Intracellular ion and protein nanoparticle-induced osmotic pressure modify astrocyte swelling and brain edema in response to glutamate stimuli

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A R T I C L E   I N F O

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A B S T R A C T

Intracellular tension activity plays a crucial role in cytotoxic brain edema and astrocyte swelling. Here, a few genetically encoded FRET-based tension probes were designed to detect cytoskeletal structural tension optically, including their magnitude and vectors. The astrocyte swelling resulted in GFAP tension increment, which is associated with the antagonistic effect of inward microfilaments (MFs) and microtubules (MTs) forces. In glutamate-induced astrocyte swelling, GFAP tension rise resulted from outward ion and protein nanoparticle-induced osmotic pressure (PN-OP) increases, where PN-OP could be elicited by MF and MT depolymerization, protein nanoparticle production, and activation of cofillin and stathmin-1. Attenuation of both ion osmotic pressure and PN-OP by drug combinations, together with free-radical scavenger, relieved cerebral edema in vivo. The study suggests that intracellular osmotic pressure (especially PN-OP) has a pivotal role in glutamate-induced astrocyte swelling and brain edema. Recovery of cytoplasmic potential is a promising target to develop new drugs and cure brain edema.

1. Introduction

Brain edema is defined as an increase in brain water content, which results either from an opening of the blood-brain barrier (vasogenic brain edema) or cell swelling (cytotoxic brain edema) [1]. The cytotoxic brain edema was believed to be mainly caused by swelling of astrocytes, but also includes neurons swell [2–4]. Astrocytes undergo rapid swelling after various acute pathological states, such as cerebral ischemia and traumatic brain injury [5]. The main reason for ischemia-induced astrocytic cell swelling is a disruption of ionic and neurotransmitter homeostasis, particularly the accumulation of glutamate and K⁺ in the extracellular space [6–9]. Moreover, free radicals exert their deleterious actions during cytotoxic edema [10]. The extracellular water can flow into the astrocytes predominantly through transmembrane aquaporin (AQP) channels [11], which is driven by the ion (e.g., Na⁺, Ca²⁺) influx and osmotic gradient [12]. Therefore, intracellular tension activity has a crucial role in astrocyte swelling.

Intracellular tension relies on cytoskeletal structure and involves regulation of cell volume and mechanical load bearing [13,14,67]. Osmotic pressure (OP) is generated by differences in chemical potential of ions and colloidal protein particles between the cytoplasm and extracellular fluid [15]. During astrocyte swelling, high OP acts on the membrane and strains the lipid bilayer, leading to an outward pull of intermediate filaments (IFs) in the cytoskeleton through transmembrane binding proteins [16,17]. Other than IFs, microfilaments (MFs) and microtubulons (MTs) are highly polarized cytoskeletal structures. The positively charged ends of MFs are joined to the membrane, and negatively charged ends dissociate in the cytoplasm [18,19]. MF tension is generated by the negatively charged-directed motion of myosin, whereas MT tension is dependent upon the movement of dynein and...
kinetics [20–22]. Notably, the intracellular structural tension is a vector [23]. Apart from their magnitude, the direction of these tensions may have a greater impact on astrocyte swelling, whereas the individual direction of tensions within the cytoskeletal structure is not known. Moreover, tension due to MFs and MTs could be eliminated if MFs and MTs are depolymerized into actin and tubulin monomers or macro-molecular polymers of size 1–100 nm [24,25]. Generation of these proteins may increase colloid OP [26,27]. The influx of ions and water, as well as adjustment of intracellular structural tensions, is involved in astrogliial swelling. However, a method to measure the tensions dependent upon the cytoskeleton at the subcellular level has not been well established. Hence, elucidating how intracellular structural tensions participate in mediating astrocyte swelling has been challenging. Recent studies have focused on the molecular mechanisms underlying astrocytic swelling and their signaling pathways, such as aquaporin. Nevertheless, mechanisms of intracellular structural tensions underlying astrocytic swelling remain unclear [28–30].

Giall fibrillary acidic protein (GFAP) is an intermediate filament protein integral to cytoskeletal dynamics regulating the morphology and function of astrocytes [31,32]. We developed a genetically encoded Förster resonance energy transfer (FRET)-based tension probe named “GFAP-cpst-GFAP (GcpG)”, where cpst is circularly permuted stretch sensitive FRET. Using the GcpG probe, we measured intracellular tension during astrocyte swelling following the formation of ischemic lesions simulated by glutamate stimuli [33]. Our study affords mechanistic insights into the role of intracellular tension in astrocytic swelling and provides innovative ideas for the clinical treatment of brain edema.

2. Materials and methods

2.1. Cell cultures and reagents

A commercial human glioblastoma U87 cell line was obtained from the American Type Culture Collection (U87-MG, ATCC HTB-14; Manassas, VA, USA). Primary astrocyte cultures were isolated from the cerebral cortex of 24 h-old sucking Sprague–Dawley rats. Before experimentation, cells were removed by tissue flakes with trypsin-Epidermal growth factor (EGF)-based culture media (Invitrogen, Carlsbad, CA, USA) and plated on glass coverslips (diameter = 22 mm) coated with poly-L-lysine. Cells were cultured in EDTA (0.25%) (Gibco, Billings, MT, USA) and plated on glass coverslips for experimentation, cells were removed from culture with trypsin-EDTA (0.25%) (Gibco, Billings, MT, USA) and plated on glass coverslips (diameter = 22 mm) coated with poly-L-lysine. Cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Billings, MT, USA) supplemented with 10% fetal bovine serum (Gibco, Billings, MT, USA), penicillin and streptomycin (Gibco, Billings, MT, USA). Cells were maintained in 5% CO2 in a humidified atmosphere at 37 °C.

Cytochalasin D and nocodazole were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Glutamate, nimodipine, jasplakinolide, taxol, and function of astrocytes [31,32]. We developed a genetically encoded Förster resonance energy transfer (FRET)-based tension probe named GcpG. The pCMV-tubulin

2.2. Probe construction and transfection

Mouse anti-β-actin or α-tubulin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-GFP antibody, Rabbit anti-cofilin antibody, rabbit anti-stathmin-1 antibody, rabbit anti-phospho-cofilin (Ser3) antibody and rabbit anti-phospho-stathmin-1 (Ser16) antibody were from Abcam (Cambridge, UK).

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Cytochalasin D and nocodazole were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Glutamate, nimodipine, jasplakinolide, taxol, sennisade A, cantharidin, tamoxifen, and bumentanide were obtained from Sigma-Aldrich (Saint Louis, MO, USA). 2.3-Butanenedione mono-xime, acetazolamide, caffeine, NaN3, Y-27632, verapamil, and cine-pazide maleate were purchased from Invitrogen (San Diego, CA, USA). Edaravone and oxiracetam were obtained from Ark Pharmaceuticals (New Delhi, India). Fasudil (HA-1077) was purchased from Shanghai Macklin Biochemicals (Shanghai, China). Cilibravin B and isopisines (SB-715992) were from MedChem Express (Princeton, NJ, USA).

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2.2. Probe construction and transfection

Sensors were created using restriction endonuclease cloning methods, in accordance with previous reports [13]. We constructed fluorescent sensors with circularly permuted cpVenus and cpCerulean (cpVenus−7aa−cpCerulean[cpstFRET]). pET-cpstFRET was created by connecting cpVenus to the C-terminal of cpCerulean in pET-52b (+) vector; primers used were: 5′-GGCGGCGAGTCTAGTCGGCGGCGGCGCAGCTCGCC-3′ with BgII and 5′-CCAGAGCGGAGTCGCTTCCATGTTGTGCGCGG-3′ with SacI.

We used NheI and KpnI restriction endonucleases to cleave the viscous end of the PCR amplified fragment, and then purified the amplified fragment with DNA purification kit. Then, the vector plasmid pEGFP-C1 of E. coli DH5α alpha was amplified, the fragments were separated by electrophoresis, and the fragments were extracted and purified by ORGA Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) and Plasmid DNA Purification Kit (BioVision, Milpitas, CA, USA). Finally, GFAP protein and fluorescent protein probe were linked to pEGFP-C1 and transformed into E. coli culture. Then, we cloned GFAP and incorporated it at each side of cpstFRET to create pCMV-GFAP-cpst-GFAP (GcpG). The pCMV-tubulin-cp-tubulin-β (TcpT) was created as well, following the same method. Positive clones were obtained by G418-selection. Moreover, the plasmids encoding Actin-cpst-Actin (AcpA) were obtained from Frederick Sachs and Fanjie Meng (University at Buffalo, Buffalo, New York, USA).

We used an E.Z.N.A.™ Endo-free Plasmid DNA Mini Kit II (Omega Bio-Tek, Norcross, GA, USA) to extract single-colony plasmids according to manufacturer instructions. The integrity of all expression constructs was confirmed by DNA sequencing. Plasmids encoding the FRET sensor were transfected into U87 cells with FuGENE® 6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland) and OptiMEM™ media (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. The transfection efficiency was about 60%. Transfected U87 cells were sorted using a MoFlo™ XDP Cell Sorter with Summit 5.3 software (Beckman Coulter, Fullerton, CA, USA), for which a cyan (458 nm) laser and 474/60 bandpass filter and yellow (514 nm) laser and 580/23 bandpass filter were employed. Cells were sorted when the cyan and yellow emission wavelengths were detected simultaneously. The sorted U87 cells were diluted into single cells and then seeded onto 96-well plates. In all experiments, the sorted single cell lines were trypsinized, and then the cells were incubated with cell culture media for 24–36 h.

2.3. cpstFRET analyses

The dipole angle between donor/eCFP and acceptor/eYFP determined the effectiveness of FRET. Cells were imaged using a confocal microscope (SP5; Leica, Wetzlar, Germany) equipped with a ×63 oil-immersion objective lens. The donor and acceptor were tested by argon lasers at 458 nm and 514 nm, respectively. CFP/FRET ratios were calculated using the equation 1/1 = cerulean donor/venus acceptor.

2.4. FRET-AB and FRAP analyses

LAS AF Application Wizard v1.7.0 (Leica) was used for detailed analyses of probes, including live cell acceptor photobleaching FRET (FRET-AB) experiments and fluorescence recovery after photobleaching (FRAP) experiments. We bleached the acceptor in the whole cell and calculated the efficiency of FRET. The recovery curve was used to estimate probe activities.

2.5. Cells area ratio analysis

The cell area was estimated using Image J package 1.48 v (National Institute of Health). CFP/FRET image was converted to the gray-scale image. Based on the pixel distinction between the objects of interest (cells) and the background of objects, area of cell cross section was automatically calculated by the software.

2.6. Calcium fluorescence imaging

Prior to the experiment, cells were mounted in a closed chamber on the stage of an inverted confocal laser scanning microscope (SP5; Leica, Wetzlar, Germany) equipped with a ×63 oil-immersion objective lens. The donor and acceptor were tested by argon lasers at 458 nm and 514 nm, respectively. CFP/FRET ratios were calculated using the equation 1/1 = cerulean donor/venus acceptor.
Wetzlar, Germany) in isosmotic, 300 mM HEPES, loaded with 10 µM calcein-AM (Molecular Probes, Invitrogen, USA) for 30 min at room temperature, and then washed with HEPES. Fluorescence images were acquired every 60 s with an epifluorescence microscope with a GFP filter. Calcein fluorescence time series were calculated by normalizing calcein fluorescence at each time point (Ft) by the calcein fluorescence at time 0 (F0). Fluorescence intensity in the cell in question was quantified from these data by Image Pro-Plus (Media Cybernetics, Rockville, MD, USA) in the chosen area of interest.

2.7. Measurement of cytoplasmic OP and Count Rate of protein particles

Cell culture medium, HEPES isosmotic solution, and trypsin solution were standardized. Cells were cultivated in 90-mm Petri dishes. When the density was > 95%, drugs were added for a certain duration. Then, specimens were transferred into 1.5-mL microcentrifuge tubes. The density was > 95%, drugs were added for a certain duration. Then, specimens were transferred into 1.5-mL microcentrifuge tubes. Ultrasounification (75% amplitude, five times, 5 s) (Sonics and Materials, Connecticut, CT, USA) and ultracentrifugation (21,000 g, 10 min, room temperature) were undertaken. Then, 50 µl of the supernantant solution (cytoplasm) was placed into 0.5-mL test tubes. An Osmomat 3000 Freezing Point Osmometer and 050 Membrane Osmometer (Gonotec, Berlin, Germany) were calibrated thrice before use. Then, cytoplasmic OP was recorded, the supernatant was diluted, and the kilocycles per second (Kcps) of cytoplasmic nanoparticles was detected (Nanognost NS300; Malvern Instruments, Malvern, UK).

2.8. Western blotting

The extracted total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the protein bands were transferred to Nitrocellulose filter membranes via wet electro-transfer. Subsequently, the membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% tween 20 (TBST) at room temperature for 1 h and incubated with primary antibodies, including GFAP, actin-β, tubulin-α, coflin, stathmin-1, phospho-cofilin (Ser3) and phospho-stathmin-1 (Ser16), at 4 °C overnight. The membranes were then probed with peroxidase-labeled affinity-purified antibodies against rabbit or mouse IgG (H + L) from ImmunoWay (Plano, TX). Target bands were visualized after the addition of Super ECL Detection Reagent Kit from Yeasen (Shanghai, China). Results were normalized to β-tubulin or β-actin levels.

2.9. Immunofluorescence

Astrocytes were fixed with 4% paraformaldehyde. Astrocytes were incubated with blocking buffer (0.1% Triton X-100, 1% bovine serum albumin) for 45 min at 37 °C, and with antibodies overnight at 4 °C. Washing with PBS was followed by addition of goat anti-mouse-FITC secondary antibody (1:200 dilution), goat anti-mouse-TRITC secondary antibody (1:200), and FITC-phalloidin and incubation for one hour at room temperature. Finally, 4’,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. Fluorescent cells were examined with an inverted fluorescence microscope to appraise cell frameworks.

2.10. Animals

All procedures were conducted according to guidelines established by the National Institutes of Health (Bethesda, MD, USA). The study protocol was approved by the Research Animal Care Committee of Nanjing University of Chinese Medicine.

Adult male Sprague-Dawley rats (240–260 g) were obtained from the Model Animal Research Center of Nanjing University of Chinese Medicine (Nanjing, China) and housed in a 12 h light/dark cycle room. All rats were allowed free access to food and water under conditions of controlled humidity and temperature (24 ± 0.5 °C).

2.11. Water content in rat brains

The water content of rat brains was measured using standard wet-dry methods. Rats were placed in a stereotactic frame after inhaled anesthesia with a mixture of anesthetic gases isoflurane (1.5−2.0%) and oxygen. The needle of a 10-µl Hamilton syringe (Shanghai Gaige Industry & Trade Co., Ltd., Shanghai, China) was inserted into the right lateral ventricle through a burr hole using the following coordinates: right side of a sagittal suture (3.5 mm) and coronal suture (2 mm). Glutamate (1.67 mol/L) was injected into the right lateral ventricles at a rate of 0.5 µl/min with a 10-µl Hamilton syringe. In the meantime, drugs were injected via the intraperitoneal route. The body temperature was controlled at 37.0 ± 0.5 °C throughout the surgery using a heating pad. Then rats were killed by decapitation after 4 h. The brainstem was discarded, while the tissue of ipsilateral cortex, the contralateral cortex, and the cerebellum was harvested. The wet weight of each cortical tissue was measured and then dried for 72 h at 80 °C and the dry weight determined. Water content was calculated as a percentage using the formula:

Water content = (wet weight − dry weight)/wet weight × 100%

2.12. IHC analyses and H&E staining

Brains were fixed in 4% paraformaldehyde in PBS over 24 h at 4 °C and then dehydrated in a graded series of alcohol solutions by xylene and embedded in paraffin. Brain tissues were cut at 5 µm, stained for expression of p-cofilin and p-stathmin-1 by H&E, and viewed under a microscope (CM3050 S; Leica). Images were captured per slide at 40 × magnification using a microscope (DMiB; Leica). Semi-quantitative analyses of IHC images was conducted using Image Pro-Plus v6.0, from which integral optical density (IOD) and area data were collected. Then, the average optical density was calculated as IOD/area, which represented the staining intensity.

2.13. Statistical analyses

The YFP/CFP ratio was calculated using ImageJ (San Diego, CA, USA). The FRET value in each subcellular region was measured for each cell and then averaged over multiple cells. Images were pseudo-colored using the 16-color map in ImageJ. Data were presented as mean ± SEM. One-way ANOVA with the least significant difference test was used to determine statistical significance and P < 0.05 was considered significant. Each experiment was repeated at least three times, > 10 cells were imaged, and each condition was analyzed. The R-value of U87 cells is presented as the pixel count distribution from > 9 cells.

3. Results

3.1. The probe could be used to detect cell swelling under extracellular stress

Astrocyte swelling is an integral component of cytotoxic brain edema and can be caused by water movement into intracellular compartments [34,35]. We employed the probe to explore the intracellular function of GFP in U87 cell swelling. The probe demonstrated resonant angle twisting toward a perpendicular configuration through a reduction in energy transfer. The periphery of the GFP backbone was embedded with a FRET module (Fig. 1A). The GFP probe was transfected into U87 cells, followed by cell sorting with fluorescence (Fig. S1A). The FRET signals of GFP tension in astrocytes under isotonic conditions was detected optically using laser confocal Z-axis scavenging (Fig. 1B, row one) and the 16-bit images were saved (Fig. 1B, row two). Results suggested that GFP tension rarely changed from the bottom layer to the top layer. The acceptor fluorescence (eYFP) was decreased dramatically upon acceptor photobleaching (AB). Concurrently, the
fluorescence (eCFP) was increased due to the unacceptable energy transfer from donor to acceptor after photobleaching (GcpG FRET AB = 21.36%; Fig. 1C). Fluorescence recovery after photobleaching (FRAP) was measured and showed that the recovery rate for GFAP was 48.52% after 500 s (Fig. 1D). The molecular weight of GcpG, Actin-cpsT-Actin (AcpA), and Tubulinα-cpsT-Tubulinβ (TcpT) tension probes were determined (Fig. S1B and S1C). As for the negative control, Gcp, the FRET index was very high compared with the tension probe (Fig. S1 D-F). All the results showed that the genetically encoded FRET-based tension probes were established and effective.

The outward tension induced by hypo-OP results in the "pulling" of cytoskeletal filaments, which is involved in cell swelling, increased volume, and altered cytoskeletal structural tension [36]. To measure FRET tension during cell swelling, we exposed U87 cells to hypo-osmotic stress through switching the 300 mOsm/kg bath (isotonic HEPES buffer) to hypo-osmotic conditions, of which follows 200, 100, 50, and 0 (pure water) mOsm/kg (Fig. 1E). Cells started to swell with the reduced osmotic pressure (Fig. 1G), at the same time the GFAP tension increased gradually (Fig. 1F). Also, cells disintegrated along with exudation of cytoplasm when OP decreased to 0 mOsm/kg, and GFAP tension declined rapidly. These data suggested that increased GFAP tension was involved in cell swelling and cell volume enlargement.
Therefore, our probe could be used to detect astrocyte swelling.

3.2. OP, MF, and MT forces are involved in regulation of GFAP tension

First, we explored the role of MF and MT forces in mediating GFAP tension and the relationship between cellular morphology and skeletal tension. Cytochalasin D (Cyto D) (10 μM) and nocodazole (Noc) (100 μM) were used to depolymerize MFs and MTs, respectively. MF depolymerization induced a relative increase of GFAP and MT tensions compared with isotonic treatment. MT depolymerization aggravated GFAP and MF tensions. Co-treatment of cytochalasin D and nocodazole increased GFAP tension enormously, and U87 cells expanded slightly (Fig. 2A–D).

Depolymerization of MFs, MTs or both could increase OP markedly (Fig. 2E). Experiments using Zetasizer Nano instruments suggested that nanoparticles of diameter < 10 nm were produced after depolymerization of MFs and MTs within 15 min. (Fig. S3B–D). We also test the control cells which cultured in the isotonic environments using the same methods. The nanoparticle size experiments showed that there was no obvious sub-100 nm granule generating (Fig. S3A). The value of osmotic pressure of the control cells was in close proximity to 300 mOsm/kg (Fig. 2E), which accorded with the average magnitude of cytoplasm OP ever reported [37]. Besides, the colloid OP, as determined by a colloidal osmometer, was increased after 2-h co-treatment with depolymerizing agents (Fig. S3E). This result suggested that depolymerization of MFs and MTs could increase the GFAP tension and involved in cell expansion, while protein nanoparticle produced from skeleton depolymerization could generate colloid OP.

Inhibitors of myoinositol, dynein, and kinesin were employed to ascertain if astrocyte swelling was related to cytoskeletal molecular motors; the inhibitors were 2,3-Butanedione monoxime (BDM) (10 mM), cytochalasin D (Cyto D) (20 μM), and ispinesib (SB-715992) (1 μM), respectively. MF, MT, and GFAP tension increased as cells swelled after hypotonic stimulation (Fig. 2F–J). GFAP tension increased and MT tension decreased when myoinositol was inhibited. Dynein inhibition could reduce GFAP tension and increase MT tension. However, kinesin inhibition resulted in the opposite result. Inhibition of dynamic molecules on MFs and MTs could increase GFAP tension more distinctly (Fig. 2F–I). These results suggested that the inward forces of MFs and MTs antagonized GFAP tension in response to outward OP in swelling U87 cells and that the intracellular mechanical system had an essential role in cell swelling.

3.3. Construction of a model of astrocyte swelling: GFAP tension participated in glutamate-induced astrocyte swelling

To investigate the role of cytoskeletal structural tension during astrocyte swelling, we constructed three U87 cells models treated by glutamate (Glu) (2 mM), caffeine (5 mM), and NaN3 (20 mM) respectively (Fig. 3A).

When stimulated with glutamate, GFAP tension increased markedly within 15 min. Glutamate could elicit Ca2+ influx and activation of molecular motors [38]. However, the Ca2+ level in cytoplasm increased in Caffeine-treated U87 cells, but the cell volume and GFAP tension did not change evidently. After depletion of cellular energy, vesicles were observed in cells [39], but GFAP tension and cellular area decreased (Fig. 3B). Overall, these results showed that glutamate-induced astrocyte swelling participated in glutamate-induced astrocyte swelling.
Glutamate-induced astrocyte swelling is thought to be closely associated with cytoskeletal rearrangement [40]. To assess the roles of the cytoskeleton in U87 cell swelling, we employed the MF stabilizer jasplakinolide (JK) (10 μM) and MT stabilizer taxol (TAX) (15 μM).

We found that jasplakinolide treatment could reduce GFAP tension and cell edema compared with the model. Similarly, taxol treatment also reduced GFAP tension and the relative fluorescence intensity of calcine. Upon co-treatment with jasplakinolide and taxol, the decrease in GFAP tension and edema was more apparent (Fig. 4A, B, D, and E). Studies (Figs. 1–3) have suggested that the cytoskeleton and OP are closely related, so stabilizers of MFs and MTs were employed to observe OP changes during U87 cell swelling. The value of OP decreased in the jasplakinolide, taxol or co-treatment group (Fig. 4C). Meanwhile, nanoparticles of diameter 1–10 nm were eliminated, and the cytoplasmic OP and colloid OP decreased (Fig. 6C; Fig. S3E). These results confirmed that glutamate-induced U87 cell swelling was caused by the increased cytoplasmic OP, colloid OP, cytoskeleton depolymerization, and nanoparticle production. Meanwhile, stabilization of MFs and MTs could recover cytoplasmic OP (particularly the colloid OP) and reduce cell swelling.

Inhibitors of myosin, dynein, and kinesin were employed to ascertain if motor molecules had effects on cell swelling. Inhibition of myosin could increase GFAP tension and decrease MF tension (Fig. 4F and H). GFAP tension was weakened and MT tension increased when dynein was inhibited, but the opposite results were observed when kinesin was inhibited. GFAP tension increased more significantly when all the molecular motors on MFs and MTs were inhibited simultaneously (Fig. 4F–I). However, cell edema was not reduced upon treatment with molecular-motor inhibitors within 15 min (Fig. 4J and K).

In summary, OP cooperated with inward MF and MT tension and had essential roles in U87 cell swelling. Molecular motors could regulate cytoskeletal structural tensions, whereas inhibiting their function did not help to reduce cell edema.

3.5. Mechanisms underlying depolymerization of MFs and MTs in response to glutamate stimulation

Astrocyte swelling has been demonstrated to be related to the dis-aggregation of the cytoskeleton, but this mechanism must be studied...
further. Cofilin and stathmin-1 are depolymerization factors for MF and MT, respectively [41,42]. We found that glutamate activation was correlated directly with the dephosphorylation of p-cofilin and p-stathmin (Fig. 5A–D). Moreover, significant activation of cofilin and stathmin was also detected by immunohistochemical (IHC) staining in vivo (Fig. 5E and F). These data suggested that glutamate could lead to the depolymerization of MFs and MTs through activation of cofilin and stathmin-1.

To explore the signaling pathways involved in this process, we used a calcium antagonist (nimodipine (NMDP), 30 μM), Shingshot (SSH) inhibitor (sennoside A (Sen), 100 μM), and Protein phosphatase 2a (PP2A) inhibitor (cantharidin (Can), 2 μM). Inhibition of calcium signaling and SSH could recover the phosphorylation levels of cofilin, while inhibition of calcium signaling and PP2A could recover stathmin-1 phosphorylation (Fig. 5G–J). Similar results were observed in IHC staining in vivo (Fig. 5K and L).

To summarize, activation of calcium signaling, SSH, and PP2A is involved in depolymerization of MFs or MTs during glutamate-induced cytotoxic edema. Hence, the hypothesis that inhibition of calcium signaling, SSH or PP2A could reduce OP and ease brain edema merited further research.
3.6. Certain drugs reduced the levels of intracellular ions or protein nanoparticles-induced OP during astrocyte swelling

β-actin and α/β-tubulin are abundant in astrocytes. Typically, β-actin and α/β-tubulin can help to construct the cytoskeletal structure to maintain the morphology and function of cells by polymerization [43]. Under normal conditions, the size distribution of cytoplasmic particles was > 100 nm (Fig. 6A). Under the action of depolymerizing agents and glutamate treatment, monomers of actin and tubulin or macromolecular polymers were produced, the composition and magnitude of cytoplasmic OP changed. Here, we termed the cytoplasmic OP changes induced by these granules as protein nanoparticles-induced osmotic pressure (PN-OP) but not colloid OP in view of the theory of Van’t Hoff [37,44,45], which would be illustrated detailedly in the discussion part. A standard curve was generated using different concentrations of depolymerization agents to produce different OP values after 2-h treatments (Fig. 6D). The formula for the PN-OP was: \( \Delta C_{\text{PN-OP}} = 0.93x - 218.69 \) \( (R^2 = 0.97) \). The results suggest that production of protein nanoparticles and OP increment have a linear relationship significantly.

To explore molecular mechanisms underlying OP in swollen U87 cells, we screened the impact of different drugs and their effective concentrations on the ion osmotic pressure (IOP) and PN-OP on swelling cells after two hours treatment by glutamate. The optimum level of the SSH inhibitor sennoside A was discovered to be 100 μM (Fig. 6E). PP2A inhibitor cantharidin (2 μM) could reduce OP more effectively (Fig. 6F). Nimodipine is a Ca\(^{2+}\) channel blocker that can improve brain metabolism by increasing blood flow in cerebral blood vessels. Cinepazide maleate worked best at 10 μM (Fig. 6I). Cinepazide maleate can inhibit p-stathmin dephosphorylation by inhibiting Ca\(^{2+}\)-dependent PP2B release and reduce Ca\(^{2+}\)-induced toxicity [47]. Verapamil (Verap) can attenuate Ca\(^{2+}\) in cerebral blood vessels. Cinepazide maleate worked best at 10 μM (Fig. 6I). Cinepazide maleate can inhibit p-stathmin dephosphorylation by inhibiting Ca\(^{2+}\)-dependent PP2B release and reduce Ca\(^{2+}\)-induced toxicity [47]. Verapamil (Verap) can attenuate Ca\(^{2+}\) influx, but its effect in astrocytes was not distinct (Fig. S4D). The ROCK inhibitors dihydrochloride (Y-27632) and fasudil (HA-1077) inhibited profilin-1 phosphorylation and promoted MF polymerization, whereas Y-27632 was more effective than HA-1077 (Fig. S4A and S4B). Edaravone (ED) is a commonly used brain-protective agent and acts as a free-radical scavenger [48]. The optimal concentration of edaravone was 50 μM (Fig. 6H). Oxicetam (Oxira) is also a commonly used neuroprotective drug for brain injury, but its effect was suboptimal (Fig. S4C). Tamoxifen (Tam) inhibits volume-regulated anion channels and reduces CI\(^-\)resorption [49]. Tamoxifen could not attenuate OP effectively (Fig. S4F). Blockade of Na-K-Cl cotransporter with bumetanide (Bum) prevents uptake of Na\(^+\), K\(^+\), and Cl\(^-\) in neural cells, but OP was reduced only slightly (Fig. S4F).

Next, drug-combination experiments were conducted based on the concentrations of single drugs that elicited the optimal effect (Fig. 6H and I). The most effective combination of drugs was sennoside A + cantharidin + nimodipine + edaravone + cinepazide maleate (Fig. 6G). Cinepazide maleate (CM) is a Ca\(^{2+}\) channel blocker that can improve brain metabolism by increasing blood flow in cerebral blood vessels. Cinepazide maleate worked best at 10 μM (Fig. 6I). Cinepazide maleate can inhibit p-stathmin dephosphorylation by inhibiting Ca\(^{2+}\)-dependent PP2B release and reduce Ca\(^{2+}\)-induced toxicity [47]. Verapamil (Verap) can attenuate Ca\(^{2+}\) influx, but its effect in astrocytes was not distinct (Fig. S4D). The ROCK inhibitors dihydrochloride (Y-27632) and fasudil (HA-1077) inhibited profilin-1 phosphorylation and promoted MF polymerization, whereas Y-27632 was more effective than HA-1077 (Fig. S4A and S4B). Edaravone (ED) is a commonly used brain-protective agent and acts as a free-radical scavenger [48]. The optimal concentration of edaravone was 50 μM (Fig. 6H). Oxicetam (Oxira) is also a commonly used neuroprotective drug for brain injury, but its effect was suboptimal (Fig. S4C). Tamoxifen (Tam) inhibits volume-regulated anion channels and reduces CI\(^-\) resorption [49]. Tamoxifen could not attenuate OP effectively (Fig. S4F). Blockade of Na-K-Cl cotransporter with bumetanide (Bum) prevents uptake of Na\(^+\), K\(^+\), and Cl\(^-\) in neural cells, but OP was reduced only slightly (Fig. S4F).
To summarize, the cytoskeletal depolymerization of astrocytes induced by glutamate could result in the production of protein granules, and then generate protein nanoparticles-induced osmotic pressure (PN-OP). Administration of Ca$^{2+}$-channel inhibitors, neuroprotective agents, combined with specific drugs, and agents that inhibit the depolymerization factors of MFs and MTs reduce the ion OP and PN-OP of U87 cells effectively in vitro.

3.7. Effective drug combinations reduced GFAP tension and cell volume during astrocyte swelling

We showed that single drugs (sennoside A, cantharidin, nimodipine, edaravone, and cinepazide maleate) and their combinations were effective in reducing cytoplasmic OP. Meanwhile, their influences on GFAP tension during cell swelling were observed on U87 cells (Fig. 7A–D). The five drugs stated above given alone could reduce GFAP tension, while combinations of these drugs were more effective. Measurement of the intensity of calcein fluorescence confirmed that a
combination of these drugs could alleviate edema in U87 cells (Fig. 7E).

Overall, these data suggested that administration of Ca\(^{2+}\)-channel inhibitors, neuroprotective agents combined with specific drugs, and agents that inhibit the depolymerization factors of MFs and MTs could attenuate GFAP tension effectively and reduce cell swelling.

### 3.8. Drug-combination treatment could decrease cytoplasmic OP and reduce cell volume in glutamate-induced astrocyte swelling in primary cultures or in vivo

The potential roles of drug combinations for relieving astrocyte swelling were studied in primary murine astrocytes. Immunofluorescence assays with FITC-labeled actin and tubulin, and TRITC-labeled GFAP was used to identify primary astrocytes (Fig. 8A). The primary glial cells transfected with GFAP probe were applied to detect the effect of combined drugs on cytoskeletal tension. We also detected the changes in cytoplasmic OP and count rate after 2 h of drug action (Fig. 8D). The primary glial cells with transfected GFAP probe were used to detect the effect of drugs combination on cytoskeletal tension. Effective drug combinations could markedly reduce GFAP tension within 15 min (Figs. 8B and 8C). We also detect the changes in cytoplasmic IOP and PN-OP after 2 h of treatment (Fig. 8D). A combination of sennoside-A, cantharidin, nimodipine, edaravone, and cinepazide maleate reduced PN-OP (decreased by 86.8 ± 16.89 mOsm/kg) and IOP (decreased by 59.5 ± 18.26 mOsm/kg) in primary astrocytes.

To further ascertain the role of drug combinations in astrocyte swelling, we measured the volume of primary astrocytes treated with glutamate for 2 h using the calcein imaging (Figs. 8E and 8F). The optimized drug combination was found to reduce edema by ~65% compared with the control group. Besides, to verify the mechanisms described above in vivo, we employed models of cytotoxic edema (Figs. 8G and 8H). Staining with hematoxylin and eosin showed, in the model group, karyopyknosis, hyperchromatic cytoplasm, and triquetrous cells, with surrounding tissues showing fissures. Cells in drug groups had remission of the features mentioned above (Fig. 8H).

Overall, these results suggested that the drug combination could mitigate glutamate-induced edema in primary astrocytes or in vivo effectively by attenuating cytoplasmic OP, among which PN-OP and IOP were attenuated.

### 4. Discussion

Cytotoxic astrocyte swelling is a causal effect common to the pathogenesis of brain edema in trauma, ischemia, hemorrhage, inflammation or hepatic encephalopathy [50–53], swelling of the neural cells involves a shift of water from interstitial compartments to intracellular compartments. Water flux is dependent upon its potential in cells [54]. The aqueous influx into astrocyte is based on the premise of intracellular hyper-osmotic pressure or extracellular hypo-osmotic pressure [23,55]. Notably, intracellular tension activity including OP is dependent upon the cytoskeletal structure.

In the present study, we designed a tension sensor module (cpstFRET) that is physically smaller, less invasive, more biocompatible, and with a higher signal-to-noise behavior than linear distance-dependent probes, and its FRET efficiency decreases under pull tension. The dipole orientation-based FRET probes GcpG, AcpA, and TcpT can be incorporated into cytoskeletal proteins to provide a real-time three-dimensional measurement of tension in IFs, MFs, and MTs when they “hang” on the cytoskeleton in astrocyte and U87 cells.

Intracellular steerable tension activity consists of OP, MF, and MT forces, while the GFAP tension can be regulated through their interplay [15]. Responding to an extracellular, gradually lowered hypo-osmotic gradient, the GFAP tension was enhanced progressively. When MFs and MTs were depolymerized, or molecular motors were inhibited, the GFAP tension was modulated accordingly. Furthermore, the GFAP tension probe varied accordingly in a glutamate-induced model of astrocytic swelling and could function as a rapid and valid indicator for the assessment of intracellular tension activity upon alterations of cell volume. Apart from the magnitude, the vector is another crucial property of intracellular tension activity and can be deduced through attenuating or eliminating certain cytoskeletal structural tensions under different conditions and comparing it with mechanical tensions. The interplay (vector relation) between the intracellular tensions during astrocytic swelling has been shown in Table 1. The studies suggest that inward contractile tensions of MF and MT act synergistically against outward OP in swollen astrocytes, contributing to protection during astrocyte swelling.
Glutamate is the primary excitatory amino acid neurotransmitter in the central nervous system and shows high levels after ischemic brain injury [29]. Glutamate stimulation results in cytotoxic astrocyte swelling in vitro and in vivo, accompanied by an increase in cytoplasmic OP. We found that glutamate signals resulted in depolymerization of MFs and MTs as well as the production of nanoparticles (actin and tubulin monomers or macromolecular polymers) that were involved in the production of cytoplasmic OP and PN-OP. This activity resulted from activation of their depolymerizing factors coflin and stathmin-1, which are distributed widely in various tissues and dephosphorylation of which regulates their activation [56,57]. Coflin was activated by SSH and calcium signals, whereas stathmin-1 was activated by PP2A, calcium signals, and PP2B [58–60]. Their activation contributes to an increase in cytoplasmic OP and PN-OP. Stabilization of MFs and MTs

Fig. 8. Application of drug combinations after glutamate-induced astrocyte swelling in primary cultures. (A) Confocal laser microscopy was employed to analyze immunofluorescence staining for β-actin (FITC), α-tubulin (FITC), and GFAP (TRITC) in primary astrocytes. (B) GFAP tension was analyzed in primary astrocytes subjected to isotonic solution alone (row 1) and glutamate (row 2). The astrocyte swelling models were respectively treated with three drugs (row 3), four drugs (row 4 and 5), and five drugs combinations (row 6). The calibration bar was set from 0.08 to 2.96. (C) Normalized CFP/FRET signals of GFAP in the primary astrocyte swelling model with the treatment in Figure (B). (D) Effects of different drug combinations on glutamate-induced swelling of astrocytes in primary cell culture. The cytoplasmic ion osmotic pressure (blue column) and protein nanoparticles-induced osmotic pressure (red column) were measured. (E) Calcein-fluorescence micrographs of primary glial cells at initially (F0) and at 2 h (F2h) in experiments with glutamate showing decreased calcein fluorescence in membranes upon application of drug combinations. (F) Relative calcein fluorescence intensity compared with the effects of drugs. (G) The effect of drugs on neuroprotection was evaluated by brain water content after cytotoxic edema in rats. (H) Microscopic findings of cerebrum tissues revealed dense pericellular spaces, interstitial laxity, cytoplasmic condensation, and nuclear shrinkage. (H&E staining, ×20, arrowhead). Average of ≥3 experiments ± SEM.*p < 0.05, **p < 0.01, ***p < 0.001. (Scale bar = 20 μm.).
structure recovered the intracellular PN-OP and reduced the astrocyte swelling in response to glutamate stimulation, whereas attenuation of MF and MT tensions elicited by inhibitors of molecular motors exhibited no obvious effect. These data showed that cytoplasmic OP and PN-OP could be involved in glutamate-induced astrocyte swelling. Hence, cytoplasmic OP, especially PN-OP, is treated as drug targets to cure astrocyte swelling and brain edema.

Although, the PN-OP (used to known as colloid OP) had been thought to involved in intracellular tension activity according to the Van’t Hoff theory, where protein granules enable generate osmotic pressure [61]. The Donnan effect might provide a reasonable explanation for the amplified role of protein nanoparticles-induced OP, which has been thoroughly discussed in the literature [37–45]. Disruption of the Donnan equilibrium generates the osmotic gradient across the plasma membrane. In physiologic pH circumstance, the protein nanoparticles like actin and α/β-tubulin carry negative charges and impart negative fixed charge density (FCD), which can strongly absorb mass positive ions near the colloidal surface [62,63]. Intracellular decreased free cation forms the osmolarities imbalance, drawing extracellular cations into the astrocytes. The cations accumulation would induce a charge gradient which leads to subsequent anions influx, ultimately brings about intracellular hyperosmosis and liquid inflow. Multiple numerical methods have been reported to support this theory, which evaluates how cell volume is affected by fixed charge density and the ion concentration in solutions [64–66]. The viewpoint also is supported by the present data. Firstly, production of protein nanoparticles can results markedly in upregulation of ion OP in response to depolymerization of MF and MT (Fig. 2). Secondly, MF and MT stabilizer or inhibitor of MF and MT depolymerization can effectively inhibit the increment of ion OP elicited by Glu stimuli (Fig. 4). Thirdly, the amount of intracellular protein nanoparticle produced and the OP increment were observed upon administration of the ROCK inhibitor Y-27632. Moreover, the free-radical scavenger edaravone and neuroprotective agent oxiracetam decreased ion OP and PN-OP distinctly, suggesting that elimination of intracellular toxic substances such as reactive oxygen species induced by glutamate could contribute to a reduction in cytoplasmic OP [10,68,69]. We speculated that cytotoxic astrocyte swelling might be a self-protection measure for diluting the toxic substances generated, similar to an inflammatory reaction. Our hypothesis was consistent with the results showing that elimination of accumulated Ca^{2+} toxicity via inhibition of Ca^{2+} channels contributed to relieve astrocyte swelling. The present study also suggests that the ratio of intracellular ion OP and PN-OP might be a crucial indicator for evaluating the drug therapy even if the value of total OP is unchanged.

The intracellular osmotic pressure, including ion OP and PN-OP, has been shown to be regulated synthetically by multi-factor. As a consequence, a plurality of drug targets should be simultaneously selected. A notable finding was that combined administration of regulators of PN-OP and ion OP could eliminate the cytoplasmic total OP effectively and cure astrocyte swelling. In the present study, we targeted at the MF depolymerizing factor coflin, MT depolymerizing factor stathmin-1, and cellular toxicity induced by calcium overload and production of oxygen-derived free radicals. The most effective drugs combination was sennoside A + cantharidin + nimodipine + edaravone + cinepazide maleate, of which the optimum concentration (in μM) was 100, 2, 30, 50, and 10, respectively. This drug combination induced a remarkable decrease in the cytoplasmic OP, GFAP tension, and the volume of swollen astrocytes. This drug combination is also valid for primary glial cells and practical for rodent models of cerebral edema. The combination of the drugs can provide a new program for clinical treatment of cerebral edema.

5. Conclusions
The present study was designed to determine the role of intracellular tension activity in glutamate-induced astrocyte swelling. The magnitude and vectors of intracellular tension activity during astrocyte swelling was identified, in particular, protein nanoparticle-induced OP. Cytoplasmic OP and PN-OP could be generated by depolymerization of MFs and MTs, and production of protein nanoparticles resulting from activation of coflin and stathmin-1. Also, ion OP and PN-OP could adjust reciprocally. Combined administration of regulators of the PN-OP and ion OP, as well as free-radical scavenger, could reduce cytoplasmic OP, recover cell volume, and improve ischemic brain lesions effectively. Recovery of cytoplasmic potential energy is a promising target to develop new drugs and cure brain edema. Further studies must be carried out to explore the mechanisms underlying intracellular ion OP and colloid-related OP and their interactions. The present study has provided more profound insights into the progression of astrocytic swelling and shed new light on treatment methods.

Table 1
Vector relation between OP, MF, MT, and GFAP tensions. Vector analysis of the intracellular tension activity during astrocyte swelling in response to hypo-osmotic or glutamate treatment. In both circumstances, the inward MF and MT tension increase to antagonize the increment of outward GFAP tension in answer to OP, as indicated.

| Treatment                     | Parameter | OP    | MF tension | MT tension | GFAP tension | Phenomenon |
|-------------------------------|-----------|-------|------------|------------|--------------|------------|
| Hypo-osmotic pressure         | Magnitude | ↑     | ↑          | ↑          | ↑            | Swell      |
|                               | Vector    | ←     |            | →          | ←            |            |
| Glutamate-induced swelling    | Magnitude | ↑     | ↑          | ↑          | ↑            | Swell      |
|                               | Vector    | ←     |            | →          | ←            |            |

†Indicates an increase; → shows an inward direction; ← shows an outward direction.
CRediT authorship contribution statement

JiaRui Zhang: Methodology, Data curation, Writing - original draft.
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ZiHui Zheng: Writing - review & editing, Data curation.
XiaoHe Sun: Methodology, TingTing Chen: Methodology. Chen Li: Methodology.
XiaoLong Zhang: Methodology. Jun Guo: Project administration, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare no competing interests

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2019.101112.

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