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The changes of intracellular H_2O_2 are an important factor maintaining mitochondria membrane potential of antimycin A-treated As4.1 juxtaglomerular cells

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ABSTRACT

We investigated an involvement of ROS, such as H_2O_2 and $O_2^{\bullet-}$ and GSH in the As4.1 cell death by antimycin A and examined whether ROS scavengers rescue antimycin A-induced As4.1 cell death and its mechanism. Levels of intracellular H_2O_2 and $O_2^{\bullet-}$ were markedly increased in antimycin A-treated cells. Antimycin A reduced the intracellular GSH content. A ROS scavenger, Tiron down-regulated the production of intracellular H_2O_2. However, the reduction of intracellular H_2O_2 level did not change the apoptosis parameters, such as sub-G1 DNA content and annexin V binding. Interestingly, treatment of Tiron could partially prevent the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Treatment of SOD and catalase also reduced the intracellular H_2O_2 and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) without reducing $O_2^{\bullet-}$ level and apoptosis in antimycin A-treated As4.1 cells. All the ROS scavengers, SOD and catalase did not inhibit GSH depletion induced by antimycin A, resulting in failure of preventing the apoptosis. In addition, all the reagents including antimycin A did not induce any specific phase arrest of cell cycle in As4.1 cells. In summary, these results demonstrate that antimycin A generates potently ROS, H_2O_2 and $O_2^{\bullet-}$ and induces the depletion of GSH content in As4.1 JG cells, and that Tiron, SOD and catalase inhibited partially the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) via the reduction of intracellular H_2O_2 level.

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1. Introduction

Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$). ROS have recently been implicated in the regulation of various important cellular events, including transcription factor activation, gene expression, differentiation and cell proliferation [1–3]. ROS are formed as byproducts of mitochondrial respiration or precise oxidases including nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO) and certain arachidonic acid oxygenases [4]. An alteration on the redox state of the tissue implies a change in ROS generation or metabolism. Principal metabolic pathway involves superoxide dismutase

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Abbreviations: AMA, antimycin A; ROS, reactive oxygen species; NADPH, nicotine adenine diphosphate; XO, xanthine oxidase; SOD, superoxide dismutase; JGCT, juxtaglomerular cell tumors; FBS, fetal bovine serum; PBS, phosphate buffer saline; FITC, fluorescein isothiocyanate; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; NAC, *N*-acetylcysteine; CMFDA, 5-chloromethylfluorescein diacetate

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(SOD), which is expressed as extracellular, intracellular and mitochondrial isoforms, and metabolizes $O_2^{\bullet-}$ to H_2O_2. Further metabolism by peroxidases that include catalase and glutathione peroxidase yields O_2 and H_2O [5]. Cells possess antioxidant systems to control the redox state, which is important for their survival. Excessive production of ROS gives rise to activation of events, which lead to death and survival in several types of cells [6–9]. The precise mechanisms involved in cell death induced by ROS remain an open question and the protective effect of some antioxidants on cell death is still controversial.

Antimycin A (AMA) is a mixture product of predominantly antimycin A1 and A3 derived from *Streptomyces kitazawensis* [10]. The compound inhibits the activity of succinate oxidase and NADH oxidase and also blocks mitochondrial electron transport specifically between cytochromes *b* and *c* [11–14]. Inhibition of electron transport causes a collapse of the proton gradient across the mitochondrial inner membrane, thereby collapsing the mitochondrial membrane potential ($\Delta\Psi_m$) [11,13,15]. This inhibition also causes the production of ROS [15,16]. There is evidence that either ROS or the collapse of mitochondrial membrane potential ($\Delta\Psi_m$) opens the mitochondrial permeability transition pore, accompanied by the release of proapoptotic molecules, such as cytochrome *c* into the cytoplasm [17–19]. In fact, because AMA acts directly on the mitochondria, AMA-induced apoptosis has been reported in many experiments [20–23].

Juxtaglomerular cell tumors (JGCTs; also known as reninomas), first described in the late 1960s [24,25], are rare benign tumors of the kidney. About 100 cases have been described to date. Reninomas are understood to arise from juxtaglomerular cells. Clinically, the patients suffer from headaches, polyuria, nocturia and dizziness including other symptoms. Hypertension is a sign in almost all patients and laboratory findings of hyperreninemia, hyperaldosteronism and hypokalemia are characteristic. Recently, a malignant JGCT was described [26].

As4.1 cells have been used as a model for the JG cell. This cell line was isolated from kidney neoplasm in a transgenic mouse containing a renin SV40 T-antigen transgene [27]. However, the role of ROS in kidney cell death, especially JG cell, has not been evaluated. Therefore, understanding the molecular mechanism of kidney cell death by ROS generator, especially AMA is an important subject. In the present study, we evaluated the involvement of ROS, such as H_2O_2 and $O_2^{\bullet-}$ and GSH in the AMA-treated As4.1 cells and investigated whether ROS scavengers could rescue AMA-induced As4.1 cell death and its mechanism.

2. Materials and methods

2.1. Cell culture

As4.1 cells (ATCC No. CRL-2193) are a renin-expressing clonal cell line derived from the kidney neoplasm of a transgenic mouse [27]. As4.1 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (GIBCO BRL, Grand Island, NY). Cell cultures were maintained in a humidified incubator containing 5% CO_2 at 37 °C. Cells were routinely grown in 100-mm plastic tissue culture dish (Nunc, Roskilde, Denmark) and were passed when they were in logarithmic phase of growth, and maintained at the above-described culture conditions for all experiments.

2.2. Reagents

AMA (Sigma–Aldrich Chemical Company, St. Louis, MO) was dissolved in ethanol at 2×10^{-2} M as a stock solution. The cell permeable $O_2^{\bullet-}$ scavengers, 4-hydroxy-TEMPO (4-hydroxyl-2,2,6,6-tetramethylpierydine-1-oxyl) (Tempol), (4,5-dihydroxyl-1,3-benzededisulfonic acid) (Tiron), (1-[2,3,4-trimethoxibenzyl]-piperazine) (Trimetazidine) and (*N*-acetylcysteine) (NAC) were obtained from Sigma. These were dissolved in designated solution buffer at 1×10^{-1} M as a stock solution. All of stock solutions wrapped in foil were kept in 4 or −20 °C.

2.3. Cell cycle and sub-G_1 analysis

Cell cycle and sub-G_1 distribution were determined by staining DNA with propidium iodide (PI; Sigma–Aldrich) as described used [28]. Briefly, 1×10^6 cells were incubated with the designated doses of AMA with or without ROS scavenger, SOD or catalase for 48–72 h. Cells were then washed with phosphate buffer saline (PBS) and fixed in 70% ethanol. Cells were again washed with PBS and then incubated with PI (10 μg) with simultaneous treatment of RNase at 37 °C for 30 min. The percentages of cells in the different phases of the cell cycle or having the sub-G_1 DNA content were measured with FACStar flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using lysis II and cellfit software (Becton Dickinson) or ModFit software (Verity Software Inc.).

2.4. Annexin V/PI staining

Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) or -phycoerythrin (PE) and PI labeling. Annexin V was used to detect early apoptotic cells during apoptosis. Briefly, to quantitate the apoptosis of cells, 1×10^6 cells were incubated with the designated doses of AMA with or without ROS scavenger, SOD or catalase for 48–72 h. The cells were washed twice with cold PBS, and suspended in 500 μl of binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Then 5 μl of annexin V-FITC or -PE (PharMingen, San Diego, CA) and PI (1 μg/ml) were added to these cells at 37 °C for 30 min. The cells were analyzed with FACStar flow cytometer (Becton Dickinson). Viable cells were negative for both PI and annexin V, and apoptotic cells were positive for annexin V and negative for PI, whereas late apoptotic dead cells displayed both high annexin V and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI and negative for annexin V.

2.5. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane was monitored using a cell permeable cationic, fluorescent dye Rhodamine 123, which preferentially enters into mitochondria due to the highly negative

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