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NEPHROLOGY - ORIGINAL PAPER

Allopurinol protects human glomerular endothelial cells from high glucose-induced reactive oxygen species generation, p53 overexpression and endothelial dysfunction

Theodoros Eleftheriadis[1] · Georgios Pissas[1] · Georgia Antoniadi[1] · Vassilios Liakopoulos[1] · Ioannis Stefanidis[1]

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Abstract
Purpose Mitochondrial reactive oxygen species (ROS) overproduction in capillary endothelial cells is a prerequisite for the development of diabetic nephropathy. Inhibition of xanthine oxidase, another ROS generator, ameliorates experimental diabetic nephropathy. To test the hypothesis that the initial high glucose-induced ROS production by the mitochondria activates xanthine oxidase, which afterward remains as the major source of ROS, we cultured primary human glomerular endothelial cells (GEnC) under normal or high-glucose conditions, with or without the xanthine oxidase inhibitor allopurinol.
Methods ROS generation and nitric oxide synthase (NOS) activity were assessed by chemiluminescence or colorimetrically. Levels of intercellular adhesion molecule 1 (ICAM-1), p53 and phosphorylated p53 (p-p53) were assessed by western blotting.
Results Allopurinol prevented high glucose-induced ROS generation indicating that xanthine oxidase is the major source of ROS. Allopurinol protected GEnC from endothelial dysfunction since it prevented the high glucose-induced decrease in NOS activity and increase in ICAM-1 expression. Allopurinol reduced p53 and p-p53 levels induced by high glucose suggesting an axis of xanthine oxidase-derived ROS, DNA damage, p53 stabilization and endothelial dysfunction that may contribute to the pathogenesis of diabetic nephropathy.
Conclusions Allopurinol protects GEnC from high glucose-induced ROS generation, p53 overexpression and endothelial dysfunction. These data provide a pathogenetic mechanism that supports the results of experimental and clinical studies about the beneficial effect of xanthine oxidase inhibitors on the development of diabetic nephropathy.

Keywords Xanthine oxidase · Reactive oxygen species · p53 · Endothelial dysfunction · Diabetic nephropathy · Allopurinol

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease, and renal endothelium plays significant role in its emergence and progression [1, 2]. Diabetic nephropathy is a microvascular complication of diabetes mellitus, and reactive oxygen species (ROS) overproduction has been incriminated for its development [3]. Elegant experiments have shown that under hyperglycemic conditions the capillary endothelial cells are unable to restrict glucose entry into the cells. The consequent hypermetabolism of glucose through the Krebs' cycle provides more electron donors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) than those the oxidative phosphorylation chain is able to transport. The excess electrons are transferred to oxygen giving rise to superoxide and other ROS [3].

Although mitochondrial ROS overproduction is a prerequisite for the development of diabetic nephropathy [3, 4], experimental studies have shown that inhibition of another source of ROS, the enzyme xanthine oxidase, ameliorates diabetic nephropathy [5–7]. Importantly, a prospective randomized controlled clinical trial supported the beneficial role of urate-lowering treatment with the xanthine oxidase

✉ Theodoros Eleftheriadis
teleftheriadis@yahoo.com

[1] Department of Nephrology, Faculty of Medicine, University of Thessaly, Neo Ktirio, Mezourlo Hill, 41110 Larissa, Greece

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inhibitor allopurinol in the course of diabetic nephropathy [8]. Xanthine oxidase produces superoxide and hydrogen peroxide by transferring electrons to molecular oxygen. Xanthine oxidase is one of the catalytic active forms of xanthine oxidoreductase, with xanthine dehydrogenase being the other one, which transfers electrons to NAD^+. The two active catalytic forms of xanthine oxidoreductase are interconvertible, yet under inflammation and oxidative stress the xanthine oxidase form prevails [9]. The latter has been confirmed in endothelial cell cultures where treatment of endothelial cells with hydrogen peroxide, which is released at large amounts by phagocytes in case of inflammation, increased xanthine oxidase activity and induced endothelial dysfunction [10, 11]. Interestingly, in endothelial cells under oscillatory shear stress, the initial ROS production by NADPH oxidase activates xanthine oxidase, which in turn becomes the main source of ROS [12].

In case of diabetic nephropathy, it is reasonable to assume that after the initial ROS overproduction by the mitochondria; xanthine oxidase is activated producing more ROS. Thus, xanthine oxidase may act as a feed-forward mechanism whereby an initial ROS generation may stimulate further ROS production. Such a scenario can explain the fact that although ROS overproduction by the mitochondria is a prerequisite for microvasculature endothelial dysfunction under high-glucose conditions [3, 4], inhibition of xanthine oxidase ameliorates the course of diabetic nephropathy both in experimental and clinical studies [5–8].

In this study, we evaluated the above hypothesis by assessing the effect of allopurinol on ROS production in cultures of primary human glomerular endothelial cells (GEnC) under conditions of normal or high glucose. In addition, we assessed endothelial dysfunction by measuring the activity of nitric oxide synthase (NOS), since decreased production of NO occurs early in endothelial dysfunction [13], as well as endothelial activation by evaluating the expression of intercellular adhesion molecule 1 (ICAM-1), which facilitates white blood cell adhesion and migration into the subendothelial space [14]. Finally, we evaluated the expression of the transcription factor p53, which is upregulated under high-glucose conditions and may be implicated in endothelial dysfunction [15].

Materials and methods

Cell culture

Primary human GEnC (Sciencell Research Laboratories, San Diego, CA) were grown in Dulbecco's Modified Eagle Medium (DMEM) low glucose (Thermo Fisher Scientific Inc., Rochford, IL), which contains 5.55 mM of D-glucose, with 20% fetal bovine serum (FBS) (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and antibiotic–antimycotic solution (Sigma-Aldrich; Merck Millipore). Cultures were incubated at 37 °C in an atmosphere of 95% relative humidity and 5% CO_2.

The number of the cells for each experiment varied according to the type of well-plate per se: 3×10^5 cells per in 6-well plates and 1×10^4 cells in 96-well plates. Cells were incubated for 24 h at basal medium and then washed and cultured under the following conditions for 48 h. Cells cultured in complete DMEM low glucose medium served as control. In these control cell cultures, L-glucose (Sigma-Aldrich; Merck Millipore) was added at a final concentration of 19.45 mM. Cells cultured in complete DMEM high glucose medium (Thermo Fisher Scientific Inc.), which contains 25 mM of D-glucose, corresponded to cells cultured under high-glucose conditions. L-glucose was added to the control cells in order to achieve equal osmolarity. Two more groups were generated that involved the addition of the xanthine oxidase inhibitor allopurinol (Selleck Chemicals, Munich, Germany) at a concentration of 100 μM in either control or high-glucose conditions. This allopurinol concentration has been used in previous studies, and its protective effect on endothelial cell damage or activation has been confirmed [16, 17]. All experiments were repeated six times.

Assessment of high glucose or allopurinol cytotoxicity

Cytotoxicity of high glucose or allopurinol was assessed in cells cultured in 96-well plates under normal or high-glucose conditions and in the presence or not of 100 μM allopurinol. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release assay using the Cytotox Non-Radioactive Cytotoxic Assay kit (Promega Corporation, Madison, WI).

Assessment of ROS production

Cellular oxidative stress was assessed in cells cultured in 96-well plates. Once incubation period was over, cells were stained with 5 μM of the fluoro genic probe CellROX® Deep Red Reagent (Invitrogen, Life Technologies, Carlsbad, CA) by adding the probe to the complete medium and incubating the cells at 37 °C for 30 min. The cells were then washed with PBS, and fluorescence signal intensity was measured and analyzed on an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA).

Assessment of NOS activity

Nitric oxide synthase (NOS) activity was assessed with Nitric Oxide Synthase Assay Kit, Colorimetric (Merck Millipore). Cells cultured in 6-well plates were lysed using the T-PER tissue protein extraction reagent (Thermo Fisher Scientific Inc.) supplemented with protease and phosphatase

inhibitors (Sigma-Aldrich; Merck Millipore). Bradford assay (Sigma-Aldrich; Merck Millipore) was performed, and the lysate volume of the samples was modified in order to contain equal concentration of protein. Then the manufacturer protocol was applied in order to quantify NOS activity by assessing the concentration of nitrate and nitrite as the end products of NO metabolism.

Assessment of ICAM-1, p53 and phosphorylated p53 expression

For assessing the level of the proteins of interest, GEnC cultured in 6-well plates were lysed using the T-PER tissue protein extraction reagent supplemented with protease and phosphatase inhibitors. Protein was quantified via Bradford assay, and 10 μg was used for western blotting. The primary antibodies were specific for ICAM-1 (Cat. no. 4915; Cell Signaling Technology, Danvers, MA), tumor suppressor p53 (Cat. no. 9282; Cell Signaling Technology) and p53 phosphorylated at Ser15 (p-p53) (Cat. no. 9284; Cell Signaling Technology). All western blot results were normalized to β-actin (Cat. no. 4967; Cell Signaling Technology). Blots were incubated with the primary antibody for 16 h, followed by the anti-rabbit IgG, HRP-linked Antibody secondary antibody (Cat. no. 7074; Cell Signaling Technology) incubation for 30 min. In case of reprobing PVDF blots, the previous primary and secondary antibodies were removed via the use of the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The PVDF blot was then reused and western blotting resumed as previously described, using a different primary antibody. Bands were visualized by enhanced chemiluminescent detection using the LumiSensor Plus Chemiluminescent HRP Substrate Kit (GenScript, Piscataway, NJ), and analysis was performed using the ImageJ software (version 1.49; National Institute of Health, Bethesda, MD).

Statistical analysis

The SPSS software (version 13; SPSS Inc, Chicago, IL) was used for statistical analysis. For comparison of means, one-way analysis of variance (ANOVA) followed by Games-Howell post hoc multiple comparisons test were used. Results were expressed as mean ± standard deviation (SD) and a $p < 0.05$ considered statistically significant.

Because in case of the western blotting, results were expressed as arbitrary optical density (OD) values, p values were calculated by comparing the means of OD values. Statistical analysis after normalization to the controls' OD values was avoided for preventing violation of the prerequisite for normal distribution of the compared variables when applying parametric statistical tests. However, for reader's convenience, these results were expressed in the text and depicted in the figures after normalization of means for the control group. The same was applied for the results derived from the assay performed for the assessment of ROS production.

Results

High glucose was slightly cytotoxic, whereas allopurinol was not cytotoxic at all

Compared to normal D-glucose concentration (5.5 mM), high D-glucose (25 mM) concentration increased cytotoxicity in GEnC only slightly (10.00 ± 0.45% vs. 12.00 ± 0.77%, $p < 0.001$) (Fig. 1).

Allopurinol at a concentration of 100 μM proved not cytotoxic for cells cultured under either normal or high-glucose conditions (10.00 ± 0.45% vs. 10.00 ± 0.77%, $p = 1.0$ and 12.00 ± 0.77% vs. 12.00 ± 0.45%, $p = 1.0$, respectively) (Fig. 1).

Allopurinol prevents high glucose-induced increase in ROS generation

Under normal-glucose conditions, allopurinol did not affect ROS levels in GEnC. Their levels were at the 103.25 ± 6.25% of the levels found in untreated cells ($p = 1.0$). Compared to GEnC cultured under normal-glucose conditions, high

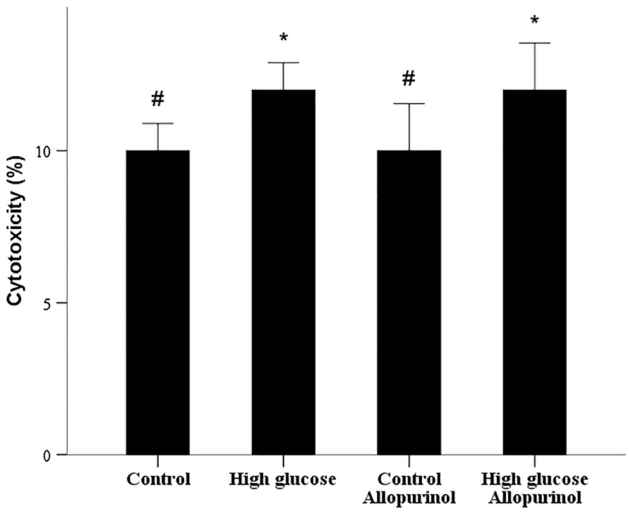

Fig. 1 Cytotoxicity of high glucose and allopurinol. GEnC were cultured under normal (5.5 mM) or high D-glucose (25 mM) conditions in the presence or not of 100 μM allopurinol. LDH release assay revealed only a slight increase of 2% in cytotoxicity when cells were cultured under high-glucose conditions. Allopurinol was not cytotoxic for GEnC cultured either under normal or high-glucose conditions. Error bars correspond to SD. Asterisk indicates a $p < 0.001$ compared to the first and third conditions, whereas hash tag a $p < 0.001$ compared to the second and fourth conditions

glucose increased ROS production by 306.82 ± 49.17% ($p < 0.001$). Treatment of GEnC cultured under high-glucose conditions with allopurinol prevented the increase in ROS levels ($p = 0.793$). In this case ROS levels were at the 117.57 ± 10.21% of the control condition (Fig. 2).

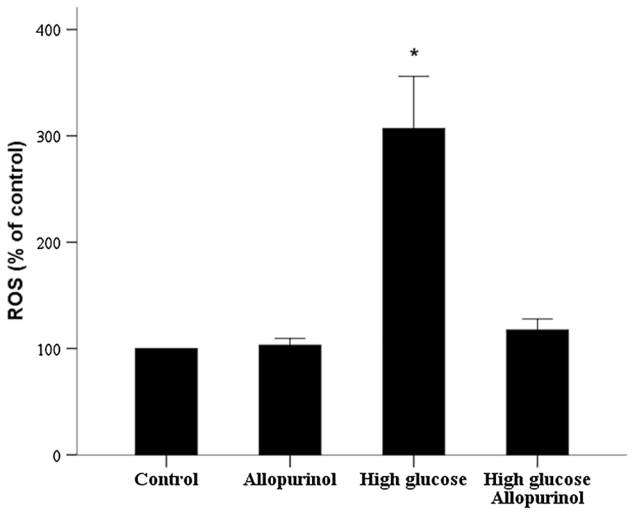

Fig. 2 Effect of high glucose and allopurinol on ROS production. GEnC were cultured under normal or high-glucose conditions in the presence or not of 100 µM allopurinol. High glucose increased ROS production, an effect that was prevented by treatment with allopurinol. Error bars correspond to SD and asterisk indicates a $p < 0.001$ compared to all other conditions

Allopurinol prevents high glucose-induced increase in NOS activity

NOS activity was assessed by measuring the concentration of nitrate and nitrite, the end products of NO metabolism. Under normal-glucose conditions, allopurinol did not affect NOS activity in GEnC (39.33 ± 4.03 µM vs. 43.67 ± 1.86 µM, $p = 0.237$). Compared to GEnC cultured under normal-glucose conditions, high glucose decreased NOS activity (10.67 ± 3.72 µM, $p < 0.001$). Treatment of GEnC cultured under high-glucose conditions with allopurinol prevented the decrease in NOS activity ($p = 1.0$). The concentration of end products of NO metabolism was 32.67 ± 13.58 µM (Fig. 3a).

Allopurinol prevents high glucose-induced increase in ICAM-1 expression

Under normal-glucose conditions, allopurinol did not affect ICAM-1 expression in GEnC. ICAM-1 level was 1.14 ± 0.12 times the level found in untreated cells ($p = 1.0$). Compared to GEnC cultured under normal-glucose conditions, high glucose increased ICAM-1 expression by a factor of 2.61 ± 0.77 ($p = 0.004$). Treatment of GEnC cultured under high-glucose conditions with allopurinol prevented the increase in ICAM-1 expression ($p = 1.0$). In this case ICAM-1 level was 1.36 ± 0.46 times the level found in the control condition (Fig. 3b).

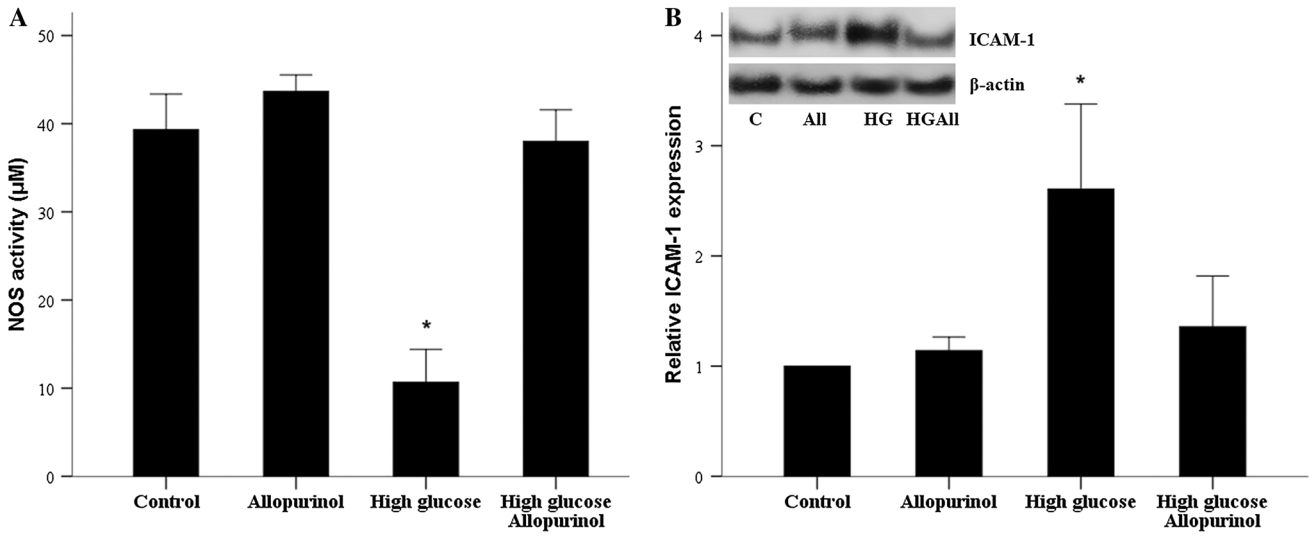

Fig. 3 Effect of high glucose and allopurinol on NOS activity and ICAM-1 expression. GEnC were cultured under normal or high-glucose conditions in the presence or not of 100 µM allopurinol. High glucose enhanced NOS activity, an effect abrogated by treatment with allopurinol (**a**). High glucose increased ICAM-1 expression, an effect that was prevented by treatment with allopurinol (**b**). At the upper left corner of panel B a representative western blot from the six performed experiments is depicted. C stands for control, All for allopurinol treatment under normal-glucose conditions, HG for high-glucose conditions and HG All for allopurinol treatment under high-glucose conditions. Error bars correspond to SD and asterisk indicates a $p < 0.001$ compared to all other conditions

Allopurinol prevents high glucose-induced increase in p53 and p-p53 levels

Under normal-glucose conditions, allopurinol did not affect p53 expression in GEnC. The level of p53 was 1.09 ± 0.35 times the level found in untreated cells ($p = 1.0$). Compared to GEnC cultured under normal-glucose conditions, high glucose increased p53 expression by a factor of 2.17 ± 0.51 ($p < 0.001$). Treatment of GEnC cultured under high-glucose conditions with allopurinol prevented the increase in p53 expression ($p = 1.0$). In this case, p53 level was 0.95 ± 0.28 times the level found in the control condition (Fig. 4a).

In GEnC cultured under normal-glucose conditions, the level of p-p53 was not affected by allopurinol treatment. The level of p-p53 was 1.08 ± 0.14 times the level found in untreated cells ($p = 1.0$). High glucose increased p-p53 by a factor of 1.67 ± 0.20 ($p < 0.001$). Treatment of GEnC cultured under high-glucose conditions with allopurinol decreased p-p53 level to 0.52 ± 0.22 times the level found in the control condition. This level was statistically significantly lower than the level observed in any other conditions ($p < 0.001$ in all cases) (Fig. 4b).

Discussion

Diabetes mellitus, especially type 2, affects almost 10% of the adult population worldwide, and its macro- and microvascular complications restrict both quality and expectancy of life [18]. Diabetic nephropathy, a microvascular complication of diabetes mellitus, is the leading cause of end-stage renal disease [1]. Thus, understanding the pathogenetic mechanisms involved in the pathogenesis of the vascular complications of diabetes mellitus is imperative since they may lead to new therapeutic approaches.

The aim of this study was to evaluate the possible protective effect of allopurinol on GEnCs dysfunction and activation due to hyperglycemic conditions. At the concentration of 100 μM, allopurinol did not add to the cytotoxicity of the high glucose concentration. It should be noted that although high glucose concentration has been found to be cytotoxic for human endothelial cells obtained from umbilical veins [19], in our study high glucose altered cytotoxicity only by 2% from 10 to 12%. This concentration of allopurinol has been selected because it protected endothelial cells from damage or activation in previous studies [16, 17]. The results from the LDH release assay confirmed the validity of our choice. Although allopurinol was not able to reverse the slight toxicity of the high glucose concentration for GEnCs, indicating that other pathways are also involved, it ameliorated both their dysfunction and activation, as discussed below.

Under hyperglycemic conditions, mitochondrial ROS overproduction in endothelial cells induces DNA strand breaks initiating a cascade of events that eventually lead to endothelial dysfunction and the vascular complications of diabetic mellitus [3, 4, 20]. However, experimental and clinical studies have shown that inhibition of another ROS producing system, the xanthine oxidase, prevents or ameliorates the course of diabetic nephropathy [5–8].

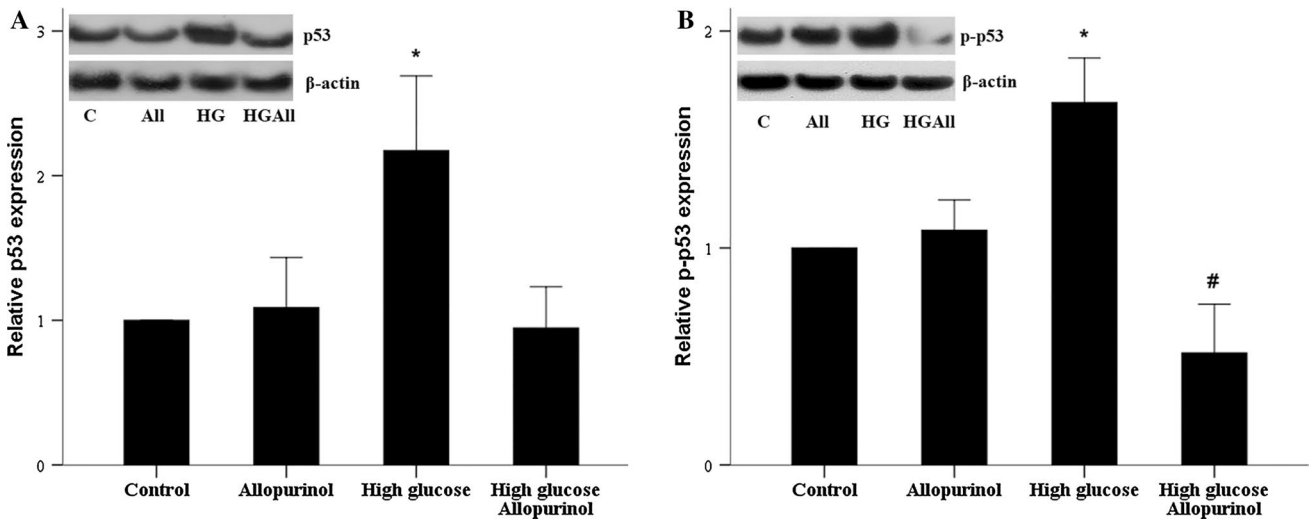

Fig. 4 Effect of high glucose and allopurinol on the levels p53 and p-p53. GEnC were cultured under normal or high-glucose conditions in the presence or not of 100 μM allopurinol. High glucose enhanced p53 expression, an effect prevented by treatment with allopurinol (**a**). High glucose increased p-p53 levels, whereas concurrent treatment with allopurinol reduced them (**b**). At the upper left corners of panels, **a** and **b** representative western blots from the six performed experiments are depicted. C stands for control, All for allopurinol treatment under normal-glucose conditions, HG for high-glucose conditions and HG All for allopurinol treatment under high-glucose conditions. Error bars correspond to SD. Asterisk and hash tag indicate a $p < 0.001$ compared to all other conditions

Considering that xanthine oxidase is one of the two catalytic forms of xanthine oxidoreductase, which prevails in the presence of oxidative stress [9–12], it is tempting to assume that under high-glucose conditions the initial mitochondrial ROS production activates xanthine oxidase, which subsequently becomes the major source of ROS. In accordance with the above hypothesis, our results showed that culture of GEnC under high-glucose conditions results in increased ROS generation, whereas the xanthine oxidase inhibitor allopurinol prevents ROS overproduction completely. Thus, it is likely that under hyperglycemic conditions xanthine oxidase acts as a feed-forward mechanism whereby an initial ROS generation stimulates further ROS production.

Then we evaluated if inhibition of ROS overproduction by allopurinol prevents endothelial dysfunction and activation. Indeed, while endothelial dysfunction, assessed by the production of NO [13], occurs in GEnC cultured under high-glucose conditions, allopurinol abrogated it completely. Similarly, allopurinol also fully prevents high glucose-induced endothelial activation, as assessed by ICAM-1 expression [14]. Therefore, it is conceivable that under hyperglycemic conditions inhibition of ROS overproduction by a xanthine oxidase inhibitor may protect endothelial cells from high glucose-induced damage, a fact that may explain the observed beneficial effect of allopurinol or febuxostat during the course of diabetic nephropathy [5–8].

Interestingly, besides glycemic control, effective treatment of hypertension also plays a significant role in the management of diabetic nephropathy [21]. Considering that in endothelial cells under mechanical stress the initial ROS production by NADPH oxidase activates xanthine oxidase, which in turn becomes the main source of ROS [12], it is possible that xanthine oxidase inhibitors may also exert their beneficial effect by preventing ROS overproduction due to hypertension.

As it is already noted, ROS-induced DNA damage is involved in the pathogenesis of endothelial cell damage under high-glucose conditions [3, 4, 20]. Elegant experiments have confirmed that DNA damage activates poly-ADP-ribose polymerase, which inactivates the enzyme glyceraldehyde 3-phosphate dehydrogenase [20]. Inactivation of glyceraldehyde 3-phosphate dehydrogenase results in accumulation of upstream glycolytic products that are diverted to certain noxious metabolic pathways resulting in activation of protein kinase C, protein modification by O-linked *β-N*-acetyl glucosamine and production of advanced glycation end products [3, 4, 20, 22–25]. Based on such studies, Brownlee et al. proposed a pathway for the microvascular complications of diabetes mellitus that sequentially includes mitochondrial ROS overproduction, DNA damage, poly-ADP-ribose polymerase activation, glyceraldehyde 3-phosphate dehydrogenase inactivation, diversion of accumulated upstream glycolytic products to certain noxious metabolic pathways and finally endothelial dysfunction [3].

However, it is also known that DNA damage by inducing phosphorylation of p53 prevents its interaction with the E3 ubiquitin-protein ligase Mdm2 and ultimately its degradation by the proteasome [26]. Interestingly, the ability of xanthine oxidase-derived ROS to induce DNA strand breaks and p53 upregulation has been demonstrated in primary human lung microvascular endothelial cells exposed to tobacco extracts [16]. Among other functions, p53 downregulates endothelial NOS expression [27, 28], or activity [15]. Also, in endothelial cells p53 up-regulates ICAM-1 expression [23, 29, 30]. In our study, the increased ROS production under high-glucose conditions was accompanied by increased p-p53 levels, which indirectly indicates DNA damage. Treatment of cells with allopurinol decreased both ROS production and p-p53 level. As expected by the fact that p53 phosphorylation prevents its degradation [26], p53 is accumulated in GEnC cultured in a high glucose medium, while allopurinol prevented the high glucose-induced p53 accumulation. Thus, our results suggest another one pathogenetic pathway that may contribute to the pathogenesis of diabetic nephropathy. This pathway sequentially includes high glucose-induced mitochondrial ROS production, xanthine oxidase activation, further ROS production, DNA damage, p53 accumulation, endothelial dysfunction and activation.

According to the results of the present study, xanthine oxidase activation could be also added immediately after the initial mitochondrial ROS production in the pathway proposed by Brownlee et al. [3, 4, 20]. Besides the common starting point, that is the mitochondrial ROS overproduction, the interplay between these two pathways may occur at many points. For instance, advanced glycation end products modify and impair proteasome function [31], which may result in further p53 accumulation [26], while endothelial NOS activity can be downregulated not only due to p53 [15, 27, 28], but also because of modification of this enzyme by O-linked *β-N*-acetyl glucosamine [24].Thus, it is likely that xanthine oxidase plays a central role in the generation of ROS and in the pathogenesis of diabetic nephropathy and possibly of the other microvascular complications of diabetes mellitus.

Certainly, as regards the observed benefit from xanthine oxidase inhibitors in the course of diabetic nephropathy [5–8], a role for the decrease in serum urate levels per se cannot be excluded. Immune cells contribute to the pathogenesis of diabetic nephropathy [32], and urate at concentrations above its crystallization threshold boosts cellular and humoral immunity [33].

In conclusion, allopurinol protects GEnC from high glucose-induced ROS generation, p53 overexpression and endothelial dysfunction. These data provide a pathogenetic

mechanism that supports the results of experimental and clinical studies about the beneficial effect of xanthine oxidase inhibitors on the development of diabetic nephropathy.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

Research involving human participants and/or animals The study did not involve Human participants or animals.

Informed consent Not applicable, the study did not involve Human participants.

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