

Original Article

Toxic effects of H₂O₂ and PARP-1 inhibitor DPQ on glioma by a Ca²⁺-Mg²⁺-endonuclease pathway

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Abstract: Ca²⁺-Mg²⁺-endonucleases (CME) have been suggested to execute DNA fragmentation in several apoptotic cascades. In this study, we aim to determine if glioma expresses Ca²⁺-Mg²⁺-endonucleases DNase I-like endonuclease and the roles of the enzyme in the survival of glioma. Western Blot, RT-PCR and immunohistochemistry were used to check DNase I-like endonuclease expression in C6 glioma cell line or human glioma tissues. The CME function was detected by MTT method in the glioma cell death induced by H₂O₂ or the poly (ADP-ribose) polymerases (PARP-1) inhibitor DPQ. We found that both C6 glioma cells and human glioma tissues expressed DNase I-like endonuclease - a major isoform of the CME. Treatment with the CME inhibitor aurintricarboxylic acid decreased the glioma cell death induced by H₂O₂ or the PARP-1 inhibitor, DPQ. H₂O₂ and DPQ had additive toxic effects on C6 glioma cells. These results suggest that the CME are a key downstream component in the cell death cascade initiated in glioma by H₂O₂ and the PARP-1 inhibitor, and the CME may be a useful target for glioma treatment.

Keywords: Ca²⁺-Mg²⁺-endonucleases, DNase I-like endonuclease, glioma, aurintricarboxylic acid, cell death, PARP-1

Introduction

Hydrogen peroxide-induced oxidative stress is a key pathological factor in many diseases [1], so study of the downstream events and searches for drugs that enhance hydrogen peroxide toxicity are of great theoretical and therapeutic significance for cancer treatment [2]. The Ca²⁺-Mg²⁺-endonucleases (CME) execute DNA fragmentation in some apoptotic cascades [3]. Our previous study showed that one CME DNase-like endonuclease is expressed in both murine astrocytes and neurons. We found that both post-treatment and pre-treatment with the CME inhibitor aurintricarboxylic acid (ATA) profoundly decreases peroxynitrite-induced DNA damage and the death of astrocytes. These results suggested that CME may be a key downstream component mediating peroxynitrite-induced DNA damage and death of astrocytes and possibly other cell types [4]. Gliomas, which are derived from glial cells, are the most prevalent malignant brain tumor in adults,

causing over 1% of cancer-related deaths. Finding a new strategy for glioma treatment is clinically important [5]. This study was designed to determine if gliomas express CME, and to test our hypothesis that treatment with the CME inhibitor ATA can decrease the death of glioma cells induced by H₂O₂ or the inhibitor of poly (ADP-ribose) polymerases (PARP-1).

Materials and methods

Materials

Reagents were from Sigma Chemical Co (St. Louis, MO, USA) except where noted.

Cell cultures

The C6 glioma cell line from rat is a high-grade glioma glioblastoma multiforme. C6 cultures were plated into 24-well culture plates, and maintained in Eagle's minimal essential medium (MEM) containing 5 mM glucose and supplemented with 5% fetal bovine serum (Hyclone,

Ogden UT) and 2 mM glutamine. The cultures were used for experiments at 20-30 days *in vitro*.

Experimental procedures

Experiments were initiated by replacing the culture medium with balanced salt solution (BSS) containing (in mM) KCl, 3.1; NaCl, 134; CaCl₂, 1.2; MgSO₄, 1.2; KH₂PO₄, 0.25; NaHCO₃, 15.7; and glucose, 2. The pH was adjusted to 7.2 while the solution was equilibrated with 5% CO₂ at 37°C. Osmolarity was verified at 290-310 mOsm with a Wescor vapor pressure osmometer (Logan, UT, USA).

Reverse transcription (RT)-PCR

RNA from C6 glioma was extracted using an affinity resin column (QIAGEN, Inc., Valencia, CA). The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm, and RNA integrity was confirmed by RNA gel. PCR primers were designed by primer design software Primer 3 based on the rat DNase I-like endonuclease sequence (GenBank Accession No.). Primer sequences are: sense: 5'-GTGATTAGCTCTCGGCTTGG-3'; and antisense: 5'-CCCA ATCAGCCAAA CAA-AGT-3'. cDNA synthesis of RNA samples and PCRs were conducted using the ThermoScript System (Invitrogen, Carlsbad, CA). cDNA was prepared for PCR by random-primed reverse transcription reactions using random hexamer primers and 1 µg RNA in 10 µl reaction volume. After completion of cDNA synthesis, one-tenth of the cDNA was used for subsequent PCR. The PCRs used 50 µl PCR medium containing 5 µl of 10× PCR buffer, 1.5 mM MgCl₂, and 0.2 mM dNTP, 0.2 µM specific target gene primers, 1 µl cDNA, and 1 U Taq DNA, which underwent 35 cycles of PCR amplification under the following conditions: 94°C for 50 s, 54°C for 50 s, and 72°C for 60 s. PCR products were analyzed by agarose gel electrophoresis and visualized by fluorescent DNA gel staining.

Immunocytochemistry and immunohistochemistry

C6 glioma cell lines were cultured on slices until they reached 70% to 80% confluence. The cell and tissue slices were incubated overnight with a 1:200 dilution of DNase Y/DNASE1L3 antibody (mouse, Oncogene Research Pro-

ducts). Immunostaining was visualized using FITC-labeled secondary antibody and counterstained with DAPI or HRP-labeled secondary antibody followed by staining with DAB. The controls run omitting the primary antibody showed no staining.

Western blotting

Cells were grown in 6-well plates to 80% confluence and treated in serum-free EMEM. The medium was then aspirated and cells were rinsed twice with ice-cold PBS. Cells were then lysed with and scraped loose into ice-cold lysis buffer (M-PER_ Mammalian Protein Extraction Reagent, Pierce Biotechnology). After centrifugation at 14,000 g for 10 min at 4°C, supernatants were boiled for 5 min in Laemmli sample buffer supplemented with 50 mM DTT. Equal amounts of lysed and boiled protein (30 µg/well) were loaded and subjected to electrophoresis on 10% polyacrylamide gels (BioRad). Separated proteins were electrophoretically transferred to PVDF membranes (GE Healthcare), which were then blocked for 1 h at room temperature with 5% nonfat dried milk (BioRad). Membranes were then incubated overnight at 4°C with the primary antibody. Concentrations were chosen according to the manufacturer instructions. Secondary horseradish peroxidase-conjugated antibody was incubated for 1 h at room temperature and proteins were detected using an enhanced chemiluminescence kit (GE Healthcare). Image J software (NIH freeware) was used for densitometric analysis.

Cell death determinations

C6 glioma cell death was quantified in monotype cultures by measuring the MTT activity in cell lysates harvested 24 h after drug exposure.

Statistical analyses

All data are presented as means ± standard errors (SE). Data were assessed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. *P* values less than 0.05 were considered statistically significant. Results are presented as mean ± SE of at least three independent experiments. Statistical analysis was done using ANOVA. Signi-

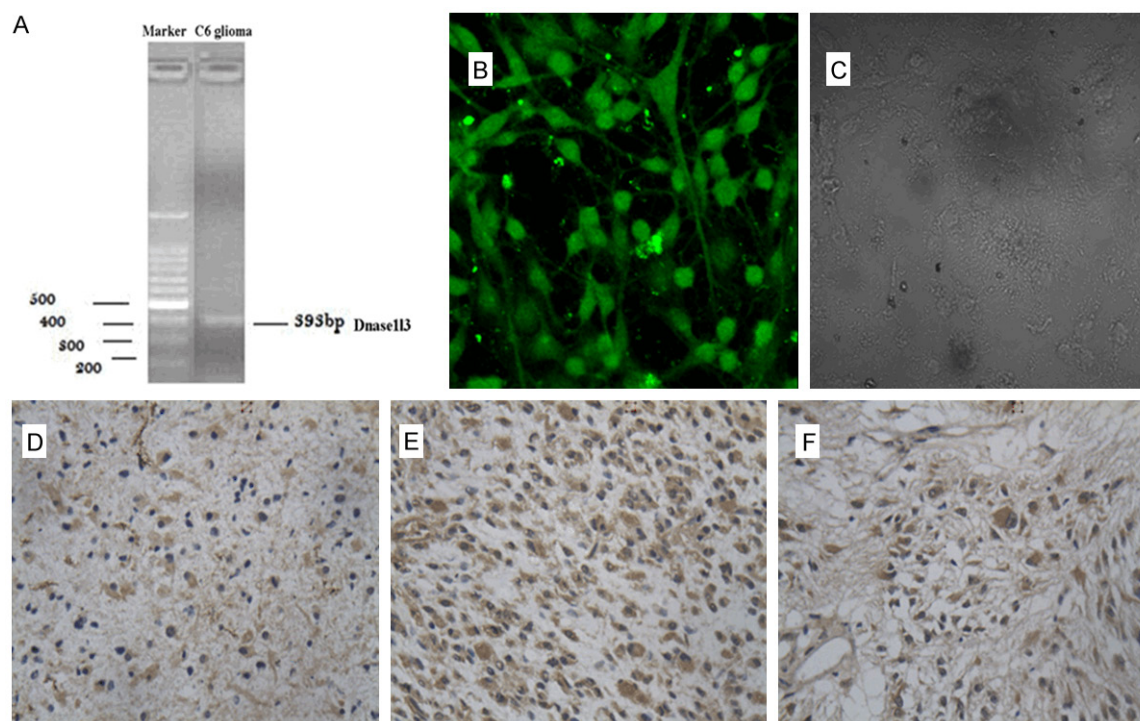


Figure 1. CME expressed in glioma cell. (A) Detection of the mRNA of DNase I-like endonuclease in murine C6 glioma cell line. There was a single, distinct PCR product at approximately 393 bp when C6 glioma cDNA was used. (B) Immunocytochemistry showed that the CME protein existed in this cell line (C. Negative control). (D-F) Immunohistochemistry confirmed DNase I-like endonuclease expression in the different grades of human glioma samples (D. grade II glioma, E. grade III glioma, F. grade IV glioma).

ficant differences are represented throughout as *P < 0.05, **P < 0.01.

Results

Glioma expresses DNase I-like endonuclease

We used RT-PCR to determine if murine glioma expresses the gene of DNase I-like endonuclease (393 bp) - a major isoform of CME. Applying the primers targeted at DNase I-like endonuclease cDNA, the RT-PCRs using rat glioma C6 cell line cDNA generated a distinct PCR product matching the size of DNase I-like endonuclease (**Figure 1A**). These results indicate that the DNase I-like endonuclease gene is expressed in murine glioma. Furthermore, C6 cell line immunocytochemistry (**Figure 1B**) and different grades human glioma immunohistochemistry (**Figure 1D-F**) all showed that CME exists in the C6 glioma cell line. C6 cell line immunocytochemistry and Western Blot results showed that DNase I-like endonuclease was inhibited by ATA (**Figure 2**).

H₂O₂-induced C6 cell death by CME pathway

Using different doses of ATA, we found that it had no toxicity on glioma C6 cells at lower doses (**Figure 3A**). H₂O₂ induced C6 death dose-dependently (**Figure 3B**), and 100 μM ATA had good protective function against H₂O₂-induced C6 cell death by inhibiting CME (**Figure 3C**). The results showed that H₂O₂-induced C6 cell death mediated by the CME pathway.

CME mediated the PARP-1 inhibitor induced C6 cell death

We found that after 24-48 h treatment, 25-100 μM DPQ induced C6 cell death in a dose-dependent manner (**Figure 4A**). ATA also had good protective function against DPQ-induced C6 cell death (**Figure 4B**), which means CME mediated the DPQ-induced cell death. We also found that DPQ and H₂O₂ induced C6 glioma cell death additionally (**Figure 4C**). Our results showed that CME involved both H₂O₂ and PARP-1 inhibitor DPQ mediated C6 glioma cell death (**Figure 5**).

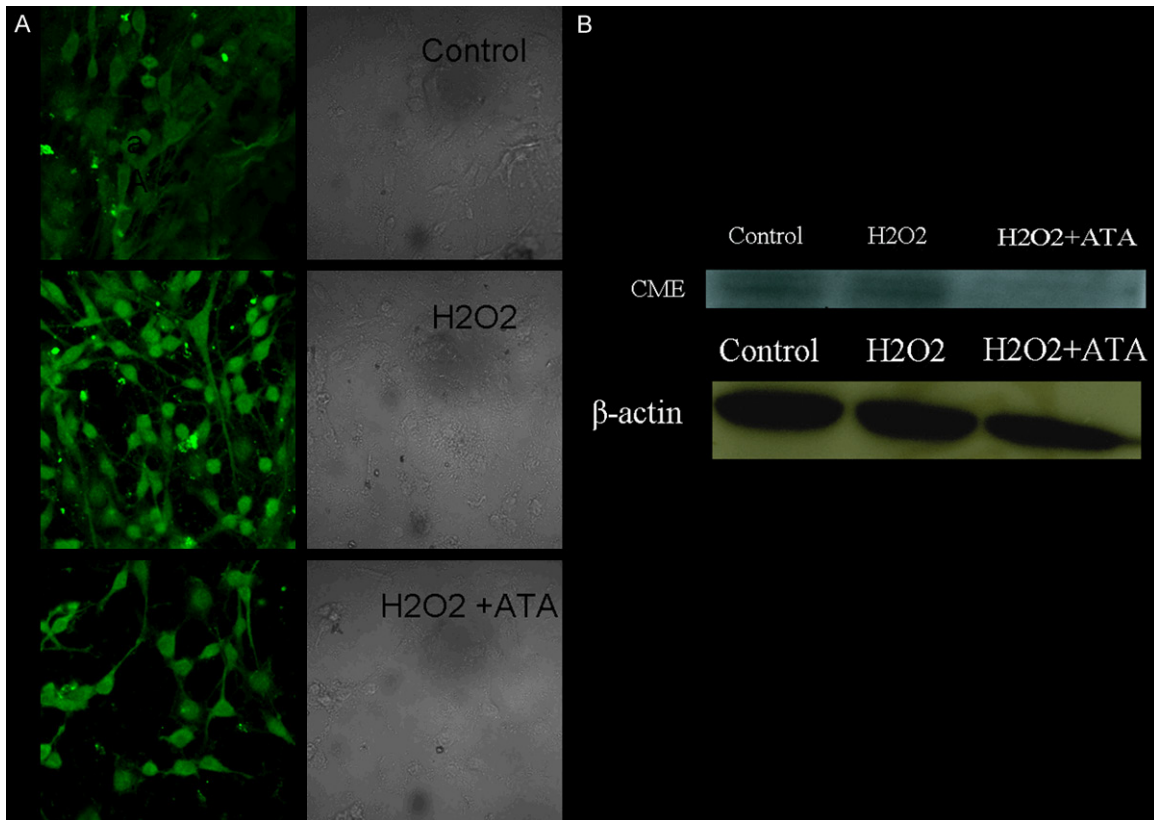


Figure 2. ATA inhibited CME expression in C6 glioma cell line. A. Immunocytochemistry showed that the CME signal decreased in the ATA treatment group. B. Western Blot showed that the CME protein decreased in the ATA treatment group.

Discussion

Our previous studies have suggested that pre-treatment with ATA is effective in decreasing nitric oxide-induced neuronal death. These studies provide important information suggesting the potentially critical roles of CME in oxidative cell death [4]. However, these studies also raised interesting questions: whether glioma expresses CME and whether ATA is effective in decreasing hydrogen peroxide toxicity in glioma, which would mean that CME may be a possible target for glioma treatment.

We found that glioma cells expressed DNase I-like endonuclease- a major isoform of the Ca^{2+} - Mg^{2+} -endonucleases [6]; and co-treatment with ATA decreased hydrogen peroxide-induced glioma cell death. Our previous study argues against the possibility that ATA decreases cell death by directly acting as an antioxidant because post-treatment with antioxidants such as Trolox and N-acetylcysteine does not reduce peroxynitrite toxicity [4]. CME has been

indicated as a major endonuclease mediating DNA fragmentation in several models of apoptosis [7]. Based on our findings, we proposed that CME may be a key mediator of H_2O_2 -induced glioma cell death.

It has also been found that CME is a substrate of PARP-1, and poly (ADP-ribosylation) of CME leads to CME inhibition [8]. PARPs are defined as a family of cell signaling enzymes present in eukaryotes, and are involved in the poly (ADP-ribosylation) of DNA-binding proteins. The best studied of these enzymes, PARP-1, is involved in the cellular response to DNA damage so that in the event of irreparable damage overactivation of PARP-1 leads to necrotic cell death. Inhibitors of PARP-1 activity in combination with DNA-binding antitumor drugs may constitute a suitable strategy in cancer chemotherapy [9]. We found that the PARP-1 inhibitor DPQ dose-dependently induced glioma death, while ATA blocked the DPQ-induced cell death. Interestingly, DPQ enhanced the H_2O_2 toxicity to glioma cells. All the above data confirm that both DPQ

ATA function on glioma by CME pathway

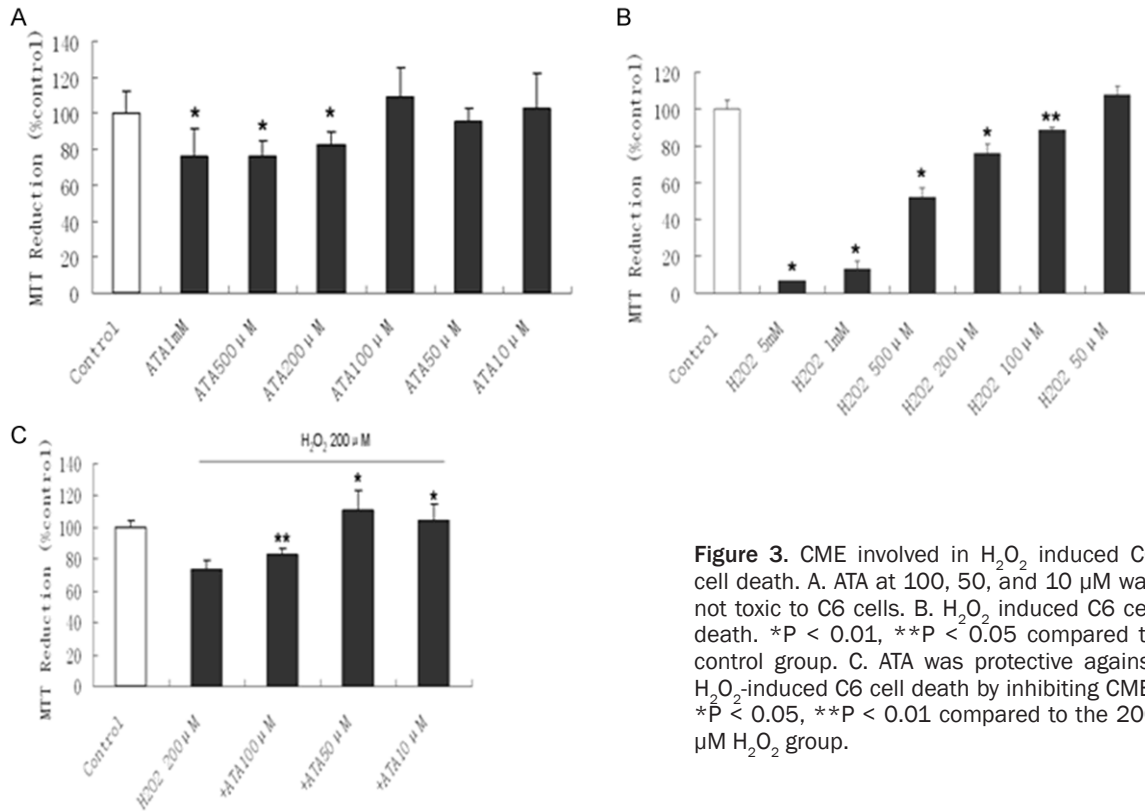


Figure 3. CME involved in H₂O₂ induced C6 cell death. A. ATA at 100, 50, and 10 μM was not toxic to C6 cells. B. H₂O₂ induced C6 cell death. *P < 0.01, **P < 0.05 compared to control group. C. ATA was protective against H₂O₂-induced C6 cell death by inhibiting CME. *P < 0.05, **P < 0.01 compared to the 200 μM H₂O₂ group.

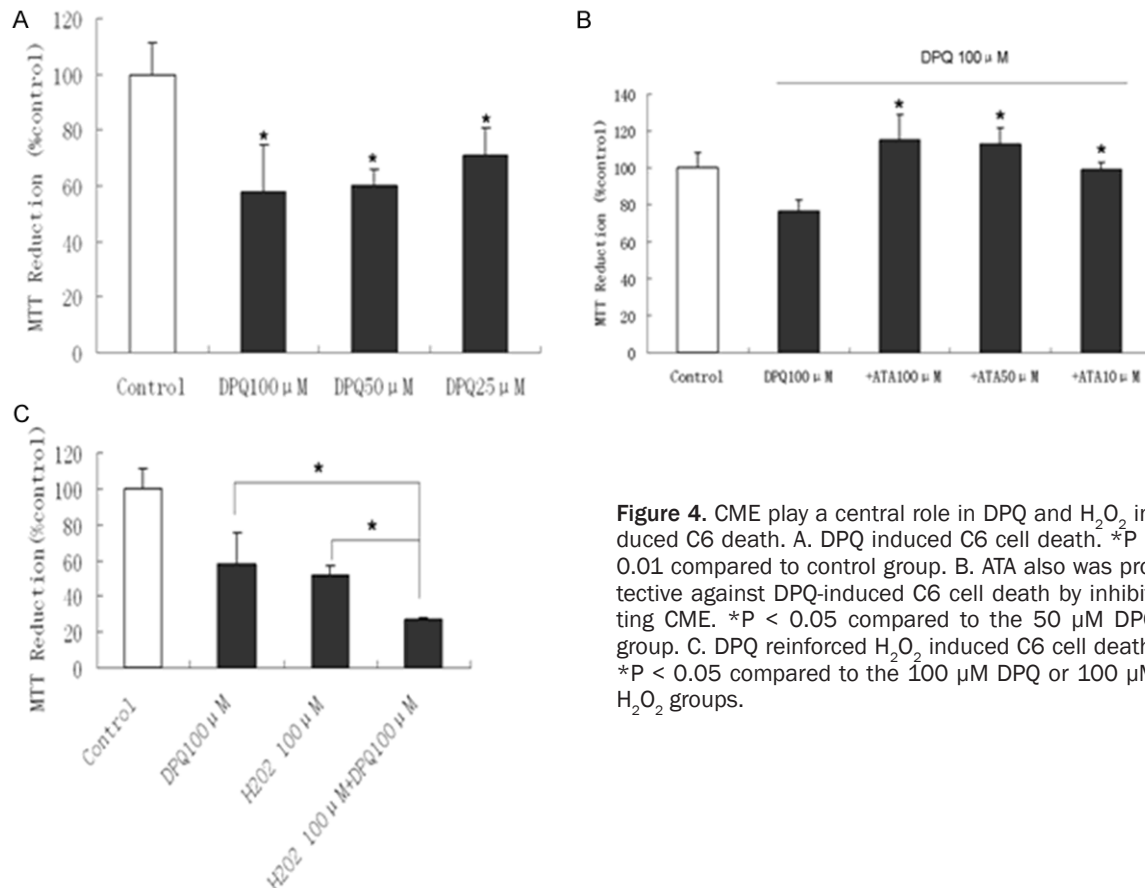


Figure 4. CME play a central role in DPQ and H₂O₂ induced C6 death. A. DPQ induced C6 cell death. *P < 0.01 compared to control group. B. ATA also was protective against DPQ-induced C6 cell death by inhibiting CME. *P < 0.05 compared to the 50 μM DPQ group. C. DPQ reinforced H₂O₂ induced C6 cell death. *P < 0.05 compared to the 100 μM DPQ or 100 μM H₂O₂ groups.

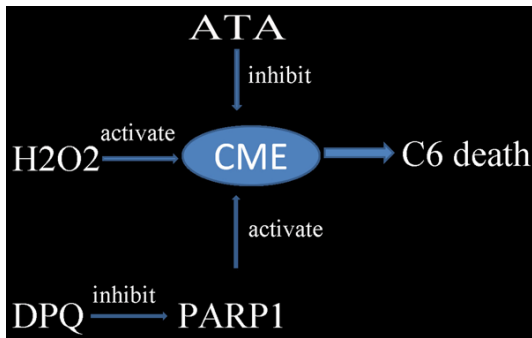


Figure 5. H₂O₂ and PARP-1 inhibitor DPQ induced C6 death by the CME pathway.

and H₂O₂ induce glioma cell death *via* the CME target, and the CME pathway may be a good target for glioma treatment [10].

ATA is the most widely used inhibitor of CME. However, like most pharmacological agents, ATA has side-effects such as affecting tyrosine phosphatases. The investigation into the roles of CME in cell injury has been severely limited by the absence of more selective inhibitors of the enzymes. With accumulating evidence suggesting significant roles of CME in DNA damage and cell death [11], future studies using molecular approaches targeting these enzymes become increasingly important.

Our current studies provide the first evidence that glioma cells express DNase I-like endonuclease- a major isoform of the Ca²⁺-Mg²⁺-endonucleases, which plays an important role in H₂O₂- and PARP-1-mediated glioma cell death. These results establish an essential basis for applying molecular approaches to determine the roles of CME in glioma treatment. Future studies are warranted to search for more selective and potent CME activators to increase glioma death.

In conclusion, our study suggests that the CME inhibitor ATA decreases hydrogen peroxide- and PARP-1 inhibitor-induced glioma cell death. CME may be a key downstream event in the glioma cell death cascade initiated by hydrogen peroxide and the PARP-1 inhibitor. Future studies using molecular approaches targeting this enzyme for glioma treatment are warranted.

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Disclosure of conflict of interest

None.

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