Low-dose arsenic trioxide enhances 5-aminolevulinic acid-induced PpIX accumulation and efficacy of photodynamic therapy in human glioma

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ABSTRACT

Among glioma treatment strategies, 5-aminolevulinic acid (5-ALA)-based fluorescence-guided resection (FGR) and photodynamic therapy (PDT) have been used as effective novel approaches against malignant glioma. However, insufficient intracellular protoporphyrin IX (PpIX) accumulation limits the application of FGR and PDT in the marginal areas of gliomas. To overcome these issues, we assessed the intracellular levels of PpIX in human glioma cell lines and rat cortical astrocytes pretreated with 0.1 μM arsenic trioxide (ATO). Apoptosis and cell viability after PDT were evaluated using Annexin V-FITC apoptosis detection kit and MTT assay, respectively. In order to find out the possible mechanism, we investigated the expression of the key enzymes in the heme biosynthesis pathway, which regulates porphyrin synthesis in glioma cells. Our findings showed that the 5-ALA-induced PpIX accumulation in glioma cell lines pretreated with 0.1 μM ATO was increased relative to the control groups. No changes in fluorescence intensity were detected in the rat cortical astrocytes pretreated using the same ATO concentration. Apoptosis following PDT in glioma cells pretreated with 0.1 μM ATO were significantly higher than in control groups, especially late apoptotic cells, while the cell viability was decreased. The expression of CPOX was upregulated in glioma cells after pretreatment with 0.1 μM ATO. We concluded that ATO was a potential optional approach in enhancing intracellular PpIX accumulation and improving the benefits of 5-ALA-induced FGR and PDT in glioma.

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1. Introduction

Malignant gliomas are the most common brain tumors and have a very dismal prognosis because of their invasiveness, after treatment with surgery, chemotherapy and radiotherapy [1]. Meanwhile, the prognosis of patients with malignant gliomas is associated with the extent of tumor resection [2]. However, intraoperatively it is difficult to distinguish tumor tissues from normal tissues. New approaches that can more precisely define intraoperative tumor borders and thus achieve maximal cytoreductive surgery without neurologic deficits might be of benefit for patients with gliomas. Recently, the efficacy and safety of 5-aminolevulinic acid (5-ALA)-induced fluorescence-guided resection (FGR) of malignant glioma has been evaluated in a prospective randomized setting [3]. In addition, 5-ALA based photodynamic therapy (PDT) is also an effective optional method to treat gliomas [4].

However, one of the major limitations of 5-ALA-induced FGR and PDT is that, in low-grade glioma and the marginal area containing infiltrating glioma cells there is insufficient 5-ALA-induced protoporphyrin IX (PpIX) accumulation. Thus, it is important to identify factors that can increase PpIX concentrations in tumor cells. This in turn would allow more precise identification of tumor cell clusters and improve selectivity of PpIX accumulation in tumor tissues from normal brain tissues. Meanwhile, it would also enhance PDT efficacy.

Recently, new regimens, methotrexate [5] and vitamin D3 [6], have been developed to improve the efficacy of 5-ALA-PDT. In addition to, there is a common alkylating agent, arsenic trioxide (ATO), against the tumor in clinical. ATO was first used for leukemias and solid tumors treatment in china based on the hypothesis of inducing terminal differentiation in cells [7].

ATO has multiple functions via different molecular targets on anticarcinogenic action. The mechanisms mainly include promoting differentiation, apoptosis and reactive oxygen species (ROS)
production. Previous research reported that ATO partially induced differentiation of primary APL cells and NB4 cells at low concentrations (0.1–0.5 μM) and promoted apoptosis at high concentrations (0.5–2 μM) [8]. It has been known that ATO can effectively down-regulate apoptosis inhibitor bcl-2 in NB4 cells at both mRNA and protein levels [9]. Meanwhile, ATO increased ROS level through inhibiting glutathione peroxidase that can eliminate ROS [10]. Therefore, these factors play an important role in anticarcinogenic action of ATO.

Meanwhile, ATO as safety and tolerability agent was demonstrated when administered concomitantly with radiotherapy for the treatment of infiltrating astrocytomas in children [11]. In addition, some authors have also reported the effects of ATO exposure on the heme biosynthetic pathway in rats and humans [12]. However, the detailed role of ATO in increasing intracellular PpIX accumulation, optimizing selectivity of PpIX accumulation in the tumor and improving the efficacy of ALA-PDT remains unclear in malignant gliomas.

Previous studies have demonstrated that several sequential enzymatic steps in the heme pathway. First, ALA are condensed to porphobilinogen and catalyzed by aminolevulinic acid dehydratase (ALAD). Four molecules of porphobilinogen are linked by porphobilinogen deaminase (PBGD) to produce the linear tetrarrole uroporphyrinogen. Uroporphyrinogen is passed directly onto uroporphyrinogen III synthase (UROS), which converts it to uroporphyrinogen III. In the common pathway, uroporphyrinogen III decarboxylase (UROD) catalyzes the sequential decarboxylation of the four acetate residues of uroporphyrinogen III to yield the corresponding methyl groups, generating the product coproporphyrinogen III. In the next step, CPOX catalyzes the transformation of coproporphyrinogen III to protoporphyrin IX. Protoporphyrin IX oxidase (PPOX) converts protoporphyrin IX to protoporphyrin IX, which could produce fluorescence. The last step of heme biosynthesis is the insertion of iron into protoporphyrin IX which is catalyzed by ferrochelatase (FECH) [13,14].

In the current study, we investigated whether or not ATO had an effect on 5-ALA-induced PpIX accumulation and PDT in glioma cells. Finally, we examined the possible mechanisms of increased PpIX accumulation in pretreated glioma cells.

2. Methods

2.1. Cell culture and cytotoxicity assay

The human glioma cell lines U87MG and T98MG were cultured in DMEM (HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified CO2 incubator. Wistar rat cortical astrocytes were cultured in wistar rat cortical astrocytes growth medium (Cyagen, USA) at 37 °C in a humidified CO2 incubator. Cytotoxicity of ATO in glioma cells was analyzed by MTT assay.

2.2. Quantitative real-time-PCR (QRT-PCR)

Total RNA was isolated from glioma cells. It was then reverse-transcribed to cDNA with random primers using a reverse transcriptase RT kit (Takara Biotechnology Co., Shiga, Japan) [15]. The mRNA levels of key enzymes in heme biosynthesis pathway were detected using QRT-PCR on a Light Cycler 480 (Roche, Basel, Switzerland) according to the manufacturer’s protocol. The primer set of ALAD-sense, 5'-GGCACATGGAATGCCAAACA-3'; antisense, 5'-TCCTAACATCCGCCAATGAT-3'; PBGD-sense, 5'-TTCGCTGATCCGCTGA AAGG-3'; antisense, 5'-GGCCAGGGAATGATGTA-3'; UROS-sense, 5'-TTTCTACTGGAATTACGG-3'; antisense, 5'-CACAGGAAATAGAAGGCGCAT-3'; UROD-sense, 5'-GACTAACACTCCCTGGTGG-3'; antisense, 5'-AGTCCAGTCACAGCAAGG-3'; CPOX-sense, 5'-TGAATC AAGAGACCGTG-3'; antisense, 5'-CAAGGGGAATGAAGAGG-3'; and FECH-sense, 5'-CACACAGATTCACAGCAAG-3'; antisense, 5'-GAGAAAACAGAATGACCACC-3'. Each sample was checked in triplicate, and parallel reactions were performed using primers to β-actin as an internal control. The data were analyzed using the Light Cycler 480 software.

2.3. PpIX fluorescence analysis in living cells

5-ALA-induced PpIX fluorescence in glioma cells was captured by means of a fluorescence microscopy (Carl Zeiss AG, Oberkochen, Germany) and flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). PpIX fluorescence in rat cortical astrocytes was analyzed by flow cytometry. For fluorescence microscopy, glioma cells were cultured on 35 mm cell culture dishes. After incubation for 72 h with or without ATO (0.1 μM, Harbin, China) pretreatment, cells were incubated with 5-ALA solution (final concentration 0.4 mM; Sigma–Aldrich, St. Louis, MO, USA) for 16 h in a cell culture incubator. PpIX-specific fluorescence in the living cells was analyzed using a fluorescence microscope. Intracellular PpIX fluorescence was excited at 488 nm and collected in the red channel through a 576 nm filter. PpIX fluorescence of individual cells was analyzed using flow cytometry. Excited at 488 nm under the same exposure time of the illumination source, fluorescence from 10,000 cells was recorded through a 613 nm longpass filter.

2.4. Pretreatment of cells with ATO and analysis of PpIX in cell lysates

Cells were plated and treated identically to the conditions detailed above for quantification. After ATO pretreatment, glioma cells were incubated in serum-free DMEM containing 5-ALA (final concentration 0.4 mM) for 16 h. The protocol for cell extraction has been described previously [16]. The PpIX content of cell extracts was assessed by fluorescence spectrophotometry (LS-55, PerkinElmer, Waltham, MA, USA). Excitation was recorded at 410 nm (slit 10 nm), and emission was set to 633 nm (slit 10 nm).

2.5. Cell viability and apoptosis assays after PDT

Glioma cells pretreated with ATO for 72 h were washed with PBS and incubated with serum-free DMEM containing 5-ALA (0.4 mM). They were then irradiated with a high-power semiconductor laser (XD-635AB; Xingda, Guilin, China). Wavelength of light was 635 ± 0.3 nm. There were the fluence rate of 30 mW/cm2 and energy of 0.3 J/cm2. After irradiation, the medium was immediately replaced and the glioma cells were cultured for another 24 h. The MTT assay was used to assess cell viability as described previously [16]. For analysis of apoptosis induced by 5-ALA-PDT, cells were harvested and identified by means of a human Annexin V-FITC Apoptosis Detection Kit (Baosea Biotechnology Co., Beijing, China) according to the manufacturer’s instructions. At least 10,000 marked cells were analyzed on a FACS Aire system (Becton Dickinson) for each sample. The data were analyzed using Cell Quest software (Becton Dickinson).

2.6. Statistical analysis

Statistical analysis was performed using Student’s test (SPSS, Chicago, IL, USA). P values <0.05 were considered statistically significant.
3. Results

3.1. Effects of ATO on intracellular PpIX levels

After exposure of U87MG and T98MG to ATO (between 0.1 and 3.2 μM) for 72 h, the cytotoxicity of ATO was analyzed. The nontoxic effects of ATO in glioma cells were detected at concentrations of ≤ 0.1 μM. Therefore, 0.1 μM was chosen as the optimal concentration for ATO treatment in U87MG (Fig. 1A) and T98MG (Fig. 1B). For successful therapy, 5-ALA-PDT requires a selective increase in 5-ALA-induced PpIX accumulation within tumor cells. Therefore, U87MG, T98MG and normal astrocytes were pretreated with a low-dose of ATO (0.1 μM) for 72 h before receiving 5-ALA. Cells were subsequently analyzed for the levels of 5-ALA-induced PpIX accumulation using flow cytometry (Fig. 2A) and fluorescence spectrophotometry (Fig. 2B). The findings showed that 0.1 μM ATO-preconditioned glioma cells had higher accumulation of 5-ALA-induced PpIX than the case in negative controls. Meanwhile, almost no change in 5-ALA-induced PpIX levels was observed in astrocytes pretreated with the same ATO concentration relative to the control groups (Fig. 2C). Thus, our findings revealed that pretreatment with 0.1 μM ATO selectively increased PpIX accumulation in glioma cell lines relative to normal astrocytes.

3.2. Enhancement of the fluorescence intensity of intracellular PpIX by ATO

We next tested if the fluorescence intensity of intracellular PpIX detected using fluorescence microscopy could be increased after exposure of glioma cells to 0.1 μM ATO. After cells were pretreated for 72 h with nontoxic concentrations of ATO, we analyzed fluorescence intensity of these cells after exposure to 5-ALA for 16 h. It was found that there was a significantly higher PpIX fluorescence signal compared with the negative control groups (Fig. 3). These data suggested that higher imaging quality due to PpIX fluorescence can be achieved in glioma cells by pretreatment with 0.1 μM ATO relative to the control groups.

3.3. Effects of ATO on 5-ALA-based PDT cell viability

To confirm that increased intracellular concentrations of PpIX accumulation were present in 0.1 μM ATO-preconditioned cells, the effects of light irradiation on the survival of these cells were evaluated. Glioma cells were pretreated with 0.1 μM ATO for 72 h, exposed to 5-ALA, and then irradiated with 635 nm light and analyzed using the MTT assay. In U87 cells pretreated with 0.1 μM ATO, viability following PDT was significantly lower than in the control groups (Fig. 4A), and similar to results obtained in T98 cells (Fig. 4B). These findings demonstrated that 0.1 μM ATO can significantly increase the phototoxicity of 5-ALA-induced PDT in glioma cells.

3.4. Effects of ATO pretreatment on PDT-induced apoptosis in human glioma cell lines

To test if PDT-induced apoptosis in glioma cells was increased after enhancement of intracellular PpIX accumulation due to pretreatment with 0.1 μM ATO, we assayed apoptosis using flow cytometry after irradiation with a high-power semiconductor laser. In cells pretreated with 0.1 μM ATO there was a significant increase in the proportion of apoptotic cells induced by 5-ALA-PDT (Fig. 5).

3.5. Semiquantitative analysis of gene expression levels in heme synthesis using qRT-PCR

To explore the mechanisms associated with the enhanced PpIX levels in glioma cells after 0.1 μM ATO pretreatment, we evaluated the change in mRNA expression levels of six porphyrin–synthetic enzymes (ALAD, PBGD, CPOX, UROD, UROS and FECH) that have been described as potentially rate-limiting enzymes in different biological systems. Fig. 6 shows the relative mRNA levels of these genes in the glioma cell lines. Among them, the mRNA levels of CPOX expression were commonly higher in U87MG (Fig. 6A) and T98MG (Fig. 6B) cells pretreated with 0.1 μM ATO than that in the control groups, showing an average 2-fold increase. However, mRNA levels were not significantly changed in other enzyme genes. Therefore, these data strongly suggest that the upregulated expression of the CPOX gene may be correlated with the accumulation of 5-ALA-induced PpIX in glioma cells exposed to 0.1 μM ATO.

4. Discussion

In the present study, it was demonstrated that 5-ALA-induced PpIX accumulation was markedly increased in glioma cells after pretreatment with 0.1 μM ATO. However, no change in PpIX accumulation was found in normal rat cortex astrocytes under the same conditions. Apoptosis was enhanced in glioma cells when 5-ALA-induced PDT was administered after pretreatment with 0.1 μM ATO, while cell viability was decreased. Therefore, enhanced accumulation of 5-ALA-induced PpIX due to pretreatment with ATO increased the efficacy of FGR and 5-ALA-PDT. In addition, we also demonstrated that the mRNA expression of CPOX, a rate-limiting enzyme and a regulatory checkpoint in porphyrin biosynthesis, was increased in glioma cells after pretreatment with 0.1 μM ATO. The change in CPOX expression is proposed to be closely related to increased intracellular accumulation of PpIX. In
addition, mRNA expression in other crucial enzymes (PBGD, ALAD, UROD, UROS and FECH) in the heme-synthetic pathway were detected using QRT-PCR and no significant changes were observed. Maximal resection of tissues containing tumor cells are critical to the prognosis of patients with malignant glioma [3,17–20]. For the character of glioma cells invading the normal tissues and making the marginal area unclear, completed tumor resection was interrupted. Therefore, new methods should be explored to solve the problem. FGR of glioma is a novel approach to effectively increase the tumor resection rate from 36% to 65% and result in...

Fig. 2. Treatment with low-dose ATO significantly increased intracellular concentrations of 5-ALA-induced PpIX detected by fluorescence microscope. Fluorescence intensity of PpIX was visualized by fluorescence microscopy in T98 glioma cells. After 72 h treated with ATO (0.1 μM), the cells were exposed to 0.4 mM 5-ALA for 16 h. Note that strong red fluorescence of glioma cells was observed in cells pretreated with ATO compared with control group. Left panels: 5-ALA group; right panels: 5-ALA + ATO group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Treatment with low-dose ATO significantly increased intracellular concentrations of 5-ALA-induced PpIX detected by fluorescence microscope. Fluorescence intensity of PpIX was visualized by fluorescence microscopy in T98 glioma cells. After 72 h treated with ATO (0.1 μM), the cells were exposed to 0.4 mM 5-ALA for 16 h. Note that strong red fluorescence of glioma cells was observed in cells pretreated with ATO compared with control group. Left panels: 5-ALA group; right panels: 5-ALA + ATO group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
statistically significant prolongation of progression-free survival for patients in controlled phase III clinical trials[3]. Although the FGR could effectively resect tumor, approximately 10% to 20% of malignant gliomas cannot show red fluorescence in surgery [21] or vague fluorescence at the margin of the tumor [22]. The possible explanation may be related with insufficient PpIX accumulation in tumor cells. Our data indicated that 5-ALA-induced PpIX accumulation in glioma cells pretreated with 0.1 μM ATO was significantly elevated. Additionally, we also found that accumulation levels of 5-ALA-induced PpIX in the rat cortex astrocytes preconditioned with the same ATO concentration under the same conditions were not significantly changed. Therefore, selectively increased accumula-
tion of 5-ALA-induced PpIX in glioma cells can also enhance visual sensitivity, making glioma cell identification easier and facilitating maximum resection of the tumor [16].

Furthermore, we assessed 5-ALA-PDT efficacy after pretreatment with 0.1 μM ATO in glioma cell lines. The efficacy of PDT depends on factors such as the type of photosensitizing agent, light dose, illumination time, oxygen concentration, photosensitizer concentration and tissue sensitivity [23]. Generally, 5-ALA-based PDT is considered a promising antitumor treatment modality approved for the management of malignant tumors [5,6,16,24], including gliomas. Unfortunately, because of insufficient accumulation of 5-ALA-induced PpIX in low-grade glioma and in the marginal area containing infiltrating cells that show vague fluorescence, 5-ALA-PDT remains unsatisfactory for treatment, with incomplete response rates and recurrence rates that are often less than ideal [25].

Previous studies have reported that manipulation of deferoxamine, FECH, CPOX, ABCG2 and ABCB6 genes in the heme biosynthetic cycle was associated with enhancement of the accumulation of 5-ALA-induced PpIX in cancer cells [5,6,16,24–28]. Some of these studies demonstrated that the efficacy of PDT was positively associated with increased intracellular accumulation of PpIX. Therefore, our experiment want to demonstrate that 0.1 μM ATO is related to the efficacy of PDT. The administration of ATO to patients with acute promyelocytic leukemia (APL) has previously been reported [29]. Meanwhile, ATO have already been used in clinical and achieve good results [29]. Our data showed that 0.1 μM ATO could increase the accumulation of PpIX in glioma cells. In our study, 0.1 μM ATO increased the efficacy of PDT in glioma cells because of obvious growth inhibition and an increased apoptotic cell index. ATO may be an ideal agent for further combination of 5-ALA-PDT in the treatment of infiltrating gliomas and other solid tumors.

Low concentration ATO has been shown to promote differentiation in acute promyelocytic leukemia cells [30], osteoblasts [31] and glioblastoma multiforme [32], and cellular differentiation associated with the enhancement of 5-ALA-induced PpIX accumulation and PDT efficacy had been reported in previous studies [5,33–37]. Whether or not ATO related with PpIX accumulation and the efficacy of PDT has not been demonstrated. Our data show that ATO at a low-dose (0.1 μM) increased 5-ALA-induced PpIX accumulation and the efficacy of PDT in glioma cells. However, this concentration of ATO did not affect apoptosis or the activity of human glioma cell lines U87MG and T98MG. The possible mechanisms for the 0.1 μM ATO-mediated accumulation of PpIX may be related with the expression of key enzymes in heme synthesis pathway. Our data show that mRNA encoding CPOX, the key enzyme in the porphyrin synthesis pathway located in mitochondria that plays a role in oxidizing coproporphyrinogen III to produce protoporphyrinogen, was up-regulated after pretreatment with 0.1 μM ATO. Similar findings have been reported by other investigators, who have demonstrated that cancer cells precultured with Vitamin D3 and methotrexate can specifically increase PpIX accumulation, while the expression of CPOX was up-regulated [5,21,30]. Some authors have also reported that high levels of CPOX mRNA expression are correlated with high intensities of 5-ALA-induced fluorescence in malignant glioma [21]. Therefore, upregulated expression of CPOX plays an important role in the enhancement of intracellular PpIX accumulation. However, the gene mRNA levels of other enzymes involved in heme biosynthesis pathway were not significantly changed in our study. These enzymes that can effectively regulate heme biosynthesis have been demonstrated in previous studies [13,14]. Taken together, these findings revealed a positive correlation between CPOX expression and accumulation of 5-ALA-induced PpIX. Thus, we assumed that CPOX may play a crucial role in 5-ALA-induced PpIX accumulation in glioma cells pretreated with a low ATO concentration; it seems to be the main checkpoint in the porphyrin metabolism pathway in our analysis.

In conclusion, our study demonstrated for the first time that the enhancement of 5-ALA-induced PpIX accumulation in glioma cells can be successfully achieved with 0.1 μM ATO, which does not influence the PpIX levels in normal cerebral cortex astrocytes. This compound helped optimize the selectivity of PpIX accumulation in the tumor from normal tissue in FGR and increased the efficacy of PDT. In addition, in our study we found that upregulated expression of CPOX mRNA after treatment with 0.1 μM ATO may be closely related to the improved accumulation of 5-ALA-induced PpIX in glioma cells.

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