Xanthine Oxidase Inhibition Reverses Endothelial Dysfunction in Heavy Smokers

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Background—Cigarette smoking causes endothelial dysfunction, possibly through increased oxidant stress. The enzyme xanthine oxidase produces oxidative free radicals. We tested the hypothesis that xanthine oxidase contributes to endothelial dysfunction in cigarette smokers by administering the inhibitor allopurinol.

Methods and Results—Fourteen cigarette smokers (31 ± 4 pack years) and 14 age- and sex-matched healthy non-smoking control subjects participated in a single-blinded, randomized, 2-phase crossover study. All subjects had no other risk factors for atherosclerosis. Inhibition of xanthine oxidase was achieved by a single oral dose of 600 mg of allopurinol on the day of the study. Stimulated nitric oxide endothelial responses were assessed by forearm blood flow responses to intraarterial administration of acetylcholine and bradykinin 4 to 7 hours later; basal nitric oxide was assessed using the nitric oxide synthase inhibitor NG-monomethyl-L-arginine (L-NMMA); and nitroprusside was used to assess sensitivity to nitric oxide. Dilatation produced by acetylcholine was significantly less in smokers (254 ± 57%) than healthy controls (390 ± 55%) (P = 0.009). Allopurinol reversed endothelial dysfunction in smokers (acetylcholine, 463 ± 78%, P = 0.001) without affecting responses in non-smokers (401 ± 80%). Bradykinin responses were also impaired in smokers (P = 0.003), and improved with allopurinol, though not significantly (P = 0.06). Responses to nitroprusside and L-NMMA were not significantly different between smokers and controls and were not altered by allopurinol.

Conclusions—Smoking-induced endothelial dysfunction of resistance vessels is rapidly reversed with oral allopurinol. These data suggest that xanthine oxidase contributes importantly to endothelial dysfunction caused by cigarette smoking. (Circulation. 2003;107:416-421.)

Key Words: atherosclerosis ■ nitric oxide ■ endothelium ■ smoking

Cigarette smoking is a risk factor for atherosclerosis and its complications. Damage to the vascular endothelium seems to contribute to complications of atherosclerosis. Endothelial dysfunction is produced by risk factors such as cigarette smoking, hypertension, hypercholesterolemia, diabetes mellitus, and hyperhomocysteinemia, possibly through production of oxygen free radicals such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Several enzymes can produce oxygen free radicals, including xanthine oxidase, NADPH oxidase, myeloperoxidase, and endotoxin.

Xanthine oxidase inhibition with oxypurinol, an active metabolite of allopurinol, has been shown to improve resistance vessel endothelial vasodilator function in hypercholesterolemic, but not hypertensive, patients. In patients with diabetes mellitus and heart failure, an improvement and reversal of endothelial dysfunction was shown after 1 month of treatment with allopurinol. No studies have tested whether inhibition of xanthine oxidase improves endothelial function in cigarette smokers. Also, no studies have examined the effects of a single oral dose of allopurinol on vascular function. We tested the hypothesis that xanthine oxidase contributes to endothelial dysfunction in cigarette smokers. We studied whether acute inhibition of xanthine oxidase with allopurinol improves endothelial dysfunction in cigarette smokers.

Methods

Subjects
Both male and female subjects were included in the study if they were aged 18 to 85 years and had low-density lipoprotein cholesterol < 130 mg/dL, total cholesterol < 250 mg/dL, systolic blood pressure < 130 mm Hg, diastolic blood pressure < 80 mm Hg, and fasting blood glucose < 126 mg/dL. We recruited 14 smokers and 14 age- and sex-matched controls with no clinical evidence of atherosclerosis. Smokers were recruited if they smoked at least a pack of cigarettes a day for at least 1 year and had a total cigarette exposure of > 20 pack years. Age- and sex-matched healthy control subjects who never had a history of tobacco use were selected for the comparison group. The studies were conducted after written informed consent was obtained from each subject and were approved by the Institutional Review Board. None of the subjects received
vasoactive drugs in the week before the study. We took extensive measures to prevent effects of acute smoking on the study. All subjects, both controls and smokers, were admitted to the General Clinical Research Center (GCRC) at least 12 hours before the study. We performed the study at 9:00 AM in the morning and the subjects were admitted by 9:00 PM on the previous evening to have a rigorous control over both diet and smoking. None of the subjects were permitted to smoke from the time of admission to the GCRC. Studies were performed in a quiet room maintained at a constant temperature between 22 and 25°C.

Measurements
Forearm blood flow was measured by venous occlusion plethysmography with the use of indium/gallium-in-silastic strain gauges. Subjects rested supine with both arms supported and with venous emptying unimpeded, and strain gauges were applied to the widest part of each forearm and attached to a plethysmographic unit. Forearm blood flows were recorded repeatedly over 3-minute periods. To measure resistance vessel endothelial function, we used forearm blood flow responses to local brachial artery administration of vasoactive agents to test resistance vessel endothelial function. The brachial artery of the left arm was cannulated anterior to the elbow by use of a 27-gauge needle under local anesthesia and aseptic conditions. Drugs were dissolved in 0.9% saline and included nitroprusside (NTP, 1 to 10 μg/min), acetylcholine (3 to 30 μg/min), bradykinin (0.03 to 0.3 μg/min), and L-NMMA (1 to 4 μmol/min). Each dose was administered for 6 minutes. Acetylcholine and bradykinin are endothelium-dependent vasodilators, and vascular responses to these drugs are mediated through the endothelium and nitric oxide. Nitroprusside is an endothelium-independent vasodilator that directly donates nitric oxide to vascular smooth muscle, and vascular responses to it are not affected by damage to endothelium. L-NMMA is an inhibitor of nitric oxide synthase and was used to assess basal nitric oxide synthesis. At least 10 minutes were allowed between drugs so that forearm blood flow returned to its basal value. Arterial pressure was measured in duplicate at intervals during the experimental protocols using a noninvasive automated oscillometric sphygmomanometer (Lifestat 200, Physio-Control). Heart rate was measured continuously using a lead II ECG.

Laboratory Assays
All the laboratory assays were performed in the clinical chemistry laboratory of the University of Iowa Hospitals and Clinics. Blood uric acid levels were measured using the uricase reaction and quantified by a colorimetric technique. Blood levels of allopurinol and oxypurinol were measured using high performance liquid chromatography with ultraviolet detection. Serum creatinine was assessed using modified Jaffe reaction and quantified by a colorimetric technique. Carboxyhemoglobin was measured using the oximetric method.

Study Design
Subjects participated in a single-blinded, randomized, 2-phase crossover study comparing allopurinol with no treatment. The subjects were not blinded to the medication and took either allopurinol or nothing. They were instructed not to reveal their treatment allocation. GCRC staff not involved in the study provided the randomization code and administered allopurinol to the subjects. However, the investigators who performed the study and scored the data were blinded to the treatment till the completion of the entire data collection. Subjects were also randomized to the order of the treatment (allopurinol versus no allopurinol). Thus, the study with allopurinol was sometimes performed before the study without allopurinol and sometimes afterward. Staff involved in the vascular study were informed of the treatment allocation only after completion of studies on all subjects. Subjects were also randomized to the sequence of intraarterial drugs and each subject received the same sequence of drugs during both their study days.

Allopurinol is converted by xanthine oxidase to its major metabolite oxypurinol. Oxypurinol has a much longer half-life (t1/2 = 17 to 21 hour) and circulates at higher plasma concentrations than allopurinol, and thus the majority of the effects of allopurinol are mediated by oxypurinol. The IC50 of oxypurinol for xanthine oxidase is around 5.0 μg/mL in healthy volunteers. After a single oral dose of 300 g of oral allopurinol, peak levels of oxypurinol of 5 to 7 μg/mL are reached at 2 to 5 hours. Pharmacokinetic data indicate that a 600 mg oral dose of allopurinol will achieve a mean concentration of 9 to 10 μg/mL, and this would inhibit more than 95% of xanthine oxidase. On each study day, allopurinol was administered in a single 600 mg dose and resistance vessel endothelial function was tested 4 hours later, at the time of expected peak of oxypurinol. We performed the studies at least a week apart because 99.75% of oxypurinol is metabolized within 1 week. Previous studies show that xanthine oxidase inhibition closely tracks oxypurinol concentrations during treatment with allopurinol, with normal xanthine oxidase activity apparent 4 days after halting a 1-week treatment course of allopurinol 300 mg.

Statistical Analysis
Power calculations were done with responses to acetylcholine as the primary endpoint, and we calculated the sample size for a power of 80% and a significance level of 0.05. Responses to bradykinin and L-NMMA were secondary endpoints. Analyses were performed by observers blinded to treatment assignment. Basal blood flow and blood pressure were taken as the average of the 3 baseline recording periods during saline infusion. In addition to absolute blood flows, we calculated percent change from baseline in the ratio of blood flow between infused and non-infused arms, because this halves variability in blood flow responses to infused agents. To test differences between baseline variables between the 2 groups, we used Students 2-sample t test. We used repeated-measures ANOVA to test for differences in forearm blood flows between the 2 groups. Differences within the group were tested with a paired t test. Data are expressed as mean±standard error, and P<0.05 has been taken as statistically significant.

Results
Baseline Criteria
Analysis of baseline characteristics between the 2 groups showed no differences in age, body mass index, total cholesterol, low-density lipoprotein cholesterol, systolic and diastolic blood pressures, and blood glucose. Triglycerides and high-density lipoprotein (HDL) cholesterol were, however, significantly different between the 2 groups (Table 1). Plasma oxypurinol levels were measured in 6 subjects in each group, and the levels reached at 7 hours were 9.9±1.5 μg/mL in smokers and 8.2±0.5 μg/mL in controls. Baseline levels of uric acid were not significantly different in the smokers and the controls. Uric acid levels decreased significantly at 4 hours and 7 hours after oral allopurinol (Table 2).

Forearm Blood Flow
Baseline forearm blood flow was similar in smokers and controls on both placebo and allopurinol days (Table 3). Heart rate and blood pressure remained similar between the 2 groups at baseline and during infusion of the drugs, as shown in Table 3. Forearm dilatation to acetylcholine was significantly reduced in smokers compared with controls (P=0.009; Table 3, Figure 1). Responses to bradykinin were significantly reduced in smokers compared with controls (P=0.003; Figure 1). Blood flow responses to nitroprusside and L-NMMA were not affected by smoking (Figure 2).

After administration of allopurinol, blood flow responses to acetylcholine in smokers increased significantly to the
levels observed in controls ($P=0.001$ at peak doses; Table 3, Figure 3). Forearm blood flow responses to bradykinin also increased in smokers after allopurinol, though not significantly ($P=0.06$ at peak doses; Figure 4). Allopurinol did not affect acetylcholine or bradykinin responses in controls (Figure 3 and Figure 4), or to nitroprusside (Figure 5) and L-NMMA (Figure 1).

### Discussion

This study confirms that heavy cigarette smoking causes resistance vessel endothelial dysfunction. This is the first study to show the effects of inhibition of xanthine oxidase on endothelial function in smokers. This is also the first study to show that a single oral dose of allopurinol can have rapid and substantial endothelial effects. Studies done in subjects with hypercholesterolemia, diabetes mellitus, and heart failure have found a significant improvement in endothelial function with the administration of either intravenous oxypurinol or oral allopurinol for 1 month. In hypertension, however, inhibition of xanthine oxidase does not improve endothelial function, which suggests that xanthine oxidase is not invariably involved in endothelial dysfunction. Thus, the demonstration of effects of xanthine oxidase inhibition in smokers extends the importance of this mechanism. In our study, when a single dose of allopurinol was administered orally, endothelial function in smokers improved to control levels, but vascular responses were not affected in controls.

Cigarette smokers had no risk factors for atherosclerosis other than smoking. The lower HDL cholesterol levels and higher triglycerides were likely due to smoking. Smokers had impaired responses to acetylcholine and bradykinin, both of which are endothelium-dependent dilators. Responses to nitroprusside, which acts on vascular smooth muscle independently of the endothelium, were not affected. This indicates that the impaired responses to acetylcholine and bradykinin were secondary to endothelial impairment. Allopurinol improved responses to acetylcholine and bradykinin but did not alter nitroprusside responses. Bradykinin responses improved non-significantly with allopurinol. The variation in the effects of allopurinol on responses to acetylcholine and bradykinin, which are both endothelium-dependent, may be because of differences in the intracellular activating mechanisms, G-proteins, or possibly endothelial mediators. The study was also not designed for bradykinin vascular responses, and further studies with this as the primary endpoint are necessary to accurately evaluate its role. Endothelium-dependent blood flow responses are indicative of stimulated nitric oxide production. Thus, our data suggest that stimulated nitric oxide production is decreased in cigarette smokers and improved by allopurinol.

Vasoconstriction to L-NMMA is a marker of basal vascular nitric oxide production. L-NMMA–induced vasoconstriction was not significantly different between smokers and controls and was not affected by allopurinol. Stimulated nitric oxide responses may be a more sensitive marker of endothelial dysfunction than basal nitric oxide. This difference between effects of risk factors on basal and stimulated nitric oxide has also been observed for other risk factors, such as hypercholesterolemia and hyperhomocysteinemia. It is also possible that L-NMMA may not be the best method to test for effects of xanthine oxidase inhibition. This is because nitric oxide synthase produces superoxide radicals, which are decreased by allopurinol, and this could lead to feedback amplification of nitric oxide synthase.

Allopurinol might be acting through one of 2 pathways, both of which inhibit xanthine oxidase. First, allopurinol inhibits production of free radicals by xanthine oxidase. Xanthine oxidase, an iron-sulfur molybdenum flavoprotein, is a multifunctional enzyme present in high concentrations in endothelial cells of capillaries and sinusoids. It exists in 2 interconvertible isoforms, xanthine dehydrogenase and xanthine oxidase. The dehydrogenase form produces uric acid and reduced nicotinamide adenine dinucleotide, and the oxidase form produces oxygen free radicals, uric acid, and superoxide. Xanthine oxidase activity is increased in ischemia-reperfusion injuries, anoxia, and inflammation. This may occur by lysis of endothelial cells and increased conversion of the dehydrogenase form to the oxidase form. Superoxide radicals undergo dismutation to hydrogen peroxide, which has been proposed to be the major oxygen radical responsible for endothelial damage by the xanthine oxidase. Additionally, xanthine oxidase–derived superoxide radicals are thought to react with nitric oxide to form peroxynitrate. This causes inactivation of nitric oxide; in addition, peroxyni-
Species, and a similar mechanism may pertain in smokers. 

Thus, both scientific and epidemiological studies have been 

dicate itself is a potent oxidant and may contribute to endothelial damage. In addition, xanthine oxidase-derived oxidative stress causes increased cytosolic $\text{Ca}^{2+}$ levels, thus depleting hormone-insensitive pools of $\text{Ca}^{2+}$ and inhibiting protein synthesis. Xanthine oxidase-derived superoxide radicals produce $\text{H}_2\text{O}_2$, which enters the cell and interacts with non-ferritin iron to produce cytotoxic hydroxyl radicals. Thus, xanthine oxidase-derived oxidative stress may be a major factor responsible for endothelial damage. These findings may not, however, exclude other mechanisms of oxidative stress. For example, in hyperhomocysteinemia in rats, oxidant species seem to be generated by cyclooxygenase after activation of cyclooxygenase by reactive oxidant species produced by xanthine oxidase. Thus, xanthine oxidase may be an initiating step in the generation of reactive oxygen species, and a similar mechanism may pertain in smokers.

Second, allopurinol may be acting by decreasing uric acid levels in the blood. Uric acid levels were decreased after a single oral dose of allopurinol in our study. The role of uric acid in causing cardiovascular disease is still unclear. A few studies have reported an association between uric acid levels and hypertension, chronic heart failure, and atherogenesis. A few epidemiological studies have found uric acid levels to be an independent risk factor for cardiovascular mortality. People with uric acid levels in the highest quartile (>7.0 mg/dL) are at higher risk of heart attack and stroke after adjustment for other cardiovascular risk factors. Other studies have suggested a protective role of uric acid, however, and some epidemiological studies have not found an association between uric acid and cardiovascular mortality. Thus, both scientific and epidemiological studies have been 

<table>
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<th>MAP, mm Hg</th>
<th>Controls</th>
<th>Smokers</th>
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<tr>
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<th>FBF infused, mL · 100 mL⁻¹ · min⁻¹</th>
<th>Controls</th>
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<th>% Δ in Flow, infused/non-infused</th>
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<td>441±94</td>
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ACH indicates acetylcholine; BK, bradykinin; NTP, nitroprusside; MAP, mean arterial pressure; HR, heart rate; and FBF, forearm blood flow.

*P<0.05 vs controls; **P<0.05 vs no allopurinol.
unable to ascertain the role of uric acid conclusively. Our study supports the possibility of uric acid being a factor in endothelial dysfunction, but this needs to be investigated further.

There are some limitations to our study. We did not look at endothelial function in conduit vessels, which are more relevant to vessels affected by atherosclerosis. However, resistance vessel responses seem to be predictive of coronary endothelial physiology.\(^\text{34}\) We cannot conclusively attribute the endothelial dysfunction in the group of cigarette smokers to tobacco use. We made strenuous efforts to exclude patients with conventional risk factors for atherosclerosis, and the groups were matched for age and sex. However, HDL was somewhat lower (but within normal limits) and triglycerides somewhat higher in smokers. These changes have been well described in cigarette smokers in other epidemiological studies,\(^\text{13, 14}\) and low HDL was likely a consequence of smoking rather than an independent additional risk factor. We did not objectively confirm or rule out coronary artery disease by either a stress test or a cardiac catheterization, and thus it is possible that endothelial dysfunction was due to early atherosclerosis.

In conclusion, allopurinol rapidly reverses endothelial dysfunction in cigarette smokers, which suggests that xanthine oxidase plays a role in endothelial dysfunction. Further studies are needed to elucidate the exact mechanisms of xanthine oxidase actions on the endothelium.

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