Original Research Article

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

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Sent for review: 4 August 2021 Revised accepted: 29 January 2022

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 H₂S and protein expression of cystathionine-β-synthase (CBS) were determined. The PC12 cells pretreated 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 H₂S and CBS expression (p < 0.05). The ATP-induced cytotoxicity was significantly compromised after pretreatment with H₂S. (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 H₂S/CBS system. Supplementation with exogenous H₂S 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

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 (P2X7R) play crucial roles in triggering the activity of neural cells [3]. Under normal conditions, the levels of extracellular ATP (ATPc) are low and in micromolar amounts. However, high ATPc 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 P2X7R. 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 (H2S) 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 H2S protects cells from damage, although the actual mechanism remains unclear. In living systems, endogenous H2S 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 H2S [9].

A study on the potential influence of H2S on ATP and its receptors, and the roles of H2S 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 H2S 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 H2S/CBS system were studied using cell viability measurement, immunofluorescence staining and Western blotting. To investigate the effects of H2S on ATP-induced cell damage, PC12 cells were pretreated with exogenous H2S, CBS inhibitor and P2X7R 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, glycin, 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 450 nm. For each sample, the mean and standard deviation of 5 parallel wells were reported. Cell viability was calculated using Eq 1.

\[
\text{Cell viability} (\%) = \frac{A_{\text{ATP}}}{A_{\text{control}}} \times 100
\]

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 % HNO₃, and 75 % alcohol, and then fixed in 6-well tissue culture plates. The PC12 cells were seeded at a density of 2 × 10⁵ 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 H₂S content**

The PC12 cells were cultured in 96-well cell culture plates at a density of 5000 cells/well at 37 °C and 5 % CO₂ for 24 h. Then, the cell culture medium was carefully removed and fresh cell culture media containing ATP at 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 H₂S 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 coverslips were incubated overnight with anti-CBS antibody at 4 °C. Subsequently, the coverslips were washed again with PBS, and incubated with Cy3-conjugated secondary antibody at 4 °C 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 (Olympus 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. The cell lysis buffer and phenylmethylsulfonyl fluoride (PMSF) (100:1 v:v) was added to each culture. 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 H₂S 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\textsubscript{2}S and KN-62 co-treatments

The PC12 cells were cultured as described above. Cell culture medium with or without the P2X7R 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 3 h. The results showed that cell viability was reduced with increase in ATP concentration (Figure 1a). 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 1b). 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 1c). 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.

ATP-induced H\textsubscript{2}S/CBS system changes

Compared with the control group, no significant changes were observed in extracellular H\textsubscript{2}S 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\textsubscript{2}S contents of ATP-treated cells were significantly higher than that of control group. In particular, at the concentration of 5 mM, the H\textsubscript{2}S content increased by 3.6 folds. These results suggest that ATP treatment at high concentrations elevated the extracellular H\textsubscript{2}S 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 1 mM 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.

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). Cells treated with NaHS and 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$_2$S exerted a protective effect against ATP-induced cytotoxicity in nerve cells.

**Influence of exogenous H$_2$S on ATP-induced cytotoxicity**

In view of increases in H$_2$S content and CBS expression in ATP-treated cells, researchers speculated that ATP-induced cytotoxicity could be compromised by H$_2$S. 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$_2$S indeed protected the cells against ATP-induced cytotoxicity, while CBS inhibitor did not exert any influence on this protective effect.

**Influence of exogenous H$_2$S and KN-62 on ATP-induced cytotoxicity**

To compare the influence of exogenous H$_2$S and P2X$_7$R inhibitors on ATP-induced cytotoxicity, PC12 cells were pretreated with NaHS and/or a P2X$_7$R 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 H₂S. 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.

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 P2X₇ 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 P₁ steroid receptor, and ATP receptor is regarded as P₂ steroid receptor which has been further categorized as P2X, P2Y, P2T and other subtypes. The P₂X₇R 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 P₂X₇R, resulting in selective Ca²⁺ influx and cell depolarization [17]. Cerebral ischemia leads to release of large amounts of ATP from neurons and glial cells, activation of P₂X₇R, 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 P₂X₇R, the resultant complex opens non-selective cation channels, causing cell depolarization and Ca²⁺ 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$_2$S. Inhibitors of CBS bioactivity regulate the generation of H$_2$S. It has been reported that H$_2$S 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$_2$S inhibited HOCl-mediated intracellular oxidative damage in a concentration-dependent manner [25]. Hydrogen sulphide (H$_2$S) can also act as a neuromodulator between glial cells and neurons. In addition, H$_2$S 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$_2$S/CBS system are up-regulated, resulting in increased production of H$_2$S. In stroke, nerve cells hypoxia leads to increased release of cysteine and promotes the generation of H$_2$S by CBS. The increase in H$_2$S level leads to cerebral vasodilatation, reduced cerebral ischemia area, and alleviated brain damage [27].

In present study, the contents of extracellular H$_2$S 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$_2$S 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$_2$S. Endogenous H$_2$S/CBS expression system was involved in the ATP-induced PC12 cell damage. High concentrations of ATP up-regulated the H$_2$S/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$_2$S is a response to neuronal damage.

During cerebral ischemia-reperfusion, ATP and P2X$_7$R 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$_2$S against ATP-induced cytotoxicity. The P2X$_7$R inhibitor (KN-62) compromised the cytotoxicity induced by ATP in the nerve cells, and it exerted a synergistic effect with exogenous H$_2$S, thereby further improving cell viability. These results indicate that H$_2$S protected PC12 cells against ATP-induced cytotoxicity via regulation of the ATP/P2X$_7$R signaling pathway.

**CONCLUSION**

This study has demonstrated that ATP induces cell cytotoxicity in a concentration- and time-dependent manner via endogenous H$_2$S/CBS system. Exogenous H$_2$S protects the cells against cell damage induced by high-concentration ATP, through an underlying mechanism related to purinergic P$_2$X$_7$R receptor. The results of this study provide a new strategy for mitigating nervous disorders and treatment of CNS diseases.

**DECLARATIONS**

**Acknowledgement**

This study was supported by Major Scientific and Technological Projects of Henan Province (Grant no.192102310083,202102310081), Xinxiang City Science and Technology Research Project (Grant no. GG2019023), Henan Provincial Department of Education Project Number (Grant no. 20A310015,19A310022), Henan Province Science and Technology Research Project (Grant no. 2018020386), and major scientific research project of Henan University (Grant no. 20B310014).

**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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