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Hypoxanthine causes endothelial dysfunction through oxidative stress-induced apoptosis

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ABSTRACT

Endothelial cell injury and dysfunction caused by reactive oxygen species (ROS) are implicated in the pathogenesis of vascular diseases. ROS are generated and hypoxanthine is degraded by xanthine oxidase. Smoking and alcohol consumption are associated with an increased level of hypoxanthine. We aimed to study the direct role of hypoxanthine in endothelial dysfunction in human umbilical vascular endothelial cells (HUVECs). Hypoxanthine induced cell death and production of ROS. Furthermore, hypoxanthine induced apoptosis through regulation of protein expression related to apoptosis. When cells were pretreated with *N*-acetylcysteine or a pancaspase inhibitor (Z-VAD-fmk) and stimulated with hypoxanthine, Z-VAD-fmk and *N*-acetylcysteine prevented hypoxanthine-induced apoptosis by inhibiting the ROS production and caspase pathway. Thus, an increased extracellular concentration of hypoxanthine induces endothelial dysfunction through ROS production and regulates expression of apoptosis-related proteins in HUVECs. These effects are expected to be associated with some vascular diseases.

1. Introduction

Hypoxanthine and xanthine are oxidized by xanthine oxidase to uric acid, which is the end product of purine metabolism. During this process, reactive oxygen species (ROS) are generated by xanthine oxidase [1,2]. The hypoxanthine level is increased in plasma by cigarette smoking, which lowers hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity [3]. Heavy alcohol consumption increases plasma concentrations of hypoxanthine [4]. In addition, hypoxanthine was identified in patients undergoing hemodialysis, which is associated with oxidative stress and/or hypoxia [5]. In a recent paper, we demonstrated that hypoxanthine induces atherosclerosis with cholesterol upregulation, and these effects are partially mediated by ROS produced in response to hypoxanthine [6]. Endothelial dysfunction serves as the initiating factor of atherosclerosis via an increase in permeability of the endothelium, enhanced adhesion of leukocytes, and changes in the expression of genes in endothelial cells [7]. Atherosclerosis is a chronic inflammatory disease affecting large and medium arteries and is considered a major underlying cause of cardiovascular disease. Endothelial cells serve as a barrier between the blood vessel wall and blood. Endothelial cell apoptosis and dysfunction caused by ROS are implicated in numerous pathological processes such as aberrant angiogenesis, anomalous thrombosis, atherosclerosis, hypertension, diabetic vasculopathy, and heart failure [8]. Although hypoxanthine may play important roles in atherogenesis and cardiovascular disease in the smoking and heavily drinking population [9,10]. There is no direct evidence to support such an association between hypoxanthine and endothelial cells.

In this study, we assessed the direct effects of hypoxanthine on ROS-mediated apoptosis in endothelial cells.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HUVECs were cultured in gelatin-coated dishes in fully

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supplemented endothelial growth medium (EGM-2, Lonza, Walkersville, MD, USA) and Medium 199 (M199; Gibco, Grand Island, NY, USA), with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. HUVECs were used in experiments at passages 5–7. Hypoxanthine, *N*-acetylcysteine (NAC), and a pancaspase inhibitor (Z-VAD-fmk; Z-VAD) were used in the experiments. Hypoxanthine and NAC were purchased from Sigma-Aldrich (St. Louis, MO, USA), and Z-VAD was acquired from Merck Millipore (Calbiochem, La Jolla, CA).

2.2. Determination of cell viability

HUVECs were seeded in a 96-well plate (2×10^4/well; Nunc, Roskilde, Denmark). The culture medium was then replaced with a fresh medium containing various reagents. Media in all wells were mixed thoroughly, and the plates were placed in an incubator at 37 °C for 24 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) was added to each well. After incubation with MTT for 4 h, 200 μL of dimethyl sulfoxide (DMSO; Amresco, Solon, Ohio, USA) was added to each well. When the crystals were completely dissolved, optical density at 570 nm in the wells was measured using a microplate reader (Bio-Rad Model 550; Hercules, CA, USA).

2.3. Quantification of H_2O_2

H_2O_2 concentrations in the culture medium were determined using the Amplex Red Hydrogen Peroxide Assay Kit according to the manufacturer's protocol (Molecular Probes, Invitrogen, Eugene, OR, USA). Fluorescence was measured on a fluorescence plate reader (Molecular Devices Corp., Silicon Valley, CA, USA) at 530 nm excitation and 590 nm emission wavelengths. Standard curves were constructed using known concentrations of H_2O_2 diluted in loading buffer. Background fluorescence was measured using loading buffer alone and subtracted from each value.

2.4. Western blot analysis

After treatment with hypoxanthine and/or the antioxidant, NAC, or pancaspase inhibitor, Z-VAD, HUVECs were lysed in RIPA buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) and Protease Inhibitor Cocktail Set III (Calbiochem, Darmstadt, Germany). The lysates were centrifuged at 12000×*g* for 15 min, and protein concentration was measured by Bradford's method. Total protein (20 μg from each sample) was separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 10% nonfat dry milk in 10 mmol/L Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h, followed by overnight incubation at 4 °C with diluted primary antibodies in TBS-T. These were anti-Bcl-2 (1:1000, Cell Signaling, Beverly, MA, USA), anti-Bad (1:1000, Cell Signaling, Beverly, MA, USA), and anti-β-actin antibodies (1:10000, Sigma Aldrich, St. Louis, MO, USA). After three times washes in TBS-T, the membrane was incubated with a secondary antibody (a horseradish peroxidase-conjugated polyclonal goat anti-rabbit immunoglobulin antibody, 1:2000; or a goat anti-mouse immunoglobulin antibody; 1:20000; Dako, Glostrup, Denmark) in TBS-T for 1 h at room temperature, and specific protein bands were detected using advanced ECL reagents (Amersham Bioscience, Piscataway, NJ, USA). The levels of expression were estimated using the Scion Image software (Scion, Frederick, MD, USA).

2.5. Assessment of apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

HUVECs were seeded at 5×10^4/well on gelatin-coated 8-well chamber slides (Nunc, Roskilde, Denmark). Apoptotic cells after treating with hypoxanthine were detected by using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). Treated cells fixed for 1 h at 15–25 °C with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) were washed with PBS, incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4 °C, washed with PBS, and incubated with 100 μL of a TUNEL reaction mixture for 1 h at 37 °C. Next, the cells were washed with PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/mL) for 5 min, washed with PBS two times, and mounted with the ProLong Gold Antifade Reagent (Invitrogen, Eugene, OR, USA). The number of stained cells was assessed using the software ImageJ. The percentage of apoptotic cells was calculated from the number of TUNEL-positive cells divided by the total number of cells counted. Three to five images obtained in a systematically random manner were analyzed in each dish.

2.6. Assessment of apoptosis by flow cytometry

HUVECs were cultured in a 6-well plate (Nunc, Roskilde, Denmark). Annexin V positive cells were detected by using the FITC Annexin V Apoptosis Detection Kit (BD biosciences, USA). After treatments, the cells were collected by trypsinization and gentle centrifugation. The cell pellet was resuspend in 100 μL of 1 × Annexin binding buffer. Then, 5 μL of annexin V was added to the cells, and the mixture was incubated for 15 min. Next, 5 μL of the propidium iodide (PI) was added to the cells, with incubation at room temperature for 15 min in the dark. Next, 200 μL of PBS was added. Finally, the cell samples were analyzed on the FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The apoptotic rate was determined by calculating the ratio of annexin V-positive and annexin V/PI-double positive cells to total cells.

2.7. Fluorometric analysis of caspase 3 activity

After the hypoxanthine treatment, cells were harvested and processed for a caspase 3 assay, using the Caspase-3 Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). After incubation at 37 °C for 70 min, fluorescence was measured at 405 nm by means of a microplate reader (Bio-Rad Model 550; Hercules, Calif., USA). A standard *p*-nitroaniline solution was used for calculating caspase activity.

2.8. Statistical analysis

Results were expressed as mean ± SEM. All the experiments were repeated at least three times. Significance of differences between groups was tested by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Differences with a *P* value less than 0.05 were considered statistically significant. Statistical analysis was performed in the SPSS Statistics software, version 22.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Effects of hypoxanthine on cell viability and ROS production

To evaluate the effect of hypoxanthine on cell viability, we incubated various concentrations hypoxanthine with HUVECs, followed by an MTT assay. As shown in Fig. 1A, hypoxanthine inhibited the growth of HUVECs in a dose-dependent manner. Next, to test whether hypoxanthine increases production of ROS, HUVECs were exposed to hypoxanthine for 2 h. After that, the level of H_2O_2 in the cells significantly increased as compared to that of control cells (Fig. 1B). These results suggested that hypoxanthine at a

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