

# Xanthine Oxidase Is Involved in Free Radical Production in Type 1 Diabetes

## Protection by Allopurinol

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**The aim of this work was to study the mechanism of free radical formation in type 1 diabetes and its possible prevention. We have found oxidation of blood glutathione and an increase in plasma lipoperoxide levels in both human type 1 diabetes and experimental diabetes. Peroxide production by mitochondria does not increase in diabetes. On the contrary, the activity of xanthine oxidase, a superoxide-generating enzyme, increases in liver and plasma of diabetic animals. The increase in plasma xanthine oxidase activity may be explained by the increase in the hepatic release of this enzyme, which is not due to nonspecific membrane damage: release of other hepatic enzymes, such as the amino transferases, does not increase in diabetes. Superoxide formation by aortic rings of rabbits increases significantly in diabetes. This is completely inhibited by allopurinol, an inhibitor of xanthine oxidase. Heparin, which releases xanthine oxidase from the vessel wall, also decreases superoxide formation by aortic rings of diabetic animals. Treatment with allopurinol decreases oxidative stress in type 1 diabetic patients: hemoglobin glycation, glutathione oxidation, and the increase in lipid peroxidation are prevented. These results may have clinical significance in the prevention of late-onset vascular complications of diabetes. *Diabetes* 51:1118–1124, 2002**

**A** major concern in the clinical management of diabetes is the occurrence of severe vascular complications. Oxidative stress has been involved in the pathogenesis of such complications (1). Oxidative stress (2) occurs when the balance between oxidation and antioxidant is tilted in favor of the former. The occurrence of oxidative stress in diabetes has been extensively documented (3,4). However, the mechanism of the formation of oxidative stress has not been studied in detail. Hunt et al. (5) suggested that auto-oxidative glycosylation is important in explaining

free radical formation and protein damage in diabetes. Recently, Jain and colleagues (6–8) have proposed that ketone bodies, especially acetoacetate, are involved in free radical formation in type 1 diabetes.

In this article, we propose an enzymatic mechanism to explain the increased generation of free radicals in diabetes. We confirm that there is oxidative stress (oxidation of glutathione and an increase in lipoperoxides) in human type 1 diabetes and experimental diabetes. We show that xanthine oxidase (a superoxide-generating enzyme) is increased in plasma and liver of diabetic rats. Xanthine oxidase is shed by the liver into the plasma and is bound to vascular endothelial cells. Arterial rings from diabetic rabbits (but not from control rabbits) produce superoxide in the presence of xanthine. This process is inhibited by treatment with heparin (which releases xanthine oxidase from the endothelial surface).

The determination of an enzymatic mechanism of free radical formation in diabetes suggests a mechanism (inhibition of xanthine oxidase) to prevent oxidative stress in this disease. Indeed, we have found that allopurinol, an inhibitor of xanthine oxidase widely used in clinical practice, is effective in preventing oxidation of glutathione and lipoperoxidation in experimental and human type 1 diabetes. Given the fact that oxidative stress plays a role in the development of complications in diabetes (1), the prevention of free radical formation by allopurinol may have clinical significance.

### RESEARCH DESIGN AND METHODS

**Patients.** This prospective, randomized, double-blind clinical trial was performed at the Unit of Endocrinology and Metabolism of the outpatient clinic of the Hospital Virgen del Consuelo, Valencia, Spain. Eligible participants were enrolled among diabetic patients fulfilling the following criteria: 1) having been routinely controlled in our outpatient clinic for at least 2 years before the beginning of the trial; 2) having no acute episode of an inflammatory and/or infectious process immediately before or during the trial; and 3) having given informed consent in all cases (in pediatric patients, informed consent was signed by the parent or guardian). The control group consisted of healthy individuals who were being checked to undergo minor surgery and were matched for age and sex with the experimental groups.

Diabetic patients were assigned to the experimental or placebo group using a random number automatically generated by a computer program. Physicians, nurses, and laboratory technicians were unaware of the type of treatment received by each individual patient.

Patients received a capsule containing 300 mg of allopurinol (Zyloric) (experimental group) or 1 g of cellulose (placebo group) to be taken by mouth daily for 14 consecutive days.

Blood samples were obtained by venipuncture of a peripheral vein at 8:00

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AAT, alanine amino transferase; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; MDA, malondialdehyde.

A.M. on day 0 and day 15 after an overnight fast of 8–10 h. Blood samples were processed immediately.

Study protocols were approved by the hospital ethics committee. Investigation was conducted according to the principles expressed in the Declaration of Helsinki. All patients were informed of the proceedings. Patients had a glycemia level of  $183 \pm 67$  ( $n = 12$ ) mg/dl and a glycosylated hemoglobin of  $6.83 \pm 0.88\%$  ( $n = 12$ ). Control subjects were age-matched patients with normal glucose metabolism (glycemia,  $<100$  mg/dl; glycosylated hemoglobin,  $<6\%$ ).

**Animals.** We used 6-month-old male Wistar rats made diabetic by streptozotocin administration. The protocols were approved by the animal ethics committee. A dose of 55 mg/kg body wt dissolved in 0.8 ml of 0.1 mol/l citrate buffer, pH 4.5, was administered. After streptozotocin administration, animals were placed in metabolic cages, and their food intake and diuresis were controlled. Ten days after administration of streptozotocin, rats had polyuria, polydipsia, and weight loss. All animals with a glucosuria of  $>20$  g/l were considered diabetic; in all cases, they had a glycemia of  $\geq 200$  mg/dl. Control animals were injected with the same volume of citrate buffer as diabetic animals but without streptozotocin. Rabbits were used to measure superoxide formation by aortic rings, because the size of the aorta in rats makes their use impractical. New Zealand rabbits weighing 2.0 to 3.2 kg were sedated with 40 mg ketamine (Ketolar) intramuscularly. Diabetes was induced by injecting alloxan (100 mg/kg body wt; Sigma, St. Louis, MO) into the lateral ear vein. To prevent hypoglycemia, 10 ml of 5% glucose was injected intravenously and drinking water was supplemented with 10% glucose for the first 24 h after the alloxan injection. Thereafter, the animals were maintained on tap water and regular food ad libitum for 6 weeks. A second group of rabbits (2.1–3.0 kg) was maintained in the same conditions for the same period to serve as age-matched controls. Diabetic rabbits showed a marked increase in serum glucose and a failure to gain weight compared with controls. Glycemia of controls did not change significantly during the study and was maintained at  $100 \pm 3$  mg/dl ( $n = 16$ ). The glycemia of diabetic rabbits was  $360 \pm 23$  mg/dl ( $n = 14$ ).

**Allopurinol administration.** Allopurinol was administered intraperitoneally to a group of diabetic rats 5 days after they had shown signs of diabetes. Allopurinol (8 mg) was dissolved in 350  $\mu$ l dimethylsulfoxide and up to 1 ml water was added. Doses were given 30 and 6 h before decapitation.

**Rat liver perfusion to measure xanthine oxidase release.** Livers were washed via the portal vein with a Krebs-Henseleit saline solution, pH 7.4, for 6–8 min. Subsequently, livers were quickly removed and constantly perfused for 60 min as described in Viña et al. (9). Perfusion fluid was collected after 5, 10, 15, 30, 45, and 60 min. Enzyme activities (xanthine oxidase, lactate dehydrogenase [LDH], and alanine amino transferase [AAT]) were determined in the perfusate. No changes in LDH or in AAT were found, showing that livers are not damaged in the isolation procedure.

**Preparation and analysis of samples.** Rat blood was obtained by decapitation. Human blood was obtained by venous puncture and was immediately put into heparinized tubes. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined as previously described (10,11). Briefly, blood samples were treated with 6% (vol/vol) perchloric acid containing 1 mmol/l EDTA (1:1) to determine GSH or with 6% perchloric acid containing 50 mmol/l *N*-ethyl-maleimide and 1 mmol/l EDTA to determine GSSG by high-performance liquid chromatography (HPLC). Samples were centrifuged for 10 min at 15000g, and the acidic supernatants were neutralized and used for determination of metabolites. Blood plasma was used to measure glucose, acetoacetate, hydroxybutyrate, and enzymatic activities (12).

Xanthine oxidase was measured as previously described (13). Briefly, isoxanthopterin formation from pterine was followed by fluorimetry (excitation at 345 nm and emission at 390 nm). Malondialdehyde (MDA) levels were determined by HPLC as previously described (14).

**Determination of superoxide formation by rabbit aortic rings.** Superoxide-dependent lucigenin chemiluminescence of aortic rings was measured as described by White et al. (15). Chemiluminescence was determined using a scintillation counter set to operate in single-photon count mode. The rat thoracic aorta was excised, cleansed of fat and adhering tissue, and divided in three rings of 3–4 mm each. In one of those rings, chemiluminescence was measured in a vial containing 3 ml PBS with 0.25 mmol/l lucigenin and 50  $\mu$ mol/l xanthine. In a second ring, chemiluminescence was determined in the same conditions plus 100 mmol/l allopurinol. A third ring was preincubated with heparin (1,000 units/ml) for 10 min, and, after washing, chemiluminescence was determined in the presence of lucigenin and xanthine.

**Isolation of mitochondria.** Isolation was performed following a standard differential centrifugation protocol described by Rickwood et al. (16). After isolation, heart or liver mitochondria were suspended in ice-cold respiratory buffer (with 0.3 mol/l sucrose, 1 mmol/l EGTA, 5 mmol/l MOPS, 0.1% BSA, and 5 mmol/l  $\text{KH}_2\text{PO}_4$  adjusted to pH 7.4 with KOH).

TABLE 1

Effect of diabetes on blood glutathione redox state and on the level of hydroperoxides in plasma of type 1 diabetic patients

	Control	Diabetic
GSH ( $\mu$ mol/l)	$919 \pm 69$ (5)	$894 \pm 145$ (12)
GSSG ( $\mu$ mol/l)	$21 \pm 14$ (5)	$70 \pm 35$ (12)*
GSSG/GSH (%)	$2.31 \pm 1.57$ (5)	$7.64 \pm 4.62$ (12)†
Lipid hydroperoxides (as MDA) (nmol/ml)	$0.90 \pm 0.35$ (5)	$1.61 \pm 0.82$ (12)†

Data are means  $\pm$  SD (number of determinations). \* $P < 0.01$ ; † $P < 0.05$  vs. control.

**Flow cytometric analysis.** Flow cytometry was performed using an EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL). Fluorochromes were excited with an argon laser tuned at 488 nm. Forward-angle light scatter and side-angle light scatter were measured, and fluorescence was detected through a 488-nm blocking filter, a 550-nm long-pass dichroic, and a 525-nm band-pass or a 575-nm long-pass. For each sample, 10,000 mitochondria were counted.

To measure the production of peroxides, dihydrorhodamine 123 was used as fluorochrome during incubation at 37°C for 30 min and was excited with an argon laser tuned at 488 nm. Oxidation of dihydrorhodamine 123 is caused by  $\text{H}_2\text{O}_2$ -dependent reactions involving reactive oxygen species. Dihydrorhodamine 123 is uncharged but is oxidized to positively charged rhodamine 123 in the extramitochondrial space. The latter is taken up by mitochondria, where it accumulates.

Mitochondrial membrane potential from isolated mitochondria was measured using rhodamine 123 as described above. The gate was set on logarithm of forward and side scatter to always include mitochondria with the same morphological properties. Thus, changes in fluorescence were independent of particle size.

**Statistics.** Results are expressed as means  $\pm$  SD. The number of experiments is included in each figure and table. Statistical analyses were performed by the least-significant difference test, which consists of two steps. First, an ANOVA was performed. The null hypothesis was accepted for all numbers of those sets in which  $F$  was nonsignificant at the level of  $P \leq 0.05$ . Second, the sets of data in which  $F$  was significant were examined by the modified  $t$  test using  $P \leq 0.05$  as the critical limit.

## RESULTS

**Oxidative stress in type 1 diabetes: oxidation of blood glutathione and increased plasma lipoperoxide levels.** Table 1 shows the effect of diabetes on glutathione redox state in blood of type 1 diabetic patients. We show that diabetes causes a significant ( $P < 0.01$ ) increase in GSSG levels,  $\sim 300\%$  of the value found in control subjects. This resulted in a significant ( $P < 0.05$ ) increase in the GSSG/GSH values (glutathione redox ratio), an index of oxidative stress (17).

Table 1 also shows the effect of type 1 diabetes on plasma levels of hydroperoxide determined as MDA: diabetic patients had plasma hydroperoxide levels 50% higher than healthy control subjects.

**Oxidative stress in experimental diabetes: oxidation of glutathione and increased lipid hydroperoxide levels in blood, liver, and heart of rats.** Experimental diabetes resulted in increased GSSG levels both in blood and in organs such as heart and liver of the rat. Figure 1 shows the GSSG-to-GSH ratio in control and diabetic animals. In all cases studied, the GSSG-to-GSH ratio in diabetic animals was  $\sim 200\%$  of the values in control animals.

Figure 1 also shows the lipid hydroperoxide levels in blood plasma, liver, and heart of control and diabetic rats. Lipid hydroperoxide levels in diabetic rats were significantly higher than in control rats. Indeed, the values in tissues from diabetic animals were 190% (heart), 250%

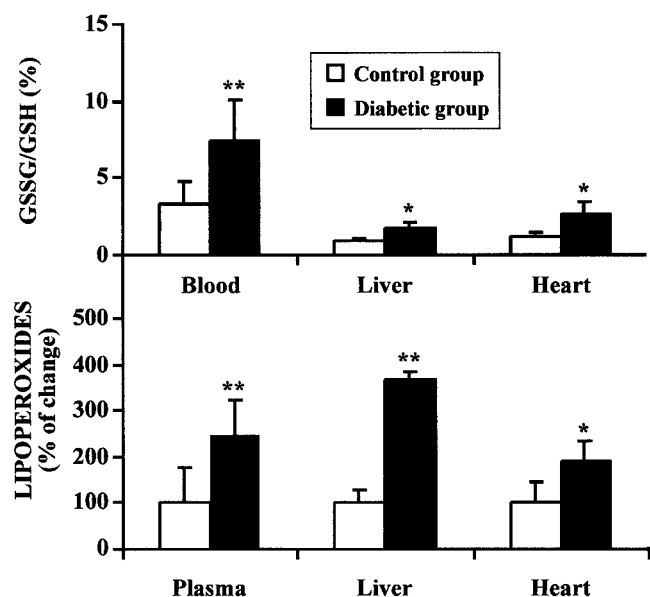


FIG. 1. Effect of diabetes on oxidation of glutathione and lipoperoxide levels in blood, liver, and heart of rats. Diabetes was induced in rats by treatment with streptozotocin, as indicated in RESEARCH DESIGN AND METHODS. All results are the means  $\pm$  SD for five experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , control vs. diabetic.

(blood plasma), and 360% (liver) higher than the corresponding control values.

**Mitochondria from liver, heart, and kidney do not directly increase levels of H<sub>2</sub>O<sub>2</sub> in type 1 diabetes.**

Mitochondria are involved in aging (18–20) and in many age-associated (21) and toxicologic (22) diseases. Thus, we studied the effect of diabetes on peroxide generation by mitochondria in liver, heart, and kidney from control and diabetic rats.

Table 2 shows that mitochondria from diabetic animals have peroxide production and mitochondrial potential similar to those of control animals. Moreover, liver mitochondria from diabetic rats have an even higher mitochondrial membrane potential than their control counterparts. Figure 2 shows a representative experiment in which experimental diabetes did not cause any detectable change in peroxide production by mitochondria.

**Xanthine oxidase activity increases in liver and plasma of diabetic rats.** Xanthine oxidase is an important source of free radicals in vivo. Indeed, we showed that this enzyme is involved in free radical production associated with exercise in patients with chronic obstructive pulmonary disease (23).

TABLE 2  
Mitochondrial membrane potential and peroxide production in liver and heart mitochondria from control and diabetic rats

	Control	Diabetes
<b>Liver</b>		
Membrane potential (AU)	43 $\pm$ 23 (6)	70 $\pm$ 26 (7)*
Peroxide production (AU)	17 $\pm$ 2 (7)	19 $\pm$ 3 (8)
<b>Heart</b>		
Membrane potential (AU)	2.9 $\pm$ 0.9 (6)	2.8 $\pm$ 0.9 (6)
Peroxide production (AU)	22 $\pm$ 2 (6)	19 $\pm$ 2 (6)

Data are means  $\pm$  SD (number of determinations). \* $P < 0.05$  vs. control. AU, arbitrary units.

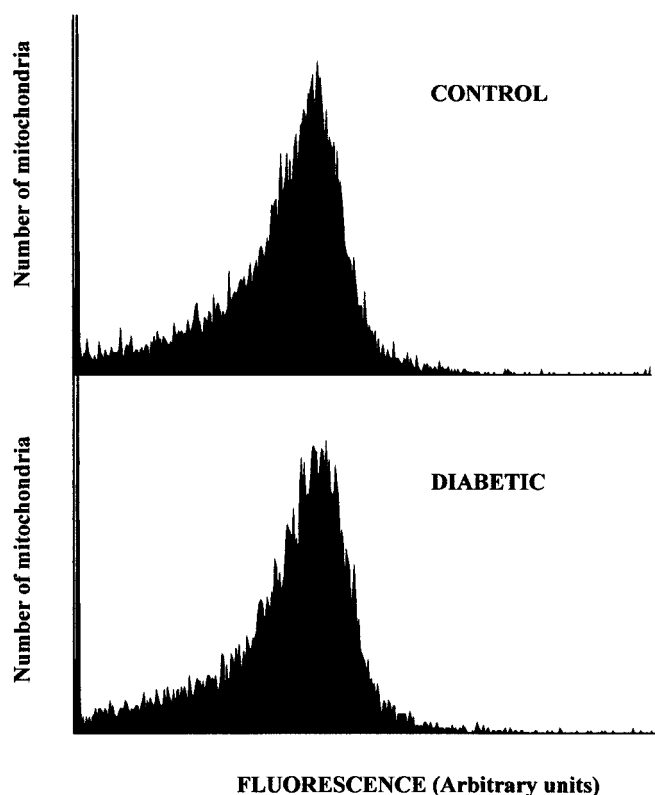


FIG. 2. Oxidant production by mitochondria from control and diabetic animals. Flow cytometric recordings indicate that oxidant production by heart mitochondria from diabetic animals is not different from that of control animals. A representative experiment of three is shown.

In this study, we found that experimental diabetes caused an increase in hepatic xanthine oxidase. Table 3 shows that diabetes causes a significant increase ( $P < 0.01$ ) in both hepatic xanthine oxidase and xanthine dehydrogenase activities. Xanthine oxidase activity in liver from diabetic rats is  $>200\%$  of the control value.

In a similar fashion, xanthine oxidase activity in plasma from control rats was  $5.9 \pm 1.0$  IU/ml ( $n = 15$ ); in plasma from diabetic rats, it was  $7.8 \pm 1.4$  IU/ml ( $n = 13$ ) ( $P < 0.01$ ). This represents an increase of  $>30\%$  over the control value.

**Origin of plasma xanthine oxidase in diabetes: increased hepatic release.** Figure 3 shows that xanthine oxidase is released from the liver of diabetic rats, but not from control rats. This hepatic release of xanthine oxidase is not the result of a nonspecific protein leakage. In fact, other hepatic enzymes, such as AAT, are not released by the liver of either control or diabetic rats (Fig. 3).

TABLE 3  
Effect of diabetes on hepatic xanthine oxidase and dehydrogenase activities in rat

	Control	Diabetes
XO (units/g protein)	0.3 $\pm$ 0.2 (4)	0.7 $\pm$ 0.1 (5)*
XDH (units/g protein)	1.3 $\pm$ 0.5 (4)	2.2 $\pm$ 0.2 (5)*
XO/XDH	0.26 $\pm$ 0.05 (4)	0.32 $\pm$ 0.02 (5)†

Data are means  $\pm$  SD (number of determinations). \* $P < 0.01$ ; † $P < 0.05$  vs. control. XO, xanthine oxidase; XDH, xanthine dehydrogenase.

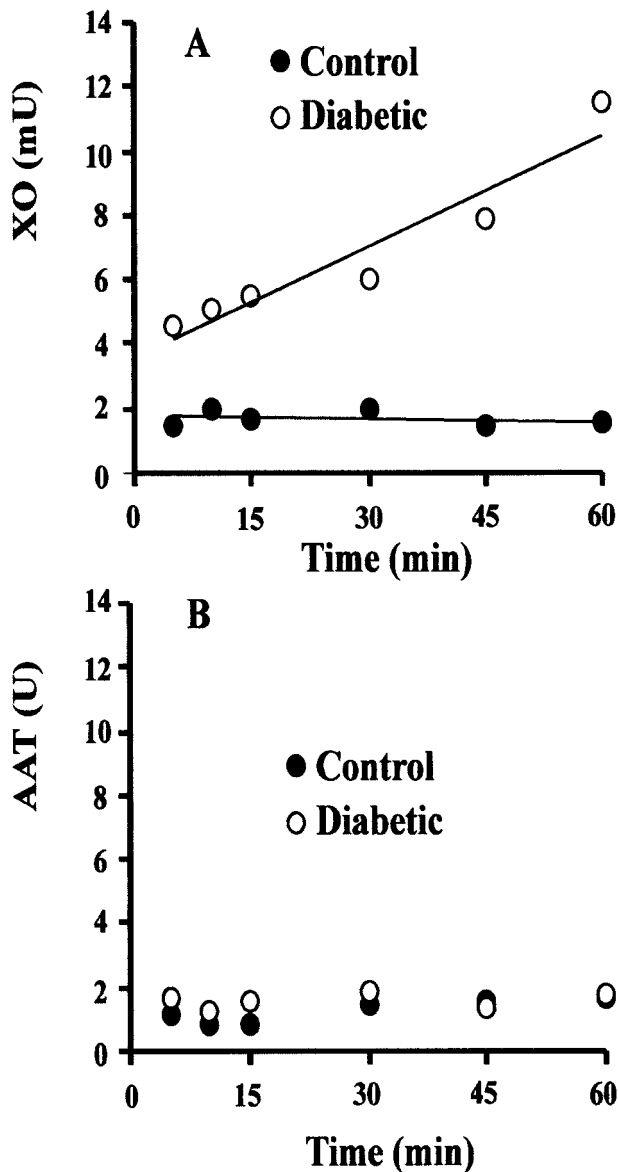


FIG. 3. Enzyme release from livers of control and diabetic rats. Livers were perfused as explained in RESEARCH DESIGN AND METHODS, and the activity of the enzymes indicated was measured at different time points. *A*: Xanthine oxidase (XO) is released from livers of diabetic animals, but not from control animals. *B*: AAT is not released from the liver of either control or diabetic rats. A representative experiment of four is shown.

**Xanthine-dependent superoxide production in aorta from normal and diabetic animals.** Xanthine oxidase binds to the surface of vascular endothelial cells (24) and produces superoxide on addition of xanthine to the suspension medium. We measured superoxide production by aortic rings of rabbits. Figure 4 shows that aortic superoxide production is significantly higher in diabetic rabbits than in control rabbits.

Xanthine oxidase is bound to endothelial cells by sulfated glycosaminoglycans, and it may be released by treatment with heparin (24). Figure 4A shows the production of superoxide in control samples. This low rate is decreased by neither heparin nor allopurinol, an inhibitor of xanthine oxidase. This result contrasts with the high rate of production of superoxide by aortic rings of diabetic

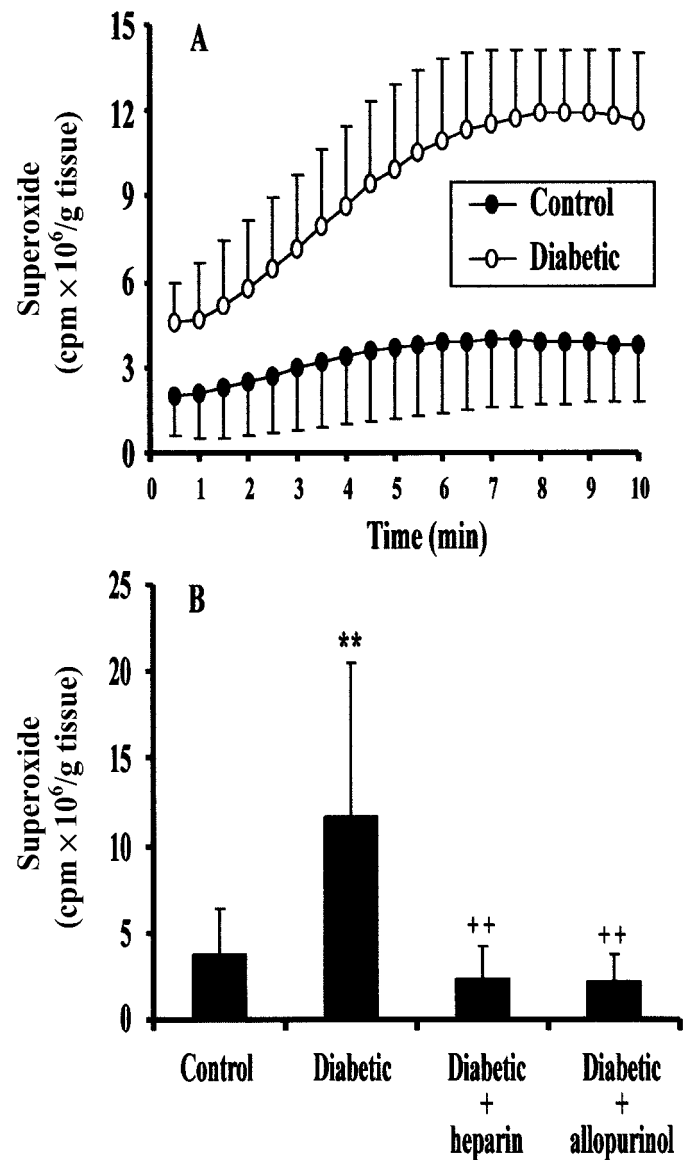


FIG. 4. Superoxide-dependent lucigenin chemiluminescence in aortic rings from control and diabetic animals. *A*: Chemiluminescence profiles of aortic rings from control and diabetic rabbits. *B*: Chemiluminescence measured at the 10-min time point in control rabbits, diabetic rabbits, and diabetic rabbits treated with heparin or allopurinol. All results are the means  $\pm$  SD for five determinations. \* $P < 0.05$ , \*\* $P < 0.01$ , control vs. diabetic; ++ $P < 0.01$ , diabetic vs. diabetic treated with allopurinol or heparin.

rabbits, which was severely inhibited by heparin or allopurinol (Fig. 4B).

**Allopurinol decreases oxidative stress in experimental diabetes.** An important consequence of the role of xanthine oxidase in the generation of oxidative stress in diabetes is that allopurinol should be able to prevent it. Table 4 shows that allopurinol is indeed able to prevent glutathione oxidation in blood, liver, and heart of diabetic rats. In all cases studied, allopurinol afforded complete prevention against the oxidation of the glutathione pool caused by diabetes.

Allopurinol was also able to prevent the increase in lipid hydroperoxide formation in experimental diabetes. Table 5 shows that the increase in hydroperoxide levels in blood

TABLE 4

Effect of allopurinol on GSSG levels in blood, liver, and heart of diabetic rats

	Control	Diabetic	Diabetic treated with allopurinol
Blood ( $\mu\text{mol/l}$ )	$37 \pm 11$ (6)	$73 \pm 26$ (7)*	$50 \pm 12$ (7)†
Liver (nmol/g)	$61 \pm 5$ (3)	$110 \pm 32$ (5)‡	$53 \pm 21$ (5)†
Heart (nmol/g)	$35 \pm 22$ (3)	$82 \pm 10$ (3)‡	$66 \pm 10$ (3)

Data are means  $\pm$  SD (number of determinations). \* $P < 0.01$ ; † $P < 0.05$  vs. allopurinol-treated; ‡ $P < 0.05$  vs. control.

plasma, liver, and heart of diabetic rats was completely reversed by treatment with allopurinol.

**Allopurinol decreases oxidative stress in human type 1 diabetes.** Table 6 shows that treatment with allopurinol prevented the oxidation of glutathione (evidenced by an increase in blood GSSG levels and blood GSSG-to-GSH ratio) that occurs in type 1 diabetes. Furthermore, allopurinol also prevented the increase in plasma lipid hydroperoxide levels. Indeed, Table 6 shows that the levels of hydroperoxides in the plasma of diabetic patients was significantly higher than in control subjects and that this increase was prevented by treatment with allopurinol. In contrast, patients who received placebo instead of allopurinol did not show changes in oxidized glutathione levels ( $21 \pm 14 \mu\text{mol/l}$  in control subjects vs.  $82 \pm 12 \mu\text{mol/l}$  in the placebo group) or in hydroperoxide levels ( $0.90 \pm 0.35 \mu\text{mol/l}$  in control subjects vs.  $1.51 \pm 0.40$  in the placebo group).

## DISCUSSION

**Oxidative stress in experimental and human diabetes.** Evidence of the occurrence of oxidative stress in diabetes has experienced an explosive growth. A search through the PubMed database of the National Library of Medicine shows that the number of publications relevant to this subject in the period 1990–1995 is about 10-fold that of the period 1980–1985.

Early observations showed a decrease in GSH content in erythrocytes from diabetic animals and an increase in plasma lipid peroxidation. The reported effects include changes in the activity of antioxidant enzymes (3), changes in hepatic glutathione metabolism (25), and changes in free radical formation due to reactions associated with heavy metals (4). It is important, however, to determine which changes are due to diabetes itself and which are a consequence of the dietary alterations associated with diabetes (25). In our study, the patients did not have any clinical sign of dietary alteration, such as polyuria, polydipsia, or polyphagia.

Using a specific method to determine the glutathione redox ratio (10,11), we have found that there is indeed glutathione oxidation in diabetes (both type 1 human diabetes and experimental diabetes). We have also found that lipoperoxide levels increase in diabetes.

The hypothesis that there is oxidative stress in diabetes has been challenged recently (26). Those authors suggested that streptozotocin causes free radical damage *per se* and that diabetes itself may not cause oxidative stress. The following facts make this unlikely: 1) we and other researchers (6) find signs of oxidative stress in human

TABLE 5

Allopurinol diminishes the increase in lipid hydroperoxide levels in blood plasma, liver, and heart of diabetic rats

	Control	Diabetic	Diabetic treated with allopurinol
Plasma (nmol/ml)	$0.4 \pm 0.3$ (6)	$1.0 \pm 0.3$ (10)*	$0.6 \pm 0.2$ (4)†
Liver (nmol/g)	$10.3 \pm 3.1$ (3)	$38.0 \pm 1.4$ (3)*	$16.0 \pm 5.9$ (3)‡
Heart (nmol/g)	$2.2 \pm 1.0$ (3)	$4.2 \pm 1.0$ (3)§	$2.5 \pm 0.4$ (3)†

Data are means  $\pm$  SD (number of animals). All results are nanomoles of MDA formed by *in vitro* degradation of lipid hydroperoxides. \* $P < 0.01$ ; † $P < 0.05$ ; ‡ $P < 0.01$  vs. diabetic; § $P < 0.05$  vs. control.

diabetes (Table 1); 2) there are signs of oxidative stress in models of experimental diabetes in which streptozotocin is not used (27); and 3) we have found that there is a period of time of about a week after streptozotocin administration before diabetes is established. In this period of time, we find no signs of oxidative stress, and only when hyperglycemia and hyperketonemia occur do we find oxidation of glutathione and an increase in plasma lipoperoxide levels.

**Mechanism of the generation of free radicals in diabetes: role of xanthine oxidase.** Different sources of free radicals in diabetes have been proposed, including the sorbitol pathway, the induction of NAD(P)H oxidases, and nitric oxide synthase. Cosentino et al. (28) reported that high glucose increases nitric oxide synthase expression in aortic endothelial cells. In a very interesting series of articles (6–8), Jain and colleagues found that hyperketonemia is associated with free radical formation in diabetes and that acetoacetate in the presence of  $\text{Fe}^{2+}$  can generate superoxide *in vitro*.

In this article, however, we report the activation of a specific enzyme activity, xanthine oxidase, which produces oxidant species and subsequently oxidative stress in diabetes.

The role of xanthine oxidase in the vascular dysfunction that occurs in atherosclerosis was studied by White et al. (15). Using aortic rings from diabetic rabbits, we have found that superoxide formation also increases in arteries from diabetic animals. This process is inhibited by allopurinol, a xanthine oxidase inhibitor widely used in clinical practice.

Xanthine oxidase is bound to endothelial cells by sulfated glycosaminoglycans (24). Treatment with heparin releases xanthine oxidase from the vessel wall. In Fig. 4, we show that treatment with heparin decreases superoxide production by aortic rings from diabetic rabbits.

The fact that the production of such a reactive molecule as superoxide is increased in the vessel wall of diabetic animals may be relevant in explaining some of the arterial complications of diabetes and underscores the importance of xanthine oxidase in this process.

**Origin of xanthine oxidase in diabetes.** The increase in xanthine oxidase activity in diabetes prompts the question of the origin of this increased activity in the diabetic animal. The tissues that express the highest activity of this enzyme are liver and intestine (29). We found that diabetes causes an increase of xanthine oxidase activity in liver. Moreover, we found that xanthine oxidase is released by the liver of diabetic animals, but not by that of controls

TABLE 6

Allopurinol decreases the oxidation of blood glutathione and the increase in plasma lipoperoxides in human type 1 diabetic patients

	Control	Diabetic	Diabetic + allopurinol	Diabetic + placebo
GSH ( $\mu\text{mol/l}$ )	919 $\pm$ 69 (5)	894 $\pm$ 145 (12)	838 $\pm$ 117 (8)	824 $\pm$ 59 (4)
GSSG ( $\mu\text{mol/l}$ )	21 $\pm$ 14 (5)	70 $\pm$ 35 (12)*	31 $\pm$ 12 (8)†	82 $\pm$ 12 (4)‡
GSSG/GSH (%)	2.31 $\pm$ 1.57 (5)	7.64 $\pm$ 4.62 (12)§	3.58 $\pm$ 1.15 (8)	10.10 $\pm$ 1.81 (4)‡
Plasma lipoperoxide (nmol/ml)	0.90 $\pm$ 0.35 (5)	1.61 $\pm$ 0.82 (12)§	0.58 $\pm$ 0.24 (8)†	1.51 $\pm$ 0.40 (4)‡

Data are means  $\pm$  SD (number of determinations). \* $P$  < 0.01 vs. control; † $P$  < 0.01 vs. diabetic group; ‡ $P$  < 0.01 vs. diabetic + allopurinol; § $P$  < 0.05 vs. control; || $P$  < 0.05 vs. diabetic group.

(Fig. 3). The release of this enzyme is not the result of a nonspecific leakage from the hepatocyte. We measured the release of other enzymes that are considered markers of hepatic damage, such as aspartate amino transferase (ALAT), and found that it is not significantly higher in diabetic versus control hepatocytes. These experiments lead us to conclude that xanthine oxidase is released from the liver of diabetic animals. This is not specific to diabetes. The release of xanthine oxidase from liver has been observed in other pathological states, such as hemorrhagic shock (30).

**Prevention of oxidative stress in diabetes by allopurinol: possible clinical implications.** Oxidative stress has been considered an important factor in the development of complications in diabetes (1). Our studies using aortic rings indicate that superoxide is formed in the vessel wall of diabetic animals (Fig. 4).

This article shows that xanthine oxidase plays an important role in the generation of free radicals in diabetes. An obvious conclusion is that inhibition of this enzyme should prevent oxidative stress in diabetes. Allopurinol inhibits xanthine oxidase in vivo, and it is used in clinical practice. Treatment of patients with allopurinol prevented glutathione oxidation and lipoperoxidation (Table 6) in human type 1 diabetes. Inhibition of xanthine oxidase has proved effective in improving endothelial vasodilator function in hypercholesterolemic, but not hypertensive, patients (31). Very recently, Butler et al. (32) reported that allopurinol protects against endothelial dysfunction in diabetic patients with mild hypertension. Here, we suggest that the liver in type 1 diabetes releases xanthine oxidase to the plasma. The enzyme binds with glucosaminoglycans to the blood vessel, inducing local oxidative stress and tissue damage.

As we report here, heparin is able to decrease peroxide levels in aortic rings from diabetic rabbits, possibly because of the release of xanthine oxidase from the vessel wall. Heparin plays an important role in the regulation of lipid levels during diabetes. Results reported here show a new potential use of heparin in diabetes.

Given the importance of oxidative stress in the development of complications in diabetes (1), the role of xanthine oxidase in the pathogenesis of such complications—and the protective effect of allopurinol that we have shown here—may have clinical relevance.

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