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Original Research Article

Effect of hydrogen sulfide on PC12 cell injury induced by high ATP concentration

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Abstract

Purpose: *To investigate the potential protective effect of hydrogen sulfide against neural cell damage induced by a high-concentration of adenosine triphosphate (ATP).*
Methods: *PC12 cells were incubated with ATP in order to induce cell damage. The extracellular level of H_2S and protein expression of cystathionine-β-synthase (CBS) were determined. The PC12 cells pre-treated with NaHS, aminooxyacetic acid (AOAA) and KN-62, prior to further incubation with ATP, and the effect of the treatments on cell viability was investigated.*
Results: *High-concentration ATP induced cell death in PC12 cells, and this was accompanied by markedly increased contents of extracellular H_2S and CBS expression ($p < 0.05$). The ATP-induced cytotoxicity was significantly compromised after pretreatment with H_2S. ($p < 0.05$). The viability of PC12 cells pretreated with NaHS and AOAA was significantly higher than that of PC12 cells treated with ATP alone. In addition, the viability of ATP-treated PC12 cells was further markedly increased after pretreatment with NaHS and KN-62 ($p < 0.05$).*
Conclusion: *ATP induced a concentration- and time-dependent cytotoxicity in PC12 cells via the endogenous H_2S/CBS system. Supplementation with exogenous H_2S mitigated the cell damage induced by high concentration of ATP via a specific mechanism which may be specifically related to P2X7R.*

Keywords: *Hydrogen sulfide, Adenosine triphosphate, Purinergic P2X7 Receptor, Cell damage, Cystathionine-β-synthase, Neural cells*

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INTRODUCTION

Central nervous system diseases such as brain trauma, Alzheimer's disease (AD), and stroke, are common and challenging brain disorders that have attracted a lot of research interest [1]. Therefore, there is need to understand the exact pathological mechanism involved in neuronal damage in order to evolve rational neuroprotective strategies [2].

Adenosine triphosphate (ATP) is an organic compound which provides energy for living cells.

It is produced by mitochondria, and exerts its functions in the cytosol. Previous studies have shown that ATP and its receptors ($P2X_7R$) play crucial roles in triggering the activity of neural cells [3]. Under normal conditions, the levels of extracellular ATP (ATPe) are low and in micromolar amounts. However, high ATPe levels induce cell death and tissue damage. Thus, ATP is also considered as a death factor [4]. Recent studies have found that ischemia, hypoxia, trauma, and inflammation in neuronal tissues are closely related to high concentrations of ATP and high expressions of $P2X_7R$. Inhibitors of ATP receptors compromise high concentration ATP-induced cellular damage, thereby protecting neuronal tissues [5]. Nevertheless, the potential applications of these ATP receptor inhibitors are limited by their poor water solubility and severe side effects. In view of the pervasiveness of highly accumulated ATP in injured central nervous systems, it is necessary to study the characteristics and roles of ATP in neurotoxicity, as well as the underlying mechanism.

Hydrogen sulfide (H_2S) is an endogenous bioactive signal molecule that freely penetrates cell membranes without the need for special transportation because of its small molecular weight and rapid diffusion rate [6]. It has a wide influence on many events such as inflammatory response, erythrocyte oxidation inhibition, vasodilatation and synaptic activity regulation [7]. In addition, recent observations have shown that H_2S protects cells from damage, although the actual mechanism remains unclear. In living systems, endogenous H_2S is produced from the sulfur amino acids cysteine (Cys) and methionine under the catalysis of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and cysteine aminotransferase [8]. In the central nervous system, CBS which is highly distributed in hippocampus and cerebellum, is the main enzyme that produces H_2S [9].

A study on the potential influence of H_2S on ATP and its receptors, and the roles of H_2S in ATP signaling system in neuron protection will not only elucidate the mechanism of neuronal damage, but also provide alternative strategies for the neuronal protection. The present study was aimed at investigating the effect of H_2S on high ATP concentration-induced damage in PC12 cells, and the mechanism involved. After treatment of PC12 cells with different concentrations of ATP, dynamic changes in endogenous H_2S/CBS system were studied using cell viability measurement, immunofluorescence staining and Western blotting. To investigate the effects of H_2S on ATP-induced cell damage, PC12 cells were pretreated with exogenous H_2S, CBS inhibitor and $P2X_7R$ inhibitor. Then, XTT assay was conducted to determine cell viability. The expectation was that the study will provide a new insight into the prevention and treatment of brain injury.

EXPERIMENTAL

Materials and reagents

Nerve growth factor (NGF) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and trypsin were bought from Invitrogen Corporation, while ATP and (XTT) were purchased from Sigma. Paraformaldehyde, glycine, tris(hydroxymethyl)aminomethane (Tris) and Tween-20 was products of Solarbio Co. Ltd. (Beijing, China). Anti-CBS antibody was purchased from Abcam.

Cell line and cell culture

The PC12 cells were cultured in DMEM culture medium supplemented with 50 ng/mL NGF for 120 h in order to induce their differentiation into neuronal phenotype. The differentiated P12 cells were used for subsequent experiments.

Cell viability assay

The culture medium was carefully removed and replaced with fresh medium containing ATP at concentrations of 1, 3, 5, 7 and 9 mM, followed by incubation for 3 h. Untreated cells served as control. Then, 50 µL of XTT (5 mg/mL) was added to each culture medium prior to incubation for 2 h. The absorbance (A) of the cell culture medium at was measured at 450 nm. For each sample, the mean and standard deviation of 5 parallel wells were reported. Cell viability was calculated using Eq 1.

$$\text{Cell viability (\%)} = A_{ATP}/A_{control} \times 100 \quad \ldots\ldots\ldots (1)$$

The PC12 cells were seeded in 96-well cell culture plates at a density of 5000 cells/well and cultured for 24 h. Thereafter, 7 mM ATP was added to each well, and the cells were incubated for 1, 2, 3, 6, 12 and 24 h. After the incubation, cell viability was measured using XTT assay as described above.

Examination of cell morphology

After incubation with ATP at different concentrations (1, 3, 5, 7 and 9 mM) for 3 h, the PC12 cells were washed thrice with PBS and

examined under a phase contrast microscope. Untreated cells served as control.

Trypan blue staining assay

The PC12 cells treated with ATP at different concentrations (1, 3, 5, 7 and 9 mM) for 3 h were treated with trypan and taken up in 2-mL tubes. Then, the cells were homogenized and incubated in 0.2 % trypan blue solution. The number of stained dead cells was counted and used to calculate the cell viability.

H&E staining

Coverslips with a diameter of 14 mm were pretreated with 5 % HCl, 30 % HNO_3, and 75 % alcohol, and then fixed in 6-well tissue culture plates. The PC12 cells were seeded at a density of 2 × 10^5 cells/well and cultured. Then, the cell culture medium in each well was carefully discarded and replaced with fresh cell culture medium containing ATP at the final concentrations of 1, 3, 5, 7 and 9 mM, followed by incubation for 3 h. Untreated cells served as control. The coverslip in each well was washed with warm PBS and the cells on coverslips were fixed with cold acetone.

The coverslips were discolored by immersing in ethanol solution, and washed with water. Thereafter, the coverslips were immersed in hematoxylin working solution for 10 min, and then in eosin working solution for 5 min. Subsequently, the coverslips were washed with 95 % ethanol solution and dehydrated with 100 % ethanol, embedded in xylene and mounted. The stained cells were examined under a microscopy and photographed.

Measurement of endogenous H_2S content

The PC12 cells were cultured in 96-well cell culture plates at a density of 5000 cells/well at 37 °C and 5 % CO_2 for 24 h. Then, the cell culture medium was carefully removed and fresh cell culture media containing ATP at a concentrations of 1, 3, 5, 7 and 9 mM were added to separate wells, followed by incubation of the cells for 3 h. Untreated cells served as control.

Then, the cell culture medium in each well was mixed with 2 % zinc acetate, 20 % trichloroacetic acid, 20 mM dimethyl p-phenylenediamine sulfate, and 30 mM ferric trichloride. The contents of each well were blended and incubated for 10 min, after which the absorbance of each well was read at 670 nm. The H_2S content of each sample was calculated from a standard calibration curve.

Immunofluorescence staining

The PC12 cells were cultured as described above, and the culture medium was replaced with fresh medium containing ATP at a concentrations of 1, 5, or 9 mM, followed by incubation for 3 h. Cells on coverslips were washed with warm PBS and fixed with cold acetone. The coverslips were then washed with PBS containing 0.2 % Triton X-100, followed by incubation with 2 % BSA solution at room temperature for 30 min. Thereafter, the cells were incubated overnight with anti-CBS antibody at 4 ºC. Subsequently, the coverslips were washed again with PBS, and incubated with Cy3-conjugated secondary antibody at 4 ºC for 1.5 h. Next, the coverslips were stained with 4´,6-diamidino-2-phenylindole (DAPI). The stained coverslips were washed with PBS and examined under a fluorescence microscope (Olympμs BX51, Japan).

Western blotting

The PC12 cells were cultured as described above. The culture medium was replaced with fresh medium containing ATP at concentrations of 1, 5, or 9 mM, followed by incubation for 3 h. Then, a mixture of cell lysis buffer and phenylmethylsulfonyl fluoride (PMSF) (100:1 v: v) was added to each culture. Then, the mixture was placed in 2-mL tubes and centrifuged to obtain supernatants. Total protein contents of the cell lysates were determined using the BCA protein assay kit. Subsequently, the proteins were transferred onto NC membranes and incubated overnight with anti-CBS antibody (1:1000 dilution, Santa Cruz) at 4 °C, followed by washing 3 times with TBST, and incubation with second antibody (1: 2000, Beyotime, USA). Finally, the stained membranes were observed using chromogenic reaction. Gray scale analysis was performed after scanning.

Cytotoxicity assay

Post- exogenous H_2S treatment

The PC12 cells were cultured as described above. Cell culture medium was removed and fresh cell culture medium with or without the CBS inhibitor, AOAA (1 mM) was added, followed by incubation for 30 min. Then, 1 μL NaHS was added at concentrations of 50, 100 and 200 μM to separate wells, and cells were incubated for another 30 min. Next, 1 μL ATP was added, following by incubation of the cells for 3 h. After the incubation, the cell morphology was examined, and cell viability was evaluated as described above.

Post-exogenous H_2S and KN-62 co-treatments

The PC12 cells were cultured as described above. Cell culture medium with or without the $P2X_7R$ inhibitor, KN-62 (5 μM) was added, then incubated with KN-62. Next, 1 μL of NaHS was added, and the cells were incubated for 30 min, followed by a 3-h incubation of all groups with 5 mM ATP. Finally, the washed cells were subjected to morphological examination and cell viability assay as described above.

Statistical analysis

SPSS software 17.0 (IBM SPSS, USA) was conducted for statistics. Statistical data was described as mean ± SD. One-way ANOVA statistical analysis was conducted to compare the differences between groups, and $p < 0.05$ was marked statistically significant.

RESULTS

ATP-induced cytotoxicity

In order to investigate ATP-induced cytotoxicity, XTT assay was carried out after incubation of PC12 cells with ATP for 3h. The results showed that cell viability was reduced with increase in ATP concentration (Figure 1 a). At concentrations of 3, 5, 7 and 9 mM, the viability values of treated cells were 87.3, 72.0, 56.8 and 57.8 %, respectively, which were much lower than that of control group. The mortality rate of PC12 cells after treatments was studied using trypan blue staining. No mortality was observed in the control and treated cells at low ATP concentration (1 mM), while cell death significantly occurred when the ATP concentration was higher than 3 mM (Figure 1 b). In particular, at ATP concentrations of 5, 7 and 9 mM, the mortality values of treated cells increased to 30.7, 45.4, and 45.5 %, respectively. These results suggest ATP induced cytotoxicity in a concentration-dependent manner, and that higher concentrations ATP may induce cell death. In addition, when PC12 cells were incubated with 7 mM ATP for different durations, it was found that with increase in incubation time, the viability of treated cells gradually decreased and reached to a minimum value of 56.8 % after 3 h (Figure 1 c). These results demonstrate that 7 mM ATP was the maximum concentration that induced cytotoxicity in PC12 cells, with 3 h as the minimum period required for induction of cell death.

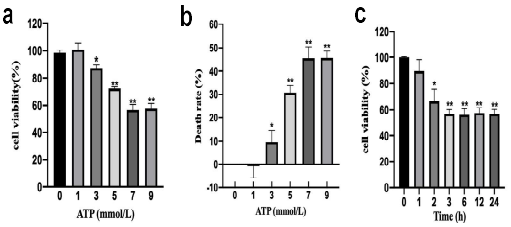

Figure 1: Effect of different concentrations of extracellular ATP on the viability of PC12 cells (a); effect of extracellular ATP on PC12 cells death (b), and effect of extracellular ATP at different durations on viability of PC12 cells (c). *$P < 0.05$, vs control group; **$p < 0.01$ vs control group

The PC 12 cells became spherical and clumped together when the ATP concentration was higher than 1 mM. In addition, compared with control group, the number of adhered cells was significantly reduced in each ATP-treated group (Figure 2). A concentration-dependent relationship was observed in the ATP-induced cytotoxicity under electronic microscopy.

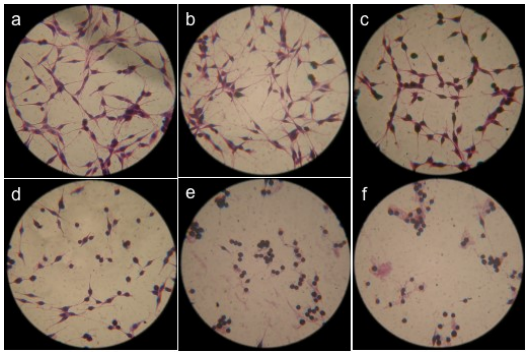

Figure 2: Hematoxylin and eosin staining of PC12 cells at different concentrations of ATP. a: control group, b: 1 mmol/L, c: 3 mmol/L, d: 5 mmol/L, e: 7 mmol/L, f: 9 mmol/L

ATP-induced H_2S/CBS system changes

Compared with the control group, no significant changes was observed in extracellular H_2S content in PC12 cells treated with a low concentration of ATP (1 mM, Figure 3a). However, when the ATP concentration was higher than 1 mM, the H_2S contents of ATP-treated cells were significantly higher than that of control group. In particular, at the concentration of 5 mM, the H_2S content increased by 3.6 folds. These results suggest that ATP treatment at high concentrations elevated the extracellular H_2S contents of PC12 cells.

The expression of CBS in cells after treatment with ATP was further studied.

Immunofluorescence staining images showed strong red fluorescence signals of anti-CBS antibody staining in cells treated with ATP at the concentrations of 5 and 9 mM, which indicated high expression of CBS (Figure 3b). In sharp contrast, similar but weaker red fluorescence signals were seen in the control group and cells treated with low ATP concentration (1 mM). These results suggest that high concentration of ATP upregulated the expression of CBS inside the cells.

The expression of intracellular CBS was further confirmed using Western-blotting. Compared to control group, the protein expression level of CBS in cells treated with 1mM ATP did not change significantly, while the protein expression levels of CBS in cells treated with higher ATP concentrations (5 and 9 mM) were significantly increased (Figure 3c). The intensity of protein bands was quantified so as to evaluate the relative expression of CBS, and the results showed that the expressions of CBS in cells treated with 5 and 9 mM ATP increased 1.3 and 1.1 folds, respectively, when compared to that in control group (Figure 3c). The results of Western blotting also showed that intracellular CBS expression was upregulated by ATP at high concentrations. This may be closely related to ATP-induced cytotoxicity.

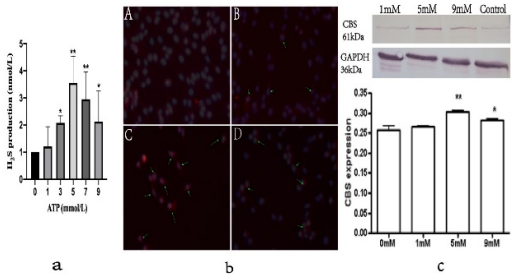

Figure 3: Effect of ATP on H_2S/CBS pathway. (a) Effect of ATP at different concentrations on H_2S production in culture medium of PC12 cells. (b) CBS immunofluorescence in PC12 cells exposed to extracellular ATP at different concentrations. (c) Effect of ATP at different extracellular concentrations on CBS expression in PC12 cells. **P* < 0.05, vs control group; ***p* < 0.01, vs control group

Influence of exogenous H_2S on ATP-induced cytotoxicity

In view of increases in H_2S content and CBS expression in ATP-treated cells, researchers speculated that ATP-induced cytotoxicity could be compromised by H_2S. To confirm this hypothesis, the influence of exogenous NaHS on ATP-induced cytotoxicity was investigated. The PC12 cells were pretreated with NaHS at different concentrations, prior to exposure to 5 mM ATP. When compared to the control group, NaHS treatment did not affect cell viability even at a high concentration of 200 mM (Figure 4). However, the viability of cells co-treated with NaHS and ATP were significantly higher than that of cells treated solely with ATP, regardless of NaHS concentration. To further study the role of CBS in ATP-induced cytotoxicity, pre-treatment of PC12 cells were with a CBS inhibitor (AOAA) did not cause obvious cytotoxicity. In addition, the viability of NaHS-pretreated PC12 cells was similar to those of cells pre-treated with NaHS and AOAA. These results clearly suggest that exogenous H_2S indeed protected the cells against ATP-induced cytotoxicity, while CBS inhibitor did not exert any influence on this protective effect.

The morphology of PC12 cells after different treatments was examined in order to further confirm the results obtained earlier. Cells treated with NaHS and AOAA exhibited normal morphologies like those in control group (Figure 4). The morphologies of PC12 cells pre-treated with NaHS and NaHS/AOAA became more normalized, with more protrusions, better refractivity and improved adherence, when compared to ATP-treated cells. Consistent with the XTT results, these data confirmed that exogenous H_2S exerted a protective effect against ATP-induced cytotoxicity in nerve cells.

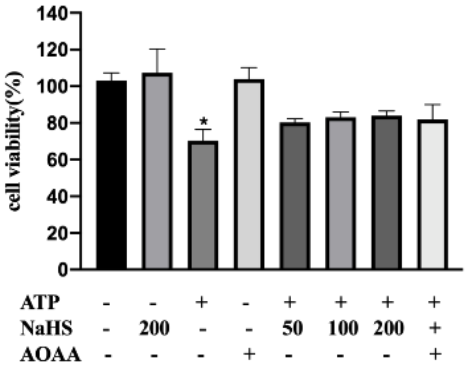

Figure 4: Effect of NaHS on viability of PC12 cells damaged by ATP. **P* < 0.05, vs control

Influence of exogenous H_2S and KN-62 on ATP-induced cytotoxicity

To compare the influence of exogenous H_2S and $P2X_7R$ inhibitors on ATP-induced cytotoxicity, PC12 cells were pretreated with NaHS and/or a $P2X_7R$ inhibitor (KN-62), and then treated with 5 mM ATP, prior to measurement of cell viability. The pre-treatments with NaHS and KN-62 increased the viability of PC12 cells, relative to

sole ATP treatment (Figure 5). The viability of PC12 cells co-treated with NaHS and ATP was 71.9 %, which was slightly higher than that of cells co-treated with KN-62 and ATP (66.3 %). These results suggest that KN-62 could also mitigate ATP-induced cytotoxicity, but its efficiency was slightly lower than that of exogenous H_2S. More importantly, the viability of ATP-treated cells pretreated with NaHS and KN-62 was as high as 88.5 %, which was significantly higher than the corresponding values for cells co-treated with NaHS/ATP and cells co-treated with KN-62/ATP. These data suggest that NaHS and KN-62 produced a synergistic action in protecting the cells against ATP-induced toxicity.

The morphology of PC12 cells treated with KN-62 was similar to that of control cells, indicating that KN-62 exerted lower cytotoxicity (Figure 5). Compared with cells treated with ATP, those pretreated with NaHS and/or KN-62 showed more normal morphology, especially cells treated with NaHS and KN-62. These observations further confirmed the protective effects of NaHS and KN-62 against ATP-induced cytotoxicity.

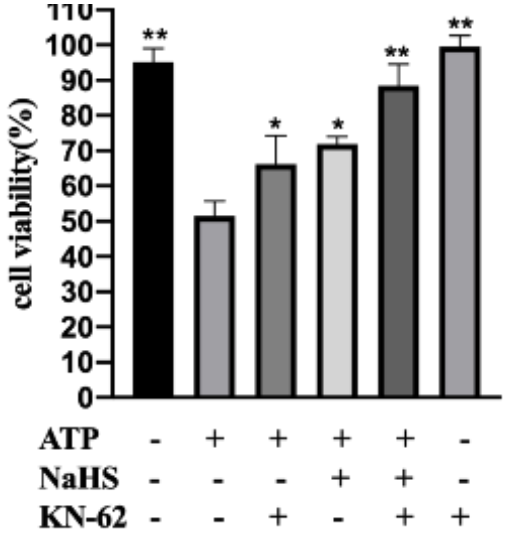

Figure 5: Effects of KN-62 and NaHS on viability of PC12 cells damaged by ATP. *P < 0.05 vs ATP, **p < 0.01 vs ATP, #p < 0.05 vs ATP + KN-62

DISCUSSION

The level of ATP demonstrates the viability and functionality of cells, and it affects the expressions of respiratory chain enzymes [10]. There are two main sources of ATP: mitochondrial oxidation and extra-mitochondrial substrate level phosphorylation. Low levels of ATP promote the proliferation and development of neurons, while inhibiting cell death [11].

It is currently believed that apoptosis is regulated by ATP, which implies that it plays a vital role in pathophysiology [13]. Studies have revealed that ATP induces apoptosis in different cells through two pathways viz extracellular-specific receptors (P2Z and $P2X_7$ receptors) and intracellular metabolites [12]. It has been suggested that high level of ATP is the principal mechanism responsible for cell death, while absence of ATP leads to cellular swelling [14]. In other studies, it was shown that cerebral cortex damage resulting in raised extracellular ATP levels, leading to free radical production, calcium overload, and increased glutamate release, eventually resulting in neuronal death [15,16].

Adenosine receptor is considered a P_1 steroid receptor, and ATP receptor is regarded as P_2 steroid receptor which has been further categorized as P2X, P2Y, P2T and other subtypes. The P_2X_7R subtype belongs to the transmitter-gated ion channel superfamily present on the cell membranes of neurons, blood vessels and visceral smooth muscle cells. It is a receptor for ATP, and also a non-selective cation channel. At an appropriate concentration, ATP activates $P2X_7R$, resulting in selective Ca^{2+} influx and cell depolarization [17]. Cerebral ischemia leads to release of large amounts of ATP from neurons and glial cells, activation of $P2X_7R$, and formation of membrane pores which allow passage of substances and cations of molecular weights below 900D, thereby causing loss of cell homeostasis and death [18]. When ATP is bound to $P2X_7R$, the resultant complex opens non-selective cation channels, causing cell depolarization and Ca^{+} influx. The agonists of P2X receptor include 2-methylene ATP (2-MeATP) and 2-methylene ADP (2-MeADP), while the commonly used antagonists for P2X receptor are isoquinoline derivatives (e.g., KN-62), pyridoxal derivative (PPADS) and suramin [19].

Low concentrations of ATP provide energy for cells, while high concentrations of ATP inhibit the expression of mtDNA. This is important for mitochondrion as a metabolically active organelle. When ATP is insufficient, the expression of mtDNA is enhanced, oxidative phosphorylation process is improved, and the production of ATP is accelerated. When energy is excessive, the expression of mtDNA is inhibited to prevent further energy production. It is an economical and effective feedback adjustment method in living systems. However, when no exogenous ATP is supplemented, some

other nucleoside triphosphates may also play compensatory roles [20,21].

In this study, the influence of ATP on PC12 cells was investigated via cell morphology evaluation, cell viability, and H&E staining. It was confirmed that the cell viability was affected by ATP in a concentration and time-dependent manner.

Hydrogen sulphide is ranked third amongst the gas signaling molecules in living systems, following NO and CO. It has similar effects as NO and CO in terms of various physiological and pathological processes. It participates in vasodilatation, synaptic activity regulation, and nerve cell protection [22-26]. In the nervous system, CBS is the main enzyme that catalyzes the production of H_2S. Inhibitors of CBS bioactivity regulate the generation of H_2S. It has been reported that H_2S protected nerve cells from oxidative stress via promoting the synthesis of intracellular glutathione which activated K-ATP channels [23]. Moreover, it has been reported that H_2S inhibited HOCl-mediated intracellular oxidative damage in a concentration-dependent manner [25]. Hydrogen sulphide (H_2S) can also act as a neuromodulator between glial cells and neurons. In addition, H_2S is involved in many neurological diseases. In febrile seizures, expressions of CBS protein and CBS mRNA are increased in the hippocampus, and the expressions of H_2S/CBS system are up-regulated, resulting in increased production of H_2S. In stroke, nerve cells hypoxia leads to increased release of cysteine and promotes the generation of H_2S by CBS. The increase in H_2S level leads to cerebral vasodilation, reduced cerebral ischemia area, and alleviated brain damage [27].

In present study, the contents of extracellular H_2S were measured using methylene blue spectrophotometry, and the expressions of CBS protein were determined with western blot assay. With increase in ATP concentration, the H_2S contents gradually increased, reaching a maximum level at an ATP concentration of 7 mM. A similar trend was observed in CBS protein expression. These results suggest that regulation of gene expression increased the expression of CBS enzyme and decreases the production of H_2S. Endogenous H_2S/CBS expression system was involved in the ATP-induced PC12 cell damage. High concentrations of ATP up-regulated the H_2S/CBS system in PC12 cells, probably due to the binding of ATP to P2X receptor. In view of this, it can be speculated that the generation of H_2S is a response to neuronal damage.

During cerebral ischemia-reperfusion, ATP and $P2X_7R$ play critical roles in inflammatory response and neuronal death. The results obtained in this study showed that AOAA (a CBS inhibitor) did not affect the protective role of H_2S against ATP-induced cytotoxicity. The $P2X_7R$ inhibitor (KN-62) compromised the cytotoxicity induced by ATP in the nerve cells, and it exerted a synergistic effect with exogenous H_2S, thereby further improving cell viability. These results indicate that H_2S protected PC12 cells against ATP-induced cytotoxicity via regulation of the ATP/$P2X_7R$ signaling pathway.

CONCLUSION

This study has demonstrated that ATP induces cell cytotoxicity in a concentration- and time-dependent manner via endogenous H_2S/CBS system. Exogenous H_2S protects the cells against cell damage induced by high-concentration ATP, through an underlying mechanism related to purinergic P_2X_7 receptor. The results of this study provide a new strategy for mitigating nervous disorders and treatment of CNS diseases.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors listed in this manuscript, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Kunli Yang, Hu Luo and Dongliang Li conceived and designed the study. Yajie Liu, Yankai Ren, Meixia Guo and Bin Wang collected and analyzed the data, while Kunli Yang wrote

the manuscript. Chihchuang Yang and Dongliang Li contributed equally to this work.

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