Allopurinol Normalizes Endothelial Dysfunction in Type 2 Diabetics With Mild Hypertension

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Abstract—Therapeutic strategies against free radicals have mostly focused on the augmentation of antioxidant defenses (eg, vitamins C and E). A novel approach is to prevent free radical generation by the enzyme system xanthine oxidase. We examined whether the inhibition of xanthine oxidase with allopurinol can improve endothelial function in subjects with type 2 diabetes and coexisting mild hypertension compared with control subjects of a similar age. We examined 23 subjects (11 patients with type 2 diabetes and 12 healthy age-matched control subjects) in 2 parallel groups. The subjects were administered 300 mg allopurinol in a randomized, placebo-controlled study in which both therapies were administered for 1 month. Endothelial function was assessed with bilateral venous occlusion plethysmography, in which the forearm blood flow responses to intra-arterial infusions of endothelium-dependent and -independent vasodilators were measured. Allopurinol significantly increased the mean forearm blood flow response to acetylcholine by 30% (3.16 ± 1.21 versus 2.54 ± 0.76 mL · 100 mL^{-1} · min^{-1} allopurinol versus placebo; $P=0.012$, 95% CI 0.14, 1.30) but did not affect the nitroprusside response in patients with type 2 diabetes. There was no significant impact on either endothelium-dependent or -independent vascular responses in age-matched control subjects. Allopurinol improved endothelial function to near-normal levels. Regarding markers of free radical activity, the level of malondialdehyde was significantly reduced (0.30 ± 0.04 versus 0.34 ± 0.05 μmol/L for allopurinol versus placebo, $P=0.03$) in patients with type 2 diabetes but not in control subjects. The xanthine oxidase inhibitor allopurinol improves endothelial dysfunction in patients with type 2 diabetes with mild hypertension but not in matched control subjects. In the former group, allopurinol restored endothelial function to near-normal levels. **(*Hypertension*. 2000;35:746-751.)**

Key Words: allopurinol ■ diabetes mellitus ■ endothelium ■ free radicals

Free radicals are highly reactive chemical entities that have 1 or more unpaired electrons within their outermost shell and can react aggressively with other biological molecules, causing tissue damage. Patients with type 2 diabetes have an increased production of free radicals,[1] which can inactivate nitric oxide (NO)[2] and increase diabetic complications.[3] Because endothelial dysfunction is thought to be a precursor of atherosclerosis, a promising therapeutic goal in patients with type 2 diabetes might be to reduce free radical activity.

There are many possible agents that act to inhibit the generation, propagation, or activity of free radicals, such as preventive antioxidants, which include catalase, superoxide dismutase, hemoglobin, and chain terminators such as vitamins E and C and glutathione. Most clinical studies to date have concentrated on the augmentation of antioxidant defense mechanisms through the administration of vitamins C and E and beta-carotene.

An intriguing alternative strategy is to inhibit the enzyme xanthine oxidase (XO), which produces superoxide, hydrogen peroxide, and the hydroxyl radical as byproducts of its normal metabolic action.[4] XO is normally present in endothelial cells, and it catalyzes the degradation of hypoxanthine to uric acid. A potent way to prevent XO-generated free radicals in the clinical setting is to use the orally active XO inhibitor allopurinol. There is evidence to suggest that allopurinol may prevent free radical–induced tissue damage; for example, allopurinol decreases reperfusion injury during coronary artery bypass graft surgery[5] and improves cardiorespiratory function in an animal transplantation model[6] and in humans.[7] Allopurinol may even speed up the repletion of high-energy phosphates during ischemia.[8] More recently, data have emerged to suggest that the acute intra-arterial infusion of oxypurinol, the active metabolite of allopurinol, may improve endothelial function in hypercholesterolemic humans.[9]

Diabetes mellitus is another disease that is characterized by higher levels of oxidative stress; therefore, with forearm venous occlusion plethysmography to assess the changes in forearm blood flow, we examined whether long-term oral therapy with allopurinol would improve endothelial function in patients with type 2 diabetes. Importantly, we compared the effect of allopurinol in

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diabetics with the effect in age-matched nondiabetic control subjects.

The forearm vascular bed was used because of established methodological advantages (with reproducibility and procedural safety being paramount) and because the results can be directly applied to the peripheral macroangiopathy that diabetics experience. Similarly, it can be applied to the coronary circulation, where there is a well established correlation between forearm vascular responses and coronary endothelium physiology[10] and coronary atherosclerosis.[11]

Methods

Two parallel groups were studied; each subject gave written informed consent to participate in the study, which was approved by the Tayside Medical Ethics Committee.

Patients With Type 2 Diabetes

Twelve patients were recruited, but 1 patient was withdrawn because of technical difficulties in cannulation of the brachial artery on the second study day. Eleven patients (10 men and 1 woman, age 65±7 years, body mass index [BMI] 26.2±3.5 kg/m^2 [values are mean±SD]) completed the study. The median duration of diabetes was 4 years (range 1 to 20 years). Ten patients controlled the diabetes with diet alone, and 1 patient received metformin. There were no other concurrent medications. Four were smokers (≈4 cigarettes/d). None of the patients had evidence of microvascular or macrovascular disease as determined by history, ECG, urinary microalbumin excretion, and dilated funduscopy.

Age-Matched Control Subjects

Twelve patients were recruited (12 men, age 58±8 years, BMI 27.4±3.0 kg/m^2). Three were smokers. None of the subjects had evidence of macrovascular cardiovascular disease as determined by history, physical examination, and ECG, and none were taking any medication.

Study Protocol

After an initial screening, volunteers were administered 300 mg/d allopurinol for 1 month in a randomized, placebo-controlled, double-blind, crossover study. After treatment, each subject attended a 3-hour morning study to evaluate endothelial function. Mornings were selected to avoid the recognized diurnal fluctuation in endothelial function.[12] On each study morning, after a 12-hour overnight fast (water was permitted), endothelial function was assessed with bilateral forearm, venous-occlusion plethysmography[13] with an intra-arterial infusion of endothelium-dependent (acetylcholine)[14] and endothelium-independent (sodium nitroprusside) vasodilators.[15] Subjects were in a temperature-controlled room (23°C) in our research unit at 8:45 AM, and after a 20-minute supine rest, baseline blood pressure measurements were recorded. The brachial artery of the nondominant forearm was cannulated with a 26-gauge cannula mounted onto a 16-gauge epidural catheter. Forearm venous occlusion plethysmography (Medasonics) was performed at baseline and then after each of three 5-minute incrementally increasing doses of acetylcholine (25, 50, and 100 nmol/mL) and sodium nitroprusside (4.2, 12.6, and 37.8 nmol/mL). Pneumatic cuffs were placed around the wrist and inflated to 200 mm Hg to isolate arterial circulation at the wrist. Intermittently, an upper arm cuff was inflated to 30 mm Hg. The change in forearm volume was estimated with mercury-filled strain gauges (stretched to forearm circumference +20%). The mean values of the final 5 plethysmographic recordings (of ≈15 recordings) were taken. The maximal dose-response was achieved within 4 minutes for both agents, and the dose effects are cumulative.

Blood flow was expressed as mL · 100 mL^{-1} · min^{-1} according to the method of Whitney,[16] and a modification of the method of Greenfield and Patterson[17] was used to express blood flow as a ratio of the blood flow in the infused arm to the blood flow in the control arm. The values are mean±SD of the average response to the 3 doses of each drug, obviously excluding baseline values, where the ratio equaled 1.

Blood was collected at baseline for measurements of serum urea, creatinine, cholesterol, and HDL-cholesterol levels. In addition, glycosylated hemoglobin levels were measured at each visit for the patients with type 2 diabetes. Samples were analyzed on the day of each visit. At each study visit, blood was collected and stored for estimation of the plasma malondialdehyde (MDA) level.

Malondialdehyde

MDA was assayed in the department according to a method developed by Tatum et al[18] with modifications. Free radical attack on plasma lipoprotein polyunsaturated fatty acids results in the formation of lipid peroxides. Acid hydrolysis of these peroxides releases MDA, which on reaction with thiobarbituric acid forms a fluorescent adduct. With the addition of antioxidants to prevent further oxidation, isobutanol extraction, and separation by high-performance liquid chromatography of interfering coproducts, this represents an indirect measure of free radical activity.

Statistical Analysis

Venous occlusion plethysmography produces 2 discrete values of blood flow (in mL · 100 mL forearm volume^{-1} · min^{-1}): 1 value for each arm. Blood flow in each arm at rest should be equivalent, and the baseline data are presented as a ratio of the blood flow in the test arm to that the control arm. At rest, this ratio equals 1. Basal blood flow is measured in mL · 100 mL^{-1} · min^{-1}, but the ratios have no units. Blood flow ratios for individual subjects were compared with a MANOVA. The calculation included blood flow ratio as a response and allopurinol treatment and dose of infusate as factors for the model. Confidence intervals were calculated with a general ANOVA and the Bonferroni method for calculation of 95% CIs.

The baseline variability of our data was <10% when blood flow was analyzed repeatedly in a steady state, in a quiet environment. The variability of repeated analysis of the same raw plethysmographic data was <5%. The values are presented as mean±pooled SE, which demonstrates the data with more clarity.

Results

There were no significant between-group differences for type 2 diabetics versus age-matched control subjects in BMI (26.2±3.5 versus 27.4±3.0 kg/m^2, P=0.40), cholesterol (5.0±0.9 versus 5.1±0.9 mmol/L, P=0.78), or HDL-cholesterol (1.0±0.3 versus 1.1±0.3 mmol/L, P=0.44). However, blood pressure was higher in the diabetic group than in the healthy age-matched control subjects (Table).

There were no significant within-group differences in glycosylated hemoglobin (HbA1c) in the type 2 diabetic group (7.1±1.6% versus 7.0±1.8%, P=0.99), in basal blood flow in the type 2 diabetic group (2.9±0.9 versus 3.1±1.5 mL · 100 mL^{-1} · min^{-1}, P=0.68 for placebo versus allopurinol, respectively) or the age-matched control subjects 3.9±1.2 versus 4.1±1.1 mL · 100 mL^{-1} · min^{-1}, P=0.71), or blood pressure in the type 2 diabetic group (155/82±20/8 versus 154/84±20/8 mm Hg, P=0.90/0.77 for placebo versus allopurinol, respectively) or the age-matched control group (138/79±15/6 and 140/80±16/8 mm Hg, P=0.79/0.73 for placebo versus allopurinol, respectively).

Basal blood flow data demonstrated a significant difference between type 2 diabetics and age-matched control subjects during the placebo study day (2.9±0.9 versus 3.9±1.2 mL · 100 mL · min^{-1} for type 2 diabetics and age-matched control subjects, respectively; P=0.03), and this became nonsignificant after allopurinol treatment (P=0.09);

Intergroup Comparison of Blood Pressure and Mean Arterial Pressure

Treatment	Variable	Patients With Type 2 Diabetes	Age-Matched Control Subjects	*P*
Placebo	SBP, mm Hg	155±20	139±15	**0.04**
Placebo	DBP, mm Hg	82±8	79±6	0.39
Placebo	MAP, mm Hg	106±11	99±8	0.08
Placebo	Basal blood flow, mL · 100 mL^{-1} · min^{-1}	2.9±0.9	3.9±1.2	**0.03**
Allopurinol	SBP, mm Hg	154±20	140±16	0.08
Allopurinol	DBP, mm Hg	84±8	80±8	0.23
Allopurinol	MAP, mm Hg	107±11	100±7.5	0.09
Allopurinol	Basal blood flow, mL · 100 mL^{-1} · min^{-1}	3.1±1.5	4.1±1.1	0.09

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; and MAP, mean arterial pressure.

there were no differences in blood pressure between groups (Table).

Forearm Blood Flow

The following section on blood flow concerns the dose-response curves of acetylcholine and sodium nitroprusside in response to oral dosing with allopurinol. There was no treatment order effect. The data are the mean response to the 3 incremental doses of each vasoactive agent.

Type 2 Diabetics

Allopurinol increased forearm blood flow response to acetylcholine by ≈30% (3.16±1.21 versus 2.54±0.76 mL · 100 mL^{-1} · min^{-1} for allopurinol versus placebo, $P=0.012$, 95% CI 0.14, 1.30) (Figure 1). Allopurinol had no effect on endothelium-independent vasodilatation with sodium nitroprusside (3.49±1.56 versus 3.36±1.40 mL · 100 mL^{-1} · min^{-1} for allopurinol versus placebo, $P=0.670$, 95% CI −0.50, 0.77) (Figure 2). Allopurinol was not associated with a change in forearm vascular resistance (42.0±18.7 versus 40.0±14.0 for control subjects: allopurinol versus placebo). However, subjects with type 2 diabetes did have higher forearm vascular resistance than the normal control subjects (allopurinol treatment: 42.0±18.7 versus 26.7±10.1 for type 2 diabetics versus control subjects, $P=0.03$; placebo: 40.0±14.0 versus 27.6±9.5 for type 2 diabetics versus control subjects, $P=0.24$). Endothelium-dependent blood flow was significantly blunted in diabetic subjects compared with control subjects on the placebo day (3.04±1.23 versus 2.54±0.76 mL · 100 mL^{-1} · min^{-1} for control subjects versus diabetics, $P=0.04$), which disappeared after allopurinol treatment (3.11±1.08 versus 3.16±1.21 for control subjects versus diabetics, $P=0.56$) (Figure 3).

Age-Matched Control Subjects

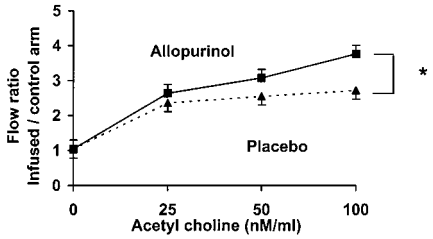
Allopurinol had no significant effect on forearm blood flow response to acetylcholine (3.11±1.08 versus 3.04±1.23 mL · 100 mL^{-1} · min^{-1} for allopurinol versus placebo, $P=0.79$) (Figure 1). Similarly, allopurinol had no effect on endothelium-independent vasodilatation with sodium nitroprusside. (3.57±1.42 versus 3.86±2.47 mL · 100 mL^{-1} · min^{-1} for allopurinol versus placebo, $P=0.49$) (Figure 2). There was no difference in forearm vascular resistance (26.7±10.1 versus 27.6±9.5 for allopurinol versus placebo)

Malondialdehyde

The level of MDA was significantly reduced by allopurinol (0.30±0.04 versus 0.34±0.05 μmol/L for allopurinol versus placebo, $P=0.02$) in patients with type 2 diabetes. There was no difference in MDA levels in age-matched control subjects (0.34±0.06 versus 0.35±0.12 μmol/L for allopurinol versus placebo, $P=0.81$).

Stepwise Regression Analysis

We investigated the relative contribution of diabetes and the covariates blood pressure, cholesterol, and BMI to the change in endothelial dysfunction. We only investigated the maximum dose of acetylcholine to simplify the model and looked at the absolute change in blood flow ratio in response to allopurinol. The model showed diabetes, cholesterol, and



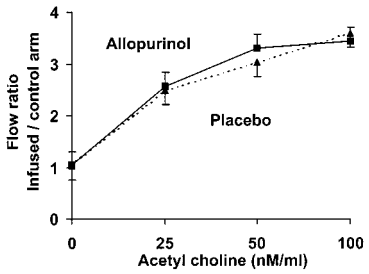


Figure 1. Endothelium-dependent vascular response for patients with type 2 diabetes (top) and age-matched control subjects (bottom) (■, allopurinol; ▲, placebo) (*$P<0.05$).

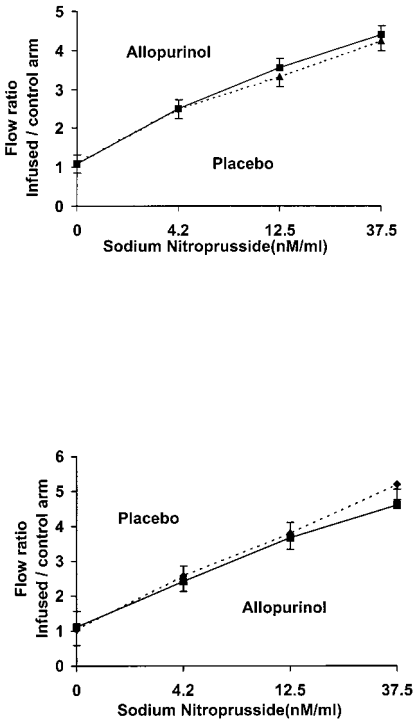


Figure 2. Endothelium-independent vascular response for patients with type 2 diabetes (top) and age-matched control subjects (bottom) (■, allopurinol; ▲, placebo).

blood pressure in descending order of importance. This relationship was consistent regardless of whether we used binary data (above and below the median value for diabetes, cholesterol, and blood pressure) or numerical data.

Discussion

These data show for the first time that treatment with allopurinol improves endothelial function in patients with type 2 diabetes and mild hypertension but has no impact in control subjects. In particular, the blunting of endothelial function seen in subjects with type 2 diabetes was abolished with allopurinol treatment (Figure 3). This is associated with a reduction in the level of MDA, a marker of lipid peroxidation, but no change in resting forearm vascular resistance.

XO is a key free radical–producing enzyme system that produces superoxide. In diabetes, other oxidoreductase enzyme systems, such as lipoxygenase, cyclooxygenase, and the aldose reductase pathways, may also contribute to the excess oxidative stress. However, XO is the easiest of these enzymes to target because allopurinol, which probably reduces oxidative stress by reducing superoxide anions, would otherwise scavenge endogenous NO.[19] Our results are important because they demonstrate in vivo free radicals may be an important and reversible cause of endothelial dysfunction in patients with type 2 diabetes. Our group has previously demonstrated that enhanced oxidative stress is present in subjects with diabetes mellitus.[20]

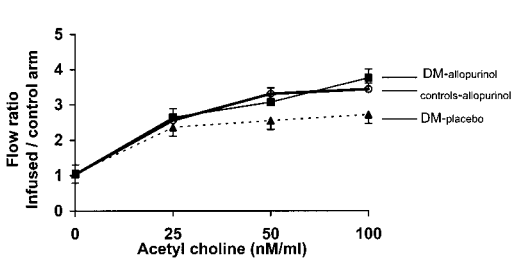


Figure 3. Endothelium-dependent vascular response for patients with type 2 diabetes (allopurinol and placebo) and control subjects (allopurinol) (mean±SEM).

The direct measurement of free radicals is difficult, relying on either spin-trapping or freezing in liquid nitrogen. Another technique is to use surrogate markers of free radical activity, such as MDA, which is a marker of lipid peroxidation that has been validated and previously demonstrated a reduction in free radical activity.[6] However, the baseline MDA results were roughly equivalent in our study and control groups. This may be because the diabetes was well controlled in our study population or because the samples from the diabetic and nondiabetic groups were not analyzed in the same batch but instead analyzed in 2 separate runs. This means that we cannot make valid comparisons between groups and that only within-group analyses of the MDA data are appropriate.

There are 2 possible mechanisms via which allopurinol would have the beneficial effects that we found. First, XO activity generates superoxide, hydrogen peroxide, and the hydroxyl radical,[4] which can directly cause tissue damage. Second, ischemia results in the dephosphorylation of ATP to AMP and then to hypoxanthine.[21] Therefore, there is a potential to prevent the irreversible loss of hypoxanthine (and therefore ATP) with allopurinol. Some data[22] support this idea and have shown that the beneficial effect of allopurinol may occur through the maintenance of high-energy phosphate levels.

In vitro work has shown that in cultured human aortic endothelial cells, XO is the major source of free radicals[23] and that hypoxanthine and XO increase endothelial dysfunction, which can then be improved by allopurinol.[24]

Allopurinol has been shown to reduce ventricular arrhythmia and to improve coronary artery blood flow,[25] to improve endothelial dysfunction,[26] and to reduce MDA levels[6] in animal studies. In humans, allopurinol has been investigated in coronary artery bypass graft surgery, where it has reduced ischemic events, produced less ST-T segment depression, and lowered the use of postoperative inotropic support.[27] This is supported by other reports[7,28,29] but not by all.[30]

More recently, Cardillo et al[9] demonstrated an improvement in forearm endothelial function in hypercholesterolemic subjects after an infusion of intra-arterial oxypurinol. This prompts 2 conclusions: that oxidative stress in hypercholesterolemia is amenable to XO inhibition and that the effects of XO inhibition manifest quickly. Although Cardillo et al[9] and we (present study) considered separate disease states, both of these disease states (diabetes and hypercholesterolemia) are characterized by oxidative stress and endothelial dysfunction, which supports the probability that XO inhibition is likely to lead to prompt and sustained improvements in vascular function under conditions of raised oxidative stress.

The work in hypercholesterolemia[9] suggests that XO inhibition can quickly improve endothelial function over a matter of minutes. Our data also are encouraging in that the benefits remained at 1 month. However, we cannot say how long the

improvement began before the month or how much longer it would have lasted. Future studies will need to be conducted to examine this issue. Certainly we found no order effect with our randomization, suggesting that the benefit does not persist for 1 month after the allopurinol is stopped.

Patients with type 2 diabetes have significantly impaired endothelial dysfunction,[31] and persistent hyperglycemia may be the key factor[32] by activating the polyol pathway,[33] increasing the level oxidation of LDL fractions,[34] and stimulating advanced glycation end products.[35] Others factors may also be important, such as insulin itself, triglycerides, and small dense LDL-cholesterol. However, the impact of such factors is difficult to assess because we did not measure LDL subfractions, advanced glycation end products (AGEs), or insulin, and therefore firm conclusions are difficult to draw. However, in the present study, the levels of hyperglycemia were well controlled, which suggests that the prevailing blood glucose may not be the only factor involved in producing endothelial dysfunction in diabetes.

There are some previous studies on therapies designed to improve vascular function in type 2 diabetics. An acute intra-arterial infusion of vitamin C improves endothelial function by 50%,[36] and vitamin E decreases lipid peroxidation products,[37] suggesting that free radicals play an important role in the development of endothelial dysfunction. ACE inhibitors appear to improve endothelial function in type 1 diabetics by 70%[38] but not in type 2 diabetics.[39] Others have found no benefit with L-arginine in type 2 diabetes.[40] There are no conclusive data concerning the benefits of angiotensin II receptor antagonists, beta-carotene, or estrogen therapy in type 2 diabetics. However, one would expect the benefits of lipid-lowering therapy on endothelial function to be similar or greater in diabetics than in nondiabetic subjects, although this has not been studied specifically.

Blood pressure and baseline forearm blood flow were unaffected by allopurinol, which suggests that hemodynamic factors are unlikely to be responsible for the observed changes. Other accepted causes for altered endothelium-dependent vasodilatation in patients with type 2 diabetes include raised cholesterol leveles and hyperglycemia. All of these factors were identical between treatment days in both type 2 diabetic subjects and age-matched control subjects. Of particular relevance, in light of the data from Cardillo et al,[9] the cholesterol levels were well within the normal range, thus excluding the possibility that we may in fact be seeing an effect of XO inhibition in patients with high cholesterol levels.

There were important, although not unexpected, differences between the diabetic and the control group. Although BMI and cigarette smoking were well matched, blood pressure was higher in type 2 diabetic patients, although the degree of hypertension was mild. Hypertension is associated with endothelial dysfunction in most, but not all, studies.[41] However, there are 2 reasons for believing that the mild hypertension seen in our diabetic subjects did not contribute much to the allopurinol-induced improvement in endothelial dysfunction. The first reason is that Cardillo et al[9] demonstrated no significant effect of oxypurinol on endothelial dysfunction in hypertensive subjects. Second, in stepwise regression analysis of our data, we found that diabetes, then cholesterol, and finally blood pressure, in that order, were predictors of the response to allopurinol. This is in fact what one might expect because much of the endothelial dysfunction of diabetes and hypercholesterolemia is thought to be directly attributable to oxidative stress, whereas oxidative stress is not a key feature of hypertension per se. In fact, our stepwise regression analysis and the work of Cardillo et al[9] tentatively suggest that mild hypertension does not produce a form of endothelial dysfunction that is amenable to treatment with allopurinol.

Conclusions

The present data show for the first time that allopurinol improves endothelial function in patients with type 2 diabetes and associated mild hypertension. In this group, allopurinol may improve endothelial function to near-normal levels. A likely mechanism is that allopurinol decreases free radical generation through inhibition of the XO enzyme system. The lower free radical burden improves the availability of NO, leading to an improvement in endothelial vasodilatation. These benefits are not apparent in healthy age-matched control subjects.

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