Mechanism of cytotoxicity of human embryonic kidney cells induced by gliotoxin

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Abstract: AIM To study the mechanism underlying gliotoxin-induced cytotoxicity of human embryonic kidney (HEK) cells. **METHODS** Crystal violet assay was used to determine cell viability. DNA fragmentation of HEK cells was measured based on Burton's method. The activity of caspase-3-like proteases was measured as increases in hydrolysis of fluorogenic tetrapeptide substrate, Ac-DEVD-7-amino-4-methylcoumarin and caspase-3 protein abundance was observed by Western blot. Based on fluorescence probe label method, DNA content and reactive oxygen species (ROS) of HEK cells were detected by flow cytometry. **RESULTS** Gliotoxin induced HEK cell death in a concentration-dependent manner within 0.4 – 1.0 mg·L^{-1}. Under gliotoxin treatment at 1.0 mg·L^{-1}, cell membrane of HEK cells kept intact associated with hypodiploid nuclei and DNA fragmentation which suggested gliotoxin killed HEK cells *via* apoptosis. Boc-aspartyl (OMe)-fluoromethylketone (BAF) and z-DEVD. fmk, commonly used as caspase-3-like proteases inhibitor, significantly abolished gliotoxin-induced cell death at 100 and 200 μmol·L^{-1}, respectively, suggesting the cytotoxicity induced by gliotoxin was mediated by caspases. *N*-acetylcysteine concentration-dependently attenuated the HEK cells death induced by gliotoxin, significantly inhibited the generation of ROS of HEK cells upon exposure with gliotoxin, which indicated that ROS was involved in the cytotoxicity of HEK cells induced by gliotoxin. **CONCLUSION** Gliotoxin-induced cytotoxicity of HEK cells proceeded *via* apoptosis, which was mediated by caspases and ROS.

Key words: gliotoxin; apoptosis; cells, human embryonic kidney; caspases; reactive oxygen species

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Gliotoxin is a fungal metabolite produced by a number of *Aspergillus* and *Penicillium* species. Recently, renal failure was frequently reported to be associated with invasive aspergillosis with a very high rate of mortality[1]. However, the underlying pathophysiology mechanism of it remains largely unknown.

Apoptosis is an active mode of cell death in physiological and pathogenic processes characterized by chromatin condensation, membrane blebbing, DNA fragmentation and apoptotic body formation[2]. Caspases are regarded as important mediators of apoptosis. Among more than 10 kinds of caspases, caspase-3 is known as the most important effector caspase in most kinds of apoptosis[3]. Another pathway that transduces cell apoptosis is mediated by reactive oxygen species (ROS), ROS may induce cell death by themselves or act as intracellular messengers during the cell death induced by various other kinds of stimuli[4].

Several lines of evidence has demonstrated that gliotoxin causes apoptosis in a series of cell lines, including thymocytes, peripheral lymphocytes, macrophages, fibroblast *in vitro*, spleen and LLC-PK1 cells[5]. However, the mechanism which leads to final apoptosis of those cells varies depending on different cell types and their reactivity to gliotoxin. Until recently, seldom research has been focused on the ROS and caspases-mediated cell apoptosis induced by gliotoxin. Gliotoxin has the ability to generate ROS[5], whether gliotoxin-induced ROS accounts for its toxic effect

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on human embryonic kidney (HEK) cells remains completely unknown. Therefore, the purpose of this study was to examine the cytotoxicity of HEK cell induced by gliotoxin and its mechanism.

1 METHODS AND MATERIALS

1.1 Materials

Gliotoxin, *N*-acetylcysteine (NAC), crystal violet dye, 2′, 7′-dichlorofluoresein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). Boc-aspartyl (OMe)-fluoromethyl- ketone (BAF) and z-DEVD. fmk were obtained from Enzyme System Products (Livermore, CA, USA).

1.2 Cell culture and treatment

HEK 293 cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were kept in DMEM culture medium plus 10% fetal bovine serum, 100 kU·L^{-1} penicillin and 100 mg·L^{-1} streptomycin in a 37℃ incubator supplied with 5% CO_2. For assay of cell viability, the cells were placed down at 1.2×10^5 cells per well (confluent) in a 96 well plate and cultured for 18 – 22 h before treatment.

1.3 Crystal violet assay

Cell viability was measured according to methods in literature[6].

1.4 DNA fragmentation assay

The fragmentary DNA in cytoplasma was detected according to Sei, *et al*[7]. Briefly, after treatment until 80% of the cells detached from dishes, the cells were lysed and centrifuged. The supernatant was digested with 0.1 g·L^{-1} proteinase K and DNA was extracted with phenol/chloroform method. The DNA was resolved in 2% agarose gel. The fragmentary DNA in the cytosol was also quantified according to Burton[8].

1.5 Measurement of caspase-3-like activity

The activity of caspase-3-like proteases was measured as increases in hydrolysis of fluorogenic tetrapeptide substrate, Ac-DEVD-7-amino-4-methylcoumarin (Ac-DEVD-AMC), according to the manufacturer′s instructions (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA). Because caspase-7 also cleaves the substrate, the activity obtained here represents as caspase-3-like! proteases activity. Briefly, the cells were lysed, then the supernatants were taken for measurement of hydrolysis of Ac-DEVD-AMC as a function of time at 22℃.

1.6 Immunoblot analysis

HEK cells were lysed and collected after treatment with gliotoxin for 2 h. After they were resolved by SDS-PAGE, the protein was electrophoretically blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were first hybridized with primary antibodies and then with horseradish-peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO, USA). The primary antibody against the caspase-3 was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA) and the secondary antibody was from Pierce Chemical Co. (Rockford, IL, USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The immune complexes were detected using chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

1.7 Cytofluorometric analysis of propidium iodide (PI) staining

Cytofluorometric analysis of PI staining was performed according to Nicoletti, *et al*[9]. Briefly, cells were collected and incubated in a hypotonic fluorochrome solution overnight at 4℃. The PI fluorescence was measured with excitation at 488 nm and emission of 620 nm. The cell debris were excluded from analysis by adjusting the forward scatter threshold. Hypodiploid nuclei due to the condensation of nuclear chromatin appeared at sub G_0/G_1 position.

1.8 Flow cytometry for reactive oxygen species formation

DCFH-DA was used as an indicator for the formation of intercellular ROS. The cells were preloaded with 20 μmol·L^{-1} freshly prepared DCFH-DA, then treated with gliotoxin for 40 min. Once ROS was generated, the DCFH oxidation product, DCF fluorescence can be detected by flow cytometer

(FACScan, Becton Dickinson). The fluorescence was assessed by counts of FL1-H[10].

1.9 Statistical analysis

All numerical data were expressed as $\bar{x} \pm s$ unless indicated otherwise. Statistical analysis was performed by unpaired t test or analysis of ANOVA as appropriate. Multiple post comparisons were made by Dunnnett analyses.

2 RESULTS

2.1 Gliotoxin-induced HEK cells apoptosis

Our previous work has shown that gliotoxin (800 $\mu g \cdot L^{-1}$) exerted its maximal cell death effect in HEK cell at 2 h after treatment, with cell viability 31.2% (data not shown). Therefore, 2 h was assigned as optimal incubation time for gliotoxin in following studies. Gliotoxin induced cell death in a concentration-dependent manner with minimal 29.6% survival rate at 1.0 $mg \cdot L^{-1}$ (Fig 1A). In accordance with cell viability, gliotoxin concentration-dependently increased DNA fragmentation of cell, which were detected by DNA fragmentation assay (Fig 1B). In order to further examine whether gliotoxin induced cell death *via* apoptosis, flow cytometry was applied to determine DNA content of HEK cell treated with gliotoxin, the result showed that occurrence of gliotoxin-induced cell death was associated with

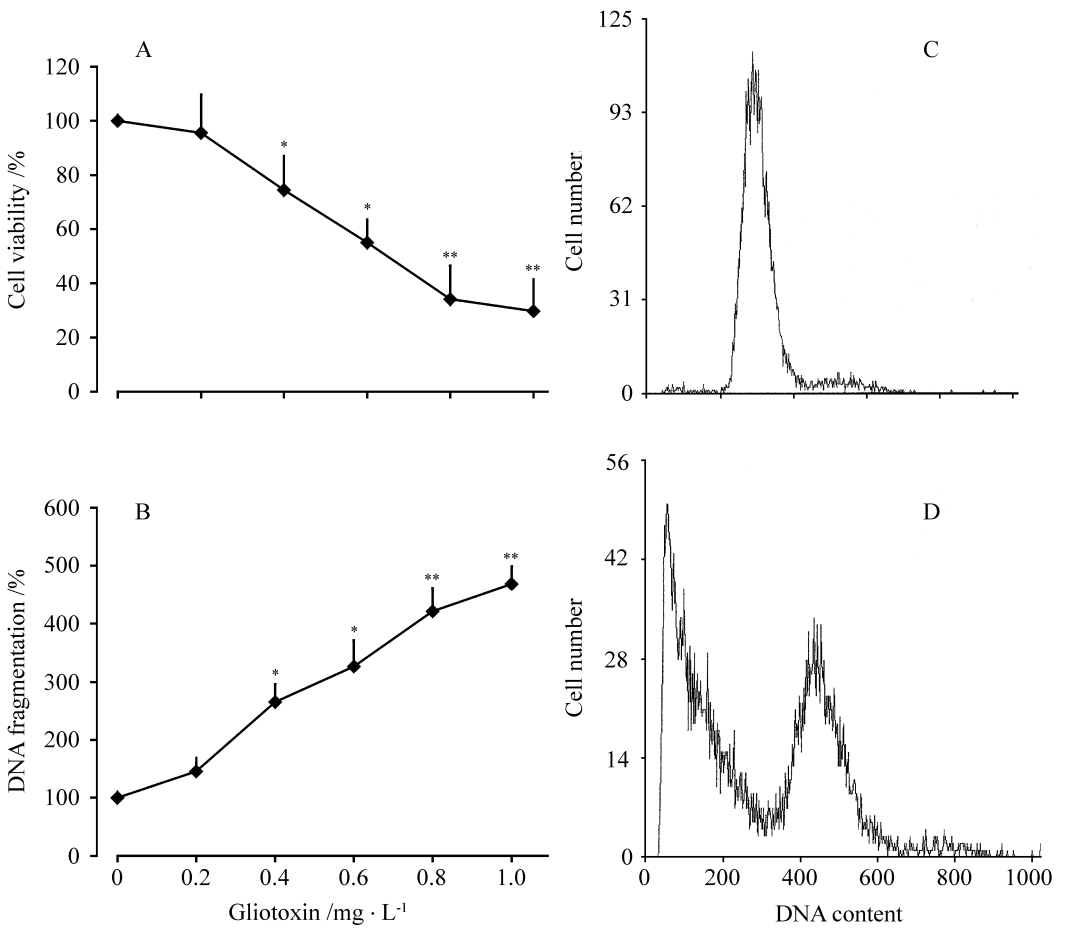

Fig 1. Cell viablity and DNA fragmentation following incubation with gliotoxin for 2 h. The cell viability was determined by crystal violet assays. DNA fragmentation in cytoplasma was quantified by diphenylamine assays, each experiment was performed in duplicate. Absorbance, representing cell viability (A) or DNA fragmentation (B) at 0 $mg \cdot L^{-1}$ was set as 100%. $\bar{x} \pm s$, $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. (C, D) Flow cytometry analysis of DNA content of HEK cells treated without (C, control) or with gliotoxin 1.0 $mg \cdot L^{-1}$ (D), respectively. Cells were treated until 60% – 80% of them were detached from the bottom of well. Both detached and attached cells were collected, the DNA content of which was assayed by using PI staining method. Each panel was a representative of 5 independent experiments. At least 15 000 cells were analyzed in each experiment.

hypodiploid nuclei (Fig 1D). Concentration-response as well as time course effects of gliotoxin on lactate dehydrogenase (LDH) activity revealed that the plasma membrane of HEK cell remained largely intact after gliotoxin treatment (data not shown), which further substantiated that cell death induced by gliotoxin was proceeded by apoptosis.

2.2 Effects of caspases on gliotoxin-induced cell death

BAF, a general caspase inhibitor, and z-DEVD. fmk, a tetrapeptide-specific inhibitor of caspase-3-like protease, significantly abolished gliotoxin (1.0 mg·L^{-1}) induced cell death at 100 and 200 μmol·L^{-1}, respectively (Fig 2A). Time course and concentration-response of gliotoxin on hydrolysis of Ac-DEVD-AMC revealed that gliotoxin exerted maximal activation of caspase-3-like proteases activity at 0.6 – 1.0 mg·L^{-1} with peak time at 1 h after treatment (Fig 2B, 2C). Western blot results revealed that BAF at 100 μmol·L^{-1} significantly inhibited the caspase-3 protein abundance stimulated by gliotoxin (Fig 2D). The data suggested caspases be involved in the cell death induced by gliotoxin.

2.3 Effects of ROS on gliotoxin-induced cell death

NAC concentration-dependently attenuated the cytotoxicity induced by gliotoxin as shown in Fig 3A. Furthermore, NAC at 4 – 12 mmol·L^{-1} dramatically abolished the increase in DNA fragmentation induced by gliotoxin (Fig 3B). As little as 12 mmol·L^{-1}, gliotoxin completely decreased the hydrolysis of Ac-DEVD-AMC elicited by

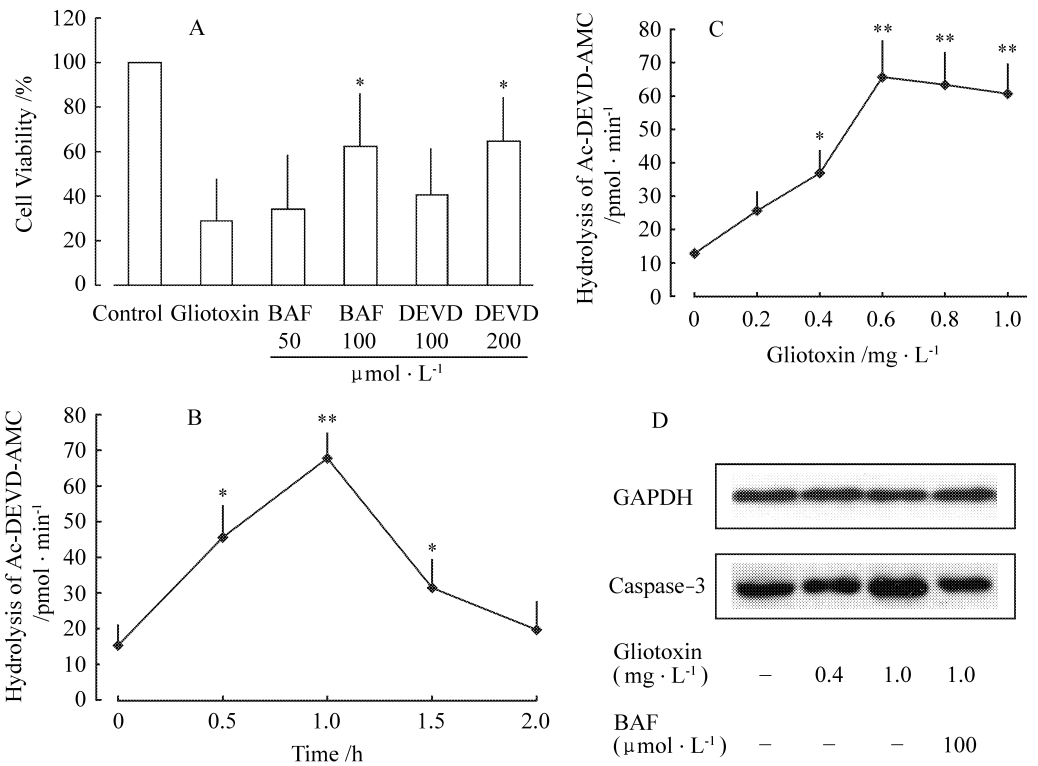

Fig 2. **(A) Boc-aspartyl (OMe)-fluoromethylketone (BAF) and z-DEVD. fmk (DEVD) inhibited gliotoxin induced cytotoxicity.** The cells were pretreated with BAF and DEVD for 30 min prior to addition of gliotoxin (1.0 mg·L^{-1}), then incubated for additional 12 h. **(B) The time course of gliotoxin (1.0 mg·L^{-1}) on the hydrolysis of Ac-DEVD-AMC in 10^5 cells. (C) Gliotoxin induced concentration-dependent hydrolysis of Ac-DEVD-AMC in 10^5 cells.** The cells were incubated with gliotoxin for 1 h. **(D) BAF inhibited the stimulation of caspase-3 protein abundance elicited by gliotoxin (1.0 mg·L^{-1}, 1 h).** GAPDH was used as internal standard to normalize loading sample volume. BAF was added 30 min prior to gliotoxin. $\bar{x} \pm s$, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control.

gliotoxin (Fig 3C). DCFH-DA was used as ROS sensitive fluorescence probe to examine whether the generation of ROS was a crucial step in gliotoxin-induced cell death. As shown in Fig 4B, the intracellular levels of ROS increased upon exposure to gliotoxin, and NAC significantly inhibited the generation of ROS induced by gliotoxin due to left shift of fluorescent curve shown in Fig 4C as compared with Fig 4B. Taken together, cytotoxicity and caspase-3-like proteases activity increase induced by gliotoxin were mediated by ROS.

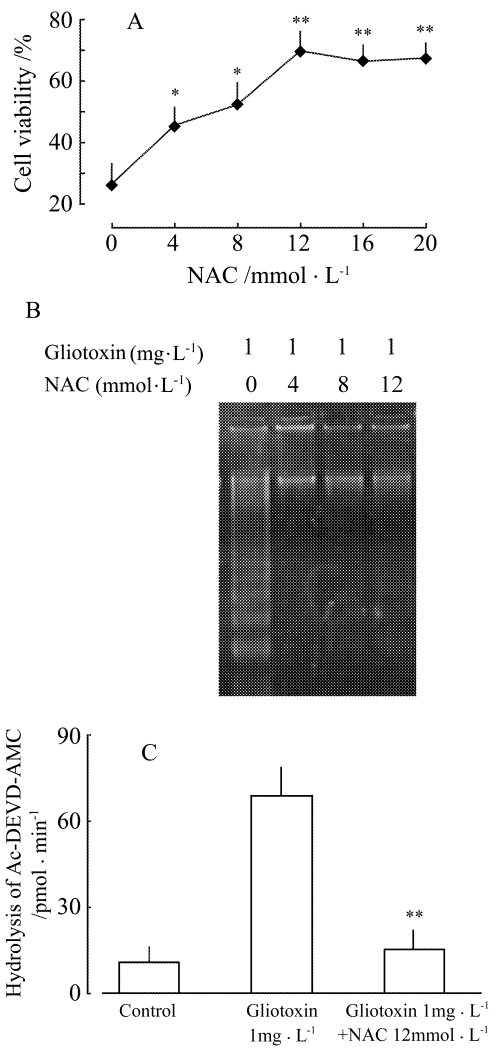

Fig 3. Reactive oxygen species were involved in the cytotoxcity of HEK cell induced by gliotoxin. (A) *N*-acetylcysteine (NAC) concentration-dependently inhibited gliotoxin (1.0 mg·L^{-1}) induced cytotoxicity. (B) NAC abolished the DNA fragmentation induced by gliotoxin (1.0 mg·L^{-1}). (C) NAC 12 mmol·L^{-1} inhibited gliotoxin-induced hydrolysis of Ac-DEVD-AMC in 10^5 cells. Cells were pretreated with NAC for 30 min, then co-incubated with gliotoxin for additional 12 h for A, 2 h for B and 1 h for C. $\bar{x} \pm s$, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control.

3 DISCUSSION

Opportunistic infections such as aspergillosis can be fatal to patients with acute renal failure[11]. One possible etiologic agent was isolated and identified as the secondary metabolite gliotoxin. Until now, the link between gliotoxin and renal disease has not been precisely demonstrated, our present study revealed that gliotoxin concentration-dependently increased DNA fragmentation of HEK cells, induced cell death associated with hypodiploid nuclei and intact cell membrane, all of which showed gliotoxin induced HEK cell death *via* apoptosis.

Until recently, the precise mechanism underlying gliotoxin-induced apoptosis is still under active investigation. Through phosphorylation of histone H_3, gliotoxin was shown to increase the sensitivity of chromatin to nuclease digestion, which led to DNA ladder. The effect of gliotoxin was also mediated by protein kinase A[5]. In our study, gliotoxin induced HEK cell apoptosis mediated by ROS and caspases.

Based on the inhibitory effect of z-DEVD. fmk and BAF, stimulation of caspase-3 protein abundance and the increase in hydrolysis activity of caspases substrate, gliotoxin-induced HEK cell death was mediated by caspases, which shared the same mechanism with gliotoxin-induced apoptosis in human granulocytes[12]. By comparing Fig 1A and Fig 2B, it was interesting to find that although gliotoxin at 200 $\mu g \cdot L^{-1}$ did not significantly induce cell death, it increased the hydrolysis of Ac-DEVD-AMC. Actually, this is not a unique phenomena observed only here. The activation of caspases has been reported not to be indispensable for occurrence of cell apoptosis[13]. So, it is not reasonable to rule out the existence of checkpoints further downstream caspases cascade, the function of which in cell apoptosis needs to be further investigated.

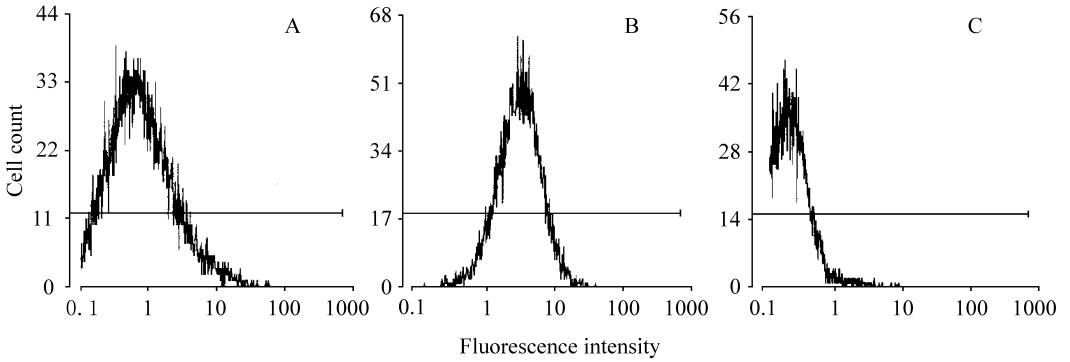

Fig 4. ***N*-acetylcysteine inhibited the generation of reactive oxygen species induced by gliotoxin (ROS).** (A) Control group without gliotoxin. (B) ROS was increased upon exposure with gliotoxin at 1.0 mg·L^{-1}. (C) NAC 12 mmol·L^{-1} inhibited the generation of ROS induced by gliotoxin. The cells (10^6) were preloaded with freshly prepared 20 μmol·L^{-1} 2′7′-dichlorofluorescin diacetate (DCFH-DA) at 37℃ for 15 min, then treated with gliotoxin for additional 10 min before being analyzed. The data were representatives of 4 independent experiments. At least 15 000 cells were analyzed in each experiment.

A variety of toxins and chemicals has been demonstrated to induce cytotoxicity *via* ROS[14], so does gliotoxin. Our results demonstrated that HEK cell death, DNA fragmentation, generation of ROS induced by gliotoxin was abolished by antioxidant NAC, substantiating that apoptosis induced by gliotoxin was tightly related to the increase in intracellular ROS. Furthermore, ROS was the cause of cell death induced by gliotoxin rather than consequence.

To date, there is no direct evidence indicating that caspase activation requires ROS. Our observation that NAC completely abolished the activity of caspase (Fig 3C) indicated that ROS production was upstream of caspase activation in gliotoxin-induced HEK cell apoptosis. These results are consistent with previous work in gliotoxin-induced apoptosis of LLC-PK1 cells[2]. However, activation of caspases for LLC-PK1 cell appeared to be a time lasting process, whereas a short phase for HEK cell, this distinction including others could be ascribed to different species and sensitivity of LLC-PK1 cell and HEK cell to gliotoxin. In addition, it was noteworthy that in present study, NAC exerted more potent cytoprotection effect as compared with BAF which indicated that perhaps there were at least one ROS signal pathway involved in the HEK cell apoptosis induced by gliotoxin, one of which was participated with caspases.

Gliotoxin has been reported to decrease mitochondrial membrane potential[5], meanwhile, gliotoxin itself has an ability to generate ROS in the presence of an appropriate reducing agent[5]. Therefore, there is a possibility that ROS produced by gliotoxin attack mitochondria, which cause the decrease in mitochondria potential and followed by release of cytochrome C, finally leads to activation of caspase-3. However, this speculation needs to be further investigated.

In recent years, Bcl-2 family has increasingly been shown to play an important role in cell apoptosis. The ratio Bax/Bcl-2 has been an effective indicator to determine the cell destiny. Also, Bcl-2 can prevent ROS formation as antioxidant[15]. However, the participation of Bcl-2 family in gliotoxin-induced cell apoptosis has not, thus far, been fully appreciated. Further investigations are needed in this issue.

In summary, gliotoxin induced the intracellular elevation of ROS in HEK cell after treatment followed by activation of caspase-3-like proteases, then triggered cell apoptosis.

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曲霉菌素诱导人胚肾细胞毒性机制

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摘要：目的　研究曲霉菌素诱导人胚肾细胞毒性作用机制。**方法**　结晶甲紫法用于细胞存活率研究。琼脂糖凝胶及 Burton 法研究细胞核 DNA 断裂片段。以水解特异性底物 Ac-DEVD-AMC 活性为研究指标，测定胞浆半胱天冬酶(caspase)-3 类蛋白酶活性。采用蛋白质印迹法检测细胞半胱天冬酶-3 蛋白表达。采用荧光标记探针、流式细胞仪技术研究细胞核 DNA 核型及细胞活性氧的产生。**结果**　曲霉菌素浓度依赖性地诱导人胚肾细胞凋亡，最大效应浓度为 1.0 mg·L^{-1}。BAF，半胱天冬酶-3 蛋白抑制剂和 *N*-乙酰半胱氨酸(活性氧抑制剂)能显著性抑制曲霉菌素诱导人胚肾细胞凋亡作用。**结论**　半胱天冬酶类及活性氧调节曲霉菌素诱导的人胚肾细胞凋亡。

关键词： 曲霉菌素；凋亡；细胞，人胚肾；半胱天冬酶类；活性氧

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