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ORIGINAL ARTICLE

Imbalance of Ca^{2+} and K^+ fluxes in C6 glioma cells after PDT measured with scanning ion-selective electrode technique

Sheng-Li Hu · Peng Du · Rong Hu · Fei Li · Hua Feng

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Abstract Photodynamic therapy (PDT) possesses the capacity to lead to death of C6 glioma in vitro and in vivo. The purpose of this study was to investigate whether Ca^{2+} and K^+ homeostasis of C6 glioma cells were affected by PDT. C6 glioma cells were randomly divided into five groups: control group, Hematoporphyrin derivative (HpD) group (10 mg/l, without irradiation), PDT group (HpD 10 mg/l + irradiation), PDT&6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) group (HpD 10 mg/l + CNQX 50 mol/l + irradiation), and HpD&CNQX group (HpD 10 mg/l + CNQX 50 mol/l, without irradiation). Glioma cells in PDT and PDT&CNQX group were subjected to PDT. Cells in PDT&CNQX group were administered α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor antagonist CNQX prior to PDT on C6 glioma cells. The changes of Ca^{2+} and K^+ fluxes were studied by using a non-invasive scanning ion-selective electrode technique (SIET). Morphology of C6 cells was observed with optical microscopy. PDT induced Ca^{2+} influx and K^+ efflux significantly, which resulted in death of C6 cells. When AMPA glutamate receptor antagonist CNQX was applied, Ca^{2+} influx and K^+ efflux were partly blocked up and viability of C6 cells increased. These results indicate that Ca^{2+} influx and K^+ efflux may correlate with the treatment effects of PDT on C6 glioma cells.

Sheng-Li Hu and Peng Du contributed equally to the article.

S.-L. Hu · P. Du · R. Hu · F. Li · H. Feng (✉)
Department of Neurosurgery, Southwest Hospital, Third Military Medical University, No. 29, GaoTanYan Street, ShaPingBa District, ChongQing 400038, The People's Republic of China
e-mail: fenghua8888@vip.163.com

P. Du
Department of Neurosurgery, Shengjing Hospital of China Medical University, No. 36, SanHao Street, HePing District, ShenYang 110004, LiaoNing Province, The People's Republic of China

Keywords Non-invasive scanning ion-selective electrode technique · photodynamic therapy · glioma · Ca^{2+} flux · K^+ flux

Introduction

Gliomas are the most common primary brain tumors. Although, accumulated research over the past two decades have led to considerable advances in the basic biology, pathogenesis, and therapeutic technique of glioma. It is still one refractory disease that causes a high level of morbidity and mortality across the world [1].

Among the various treatment methods for tumors, photodynamic therapy (PDT) is an emerging adjuvant intervention. PDT means tumor cells are dealt with irradiation by using light of appropriate wavelength to activate photosensitizer (PS) uptaken by the cells, which leads to cytotoxicity [2]. With the development of PS and light source (radiator), PDT has been applied clinically in several cancers originated from different sources such as head, neck, ovary, and skin [3–5]. The effects of PDT on intracranially implanted VX2 tumors in rabbits [6] and G422 glioma in mice have also been investigated in previous research [7]. Moreover, recent studies have shown PDT can lead to death of C6 glioma in vitro and in vivo [8, 9]. Especially in patients with gliomas, PDT could prolong their survival time [10, 11]. All these results show that PDT has the ability of anti-tumor effects.

Past studies have revealed that the proliferation, migration, and invasion of glioma cells correlated closely with high concentration of extracellular glutamate and its receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in physiological conditions [12–15]. However, the recent research found that increased extracellular glutamate and expression of AMPA might be correlated with the anti-glioma effects of PDT on C6 glioma cells [16]. AMPA

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receptors are composed of subunits taken from a set of four proteins, GluR1 through GluR4. There are two editing variants in the GluR2 subunit, the edited and unedited form (GluR2Q). AMPA receptors are permeable to Ca^{2+} only when AMPA receptors are composed of the unedited GluR2Q. Previous results have indicated that the increased expression of GluR2 proteins is possible unedited form (GluR2Q) [16]. Furthermore, it is a common brief that AMPA glutamate receptor is permeable to K^+. Thus, the purpose of this study is to prospectively evaluate the influence of PDT on the ability of C6 glioma cells to maintain Ca^{2+} and K^+ homeostasis by using a non-invasive scanning ion-selective electrode technique (SIET).

SIET is a non-invasive method by using a machine to obtain the information of ions molecules across cells membranes. This technique can measure the absolute concentration of ions and molecules, and also their fluxes and directions of movement. The samples to be analyzed can be single cell and a piece of tissue.

Materials and methods

Chemicals

Hematoporphyrin derivative (HpD), a PS [9], was purchased from HuaDing Corp. Ltd. (Chongqing, China). It was stored at −20 °C, avoiding exposure to light. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, AMPA glutamate receptor antagonist) was purchased from Sigma (St. Louis, MO) and stored according to the manufacturer's instructions.

Cell culture

C6 glioma cells (a generous gift of professor XiuWu Bian, Department of Pathology, Southwest Hospital, Third Military Medical University, Chongqing, China) [17] were grown in RPMI-1640 (Hyclone, America) with 10 % fetal bovine serum (FBS; PAA, Austria). Cells were incubated at 37 °C with 5 % CO_2 in a fully humidified incubator. They were re-fed and passaged as necessary. C6 glioma cells were randomly divided into five groups (Table 1): control group, HpD group (10 mg/l, without irradiation), PDT group (HpD 10 mg/l + irradiation), PDT&CNQX group (HpD 10 mg/l+CNQX 50 mol/l + irradiation), and HpD &CNQX group (HpD 10 mg/l+CNQX 50 mol/l, without irradiation). CNQX (50 mol/l final concentration) was given 30 min pre-irradiation.

Table 1 Five groups of C6 glioma cells

	Control group	HpD group	PDT group	PDT&CNQX group	HpD &CNQX group
HpD	No	Yes	Yes	Yes	Yes
PDT	No	No	Yes	Yes	No
CNQX	No	No	No	Yes	Yes

PDT treatment

C6 glioma cells were seeded in culture dishes. Culture medium was changed in the following day. After another overnight incubation, the culture medium was removed and cells were washed twice with PBS, followed by incubation with HpD (10 mg/l) diluted with RPMI-1640 medium. Twenty-four hours later, the HpD solution was removed and RPMI-1640 medium with 10 % FBS was re-applied. Then, C6 glioma cells seeded in culture dishes were irradiated by red light at 628 nm, 20 mW/cm^2 for 5 min with the PDT machine (Lumacare-051, England) [6]. The light intensity at the treatment site was kept stable at 20 mW/cm^2 with good homogeneity according to our previous study which determined the appropriate parameter [18].

Morphological observation

C6 glioma cells were seeded in the 24-well cell culture plates at a density of 5×10^4/well. Morphological observations were performed at 4, 8, and 16 h after PDT irradiation.

Ion-selective flux measurements with the non-invasive SIET

Measurements of net fluxes of Ca^{2+} and K^+ were performed using non-invasive SIET according to the methods described previously [19, 20] (BIO-001A, Younger USA Sci. & Tech. Co., Amherst, MA, USA; Applicable Electronics Inc., Forestdale, MA, USA; and ScienceWares Inc., East Falmouth, MA, USA). The electrode was controlled to move with an excursion of 10 μm at a programmable frequency in the range of 0.3–0.5 Hz; this minimized mixing of the bathing saline.

To construct the microelectrodes, borosilicate micropipettes (2–4 μm aperture, XYPG120-2, Xuyue Science and Technology Co., Ltd., Beijing, China) were silanized with tributylchlorosilane and the tips filled with selective liquid ion-exchange cocktail (Ca 21048, K 60398, Fluka, Busch, Switzerland). An Ag/AgCl wire electrode holder (XYEH01-1) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. Prior to fluxes measurement, the microelectrode was calibrated with different concentrations of ion buffer (Ca 0.05, 0.1, and 0.5 mM; K 0.05, 0.1, and 0.5 mM). Only electrodes with Nernstian slopes >25 mV were used. Ion fluxes were calculated by Fick's law of diffusion: $J_0=-(D\times(d_C/d_X))$ where J_0 represents the net ion flux (in μmol/cm^2 per second), D is the self-diffusion coefficient for ion (in cm^2/s), d_C is the difference value of ion concentrations between the two positions, and d_X

is the 10 μm excursion over which the electrode moved in this experiments. Data and image acquisition, preliminary processing, control of the three-dimensional electrode position, and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software [Science Wares (East Falmouth, MA) and Applicable Electronics].

As shown in Fig. 1a, the ion (Ca^{2+} or K^{+}) selective microelectrode moved between two positions close to the tested cells constantly to acquire experimental data. Primitive data were acquired at a rate of 12 values/min; the fluxes curve were shown in Fig. 1b.

Ion fluxes (pmol cm^{-2} s^{-1}) were calculated using MageFlux software, developed by XuYue Science and Technology Co., Ltd., Beijing, China (http://xuyue.net/mageflux).

Experiments were performed in five treatment groups with at least n=4 independent experimental repetitions in each treatment group. Stable ion fluxes were recorded for about 30 min at each single experiment, in which 12 values per min were acquired resulting in 120 values per 10 min. These 120 values were consecutively sub-grouped to packages of 10 values starting from the beginning and taking adjacent values. The sub-groups were averaged thus getting 48 sub-group average values during the 10 min for each treatment group. Finally, the corresponding sub-group average values of the four independent experiments in each group were averaged resulting in 12 average values per 10 min. This time dependent correlation of the ion fluxes according to the different treatment groups was graphically depicted. In a further evaluation, the 12 average values per 10 min were averaged again to get 10 min-condensed average values for each 10 min interval during the experimental period of 30 min. Finally, the corresponding 10 min-condensed average values for the treatment groups were compared.

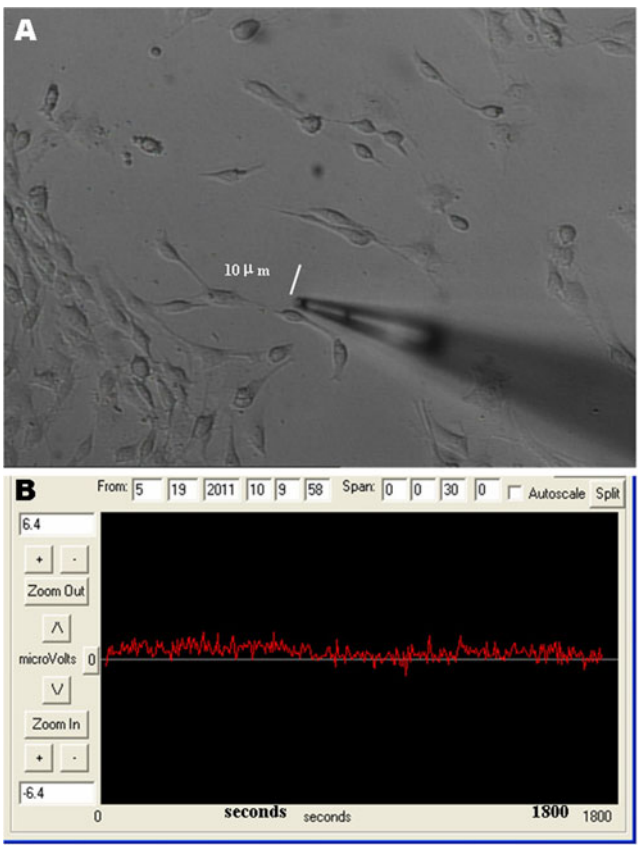

Fig. 1 Measurement of ion fluxes of single C6 glioma cell by non-invasive SIET. Selective microelectrode moved between two positions close to the cells with an excursion of 10 μm to acquire experimental data (**a**). Primitive data were acquired at a rate of 12 values/min for about 30 min (**b**)

Statistical analysis

Data were presented in graphs showing means±standard deviation (SD). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison tests. A p value of ≤0.05 was considered significant.

Results

Morphological observation showed that C6 glioma cells in the control group displayed a fibroblast-like or spindle-shaped morphology (live cells) in confluent wave-like layers in culture at 4, 8, and 1h (Fig. 2a, d, g). Cells in the HpD and HpD&CNQX group manifested the same shape as those in the control group (data not shown). Four hours after PDT, parts of cells in the PDT and PDT&CNQX group showed a spherical shape (degenerated or dead cells, Fig. 2b, c). Eight hours later, there were lots of degenerated or dead cells (round shape) in the PDT group (Fig. 2e), while fewer round cells were seen in the PDT&CNQX group (Fig. 2f). Sixteen hours post-PDT, morphological observation revealed that PDT killed all glioma cells in the PDT group (Fig. 2h). But, CNQX, the AMPA glutamate receptor antagonist, delayed this process. There were still some live cells at 16 h after PDT (Fig. 2i), while no live cells were observed at 24 h post PDT.

The Ca^{2+} fluxes were then measured by non-invasive SIET. Net Ca^{2+} fluxes were depicted in Fig. 3a. In the control, HpD, and HpD&CNQX group, C6 glioma cells manifested a minor Ca^{2+} efflux. When PDT was given, the cells showed a significant Ca^{2+} influx. However, CNQX inhibited this response. The mean Ca^{2+} fluxes during the first, second, and third 10 min duration were also analyzed. The results revealed that there was statistical significance among the control, PDT, and PDT&CNQX groups. But no statistical significance was seen among the control, HpD, and HpD&CNQX groups (Fig. 3b).

Like Ca^{2+} fluxes, the fluxes were also measured by non-invasive SIET. The SIET data showed that the dynamic patterns of net K^{+} fluxes changed significantly in the different groups (Fig. 4a). A net K^{+} influx was observed in the control, HpD, and HpD&CNQX group. When PDT was given, the

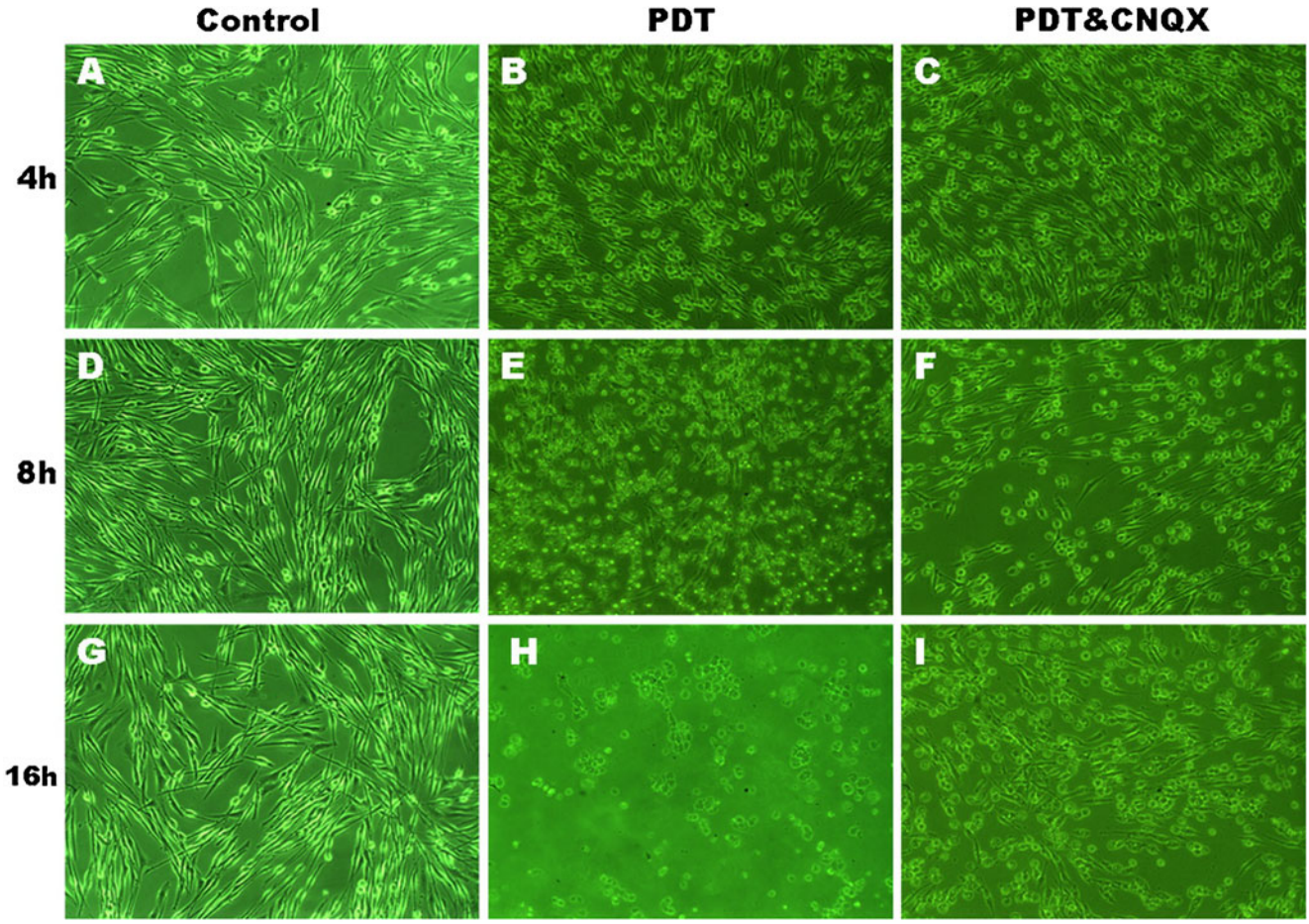

Fig. 2 PDT induced death of C6 glioma cells, while AMPA glutamate receptor antagonist prolonged the survival time. Glioma cells in *control* group demonstrated a fibroblast-like or spindle-shaped morphology (live cells) in culture at 4, 8, and 16 h (**a**, **d**, and **g**). In *PDT* group, the number of spherical cells (degenerated or dead cells) increased over time (**b** and **e**). Sixteen hours after PDT, almost all glioma cells were killed (**h**). CNQX delayed this process (**c** and **f**). There were still some live cells at 16 h after PDT (**i**). Original magnification, ×100

cells displayed a giant K^+ efflux. In the PDT&CNQX group, potassium fluxes showed a tendency for fast oscillations near the base line. The mean K^{2+} fluxes during the first, second, and third 10 min duration in the control, PDT, and PDT&CNQX group were significantly different. There was statistical significance. However, no statistical significance was observed among the control, HpD, and HpD&CNQX groups (Fig. 4b).

Discussion

The major findings of this study are that PDT can lead to death of C6 glioma cells and that the mechanism of PDT induced cells destruction may be related to its effect of impairment of the ability of C6 glioma cells to maintain Ca^{2+} and K^+ homeostasis.

In physiological conditions, glutamate and AMPA has been considered as a key factor in microenvironment which promoted tumor growth and invasion [12–15]. And many clinical trials aiming at AMPA receptors have been investigated. Most of the results showed significant outcome [21–23]. However, the recent study revealed that increased extracellular glutamate and expression of AMPA glutamate receptor might be related to the treatment potential of PDT on C6 glioma cells. When CNQX an AMPA glutamate receptor antagonist was given, anti-glioma effects of PDT were blocked up [16]. AMPA receptor is one kind of ionotropic glutamate receptor which is composed of subunit taken from a set of four proteins, GluR1 through GluR4. There are two editing variants in the GluR2 subunit, the edited and unedited form (GluR2Q). AMPA receptors are permeable to Ca^{2+} only when AMPA receptors are composed of the unedited GluR2Q. Previous results indicated that the increased expression of GluR2 proteins were possible unedited form (GluR2Q) [16]. In addition, Ca^{2+} overload of cells can induce its death. These phenomena indicate that PDT may result in Ca^{2+} influx through AMPA receptors directly. So, Ca^{2+} fluxes were measured directly by using non-invasive SIET. In the control group, C6 glioma cells manifested a minor Ca^{2+} efflux. When PDT was given, the cells showed a significant Ca^{2+} influx. However, CNQX

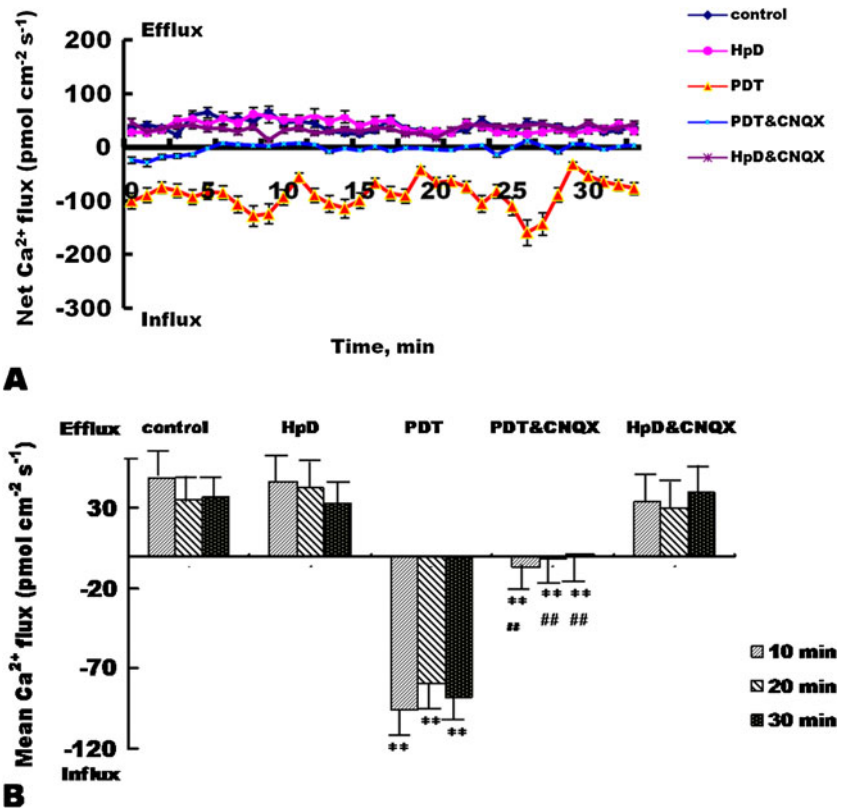

Fig. 3 PDT promoted Ca^{2+} uptake by C6 glioma cells, which was inhibited by AMPA glutamate receptor antagonist. A minor Ca^{2+} efflux was observed in control, HpD, and HpD&CNQX group. However, PDT group cells displayed a significant Ca^{2+} influx in the cells. CNQX reversed this procedure (a). There was statistical significance in the mean Ca^{2+} fluxes during the first, second, and third 10 min duration among control, PDT, and PDT&CNQX groups (b). ** $p<0.01$, compared with *control* group; ## $p<0.01$, compared with *PDT* group

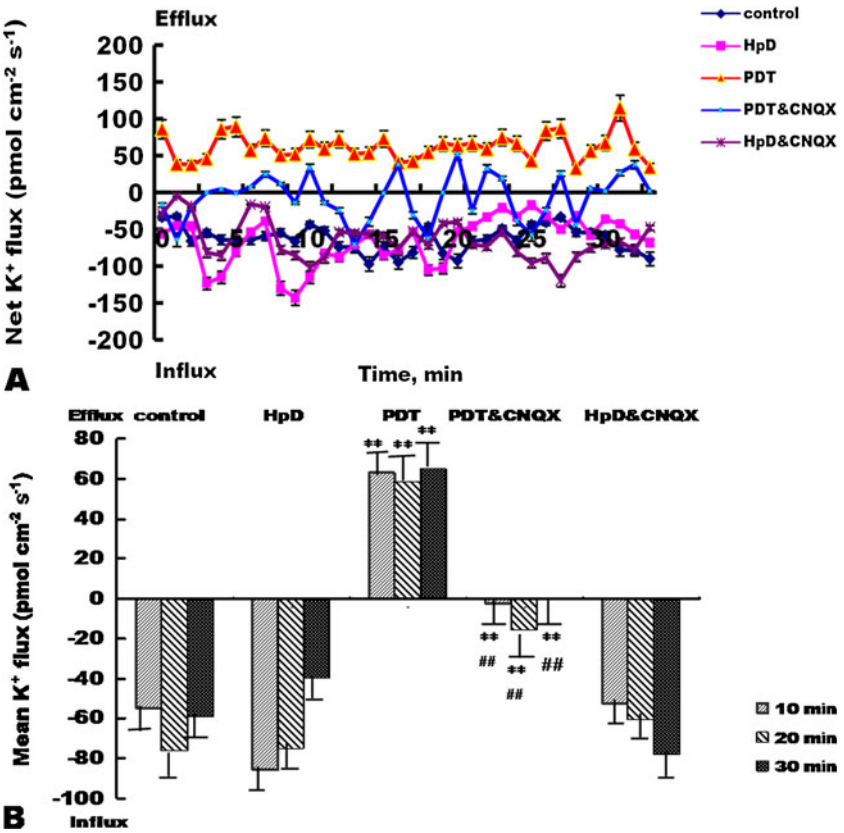

Fig. 4 PDT induced K^+ efflux of C6 glioma cells, which was reversed by CNQX. In control, HpD, and HpD&CNQX group, a net K^+ influx was observed while PDT induced a giant K^+ efflux in the cells. CNQX inhibited this response (a). The mean K^+ fluxes during the first, second, and third 10 min duration were statistically different among control, PDT, and PDT&CNQX groups (b). ** $p<0.01$, compared with *control* group; ## $p<0.01$, compared with *PDT* group

inhibited this response and induced tolerance to PDT. This study demonstrated directly that the $(Ca^{2+})_i$ increased partly through the Ca^{2+} influx via AMPA glutamate receptor, of which the function may be enhanced by increased glutamate during PDT.

It is well known that glioma cells express glutamate receptors. However, unlike granule cells, they express a subclass of Ca^{2+} permeability AMPA receptors instead of *N*-Methyl-D-aspartate (NMDA) receptors [12]. The association between Ca^{2+}-permeable AMPA glutamate receptor and cells death has been extensively investigated in oligodendrocytes and neurons [24–26]. The present work showed that PDT induced impairment in the ability of C6 glioma cells to maintain Ca^{2+} homeostasis and Ca^{2+} influx via AMPA glutamate receptor might lead to cells death when the $(Ca^{2+})_i$ exceeded certain level [27].

AMPA glutamate receptor is not only permeable to Ca^{2+} but also permeable to K^+. Therefore, The K^+ fluxes were also observed using non-invasive SIET. A net K^+ influx was observed in the control group. After PDT was given, the cells displayed a giant K^+ efflux. In the PDT&CNQX group, potassium fluxes showed a tendency for fast oscillations near the base line. The observed K^+ efflux of C6 glioma cells after PDT may correlate with cells apoptosis. Previous research demonstrated K^+ efflux provoked a substantial cellular shrinkage and affected the activation of caspases in Jurkat T lymphocytes [28]. This was further confirmed in studies using cortical and hippocampal neurons. The decrease in intracellular K^+ concentration is consistent with a decrease in cell viability since this is required for apoptosome formation and activation of caspases and endonucleases during apoptosis [29, 30]. The above results further explained the previous work in which increased apoptosis of C6 glioma cells was found after PDT [16].

The biological basis of PDT has been investigated extensively in the laboratory for hundreds of years [31]. A growing body of evidence suggests PDT can produce anti-tumor effects on C6 glioma in vitro and in vivo [8, 9, 27, 32–34]. It is a common brief that both photochemical and photobiological processes occur to result in selective destruction of cancer cells. Hence, PDT has been regarded as an effective adjuvant therapy to surgical resection of cerebral glioma [10, 11, 35, 36]. The results of this study can help us furtherly understanding the molecular mechanism of PDT anti-glioma effects. However, a lot of environmental and genetically based factors influence the outcome of PDT response. deCarvalho et al. [37] even reported that subclinical PDT may promote glioma growth. Thus, further research is needed to investigate the biological characteristics of PDT.

Conclusions

In conclusion, the study indicated that PDT could induce Ca^{2+} influx and K^+ efflux of C6 glioma cells, which might co-contribute to the cancer cells death. These results allow us to further understand the molecular mechanism of PDT on glioma. PDT might be a potentially valuable adjuvant therapy for the treatment of glioma.

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Conflict of Interest This work has no conflict of interest to declare.

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