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Persistent oxygen-glucose deprivation induces astrocytic death through two different pathways and calpain-mediated proteolysis of cytoskeletal proteins during astrocytic oncosis☆

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ABSTRACT

Astrocytes are thought to play a role in the maintenance of homeostasis and the provision of metabolic substrates for neurons as well as the coupling of cerebral blood flow to neuronal activity. Accordingly, astrocytic death due to various types of injury can critically influence neuronal survival. The exact pathway of cell death after brain ischemia is under debate. In the present study, we used astrocytes from rat primary culture treated with persistent oxygen-glucose-deprivation (OGD) as a model of ischemia to examine the pathway of cell death and the relevant mechanisms. We observed changes in the cellular morphology, the energy metabolism of astrocytes, and the percentage of apoptosis or oncosis of the astrocytes induced by OGD. Electron microscopy revealed the co-existence of ultrastructural features in both apoptosis and oncosis in individual cells. The cellular ATP content was gradually decreased and the percentages of apoptotic and oncotic cells were increased during OGD. After 4 h of OGD, ATP depletion to less than 35% of the control was observed, and oncosis became the primary pathway for astrocytic death. Increased plasma membrane permeability due to oncosis was associated with increased calpain-mediated degradation of several cytoskeletal proteins, including paxillin, vinculin, vimentin and GFAP. Pre-treatment with the calpain inhibitor 3-(4-iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid (PD150606) could delay the OGD-induced astrocytic oncosis. These results suggest that there is a narrow range of ATP that determines astrocytic oncotic death induced by persistent OGD and that calpain-mediated hydrolysis of the cytoskeletal-associated proteins may contribute to astrocytes oncosis.

Oncosis plays a critical role in the tissue/organ injury induced by a variety of different insults. In the early stages, it is characterized by plasma membrane blebbing, dilation of the ER, mitochondrial swelling, and clumping of the nuclear chromatin. These morphological changes are followed by the breakdown of the plasma membrane, release of intracellular constituents, and inflammation [19]. Oncosis occurs in numerous human diseases, such as acute myocardial infarction and acute renal and liver failure [7,21]. In previous studies, we reported that oncosis may account for the cell death of astrocytes in an ischemic region [2]. However, the mechanisms of oncosis are poorly understood. Schnellmann et al. define oncotic death as the point in the oncotic process in which respiration (mitochondrial function and the associated ATP formation) and ion homeostasis are restored [16]. For cultured mouse renal proximal tubule (RPT) cells, depletion of ATP by more than 80–85%, triggers oncosis [15]. However, the threshold of ATP depletion that triggers oncosis may vary among different cell types.

Oncosis has been characterized as an un-orchestrated event in which a cell swells beyond its capacity, resulting in bursting. However, it is now recognized that oncosis involves a series of clear, identifiable biochemical events that lead to the breakdown of the plasma membrane and cell death [30]. Several studies have suggested that the down-regulation of cytoskeleton-associated proteins, such as talin, paxillin and vinculin, precedes the formation of plasma membrane blebs and is closely associated with progressive increases in plasma membrane permeability [5,29]. However, it is unclear whether ischemia can induce changes in the cytoskeleton-associated proteins of astrocytes. The purpose of this study was to evaluate the changes in cellular morphology, energy metabolism and levels of several cytoskeletal proteins in astrocytes

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treated with persistent OGD *in vitro*. In addition, we explored the mechanism of oncosis in astrocytes.

Astrocytes were obtained from neonatal rats using a modified version of a previously described method [3]. Briefly, the dissociated cerebral cortical cells were seeded into poly-L-lysine-coated 50 cm^2 flasks (Corning, USA) and cultured in high glucose DMEM (Gibco, USA) supplemented with 20% fetal calf serum (Gibco, USA) and 2 mM glutamine (complete medium). After approximately 10 days, the confluent cultures were shaken at 200 rpm and 37 °C for 12 h to separate the astrocytes from the remaining microglia and oligodendroglia. The adherent cells were re-plated in complete medium. More than 95% of the cultured cells were astrocytes, as identified by immunofluorescent staining for glial fibrillary acidic protein (GFAP) (Santa Cruz, USA). The following experiments were performed using *in vitro* cultures between 18 and 21 days old, when they reached maximal sensitivity to OGD-induced cell death [11].

Astrocytes were used to study persistent OGD injury, as described previously [23]. The cultures were washed 3 times with glucose and serum-free DMEM. An appropriate amount of glucose and serum-free DMEM was added to cover the cells, and the cultures were placed in an anaerobic chamber (Coy Laboratory Products Inc., USA) with an atmosphere of 10% H_2, 85% N_2 and 5% CO_2. Control cells were maintained in complete medium under normal culture conditions. In the experiments with the calpain inhibitor, 100 μM PD150606 (San Diego, CA, USA) was added to the media 2 h prior to OGD [17].

Electron microscopy was used to evaluate morphological changes. After being detached from the culture dishes, cells were centrifuged at 1000 rpm for 10 min, fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C and washed three times with 6.8% Sabatini in PBS. Samples were post-fixed with 2% buffer osmium tetroxide for 2 h at 4 °C and washed three times in Sabatini's solution (PBS with 6.8% sucrose). The samples were then passed through a graded series of alcohols (30, 50, 70, 90 and 100%) for 15 min each and through a graded series of acetonum (90 and 100%) for 15 min each. This procedure was followed by treatment with propylene oxide (15 min), a 1:1 Epon-acetonum mix (2 h), and three changes in pure epon (twice for 3 h and overnight). Polymerization occurred overnight at 80 °C. Ultrathin sections (50 nm) were cut with a Leicaultracut ultramicrotome, stained with lead citrate and uranyl citrate for 10 min each, and then examined and photographed with a transmission electron microscope (FEI Tecnai G^2 12 Transmission Electron Microscope, Dutch).

Intracellular ATP levels were determined using an ATP Bioluminescence Assay kit (Beyotime, China, Cat No. S0026) according to the manufacturer's protocol. ATP content is expressed as nmol/mg protein, and the data are presented as the average of three independent experiments.

Lactate dehydrogenase (LDH) activity was assessed using a CytoTox Non-Radioactive Cytotoxicity Assay (Promega, USA) by a microplate reader (Synergy 2, Bio-tek, USA) according to the manufacturer's instructions. LDH activity was measured in both floating dead cells and viable adherent cells. Floating dead cells were collected from the culture medium by centrifugation (240 rpm) at 4 °C for 5 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp). The LDH released into the culture supernatant, referred to here as the extracellular LDH (LDHe), was used as an index of necrotic death, and the LDH present in the adherent viable cells represents the intracellular LDH (LDHi). The percentage of apoptotic and oncotic cell death was calculated as follows [1]:

$$\text{Apoptosis}\,\% = \frac{\text{LDHp}}{\text{LDHp} + \text{LDHi} + \text{LDHe}} \times 100\,\%$$

$$\text{Oncosis}\,\% = \frac{\text{LDHe}}{\text{LDHp} + \text{LDHi} + \text{LDHe}} \times 100\,\%$$

Samples of astrocytes were processed to obtain the cytoskeletal fraction as previously described [29,31]. Protein concentration was determined using a Lowry DC kit (Bio-Rad, USA) with bovine serum albumin as the concentration standards. Equal amounts of total protein (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, China, Cat No. P0012A) and then transferred onto nitrocellulose membranes (Pell, USA). The membranes were washed in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBST) 3 times followed by blocking for 1 h with 5% nonfat milk in TBST at room temperature (RT). The membranes were then incubated overnight with the anti-paxillin antibody (1:5000; Abcam, UK, Cat No. ab4832), the anti-vinculin antibody (1:2000; Abcam, UK, Cat No. ab11194), the anti-vimentin antibody (1:2000; Abcam, UK, Cat No. ab8545), the anti-GFAP antibody (1:1000; Santacruz, USA, Cat No.sc-9065) and the anti-GADPH antibody (1:500; Santacruz, USA, Cat No.sc-25778), respectively. The membranes were washed and incubated for 1 h at RT with horseradish peroxidase-conjugated anti-rabbit (JacksonImmuno, USA, Cat No. 11-GAR007) or anti-mouse antibodies (JacksonImmuno, USA, Cat No.11-GAM007) at a dilution of 1:5000. The washed blots were treated with an enhanced chemiluminiscence detection reagent (Pierce, UK, Cat No. 32109). Optical densities of individual bands were determined using NIH image software. Results are expressed as the percentage of controls.

All data were analyzed by SPSS 11.0 and are presented as means ± SD. Comparisons between multiple groups were performed using a one-way analysis of variance (ANOVA). Statistical significance was accepted at $p < 0.05$.

Although research in the past two decades has significantly advanced our understanding of oncosis, pathologists predominantly use the morphological criteria to distinguish oncosis from apoptosis, because these criteria remain the most reliable indicators of the mechanism of cell death [6,18]. This study used electron microscopy to characterize the details of morphological changes in astrocytes due to persistent OGD insult, including the features of apoptosis and oncosis (Fig. 1). Unlike the well-known process of apoptosis, many of the astrocytes subjected to OGD underwent plasma membrane blebbing, swelling of the mitochondrial and endoplasmic reticulum and ultimately rupture, which led to the release of cytosolic contents. These were typical characteristics of oncosis and were concordant with the phenomenon we observed *in vivo* [2]. In the initial phase of OGD (0.5 h), we observed swelling in the mitochondria, endoplasmic reticulum and vacuolus in some cells. However, these cells exhibited evenly distributed chromatin in the nucleus and apparently intact cellular membranes, suggesting that they were likely oncotic. After 1 h of OGD treatment, apoptotic-like as well as oncotic-like cells were evident. The apoptotic-like astrocytes showed condensed chromatin and delineated abutting on the inner surface of the nuclear envelope, whereas the oncotic-like astrocytes showed swollen cellular organs and nuclei. Interestingly, we found the co-existence of apoptotic nuclei and swollen mitochondria and vacuoles (Fig. 1F). This oncotic-apoptotic-like cell morphology may support the hypothesis that oncosis can serve as a fallback pathway for cell death if apoptosis is non-functional or cannot be completed. Notably, it has been reported that the protein kinase receptor-interacting protein 3 (RIP3) can act as a molecular switch between TNF-induced apoptosis and necrosis in NIH 3T3 cells and that RIP3 is required for necrosis in some other cells [18]. Whether there is a molecular switch between apoptosis and oncosis in astrocytes during OGD insult remains to be determined. After 2 h of OGD treatment, a small number of oncotic cells were detectable. In these cells, the integrity of the cellular membrane was lost and massive organelles were observed outside the cytoplasm (Fig. 1G). After 8 h of OGD treatment, a large number of amorphous debris and a few apoptotic bodies were found (Fig. 1J).

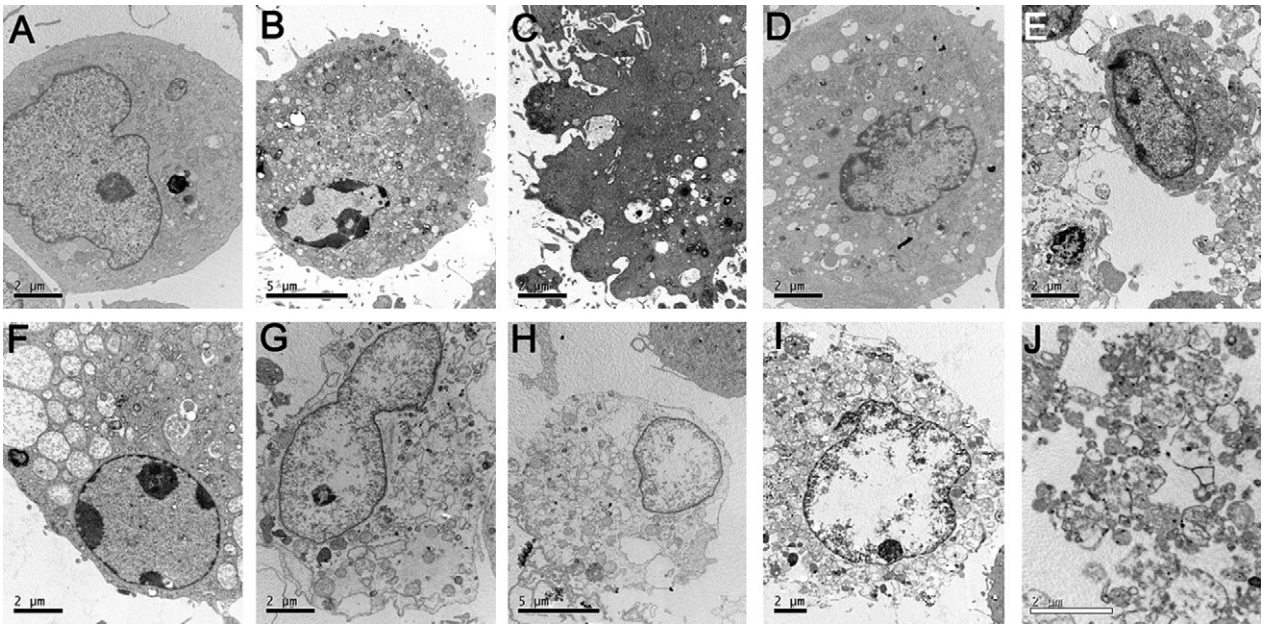

Fig. 1. Representative images of the ultrastructural changes in astrocytes induced by OGD. (A) Normal astrocyte. (B and C) Typical apoptotic cells. Apoptotic cells were found in almost all samples. (D–J) Cells after 0.5, 1, 2, 3, 4 and 8 h of OGD, respectively. (F) A typical apoptotic-oncotic-like cell (co-existence of the condensed nucleolar chromatin and swollen mitochondria and vacuoles) in a 1 h OGD sample.

Membrane damage with increased permeability is a hallmark of oncosis [19], and the release of LDH into the culture medium is an indicator of plasma membrane disruption [8,12,28]. As shown in Fig. 2B, the percentage of apoptotic cells increased gradually during the first 4 h of OGD treatment. However, after 3 h of OGD treatment, the percentage of oncotic cells was greater than the percentage of apoptotic cells.

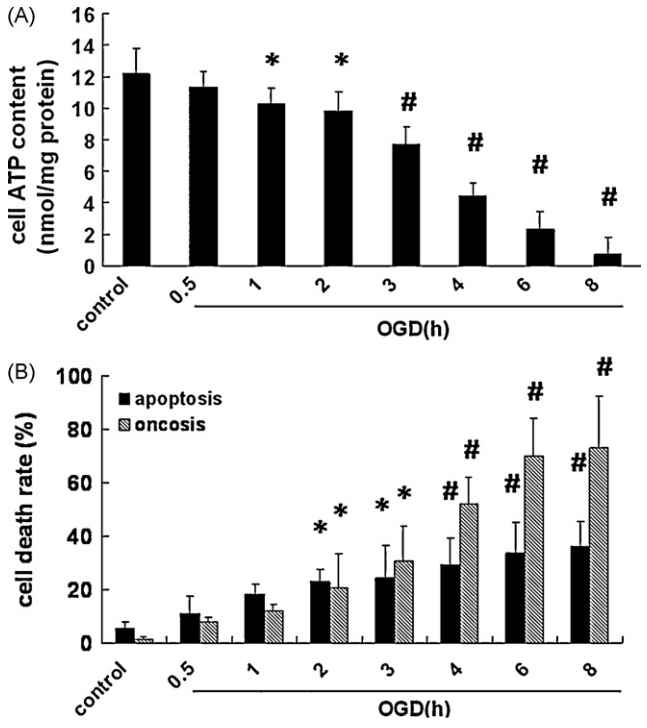

Fig. 2. OGD leads to a decrease in intracellular ATP and an increase in oncosis and apoptosis. (A) Effect of OGD on cellular ATP content in astrocytes. Astrocytes were treated with OGD for 0.5–8 h. The intercellular ATP content decreased gradually. (B) Characterization of cell death by LDH-based assay. After 3 h of OGD, the percentage of oncosis was greater than the percentage of apoptosis. Data are expressed as the mean ± SD; $n = 3$ for each group for each group; $^{*}p < 0.05$, $^{\#}p < 0.01$ compared with the control.

Lieberthal et al. have provided evidence that there is a narrow range of ATP depletion that represents a threshold for determining the mechanism of mouse RPT cell death [15]. When RPT cells are exposed to severe ATP depletion (less than ~15% of control), they die uniformly through oncosis; however, when the cells are subjected to milder degrees of ATP depletion (more than ~25% of control), they undergo apoptosis. To study the impact of persistent OGD on the cellular energy reserve, we measured intracellular ATP levels in astrocytes before and after OGD injury. The results showed that OGD decreased the intercellular ATP content in astrocytes in a time-dependent fashion (Fig. 2A). When the cellular ATP content was depleted to less than 35% of the control level, the astrocytes died primarily through oncosis. Although confirmation of these conclusions in other injury models is needed, these results reveal that the absolute decrease of ATP content required to induce astrocyte oncosis differs from that determined in other cell types, implying that astrocytes might be more susceptible than RPT cells to OGD damage.

How do the cellular levels of ATP determine apoptotic or oncotic cell death? Apoptosis is an active, energy-dependent process that requires ATP to initiate the molecular cascade [13]. Thus, when ATP is depleted past a certain point, the cell death pathway may be directed towards oncosis [13,4]. However, if the two pathways are independent, why do astrocytes within the same culture undergo cell death via different pathways simultaneous during OGD? One possible explanation is that the astrocyte populations, or portions of the astrocytes within the culture, are at different stages of the cell cycle at the time of the insult [32].

The increased permeability of the plasma membrane in oncotic cells leads to a loss of cytosolic enzymes and metabolites and the collapse of electrochemical gradients. Those are incompatible with cell viability. It has been reported that cytoskeleton-associated paxillin, talin and vinculin contribute to the maintenance of membrane integrity and that these proteins participate in important functions, such as supporting cell adhesion and migration, in astrocytes [10,14,25,26]. Depletion of these proteins can break the membrane-cytoskeleton linkage and decrease the physical support of the

basal plasma membrane, leading to bleb formation and changes in plasma membrane permeability [29]. In the present study, we used immunoblotting to observe changes in the levels of paxillin, vinculin, vimentin and GFAP in astrocytes induced by OGD. As shown in Fig. 3, OGD led to a significant decrease in paxillin, vinculin and vimentin protein levels in a time-dependent manner. By contrast, the level of GFAP markedly increased compared to the control after 0.5 h of OGD treatment, followed by a later decrease. This phenomenon could be associated with the involvement of GFAP in the morphological change of cultured astrocytes from a flattened

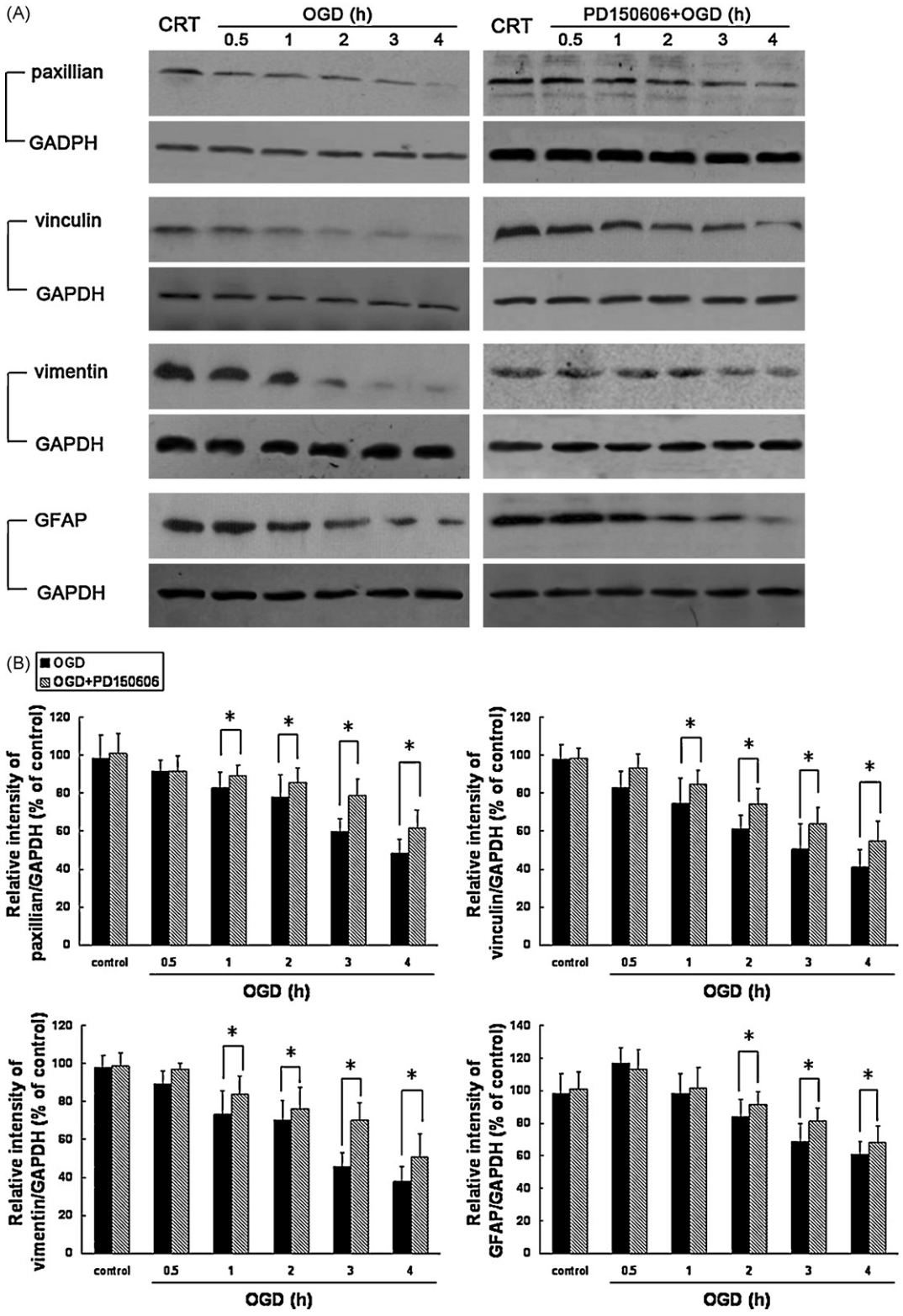

Fig. 3. Inhibition of calpain decreases the proteolysis of cytoskeleton-associated proteins in OGD treated astrocytes. The changes in cytoskeleton-associated paxillin, vinculin, vimentin and GFAP levels in astrocytes during OGD treatment and the effects of the calpain inhibitor PD150606 on the degradation of these proteins. (A) Representative western blot images. (B) Relative density of the proteins. Data are expressed as the mean ± SD; $n = 3$ for each group; $^*p < 0.05$.

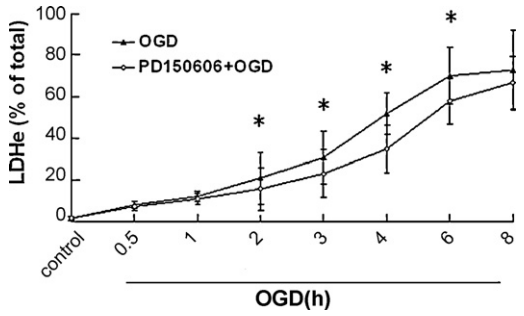

Fig. 4. Effects of the calpain inhibitor PD150606 on membrane permeability to LDH release in OGD treated astrocytes. PD150606 pre-treatment delayed the LDH release in astrocytes with OGD damage. Data are expressed as the mean ± SD; $n = 3$ for each group; $^{*}p < 0.05$ between the OGD group and the PD150606 + OGD group.

polygonal shape to their *in vivo* stellate morphology in response to different kinds of stimuli [20,24]. The calpain inhibitor PD150606 was able to ameliorate the hydrolysis of the four proteins (Fig. 3) and delay the release of LDH (Fig. 4), suggesting that the depletion of cytoskeleton-associated proteins and the increase of plasma membrane permeability were mediated by calpain during OGD-mediated oncosis. However, pre-treatment with a calpain inhibitor was unable to reverse oncosis in astrocytes once the cellular energy metabolism was compromised.

The loss of energy in astrocytes might lead to a malfunction during ischemia that exacerbates the ischemic injury to neurons [27,22]. Moreover, unlike the apoptotic process, oncotic cells are involved in tissue edema, and the release of intracellular constituents and inflammation would further worsen the disturbance [9]. Therefore, the mechanism of astrocytic death after ischemia and the corresponding protective measures are becoming important topics for many researchers.

In summary, we demonstrated that the loss of ATP production under persistent OGD damage was the immediate cause of astrocytic death. When the cellular ATP content is depleted to less than 35% of the control, astrocytes die primarily through oncosis. Increased plasma membrane permeability due to calpain-mediated hydrolysis of the cytoskeletal proteins may contribute to astrocytic oncosis. The inhibition of calpain activity was able to limit or delay, but not reverse, the astrocyte oncosis. Thus, the protection of energy metabolism should be the primary preventative task following ischemia.

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