Effects of Allopurinol on Ketone Body Metabolism and Tissue Lipid Peroxidation in Alloxan Diabetes in Rats.

Saleh A. Wohaieb

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ABSTRACT

The aim of the present study is to investigate whether or not xanthine oxidase (XO)–derived reactive oxygen species (ROS) may play a role in the pathogenesis of alloxan (ALX)–induced diabetes in rats using the specific XO inhibitor and hydroxyl radical scavenger, allopurinol

The involvement of oxidative stress in ALX – diabetes was assessed by the measurement of plasma and various tissues lipid peroxides levels (using thiobarbituric acid (TBA) reactive substances). Furthermore, the ability of allopurinol to influence these and other biochemical parameters, including plasma and urine ketones levels were also investigated in diabetic rats.

Rats were divided into four groups: control, untreated diabetic, allopurinol – treated diabetic, and insulin – treated diabetics. At the end of the one week experimental period, blood and tissue samples were obtained from anesthesized animals for the measurement of the above – mentioned parameters.

Although the single intraperitoneal (i.p.) injection of allopurinol (25 mg/kg body wt.) 1h before or 1h after ALX injection (100 mg/kg body wt., i.p.) failed to prevent the induction of diabetes, it did lower ketonuria and the incidence of early ketosis–associated mortality in diabetic animals in comparison with non–allopurinol–treated diabetic rats. Subsequent administration of allopurinol (25 mg/kg body wt., i.p.) every 48 hr for 1wk (i.e., 3 additional doses) also decreased plasma ketone bodies levels as well as plasma and tissue (heart, liver, kidney, pancreas) lipid peroxides levels in comparison with non–allopurinol–treated diabetic rats. Daily insulin injection (9–12 U/kg body wt., S.C.) for 1wk period normalized all of the above–mentioned abnormalities.

The present results suggest that XO–derived ROS play a minor role (if any) in the diabetogenic effect of ALX. On the other hand, although the mechanism (s) underlying the protective effects of allopurinol on the diabetic state is presently unknown, these effects may reflect a possible association between impaired ketone body metabolism and lipid peroxidation: and suggest an effect of allopurinol on ketone body metabolism.

خلاصة :

إن الهدف من الدراسة الحالية هو تقصي إمكانية وجود أي دور لأنواع الأوكسجين المتفاعلة ، والمتكونة من انزيم الزانثين اوكسديز، في امراضية داء السكري المحدث بواسطة الالوكسان في الجرذان ، وذلك باستخدام عقار الالوبيورينول ، المثبط الخاص لأنزيم الزانثين اوكسديز وكذلك كاسح جذور الهيدروكسيل الحرة .

وتم تقييم الدور الذي يؤديه الاجهاد التأكسدي في داء السكري المحدث بواسطة الالوكسان من خلال قياس مستويات بيروكسيدات الدهن في البلازما والعديد من الانسجة (بأستخدام المواد المتفاعلة مع حمض الثايوباربتيوريك). بالاضافة لذلك, تم دراسة قابلية عقار الالوبيورينول في التأثير على هذه المعايير وكذلك على المعايير الكيميائية الحياتية الاخرى, ومن ضمنها مستويات الكيتونات في البلازما والبول في الجرذان المصابة بالسكري. وتم تقسيم الجرذان الى اربعة مجاميع: السيطرة, المصابة بالسكري غير المعالجة, المصابة بالسكري والمعالجة بالالوبيورينول, ومجموعة المصابة بالسكري والمعالجة بالانسولين. وبعد انتهاء فترة التجربة الممتدة لاسبوع واحد, تم أخذ عينات من الدم والانسجة من الحيوانات المخدرة من اجل قياس المعايير اعلاه. وعلى الرغم من ان الحقن ، ولمرة واحدة ، لعقار الالوبيورينول (25 ملغم/كغم وزن الجسم في البريتون) قبل ساعة أو بعد ساعة من حقن الالوكسان (100 ملغم/كغم وزن الجسم في البريتون) ، قد فشل في منع حدوث السكري ، إلا إنه أدى إلى تقليل مستوى الكيتون في البول ، وكذلك إلى انخفاض حدوث الوفيات المبكرة المرافقة للكيتوزية (فرط الكيتون في الجسم) في الحيوانات المصابة بالسكري بالمقارنة مع تلك غير المعالجة بالالوبيورينول . وقد أدى الحقن اللاحق ، ولثلاث مرات ، لعقار الالوبيورينول (25 ملغم/كغم وزن الجسم في البريتون ، مرة كل 48 ساعة) لمدة أسبوع ، كذلك إلى تقليل مستويات الكيتون في البلازما ، وخفض مستويات بيروكسيدات الدهن في البلازما والعديد من الأنسجة (القلب ، الكبد ، الكلية ، البنكرياس) بالمقارنة مع تلك الحيوانات المصابة بالسكري غير المعالجة بالالوبيورينول . وقد أدى الحقن اليومي بالانسولين (9–12 وحدة/كغم وزن الجسم ، تحت الجلد) لمدة أسبوع إلى تصحيح جميع التغيرات غير الطبيعية في المعايير المذكورة أعلاه في حيوانات السكري . وتشير النتائج الحالية لوجود دور ضئيل (إن وجد) لأنواع الأوكسجين المتفاعلة والناتجة من أنزيم الزانثين أوكسديز في التأثير المحدث للسكري للالوكسان. ومن ناحية اخرى,وعلى الرغم من عدم معرفة الآلية (الآليات) المسؤولة عن التأثيرات المفيدة للألوبيورينول في الوقت الحاضر، إلا إنها قد تعكس إمكانية وجود علاقة ما بين الخلل في العملية الايضية للكيتون وبين بيروكسدة الدهن في مرض السكري,وقد تشير أيضاً إلى وجود تأثير لعقار الالوبيورينول على العملية الايضية للكيتون .

Dept. Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad – Iraq.

INTRODUCTION

There is considerable evidence that xanthine oxidase (XO) system (one source of superoxide radicals in the body) may be involved in the generation of reactive oxygen species (ROS)in several conditions associated with oxidative stress, including chronic obstructive pulmonary disease [1], ischemia–reperfusion injury [2], endothelial dysfunction in type II diabetes [3]. Furthermore, in vitro studies have suggested that ROS (generated during the oxidation of hypoxanthine by XO) may play a role in causing oxidative damage to rat pancreatic β–cells [4].

The present study was therefore undertaken to investigate whether or not XO–derived ROS may play a role in the pathogenesis of ALX–induced diabetes using the specific XO inhibitor (and consequently of superoxide generation) as well as hydroxyl radical scavenger, allopurinol [5]. If the mechanism (s) of ROS production in ALX–diabetes is mediated, at least in part, through the enzyme XO, then allopurinol should either prevent or at least ameliorate the diabetic state.

MATERIALS and METHODS

Adult female Wistar rats (200–250 g) were housed in hanging plastic cages in a room kept at 22–25°C with a 14hr light and 10hr dark cycle. Animals were allowed free access to food and water during the entire experimental period except that of the induction of diabetes where animals were fasted for 48hr prior to the administration of alloxan [6].

Diabetes was induced in ether–anesthesized animals by the intraperitoneal (i.p.) injection of alloxan (Sigma Chemicals Co, USA) at a dose of 100 mg/kg body weight as a freshly prepared solution (100 mg/ml) in saline. ALX–treated animals were allowed to drink 5% glucose solution overnight to overcome drug–induced hypoglycemia [6]. The diabetic state was monitered by the frequent daily testing for glucosuria (with Lilly Tes–Tape, Eli Lilly & Co, USA) and for ketonuria (using strips obtained from Ketostix, Ames Co, USA).

The following groups were studied :

1-Control rats (n = 6).

2-Untreated diabetic rats (n = 12). Rats of this group received the alkaline vehicle (0.2 –0.25 ml of saline, pH 12.0) i.p. 1hr before (n = 6) or 1hr (n = 6) after ALX injection.

Diabetic rats which survived this period received further alkaline vehicle injections at 48, 96 and 144 hrs after ALX injection.

3-Allopurinol–treated diabetic rats (n =12).

Allopurinol (Sigma Chemical Co, USA) was prepared at a concentration of 25 mg/ml of saline pH 12.0. Rats received a single i.p. injection of 0.2–0.25 ml of alkaline vehicle to attain a final dose of 25 mg/kg body wt. 1hr before (n = 6) or 1hr (n = 6) after ALX injection. Then allopurinol was further given at 48 hr, 96 hr and 144 hr of the ALX–diabetic period. The dose of allopurinol was ¼ of that dose (100 mg/kg body wt.) reported to provide protection against oxidative–stress–induced lipid peroxidation in various tissues of rats [7].

4-Insulin–treated diabetic rats (n = 6) :

ALX–diabetic animals were injected with insulin zinc suspension (Lente MC, Novo Industri A/S, Denmark) subcutaneously at a daily dose of 9–12 U/kg body wt. [6] immediately after detection of the diabetic state. The dosage of insulin was adjusted by daily monitering urinary glucose and ketone levels.

At the end of the 1wk experimental period, animals were anesthesized with ether, and heparinised blood samples were obtained by cardiac puncture. Animals were killed by cardiac excision, and tissue (heart, pancreas, liver and kidney) homogenates (10% w/v) were prepared in ice–cold 50 mM Tris –0.1M EDTA buffer, pH 7.6. Plasma & tissue malondialdehyde (MDA) levels (as an index of lipid peroxidation) were determined by the thiobarbituric acid (TBA) reaction [8]. The TBA–reactive substances were calculated using an exctinction coefficient of MDA of 1.56×10^5.

Plasma levels of glucose, cholesterol & triglycerides as well as uric acid (to assess xanthine oxidase activity) were measured using commercial assay kits (Sigma Chemicals Co, USA). Plasma ketone bodies levels (β–hydroxybutyrate) were measured using the method of Williamson and Mallanby [9]. β–hydroxybutyrate is quantitatively the predominant ketone body present in the blood in uncontrolled diabetes mellitus [10].

Statistical analyses were performed using ANOVA at significance level of $P < 0.05$. Further specific group differences were determined using Tukey's test.

RESULTS

General Features of Diabetes :

The administration of the alkaline vehicle (whether given 1hr before or 1hr after ALX injection) did not prevent the incidence of diabetes, and produced comparable picture in

regard to degree of ketosis and the early mortality among diabetic rats.

Induction of diabetes was confirmed by the presence of glucosuria and ketonuria within 24 after ALX –injection. During the first week of diabetes, 6 animals died in ketosis and diabetic coma, which started as early as 48 hr after induction of diabetes.

Animals which survived this period (6/12) also showed hyperglycemia , hypertriglyceridemia , and ketonemia (Table 1) at the end of 1 wk period. However, levels of plasma cholesterol and uric acid were not changed during this period (Table 2). Plasma and tissue lipid peroxide levels were also elevated in ALX–diabetic rats (Table 3).

Effect of Allopurinol Treatment

Although the single i.p. injection of allopurinol (25 mg/kg body wt.) given 1hr prior to or 1hr post ALX administration did not prevent the induction of diabetes, it did lower the incidence of early ketosis–associated mortality in these animals. Unlike the high mortality in non–allopurinol–treated diabetic animals, only 2 of the 12 allopurinol–treated diabetic rats died in ketosis and coma (Table 1). The remaining 10 animals showed glucosuria and slight ketonuria. Treatment with allopurinol for 1 wk period did also lower the associated ketonemia (Table 1) as well as the lipid peroxide levels in plasma and tissues of allopurinol–treated diabetic rats compared to the non –treated diabetics (Table 3).

Effect of Insulin Treatment

Insulin treatment (9 –12 U/kg body wt/day) started immediately after detection of diabetes prevented the ketosis–associated coma and mortality, and normalized all of the above–mentioned abnormalities (Table 1–3).

Table 1: Plasma and urine ketone body levels and mortality rate in control, untreated–ALX diabetic, allopurinol–treated–ALX diabetic, and insulin treated–ALX diabetic rats after 1week of diabetes

	Plasma ketones (β-hydroxybutyrate) (μmol/L)	Urine ketones* (acetoacetate)	Number of animals died with diabetic coma/1week
Control (n = 6)	0.68 ± 0.29 [a]	(–)	–
Untreated–diabetic (n = 12)	9.20 ± 3.60 [b]	(+ + +)	6/12 animals (50%)
Allopurinol –treated diabetic (n = 12)	3.54 ± 2.02 [c]	2 (+ +) 2 (+) 8 (–)	2/12 animals (17%)
Insulin–treated diabetic (n = 6)	0.98 ± 0.35 [a]	(–)	–

Values are expressed as mean ± SD.

- Values with different letters are significantly different (P < 0.05).

*** Urine ketone body levels were qualitatively measured using ketostix strips obtained from Ames Co, USA, as described in material and methods**

Table 2: Body weight and biochemical parameters in control, untreated–ALX diabetic, allopurinol–treated–ALX diabetic, and insulin treated–ALX diabetic rats.

Group	Body wt. (g)	Plasma level (mg/dL)			
		Glucose	Triglycede	Cholesterl	Uric acid
Control (n = 6)	231 ± 24	146 ± 9 [a]	104 ± 23 [a]	65.8±23.2	1.80 ±0.15
Untreated–diabetic (n = 6)	219 ± 26	478±75 [b]	635 ± 287 [b]	61.9±19.3	1.70 ±0.19
Allopurinol–treated diabetic (n = 10)	228 ± 18	497 ± 120 [b]	595 ± 212 [b]	52.5±11.6	1.73 ±0.22
Insulin–treated diabetic (n = 6)	238 ± 32	109±30 [a]	168 ± 53 [a]	58 ± 19.3	1.68 ±0.20

- Values are expressed as mean ± SD.
- Within each column, values with different letters are significantly different (P < 0.05).

Table 3: Plasma and tissue TBA–reactive substances (an index of lipid peroxidation) in control, untreated–ALX diabetic, allopurinol–treated–ALX diabetic, and insulin treated–ALX diabetic rats.

Group	TBA –Reactive substances				
	Plasma n mol/ml	nmol/g wet tissue			
		Heart	Liver	Kidney	Pancreas
Control (n = 6)	1.44± 0.32[a]	178 ± 25 [a]	451 ± 37 [a]	221 ± 33 [a]	184 ± 34 [a]
Untreated–diabetic (n = 6)	5.93± 2.80 [b]	600 ± 75 [b]	612 ± 95 [b]	386 ± 65 [b]	368 ± 44 [b]
Allopurinol – treated diabetic (n = 10)	2.28± 1.32[a]	351 ± 88 [c]	273 ±101[c]	298 ±60 [c]	280 ± 67 [c]
Insulin–treated diabetic (n = 6)	1.65 ±0.54[a]	156 ±13 [a]	460 ± 84 [a]	218 ± 27 [a]	172 ± 57 [a]

- Values are expressed as mean ± SD.
- Within each column, values with different letters are significantly different (P < 0.05).

DISCUSSION

The diabetic state produced in rats during the first week following ALX injection was confirmed by glucosuria , ketonuria, hyperglyemia and ketonemia. The diabetic state was also accompanied by elevated plasma and tissue lipid peroxides levels as well as increased incidence of early ketosis – related mortality.

The ability of insulin therapy to completely reverse these changes suggests that they are related to the diabetic state in this strictly insulin–dependent model of experimental diabetes [6].

In an attempt to investigate whether or not allopurinol can prevent the induction of diabetes, the single i.p. administration of 25 mg/kg body wt. failed to prevent the induction of diabetes, whether it was administrated 1hr before or 1hr after ALX injection. This fact is suggestive of a minor role (if any) of XO – generated ROS in the diabetogenic action of ALX, and disagrees with the conclusion reached by Heller et al.[4] who suggested that ROS generated by XO are responsible for the destruction of insulin –producing β–cells during insulitis.

However, the single allopurinol administration was able not only to lower ketonuria, but also the incidence of early ketosis–associated mortality in diabetic rats, a fact suggestive of a possible effect on ketone body metabolism. Furthermore, preliminary experiments revealed that after a single i.p. administration of allopurinol, urinary ketone bodies start to increase (after the initial decline in level) again within 50–60 hr in allopurinol–treated diabetic animals : and by approximately 80 hr of diabetes no significant differences could be observed between allopurinol–treated and non–allopurinl–treated diabetic animals. This finding, suggests the involvement of allopurinol and/or its metabolite alloxanthine in these protective effects, given that the half–life of allopurinol is 2–3 hr and that of alloxanthine is 18–30hr[11]. Accordingly, the present experiment also applied repeated administration of allopurinol every 48 hr for 6 days. Interestingly, these repeated administration of allopurinol did lower ketone body levels in both urine and plasma and the lowered mortality rate (due to the first single dose) was maintained till the end of experimental period (1week).

It is important to point out that although alkali treatment has been recommended to correct diabetic ketoacidosis [12], the possibility that the ability of allopurinol to lower ketonuria is in fact attributable to the high alkalinity of the dissolving vehicle (pH 12) rather than to a direct effect of allopurinol per se has been ruled out. Diabetic animals receiving the same alkaline vehicle failed to demonstrate similar protective effects.

In a computer med–line search of the literature from 1970 –2002, no comparable reports concerning the effect of allopurinol treatment on ketone body metabolism in diabetes were found : therefore, the mechanism (s) responsible for the protective effects of allopurinol against the increased levels of ketone bodies remain (s) to be investigated in further studies. It is possible that allopurinol exerts its effects through an inhibition of XO; however, the fact that allopurinol treatment did not lower plasma uric acid in ALX –diabetic rats makes it an unlikely possibility.

The ability of allopurinol to lower ketonuria and ketonemia could be explained through either increasing ketone bodies metabolism by various tissues and/or decreasing their synthesis via affecting deacylase or β–hydroxy–β–methylglutaryl–CoA formation [13]. It is unlikely that allopurinol might have enhanced endogenous insulin release because other insulin–dependent abnormalities were not corrected by allopurinol treatment.

The ability of allopurinol to lower ROS–induced lipid peroxidation provides another evidence for the potential beneficial effect of allopurinol treatment on tissue antioxidant defenses against diabetes–induced increased oxidative stress. It further supports the findings of other studies which reported that allopurinol lowered MDA levels in plasma of patients with type II diabetes with mild hypertension [3] and in various tissues of rats exposed to oxidative stress induced either by cypermethrin [7] or by experimental acute pancreatitis [14].Furthermore, the possibilities of a direct effect of allopurinol and alloxanthine on ketone body metabolism and lipid peroxidation independently of inhibition of XO, or via scavenging the powerful hydroxyl radicals [14], or may involve generalized alterations in tissue antioxidant status [2] can not be ruled out at present. Much more work must be done to assess whether there is any relationship between lipid peroxidation and ketosis in ALX diabetes and further investigate the protective effects of ALP in this regard.

In conclusion, the present study suggests that under the conditions tested and in the doses and duration of treatment used, allopurinol through its probable or apparent ability to inhibit generation of ROS (especially hydroxyl radical scavenging) rather than XO inhibition

appears to have a protective effect on both lipid peroxidation and ketogenesis and consequently early mortality in ALX–diabetes .

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