHg. Ligated and contralateral CCAs were carefully removed. The proximal third portion excluding the most proximal 0.5 mm of the ligature site of the CCAs was dehydrated and embedded in paraffin. Cross-sections located 250 μm apart and spanning the entire length of the embedded CCA of the ligated artery were prepared (5 μm thickness) and stained with hematoxylin and eosin with/without Victoria blue. External circumference, medial and intimal cross-sectional areas were measured using Scion Image software (Scion Corporation, Frederick, USA). Some sections were immunohistochemically stained with α-actin antibody (NeoMarkers, Fremont, USA).

Blood Pressure and Heart Rate Measurement

Blood pressure (BP) and heart rate (HR) were examined on day 1 (before the surgical procedure) and day 21 (before euthanasia) by tail-cuff plethysmography (KN-214 II; Natsume, Tokyo, Japan) on conscious restrained rats. Each measurement was performed three times, and the values were averaged and recorded.

Serum Analysis

Serum XO activity was evaluated as previously reported (*23*). Briefly, after preincubation for 5 min at 37°C of 240 μl 0.2 mol/l Tris-HCl containing 113 μmol/l pterin with or without 4 mmol/l nicotinamide adenine dinucleotide, the enzyme reaction was started by adding 60 μl of serum. After incubation for 10 min, 100 μl of the reaction mixture was added to 100 μl of 4% $HClO_4$. The resultant mixture was vigorously shaken with an agitator and then centrifuged. The supernatant was neutralized with 5 mol/l K_2CO_3, and neutralized supernatants were used to measure XO activity by high performance liquid chromatography (HPLC; LC-6A HPLA apparatus, RF 530 fluorescence HPLC monitor, C-R3A chromatopac recorder; Shimadzu, Kyoto, Japan). Serum uric acid levels were evaluated using a Uric Acid C Test (Wako Pure Chemical Industries, Tokyo, Japan) following the manufacturer's instructions. Serum XA levels were evaluated by the HPLC method.

Isolation and Culture of SHR Thoracic Aortic Smooth Mmuscle Cells

Thoracic aortas were removed under sterile conditions and washed three times with PBS containing streptomycin, penicillin and fungizone. The excised aortas were cut into small pieces, placed in a collagen-coated cell culture dish and cultured in DMEM containing 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. When the cells reached a sub-confluent state, they were passaged by trypsinization. The cells from passages 4 to 6 were used for the experiments. The smooth muscle cell lineage was confirmed by cell morphology and positive staining with monoclonal antibodies against rat smooth muscle α-actin.

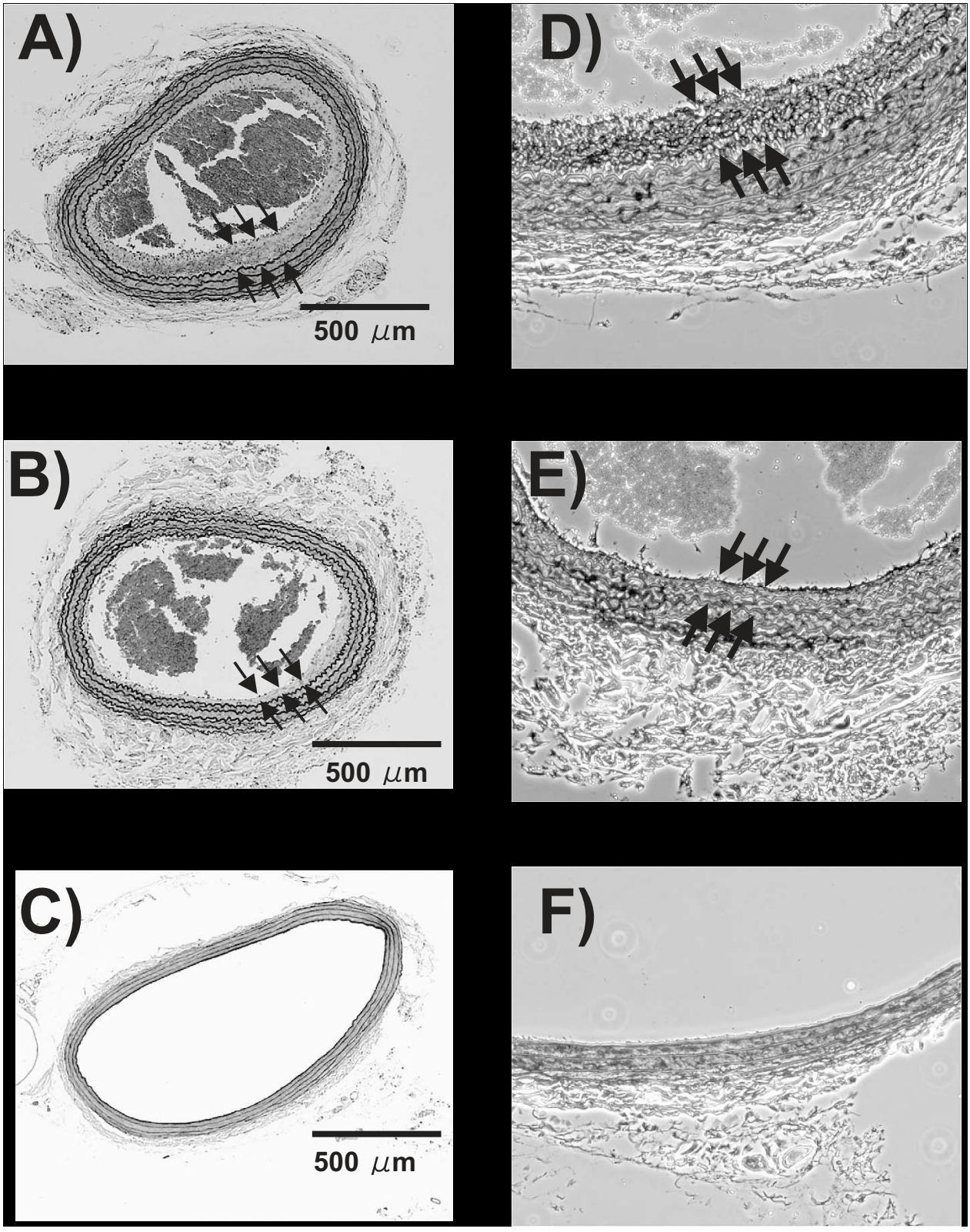

Fig. 1. *Representative sections from carotid arteries of rats 3 weeks after ligation. A, D: Ligated arteries of non-treated rats (*n = *10, control group). B, E: Ligated arteries treated with allopurinol (*n = *10, 30 mg/kg BW/day in drinking water, allopurinol group). C, F: Contralateral non-ligated arteries. Photographs are shown at ×100 (A–C) and ×200 (D–F). Arrows indicate neointimal formation.*

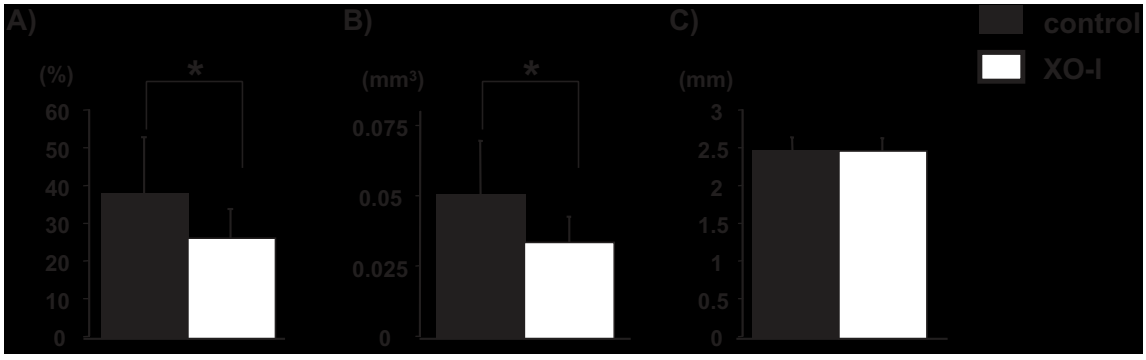

Fig. 2. *Effect of allopurinol on morphological changes in a rat carotid artery ligation model. A: Neointimal/medial area ratio. B: Neointimal formation. C: External circumference. Each value represents the mean±SD.* *p < *0.05 for the allopurinol-treated group* vs. *the control group.* n = *10 each.*

VSMC were plated at 5×10^4 cells/well in a 24-well plate. Pre-confluent VSMC were incubated in DMEM containing 0.4% FBS for 48 h to achieve quiescence. After 48 h, cells were challenged with increasing concentrations of uric acid or XA (Sigma, Tokyo, Japan) with or without allopurinol (5 μmol/l). Another 48 h later, cell numbers were counted three times under microscopy.

Statistical Analysis

All values were expressed as the mean±SD. Data were analyzed by a Student's *t*-test, assuming equal variance, to assess the difference between the two groups. Otherwise, the data were analyzed by ANOVA. StatView software Version 5 for Windows (SAS Institute Inc., Cary, USA) was used for all statistical analysis. A value of $p<0.05$ was considered statistically significant.

Results

Effects of Allopurinol on Vascular Remodeling

Ligated artery specimens demonstrated medial thickening (0.106±0.014 mm^2 in non-ligated arteries and 0.135±0.014 mm^2 in ligated arteries, respectively, $p<0.01$) and neointimal formation when compared with contralateral non-ligated artery specimens (Fig. 1A, C, D, F). Neointimal formation was attenuated in rats treated with allopurinol (0.051±0.019 mm^2 in the control group and 0.033±0.009 mm^2 in the allopurinol group, $p<0.05$), although medial thickening was not affected (0.135±0.014 mm^2 in the control group and 0.132±0.018 mm^2 in the allopurinol group) by allopurinol (Figs. 1, 2). The neointimal/medial area ratio was reduced by allopurinol treatment (38.1±15.5% in the control group and 26.0±7.6% in the allopurinol group, $p<0.05$). The external circumference of the left CCA did not differ between the two groups (2.48±0.11 mm in the control group and 2.45±0.18 mm in the allopurinol group). Cells comprising the neointima and media were determined to be smooth muscle cells by immunohistochemical staining with α-actin antibody (data not shown).

Effect of Allopurinol on BP and HR

Before carotid artery ligation, mean BP was 155.7±9.9 mmHg in control group and 156.7±10.8 mmHg in the allopurinol group, and HR was 330.7±27.7/min in the control group and 344.3±27.6/min in the allopurinol group. Three weeks after ligation, mean BP was 155.3±6.0 mmHg in the control group and 160.3±6.1 mmHg in the allopurinol group, and HR was 333.5±16.9/min in the control group and 350.0±14.3/min in the allopurinol group. There were no significant differences in these parameters between the two groups.

Serum Analysis

There were no significant differences in serum uric acid concentration or XO activity between before and after carotid artery ligation. Allopurinol treatment significantly reduced serum XO activity (1.10±0.06 μmol/mg/h in the control group and 0.09±0.035 μmol/mg/h in the allopurinol group, $p<0.01$) and serum uric acid concentration at euthanasia (2.47±0.06 mg/dl in the control group and 0.27±0.06 mg/dl in the allopurinol group, $p<0.01$). The serum XA concentration at euthanasia was below the detectable level (<0.005 mg/dl) in the control group and 0.47±0.89 mg/dl in the allopurinol group.

Uric Acid But Not XA Stimulated VSMC Proliferation *In Vitro*

One hundred and 200 μmol/l but not 50 μmol/l of uric acid resulted in increased VSMC proliferation (Fig. 3). On the other hand, none of the concentrations of XA (50, 100 and 200 μmol/l) had any influence on VSMC proliferation. In addition, allopurinol did not affect VSMC proliferation when

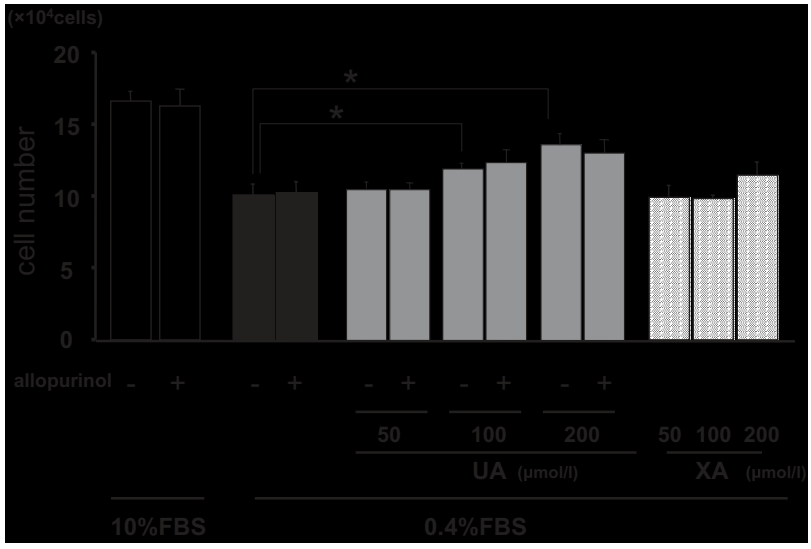

Fig. 3. *Effect of uric acid and xanthine on VSMC growth.* *$p < 0.01$ *for uric acid treated* vs. *0.4% FBS (control). UA, uric acid; XA, xanthine.* $n = 4$ *each.*

VSMC were incubated in 50, 100 or 200 μmol/l of uric acid, and 0.4% or 10% FBS (Fig. 3).

Discussion

In the present study, allopurinol suppressed neointimal formation in the carotid artery with no observable change in outer circumference in the SHR carotid artery ligation model. This inhibitory effect against inward vascular remodeling was brought about by the suppression of smooth muscle proliferation, as the thickened medial wall and neointima were mainly composed of smooth muscle cells. In addition, uric acid, but not XA, increased VSMC proliferation *in vitro*. These findings indicate that uric acid *per se* is responsible for inward vascular remodeling, and the inhibitory effect of the remodeling by allopurinol was in part the result of lowering the serum uric acid concentration.

Several epidemiological studies have suggested that hyperuricemia is an independent risk factor of cardiovascular diseases in patients with hypertension (*2–4*). However, the detailed mechanism is still unknown. Previous reports have suggested that XO pathways play key roles in the pathogenesis of hypertension and vascular dysfunction (*7*, *12*, *13*, *18–20*, *24*), but there have been no reports on the relation between XO pathways and vascular remodeling. Our results suggested that uric acid has potent proliferative effects on VSMC independent of XO activity and that the inhibitory effects on VSMC proliferation by XO inhibition *in vivo* were brought about through suppression of uric acid production. Uric acid has also been reported to play a role in atherosclerosis and endothelial dysfunction. Carotid intima-media thickness has been reported to correlate with serum uric acid level in healthy subjects and elderly subjects (*25*, *26*). Recently, we reported impaired endothelium-dependent vasodilation in hyperuricemic patients without any cardiovascular disease (*27*). Further, in a rat study, oxonic acid–induced hyperuricemia reduced the level of serum nitrates (NO), and allopurinol reversed this decrease in NO (*28*). Taken together, these results indicate that uric acid *per se* may be one of the plausible explanations for our results.

Although serum uric acid levels in SHR are higher than in normotensive rats (*19*), in both models the levels are still much lower than those in humans. However, a slight increase in serum uric acid concentration from 1.18 mg/dl (70 μmol/l) to 1.75 mg/dl (104 μmol/l) has been reported to bring about dramatic increases in the BP of rats (*29*). Therefore, small changes in serum uric acid concentration in rats may not be comparable to similar changes in humans. The different uric acid concentrations we utilized in *in vitro* studies (50–200 μmol/l) had different effects on the proliferation of VSMC (Fig. 3). In our *in vivo* study, the serum uric acid levels in pre- and post-medicated SHR were 2.47 mg/dl (147.0 μmol/l) and 0.27 mg/dl (16.1 μmol/l), respectively. We considered that this small but significant decrease of serum uric acid concentration showed a strong influence on vascular remodeling.

Uric acid has been reported to stimulate the production of monocyte chemoattractant protein-1 and platelet-derived growth factor in VSMC (*6*, *30*). We speculated that several mitogenic agents, including these cytokine and growth factors, may be involved in this mitogenic action. Also, we cannot exclude the possibility that reactive oxygen species are involved in vascular remodeling, because the purine-nucleotide pathway generates reactive oxygen species together with uric acid. However, their effects on vascular cellular proliferation are still controversial (*31–34*). In addition, while single or low-level exposure to XO/XA has been shown to

stimulate VSMC proliferation (*31*, *32*), high-level or frequent exposure to XO/XA resulted in VSMC apoptosis (*32*, *34*). Not only VSMC but also macrophages, endothelium and fibroblasts may play several roles in the production and response to the reactive oxygen species, and these interactions stimulate vascular cells to produce adhesion molecules, matrix metalloproteinases, and chemotactic factors, which may induce vascular remodeling. Further studies are required to clarify the relationship between reactive oxygen species and vascular remodeling.

In conclusion, the present study demonstrated that allopurinol inhibited neointimal hyperplasia and lowered the serum uric acid concentration in SHR, and that uric acid *per se* may cause vascular remodeling. Since hyperuricemia has been reported to be a risk factor for cardiovascular disease in patients with hypertension (*2–4*), our results support the notion that allopurinol may exert its beneficial effects in hypertension by reducing uric acid levels.

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