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## Original Contribution

Xanthine oxidase-induced oxidative stress causes activation of NF- $\kappa$ B and inflammation in the liver of type I diabetic rats

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## ABSTRACT

We previously showed that xanthine oxidase activity increases in type I diabetic animals and that this is a significant cause of the oxidative stress which occurs in the disease. The aim of this work was to search for molecular links between xanthine oxidase-induced oxidative stress and inflammation in Type I diabetes and to assess the ability of allopurinol, a drug widely used in clinical practice, to prevent both processes.

3-month-old male Wistar rats were made diabetic by injection (i.p.) of either streptozotocin or alloxan. Allopurinol (32 mg/Kg) was administered (i.p) to diabetic rats after they had shown clear signs of diabetes such as glucosuria and polyuria.

Hepatic phospho-IKK $\beta$  and phospho-I $\kappa$ B $\alpha$  contents were increased in diabetic animals. This was accompanied by increased levels of NF- $\kappa$ B (p65 protein content) in liver nuclear extracts. Hepatic expression of NF- $\kappa$ B dependent inflammatory cytokines and enzymes, namely interleukin 1 $\beta$ , iNOS and interleukin 6 were markedly increased. Both diabetes-induced activation of NF- $\kappa$ B signalling cascade and subsequent over expression of inflammatory cytokines and enzymes were abolished by administration of allopurinol. Moreover, we found a significant neutrophil infiltration in the liver of diabetic animals. These events were also prevented by administration of allopurinol.

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## Introduction

The occurrence of oxidative stress in diabetes has been well documented [1–4]. We previously reported that xanthine oxidase plays an important role in the development of oxidative stress occurring in diabetes [5]. Our results were later confirmed by other groups [6]. On the other hand oxidative stress plays a pivotal role in the pathophysiology of inflammation [7] which is a critical component of the cascade of events leading to the development of diabetic complications. In relation to this, we previously found that mice in which hepatic NF- $\kappa$ B was selectively knocked out, were protected against fat-induced insulin resistance [8]. Further, diabetes increases the risk of chronic liver diseases like hepatic necrosis [9] or hepatocellular carcinoma [10,11]. It has also been shown that diabetes predisposes to non-alcoholic steatohepatitis which progresses to cirrhosis in 5–20% of the cases [12,13].

The aim of this paper was to identify molecular links between xanthine oxidase-derived oxidative stress and inflammation in diabetes, and to prevent such an inflammatory state through the

inhibition of xanthine oxidase activity with allopurinol, a drug widely used in clinical practice.

We have found that the oxidative stress induced by xanthine oxidase is responsible for the activation of the NF- $\kappa$ B signalling pathway. This, in turn, up-regulates the levels of inflammatory proteins like interleukin 1 $\beta$ , interleukin 6 and iNOS. As a consequence of such an inflammatory state, a significant infiltration by polymorphonuclear cells was consistently observed in the liver of type I diabetic animals. Oxidative redox imbalance, inflammatory reactions and hepatic changes were all inhibited by treatment with allopurinol.

## Materials and methods

## Animals

We used 3-month-old male Wistar rats made diabetic by injection (i.p.) of either streptozotocin or alloxan. The experimental protocol was approved by the Committee on Ethics in Research of the Faculty of Medicine, Valencia.

For the streptozotocin experiments we injected i.p. the drug (55 mg/kg body wt dissolved in 0.8 ml of 0.1 mol/l citrate buffer, pH 4.5). The rats had polyuria, polydipsia, and weight loss 7–10 days after the drug administration.

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For the alloxan experiments we injected it i.p. (90 mg/kg body wt dissolved in saline). The rats had polyuria, polydipsia, and weight loss 9–11 days after the drug administration.

All animals with a glucosuria of >20 g/l were considered diabetic.

Control animals were injected with the same volume of citrate buffer or saline as the diabetic animals but without streptozotocin or alloxan.

#### *Allopurinol administration*

Allopurinol (32 mg/Kg) was administered intraperitoneally to rats 5 days after they had shown clear signs of diabetes such as glucosuria and polyuria. Doses were given 30 h and 6 h before the rats were sacrificed [5].

0.35 mL of DMSO was used as a vehicle for the allopurinol experiments. Diabetic animals were injected with the same volume of DMSO as those diabetic treated with allopurinol. Only animals made diabetic with streptozotocin were treated with allopurinol.

All the animals were killed by an overdose of sodium pentobarbitone.

#### *Determination of reduced glutathione (GSH), oxidized glutathione (GSSG) and of Malondialdehyde (MDA)*

GSH and GSSG were determined in liver from rats by HPLC following the protocol described in [14] and MDA as described in [15].

#### *Determination of XO activity*

XO activity was determined in rat liver and plasma by a fluorimetric method [16]. Frozen liver was homogenized with 5 volumes per gram of tissue of 0.25 M sucrose, 10 mM DTT, 0.2 mM PMSF, 0.1 mM EDTA and 50 mM K-phosphate, pH 7.4. Homogenates were centrifuged for 30 minutes at 15,000×g and activities measured in supernatants. XO activity was measured by calculating the slope of the increase in fluorescence after adding pterin (0.010 mmol/L), which actually measures conversion of pterin to isoxanthopterin. The reaction was stopped by adding allopurinol (50 μmol/L). To calibrate the fluorescence signal, the activity of a standard concentration of isoxanthopterin was measured. Values are expressed as nmol/min per gram of protein. Protein concentration of homogenates was determined by the Bradford assay [17].

#### *Nuclear extract preparation*

Nuclear extracts were obtained following the method of Dignam et al. [18], with slight modifications [19], and protein content was determined by the Bradford method [17].

#### *Western blot analysis*

Aliquots of liver lysate (20–40 μg) were separated by SDS-PAGE electrophoresis. Proteins were then transferred to nitrocellulose membranes, which were incubated overnight at 4 °C with appropriate primary antibodies. Antibodies against iNOS, phospho-IKKβ, phospho-IκB, P65 and α-tubulin were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). Thereafter, membranes were incubated with a secondary antibody for 1 h at room temperature. Oxidative modification of total proteins was assessed by immunoblot detection of protein carbonyl groups using the 'OxyBlot' protein oxidation kit from Intergen Company (Purchase, NY) following the manufacturer's instructions. Approximately 20 μg of total protein was loaded onto paired gels and electrophoretically separated. Immunoblots of one gel from each pair were prepared, with the other gel stained in Coomassie blue and used for verification of equality of lane loading [20,21]. Antibody anti-dinitrophenylhydrazine was purchased from Intergen Company (Purchase, NY). The procedure to quantify total protein carbonyls with the

OxyBlot kit was densitometry of the oxyblot and of the Ponceau staining, followed by finding the ratio between the total density in the oxyblot and the total density in the Ponceau [22]. Specific proteins were visualised by using the enhanced chemiluminescence (ECL) procedure as specified by the manufacturer (Amersham). Autoradiographic signals were assessed using a BioRad scanning densitometer.

#### *Interleukins*

The concentrations of IL-6 and IL-1β in rat liver was determined by ELISA (Amersham-Biosciences). For each individual cytokine the manufacturer's protocol was followed.

#### *Number of neutrophils*

Fragments of liver of an approximate size 1.0×0.5×0.3 cm were placed overnight in a solution of 4% formaldehyde in 50 mM phosphate buffer solution, pH 7.4. Polymorphonuclear leukocytes (PMN) were identified on 5-mm paraffin sections by means of the naphthol AS-D chloroacetate technique for esterase, following the method described by Moloney et al. (1960) [23] with slight modifications; the substrate was dissolved in dimethyl sulphoxide/Triton X-100 (9:1, v/v) and then 0.1% Fast Garnet dGBC in 0.1 M phosphate buffer solution, pH 8.5 was added. Red stained PMN were counted in 20 non-consecutive, randomly chosen x 500 histological fields. Results were expressed as the number of neutrophils per 40 x histological power field (hpf).

#### *Statistics*

Results are presented as mean ± standard deviation indicating the number of observations (n) in brackets. The statistical treatment of the results when comparing control, diabetic and diabetic treated with allopurinol groups, was carried out after finding normal data distribution with Shapiro-Wilk test. As several groups of data did not show normal distribution, the means of the groups were compared with a non-parametric test, Kruskal-Wallis. The null hypothesis considered the lack of differences between the group means and was rejected for *P* values < 0.05. Post-hoc comparisons were then performed with the Mann-Whitney U test. In some cases in which just 2 groups were compared, we used an Student *t* test. In all comparisons, the null hypothesis was rejected with *P* values < 0.05. Statistical treatment of the results was carried out with SPSS software version 11.01, for Windows.

## **Results**

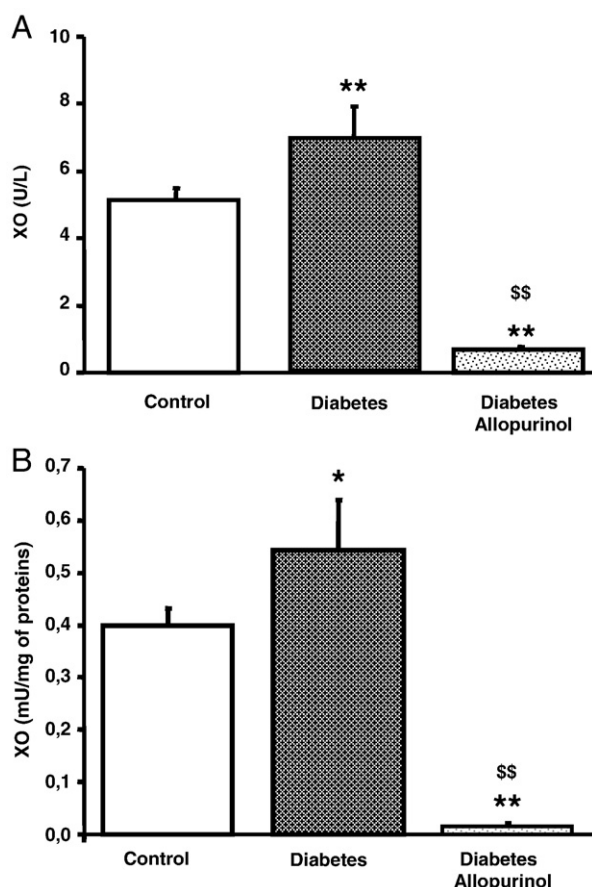
### *Increase in xanthine oxidase activity in experimental diabetes*

We first of all studied whether xanthine oxidase is activated in experimental diabetes. We used two well established experimental models of insulin-dependent diabetes: streptozotocin or alloxan. In both models diabetic rats showed a significant increase of plasma and hepatic xanthine oxidase activity, which was abolished when diabetic animals were treated with allopurinol (See Figs. 1 and 2).

### *Allopurinol prevents oxidative stress associated with diabetes*

As shown in Fig. 3, treatment with allopurinol diminished the oxidative stress associated with diabetes.

Plasma malondialdehyde (MDA) levels of diabetic rats were more than threefold higher than those of corresponding controls. This was almost completely abolished by treatment with allopurinol (panel A). Whole blood of diabetic rats showed a glutathione redox ratio (GSSG/GSH) which was approximately 50% higher than that of controls. This was completely prevented by treatment with allopurinol (panel B). Carbonylated proteins in cytosolic extracts from liver were fivefold

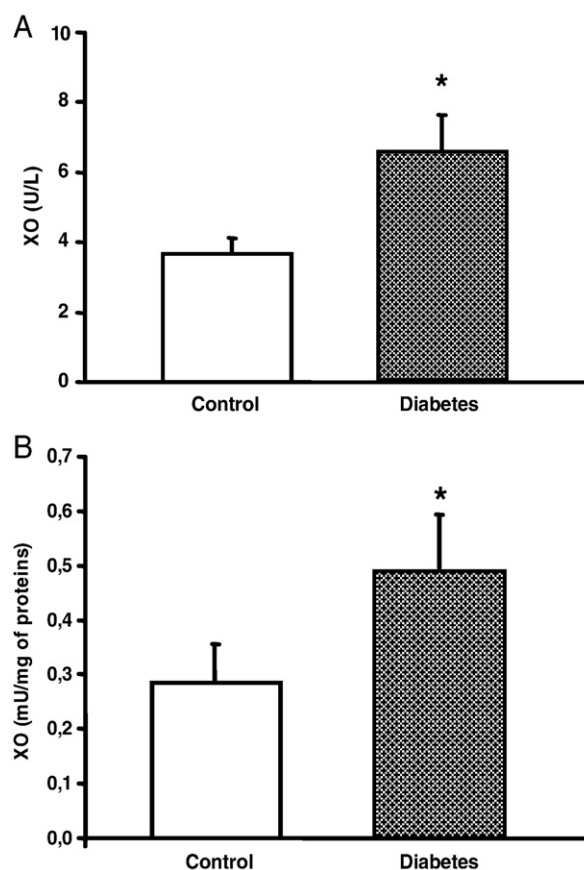


**Fig. 1.** Allopurinol prevents the activation of xanthine oxidase in streptozotocin-induced diabetes. (A) Plasma XO activity increases in streptozotocin-induced diabetes in rats. Allopurinol administration prevents this effect. Values are mean  $\pm$  SD. Control group, N=5; Diabetic group, N=4; Diabetic treated with allopurinol, N=5. (\*\*\*) indicates  $p < 0.01$  vs control. (\*\*\$) indicates  $p < 0.01$  vs diabetes. Group means were compared using the Kruskal-Wallis test. Post-hoc comparisons were performed using the Mann-Whitney U test. (B) Liver XO activity increases in streptozotocin-induced diabetes in rats. Allopurinol administration prevents this effect. Values are mean  $\pm$  SD. Control group, N=3; Diabetic group, N=4; Diabetic treated with allopurinol, N=4. (\*) indicates  $p < 0.05$  vs control. (\*\*) indicates  $p < 0.01$  vs control. (\*\*\$) indicates  $p < 0.01$  vs diabetes. Group means were compared using the Kruskal-Wallis test. Post-hoc comparisons were performed using the Mann-Whitney U test.

higher than those of corresponding controls. Administration of allopurinol significantly prevented the liver protein oxidation in the diabetic animals.

#### *Allopurinol prevents diabetes-associated activation of the NF- $\kappa$ B signalling pathway*

NF- $\kappa$ B activation is highly dependent on oxidative stress. On the basis of the results reported in Fig. 3 the activation of NF- $\kappa$ B signalling pathway was evaluated in diabetic animals and its possible prevention by allopurinol checked. As reported in Fig. 4A the level of phosphorylated IKK $\beta$  was approximately threefold higher in diabetic animals than in the corresponding controls. Such activation was fully inhibited by treatment with allopurinol, thus pointing to the involvement of xanthine oxidase in the up-regulation of the NF- $\kappa$ B pathway observed in diabetes. Downstream of IKK $\beta$  activation is the activation of I $\kappa$ B $\alpha$ , Fig. 4B also shows that the hepatic level of phosphorylated I $\kappa$ B $\alpha$ , i.e. the inhibitory protein which binds to NF- $\kappa$ B preventing its translocation into the nucleus, was increased in diabetes but significantly down-regulated by allopurinol. The next event in the activation of NF- $\kappa$ B pathway is its translocation to the nucleus, easily checked by measuring the increased level of p65 unit



**Fig. 2.** Activation of xanthine oxidase in alloxan-induced diabetes. (A) Plasma XO activity increases in an alloxan-induced diabetes in rats. Values are mean  $\pm$  SD. Control group, N=3; Diabetic group, N=3. (\*) indicates  $p < 0.05$  vs control. Group means were compared using the Student t test. (B) Liver XO activity increases in an alloxan-induced diabetes in rats. Values are mean  $\pm$  SD. Control group, N=6; Diabetic group, N=6. (\*) indicates  $p < 0.05$  vs control. Group means were compared using the Student t test.

in nuclear extracts. We found that p65 (See Fig. 4C) in nuclear hepatic extracts from diabetic animals was higher than in controls and that this was inhibited by allopurinol.

#### *Allopurinol prevents the diabetes-associated up-regulation of interleukin 6, interleukin 1 $\beta$ and iNOS*

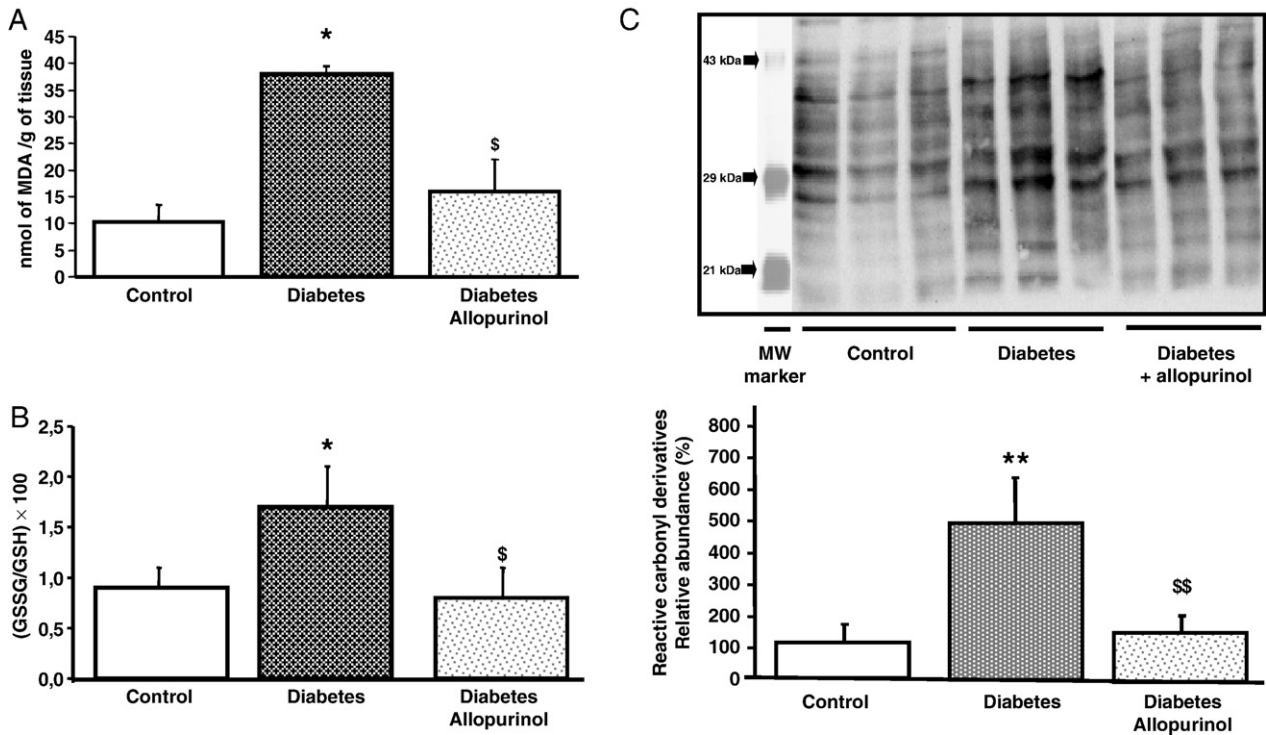
NF- $\kappa$ B is involved in the regulation of hundreds of genes, several of them involved in inflammation. Prominent among these genes are those coding for key inflammatory cytokines, like interleukin 6 (IL-6) and interleukin 1 $\beta$  (IL-1 $\beta$ ) and for the inducible nitric oxide synthase. Table 1 and the supplementary material show that the hepatic levels of these proteins were markedly elevated in diabetic animals but significantly quenched in case of allopurinol administration.

#### *Neutrophil infiltration in the liver of diabetic animals*

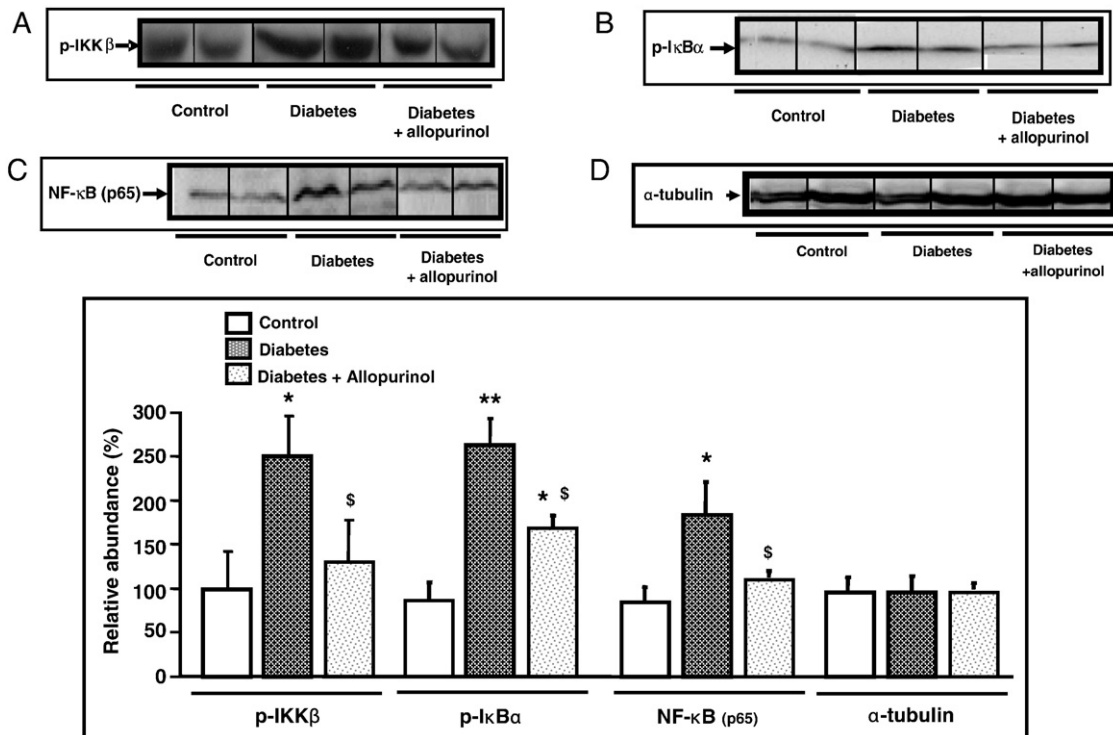
##### *Prevention by allopurinol administration*

After studying the molecular mechanisms linking xanthine oxidase activation in diabetes to the inflammatory response mediated by free radicals and the NF- $\kappa$ B signalling pathway, we turned to histology to determine the cellular effects of diabetes in liver and whether this could be prevented by treatment with allopurinol. We measured the neutrophil infiltration in liver of diabetic animals. Fig. 5 shows that the number of polymorphonuclear cells was more than fourfold in streptozotocin-injected animals than in controls, and partially but significantly reduced by allopurinol. Neutrophil infiltration is a clear





**Fig. 3.** Allopurinol prevents oxidative stress in streptozotocin-induced diabetes. (A) Liver MDA levels increase in streptozotocin-induced diabetes in rats. Administration of allopurinol prevents this effect. Values are mean  $\pm$  SD. Control group, N = 4; Diabetic group, N = 5; Diabetic treated with allopurinol, N = 4. (\*) indicates  $p < 0.05$  vs control. (\$) indicates  $p < 0.05$  vs diabetes. (B) Allopurinol prevents oxidation of liver glutathione in streptozotocin-induced diabetes in rats. (GSSG/GSH)\*100 levels in liver. Values are mean  $\pm$  SD. Control group, N = 4; Diabetic group, N = 6; Diabetic treated with allopurinol, N = 4. (\*) indicates  $p < 0.05$  vs control. (\$) indicates  $p < 0.05$  vs diabetes. (C) Allopurinol prevents liver protein oxidation in rats made diabetic with streptozotocin. Western blotting and densitometry analysis (MW: 29 kD) showing carbonylated proteins in cytosolic extracts from liver. Values are mean  $\pm$  SD. Control group, N = 3; Diabetic group, N = 3; Diabetic treated with allopurinol, N = 3. (\*\*) indicates  $p < 0.01$  vs control. (\$\$) indicates  $p < 0.01$  vs diabetes. Group means were compared using the Kruskal-Wallis test. Post-hoc comparisons were performed using the Mann-Witney U test.



**Fig. 4.** Allopurinol prevents the phosphorylation of IKK $\beta$ , I $\kappa$ B $\alpha$  and the translocation to the nucleus of NF- $\kappa$ B (p65) in liver of rats made diabetic with streptozotocin. Densitometry analysis of cytosolic p-IKK $\beta$ , p-I $\kappa$ B $\alpha$ , nuclear p65 subunit and  $\alpha$ -tubulin in liver of diabetic rats. Values are mean  $\pm$  SD. Control group, N = 3; Diabetic group, N = 3; Diabetic treated with allopurinol, N = 3. (\*) indicates  $p < 0.05$  vs control. (\$) indicates  $p < 0.05$  vs diabetes. (\*\*) indicates  $p < 0.01$  vs control. Representative experiments are shown. The content of  $\alpha$ -tubulin, a house-keeping protein marker in liver, was not altered in the various treatment groups of rats. Group means were compared with the Kruskal-Wallis test. Post-hoc comparisons were performed using the Mann-Witney U test.

**Table 1**

Interleukin levels in liver of diabetic animals. Effect of allopurinol administration. Values are mean  $\pm$  SD. Control group, N=4; Diabetic group, N=4; Diabetic treated with allopurinol, N=4. (\*) indicates  $p < 0.05$  vs control, (S) indicates  $p < 0.05$  vs diabetes

LIVER	IL-6 ( $\mu\text{g/g}$ of tissue)	IL-1 $\beta$ ( $\mu\text{g/g}$ of tissue)
Control (4)	47,1 $\pm$ 5,3	20,7 $\pm$ 5,9
Diabetes (4)	72,6 $\pm$ 13,7*	35,3 $\pm$ 5,2*
Diabetes + Allopurinol (4)	53,0 $\pm$ 06,4 <sup>S</sup>	25,2 $\pm$ 4,9 <sup>S</sup>

marker of inflammation which in turn may lead to necrotic death of cells.

## Discussion

The occurrence of oxidative stress in diabetes has been well documented. Work from several laboratories, including our own [5] has shown that diabetes, both clinical and experimental, causes oxidative stress as measured by an increased level of hydroperoxides, an oxidation of glutathione, an increase in levels of oxidised proteins and other signs of oxidation to cell components. This is of particular importance in the development of the very serious late onset complications of diabetes [24], like atherosclerosis [25], retinopathy [26], and nephropathy [27]. However, the hepatic complications of diabetes have been somewhat neglected. In fact, diabetes causes an increased risk of chronic liver diseases [10] and even of acute hepatic necrosis and failure [9].

For the majority of experiments in this paper we have used streptozotocin to induce diabetes. However, to show that the effects on oxidative stress caused by experimental diabetes were not due to streptozotocin itself, but rather to the lack of insulin caused by this toxic drug, we used another one, i.e. alloxan. When we treated rats with alloxan we found that xanthine oxidase activity, both in liver and in blood, was increased (see Figs. 2A and B). Thus, the effects that we find are not due to streptozotocin but rather to the diabetic state caused by either streptozotocin or alloxan.

Diabetes has been considered as an inflammatory disease [28]. Our guiding hypothesis was that inflammation could be important in the development of hepatic complications in diabetes [29]. This in turn, might result in changes in glucose homeostasis which may complicate the prognosis of the disease.

Several pathogenetic mechanisms have been proposed to explain the oxidative state which occurs in diabetes. Hyperglycaemia itself is one of such mechanisms, albeit not the only one [30]. For instance Jain and co-workers suggested that the ketone bodies which are increased in all forms of diabetes, but particularly in type I, cause oxidative stress in many cases by auto oxidation [31]. Advanced glycosylation end products also play a major role in the cellular toxic effects associated with diabetes [32]. Some classical enzymatic mechanisms have been proposed as causes of oxidation in diabetes. Prominent among these is NADPH oxidase [33] (whose activity is increased in diabetes). Its localisation near or at the vascular bed makes it a critically important enzyme in the local oxidation leading to atherosclerosis [34,35].

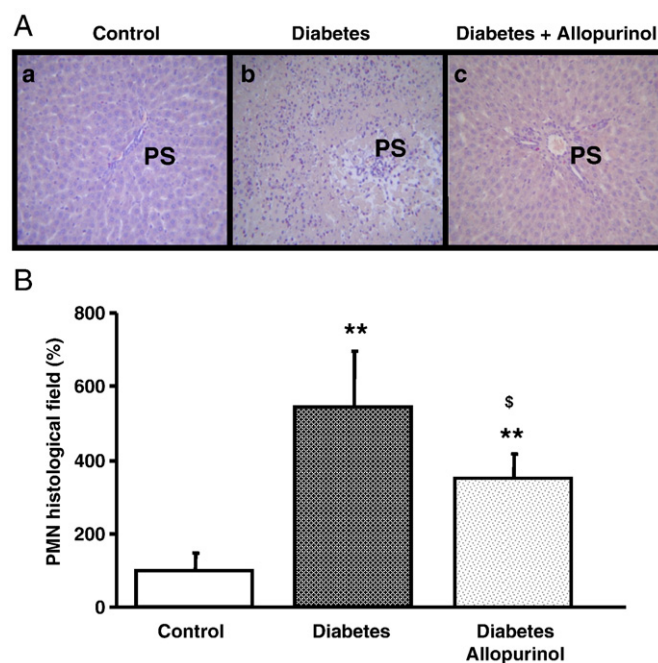
Work from our laboratory showed that xanthine oxidase, another oxidase which is active and causes oxidative stress in several pathological states such as the ischemia reperfusion syndrome, is an important determinant of the oxidation that we observe in diabetes. An important point related to this enzyme is that we can inhibit it by using a well known, quite safe drug, namely allopurinol which has been used for decades to treat hyperuricemia. Although it has been suggested that allopurinol generates superoxide radical in liver [36] we have previously shown that allopurinol prevents, to a large extent, oxidative stress which occurs in diabetes [5] and in other pathophysiological situations like exhaustive exercise [37]. Other groups

confirmed our findings pointing to the important role of xanthine oxidase in the pathophysiology of the disease [6].

We tested the role of allopurinol in the prevention of diabetic complications and in particular of those associated with the liver. We chose the liver because, as we proved earlier it is the main source of xanthine oxidase which is shed by it and released to the blood plasma and then distributed along all the vascular bed where it is anchored to the endothelium by a heparan sulphate [5]. But a major site of damage caused by this enzyme is very likely to be at the site of production, namely the liver. In this organ we have studied the role of xanthine oxidase-derived reactive oxygen species in the activation of the cascade of molecular events leading to oxidation, and eventually to liver inflammation. NF- $\kappa$ B is one of the major transcription factors involved in unleashing the cascade of events leading to inflammation [38]. Different research groups have demonstrated its activation in the diabetic liver [29,39,40].

The data we present in the Results section show that the oxidative stress condition induced by xanthine oxidase activation in the liver of streptozotocin-treated rats leads to a marked up-regulation of the NF- $\kappa$ B pathway. The latter was evaluated in terms of IKK $\beta$  and I $\kappa$ B $\alpha$  increased phosphorylation, and definitely supported by the significant rise of IL-6, IL-1 $\beta$  and iNOS protein levels observed in the hepatic tissue of the same animals. As for the two cytokines' genes also iNOS gene has mandatory NF- $\kappa$ B binding sequences on their promoter region [41]. Notably, even if secondary modifiers such as oxidants and NO itself could in principle alter NF- $\kappa$ B binding despite nuclear translocation, the net increase of the two inflammatory cytokines and of the enzyme demonstrates the actual induction of a NF- $\kappa$ B dependent inflammatory state in the diabetic rats. In addition, it has been demonstrated that iNOS plays a major role in the induction of albuminuria in diabetes and is of critical importance in the onset of inflammation as well [42].

The observed activation of liver NF- $\kappa$ B and consequent molecular events were bound to have histological repercussions. Fig. 5 shows



**Fig. 5.** Neutrophil infiltration in liver of rats made diabetic with streptozotocin. Effect of allopurinol administration. (A) A representative image showing PMN infiltration is shown for every experimental group. PS: Portal Space. (B) Values are mean  $\pm$  SD. Control group, N=4; Diabetic group, N=4; Diabetic treated with allopurinol, N=5. (\*\*) indicates  $p < 0.01$  vs control. (S) indicates  $p < 0.05$  vs diabetes. Group means were compared using the Kruskal-Wallis test. Post-hoc comparisons were performed using the Mann-Whitney U test.

polimorphonuclear infiltration in the liver of diabetic animals which is prevented by allopurinol.

We consider that the main importance of the finding reported in our manuscript lies in the fact that a widely used drug in the clinical practise, allopurinol, can be used to decrease NF- $\kappa$ B activation and inflammation associated with diabetes. We do not consider that, although the effects of oxypurinol (a metabolite which is not used in clinical practice), could reproduce or even improve the results found in our study, it could reach the clinical significance that we have obtained with allopurinol.

Inhibition of xanthine oxidase prevents signs of oxidative stress which occur in diabetes, thus confirming our previous work where we showed the relevance of this enzyme in the process of oxidation associated with diabetes.

The fact that inhibition of xanthine oxidase protects against oxidative stress associated with a number of physiological and pathological processes is well documented. For instance, we showed that allopurinol prevents oxidative stress and cell damage which occurs in strenuous physical exercise such as that of the Tour de France [37]. In 2001 Jain and co-workers showed that XO-derived free radicals contribute to liver necrosis and that it was prevented by the administration of allopurinol [43]. In a similar fashion allopurinol prevented early alcohol-induced liver injury [44] and a number of complications associated with the ischaemia reperfusion syndrome [45]. It has also recently been shown that treatment with allopurinol improves nerve and vascular function in diabetic rats [46] which points out that inhibition of xanthine oxidase could be a potential therapeutic approach to diabetic neuropathy and vasculopathy [46,47].

Work reported here shows that allopurinol prevents the cascade of cell signals which lead to inflammatory signs associated with diabetes. The possible role of allopurinol to treat late onset complications of diabetes which are sparked by inflammation should be studied in the clinical setting.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2010.03.024.

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