Is Hyperuricemia a Risk Factor for Arteriosclerosis? Uric Acid and Arteriosclerosis in Apolipoprotein E-Deficient Mice

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Although hyperlipidemia, high blood pressure, and diabetes increase the risk of arteriosclerosis, it is not clear whether hyperuricemia increases the risk of arteriosclerosis or not. We examined the effects of uric acid and curative drugs for hyperuricemia on atherosclerosis-susceptible C57BL/6J apolipoprotein E-deficient (apoE−/−) mice. Male apoE−/− mice (age: 6 weeks) were fed a normal diet (normal diet group) or a uric acid-enriched diet. Mice fed the uric acid-enriched diet were divided into three groups and administered a drinking vehicle (high uric acid diet group), allopurinol (20 mg·kg−1·d−1), or benzbromarone (20 mg·kg−1·d−1) for 10 weeks. Serum uric acid concentrations were higher in the high uric acid diet group than in the normal diet group, and concentrations in the allopurinol and benzbromarone groups were lower than in the high uric acid diet group. Serum total cholesterol and triglyceride levels were lower in the allopurinol group than in the high uric acid diet group. Oxidative stress was lower in the benzbromarone group than in the high uric acid diet group. Atherosclerotic lesion areas were smaller in the allopurinol and benzbromarone groups than in the high uric acid diet group. Thus, hyperuricemia may not be an independent risk factor for arteriosclerosis; however, the administration of allopurinol and benzbromarone prevented the development of atherosclerosis in apoE−/− mice fed a uric acid-enriched diet. The anti-atherosclerotic effect was in part due to lower total cholesterol and oxidative stress in the serum. Other possible mechanisms underlying this effect should be investigated.

Key words hyperuricemia; atherosclerosis; apolipoprotein E-deficient mouse; allopurinol; benzbromarone

Uric acid is the final product of purine metabolism in humans. The role of uric acid in cardiovascular disease remains unclear. Although some epidemiological studies have shown that hyperuricemia (gout) is an independent risk factor for cardiovascular disease,1–3 others have suggested that this association is confounded by the coexistence of conditions such as hypertension, obesity, hyperlipidemia, and diabetes mellitus, all of which are known to be independent risk factors for atherosclerosis.4–6

Hyperuricemia is the presence of high levels of uric acid in the blood (serum urate >7 mg·dL−1). It is classified into three types: increased production of uric acid, decreased excretion of uric acid, and mixed type.7 Uric acid is produced by xanthine oxidase from xanthine and hypoxanthine (Fig. 1). Allopurinol and its major active metabolite oxypurinol, which has a much longer half-life than allopurinol and is primarily eliminated by renal excretion, inhibit the activity of xanthine oxidase and suppress uric acid generation upstream.8 Benzbromarone is a uricosuric agent that blocks tubular reabsorption of uric acid. In most mammals, uric acid is further metabolized to allantoin by the enzyme uricase. In order to study hyperuricemia in an animal model, uricase can be blocked by selective inhibitors. Potassium oxonate is a well-known inhibitor of uricase. In this study, 5% potassium oxonate and 2.5%

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MATERIALS AND METHODS

Materials  Allopurinol, benz bromaron, uric acid, and potassium oxonate were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Other chemicals of analytical reagent grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Treatments Animals were handled humanely in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka. The experimental protocol and animal use procedures were approved by the Committee of the University of Shizuoka. ApoE−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and bred in our laboratory under specific pathogen-free conditions. Male apoE−/− mice at 6 weeks of age were divided into four groups. The normal diet group was fed a normal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and drinking water (0.5% carboxymethyl cellulose and 1% ethanol). The high uric acid diet, allopurinol, and benz bromaron groups were fed a uric acid-enriched diet (CRF-1 containing 5% potassium oxonate and 2.5% uric acid) and drinking water (allopurinol (20 mg·kg−1·d−1), or benz bromaron (20 mg·kg−1·d−1), respectively, for 10 weeks. Body weight and serum total cholesterol (TC) and triglyceride (TG) concentrations were measured every 2 weeks during the experimental period. At the end of the experiment, when mice were 16 weeks of age, they were anesthetized with diethyl ether and blood was collected from the abdominal aorta. The heart was perfused with phosphate-buffered saline and 10% formalin neutral buffer solution (pH 7.0–7.5), excised with the aortic arch, and placed in 10% formalin neutral buffer solution for 2 weeks.

Measurement of Serum Uric Acid Concentrations The blood was allowed to stand at room temperature for 1 h. The serum was obtained by centrifugation at 1000×g for 10 min at 4°C and kept at −80°C until analysis. Serum samples (30 µL) were mixed with 0.3 mol/L perchloric acid (1.0 mL). The samples were kept on ice for 30 min. After vortexing, the samples were centrifuged at 3000×g for 10 min. The supernatants (300 µL) were then mixed with an equal volume of 0.2 mol/L disodium phosphate and filtered through 0.45-µm Millipore filters (Millipore, Bedford, MA, U.S.A.). A 50-µL sample was injected into an HPLC unit fitted with a pump (LC-20ADVP; Shimadzu, Kyoto, Japan), autosampler (SIL-20AC; Shimadzu), and system controller (CBM-20A; Shimadzu). Separations were performed at room temperature with a 4.6×150 mm column [Phenomenex Luna 3u C18 (2) 100A; Shimadzu]. Absorbance was measured at 284 nm with an ultraviolet variable column [Phenomenex Luna 3u C18 (2) 100A; Shimadzu]. The flow rate was 1.0 mL/min and the mobile phase buffer was phosphoric acid/methanol (74:26, pH 2.2).

Measurement of Serum TC and TG Concentrations Serum TC and TG concentrations were measured with commercial kits (Cholesterol E-test and Triglyceride E-test; Wako Pure Chemical Industries, Ltd.).

Measurement of Oxidative Stress To monitor oxidative stress, including hydroperoxides, serum levels of derivatives of reactive oxygen metabolites (d-ROMs) were determined using a free radical analytical system (FRAS4; Wismerll, Tokyo, Japan). This test measured the equilibrium between free radical production and antioxidant defense. Hydroperoxides are converted into radicals that can oxidize a chromogenic substrate (N,N-diethyl-para-phenylenediamine) and be detected by spectrophotometric analysis at 505 nm. In this method, 1 unit (Carratelli units: U. CARR) represents 0.08 mg/100 mL H2O2, and is equivalent to the hydroperoxide concentration.

Histologic Analysis of Atherosclerotic Lesions The formalin-fixed hearts were cut horizontally just under the atrium. The aortic root with the top of the heart was embedded in paraffin and serially sectioned at 5-µm intervals. Cross sections were taken at the level of the aortic valves and stained with hematoxylin and eosin. The area of the atherosclerotic lesions in the hearts of the mice was measured with image analysis software (Micro Analyzer; Japan Poladigital, Tokyo, Japan). Atherosclerotic lesions were calculated as the sum of the lesion area across four cross-sections.

Statistical Analysis All values represent the mean±S.E.M. Results were analyzed by one-way ANOVA followed by a post-hoc Dunnett’s test or by two-way ANOVA followed by a Bonferroni post-test. The significance level was set at p<0.05. Statistical analyses were performed in GraphPad Prism 4.03 software (GraphPad Software, La Jolla, CA, U.S.A.).

RESULTS

No significant difference in body weight was observed between the normal diet group and the other groups (Fig. 2). Food intake was approximately 4 g/d per mouse throughout the experiment in all groups (data not shown). Serum uric acid concentrations in the high uric acid diet group were 3.2-fold and 2.3-fold higher than in the normal diet group when the mice were 11 and 16 weeks of age (Fig. 3). In addition, serum uric acid in the allopurinol and benz bromaron groups was significantly lower than in the high uric acid diet group and similar to the normal diet group (Fig. 3). Serum TC concentrations were 37%, 47%, and 44% lower in the allopurinol group than in the high uric acid diet group at 10, 14, and 16 weeks of age (Fig. 4A). Serum TG concentrations were 22–46% lower in the allopurinol group than in the high uric acid diet group after 10 weeks of age (Fig. 4B). There was no significant difference in the serum TC and TG concentrations between the normal, high uric acid, and benz bromaron groups during the experimental period (Figs. 4A, B). Oxidative stress was...
significantly lower in the benzbromarone group than in the high uric acid diet group (Fig. 5). Figure 6 shows photomicrographs of atherosclerotic lesions in the aorta. Atherosclerotic lesions were smaller in the hearts of mice in the allopurinol and benzbromarone groups than in mice in the high uric acid diet group. Total atherosclerotic lesion size was 73% and 58% smaller in the allopurinol and benzbromarone groups than in the high uric acid diet group at the end of the experiment (Fig. 7).

DISCUSSION

In this study, we examined the influence of uric acid, allopurinol, and benzbromarone on arteriosclerosis. We created hyperuricemic mice by feeding apoE−/− mice a uric acid-enriched diet. In many rodents, the serum uric acid level is low because uric acid is further metabolized by uricase. Therefore, there are few animal models for evaluating drugs for hyperuricemia. In this study, the uric acid-enriched diet contained 5% potassium oxonate, which is an inhibitor of uricase, and 2.5% uric acid, which was given to raise serum uric acid significantly lower in the benzbromarone group than in the high uric acid diet group (Fig. 5). Figure 6 shows photomicrographs of atherosclerotic lesions in the aorta. Atherosclerotic lesions were smaller in the hearts of mice in the allopurinol and benzbromarone groups than in mice in the high uric acid diet group. Total atherosclerotic lesion size was 73% and 58% smaller in the allopurinol and benzbromarone groups than in the high uric acid diet group at the end of the experiment (Fig. 7).

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As a result, the serum concentration of uric acid was significantly higher in the high uric acid diet group than in the normal diet group (about 2.5-fold) and serum uric acid concentrations in the allopurinol, benzbromarone, and normal groups were similar. This animal model is thus useful for evaluating drugs for the treatment of hyperuricemia.

Total atherosclerotic lesion size did not significantly differ between the normal and high uric acid diet groups. However, total lesion size was markedly smaller in the allopurinol and benzbromarone groups than in the high uric acid diet group. We are the first to report that allopurinol and benzbromarone control the progression of atherosclerosis in apoE−/− mice. Engberding et al. reported that allopurinol represents a potential novel strategy for preventing left ventricular remodeling and dysfunction after myocardial infarction.14) There have been no such reports for benzbromarone. It is interesting that in our study, allopurinol and benzbromarone inhibited atherosclerotic progression in an animal model similar to human metabolic syndrome.

Serum TC and TG concentrations were significantly lower in the allopurinol group than in the high uric acid diet group. At present, the mechanism behind this reduction is not clear, and further in vitro and clinical studies are needed. Allopurinol inhibits xanthine oxidase activity and suppresses uric acid biosynthesis.15,16) Xanthine oxidase generates uric acid and reactive oxygen species. Allopurinol, and its major active metabolite oxypurinol, thus reduces reactive oxygen species by inhibiting xanthine oxidase. Although the effect was not significant, allopurinol reduced oxidative stress in our study. Reactive oxygen species are characteristic of atherosclerosis,17,18) and allopurinol may control the progress of atherosclerosis by lipopenic action, decreasing the level of reactive oxygen species. On the other hand, no decrease in serum lipid was observed in the benzbromarone group, but there was a significant decrease in oxidative stress. Inokuchi et al. reported that benzbromarone enhances adiponectin production by activating peroxisome proliferator-activated receptor (PPAR) gamma.19) Adiponectin is a protein hormone that mediates suppression of metabolic derangements that may result in type 2 diabetes and atherosclerosis.20,21) Yamauchi et al. reported that globular adiponectin can protect against atherosclerosis in vivo.22) Benzbromarone might have controlled the progress of arteriosclerosis by decreasing oxidative stress and increasing adiponectin.

In conclusion, a single rise in the uric acid level did not increase the risk of atherosclerosis. This might be because...
uric acid acts as a radical scavenger and inhibits oxidation; however, allopurinol and benzbromarone administration prevents the development of atherosclerosis in apoE−/− mice fed a uric acid-enriched diet. The anti-atherosclerotic effect is partly a result of lower serum TC concentrations and lower serum oxidative stress, but other mechanisms should be investigated. Clinical studies have shown association of high levels of serum uric acid with poor coronary collateral circulation in patients with stable coronary artery disease.25 Some reports have suggested that allopurinol reduces cardiovascular risks.24–26 Higgins et al. reported that allopurinol reduces central blood pressure and carotid intima-media thickness progression at 1 year versus a placebo in patients with recent ischemic stroke and transient ischemic attack.25 On the other hand, Kok et al. reported that allopurinol therapy in patients with gout does not yield beneficial cardiovascular outcomes.27 Okuda et al. reported that hyperuricemia may not contribute to an increase in serum C-reactive protein (CRP), which is associated with increased risk for myocardial infarction, atherosclerosis, and peripheral artery diseases, but benzbromarone may have a favorable effect on CRP.28 Further research is needed to determine the possible utility of allopurinol and benzbromarone in the treatment of cardiac disease.

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REFERENCES

