Hypertonic stimulation induces synthesis and release of glutamate in cultured rat hypothalamic astrocytes and C6 cells

Rong CAO, Shan JIANG, Li DUAN, Ying-Fei XIONG, Bei GAO, Zhi-Ren RAO

Institute of Neuroscience, The Fourth Military Medical University, Xi'an 710032, China

Abstract: Objective To investigate whether hypertonic saline (HS) can induce the synthesis and release of glutamate in cultured hypothalamic astrocytes or C6 cell line. **Methods** Astrocytes were isolated, cultured, purified and identified from the hypothalamus of newborn rat (1 day). The astrocytes were randomly divided into five groups: isotonic (IS) and HS groups, astrocytes were incubated by IS and HS (320 mOsm NaCl) medium, respectively, for 1, 3, 5, 10 or 15 min; carbenoxolone (CBX) +IS and CBX+HS groups, astrocytes were pre-treated with CBX (100 mmol/L) for 1 h at 37 °C in a 5% CO₂ / 95% atmosphere, then removed to IS and HS medium, respectively, for 1, 3, 5, 10 or 15 min; Ca²⁺+HS group, astrocytes were pre-incubated with Ca²⁺ (1 000 μmol/L) for 1 h at 37 °C in a 5% CO₂ / 95% atmosphere, followed by a wash with isotonic FBS/DMEM, and then removed to hypertonic saline for 1, 3, 5, 10 or 15 min. The media of five groups were collected to analyze the medium glutamate concentration with high performance liquid chromatography. The astrocytes were fixed and double immunofluorescent stained with anti-glial fibrillary acidic protein (GFAP) and anti-glutamate. The C6 cells were divided into four groups: IS, HS, CBX+IS and CBX+HS groups, and used for quantitative measurement of glutamate in cells by flow cytometry (FCM). **Results** (1) Anti-GFAP immunofluorescent signal revealed no significant difference among various time points in each group, or among the five groups. (2) The anti-glutamate immunofluorescent signal was increased in HS group and peaked at 5 min, and decreased and returned to the level of IS group at 15 min ($P < 0.01$ vs the 5 min of HS group). In CBX+HS group, the glutamate intensity was higher than that in CBX+IS and HS groups. (3) The medium glutamate concentration had no change after treatment with HS for 1 and 3 min, while increased markedly after treatment for 5 min to 15 min ($P < 0.01$ vs 1 min and 3 min). On the contrary, the medium glutamate concentrations in the CBX+HS or Ca²⁺+HS group were significant lower than that in the HS group ($P < 0.01$). (4) FCM showed HS and CBX+HS induced glutamate increase in C6 cells. **Conclusion** HS induced cultured rat hypothalamic astrocytes or C6 cells to synthesize and release glutamate; CBX could block glutamate release, but could not disrupt glutamate synthesis.

Keywords: astrocytes; hypertonic stimulation; carbenoxolone; connexin 43; high performance liquid chromatography; immunofluorescent stain; rat

1 Introduction

Astrocytes in the supraoptic nucleus (SON) and the paraventricular nucleus play an important role in regulating osmotic pressure^[1]. Previous studies showed that an acute

hypertonic stimulation could activate SON astrocytes and increase the expression of *c-fos*, glial fibrillary acidic protein (GFAP, an astrocytic specific marker), and connexin 43 (Cx43, a gap junction protein subunit)^[2-4]. There is a glutamate-mediated astrocyte-neuron signaling in the central nervous system^[5]. Astrocytes release glutamate through various mechanisms, for example, P2X7 receptor^[6], exocytosis^[7,8], and Cx43 hemichannels^[9]. Cx43 is a main connexin protein expressed in astrocytes^[10-12] and is sensitive to various stimuli including osmotic stimulation^[13,14].

Our previous studies showed that hypertonic medium

Corresponding author: Zhi-Ren RAO
Tel: 86-29-84774505
Fax: 86-29-83246270
E-mail: zrrao@fmmu.edu.cn
Article ID: 1673-7067(2008)-06-0359-08
CLC number: Q42
Document code: A
Received date: 2008-07-09

could induce Cx43 expression in cultured rat hypothalamic astrocytes^[15]. The aim of present study was to investigate whether the hypertonic medium treated hypothalamic astrocytes could synthesize and release glutamate, and the effect of carbenoxolone (CBX, a gap junctional blocker) on the synthesis and release of glutamate.

2 Materials and Methods

2.1 Culturing of astrocytes Astrocytes from the hypothalamus of newborn rat (1 day) were isolated, cultured, purified and identified as previously described^[16]. The astrocytes were plated on 60-mm Primaria plastic culture dishes (Nunc Clone) with glass coverslips (reaching 1×10^5 cells/cm² 24 h before the experiment) and kept at 37°C in a 5% CO₂ / 95% atmosphere at nearly 100% relative humidity. The cultured astrocytes were maintained in growth medium (Dulbecco's modified Eagle's medium, DMEM) and reached confluence within 2 weeks, and more than 90% of cells were positive for GFAP (Dako, Z0334). These cultured astrocytes were typically used for experiments after culturing for 2-4 weeks. They were essentially free of neurons and judged by morphology and β -tubulin isotype III staining (Promega, Madison, WI, USA).

2.2 Osmotic stimulation Cultured astrocytes were randomly divided into five groups. (1) IS group: astrocytes were divided into 5 subgroups and incubated with the isotonic DMEM free of Ca²⁺ and Mg²⁺ and supplemented with 10% fetal bovine serum (FBS) for 1, 3, 5, 10 and 15 min, respectively. (2) CBX+IS group: astrocytes were pre-incubated with CBX (100 μ mol/L) for 1 h at room temperature^[9], then divided into 5 subgroups and removed to the isotonic FBS/DMEM free of Ca²⁺ and Mg²⁺ for 1, 3, 5, 10, and 15 min, respectively. (3) HS group: astrocytes were moved from the isotonic FBS/DMEM to the hypertonic (320 mosM) FBS/DMEM free of Ca²⁺ and Mg²⁺^[17], and cells were divided into 5 subgroups and incubated for 1, 3, 5, 10 and 15 min, respectively. (4) CBX+HS group: astrocytes were pre-incubated with CBX (100 μ mol/L) for 1 h at room temperature^[9], then divided into 5 subgroups and removed to hypertonic FBS/DMEM free of Ca²⁺ and Mg²⁺ for 1, 3, 5, 10, and 15 min, respectively. (5) Ca²⁺+HS group: astrocytes were pre-incubated with the solution containing Ca²⁺ (1 000 μ mol/L)^[9] for 1 h at 37°C in a 5% CO₂ / 95% atmosphere, followed by washing with isotonic FBS/DMEM, and then divided into 5 subgroups and removed

to hypertonic saline for 1, 3, 5, 10 and 15 min, respectively. Five culture dishes were used at every time point.

All astrocytes in the four groups of IS, CBX+IS, HS and CBX+HS were fixed with 4% paraformaldehyde for 30 min at room temperature, and the cells were used for subsequent double immunofluorescent staining.

Since it had been reported that Ca²⁺ affected the synthesis and release of glutamate, the quantitative fluorescence intensity of glutamate in the medium containing Ca²⁺ (Ca²⁺+HS group) was also measured.

The medium of the five groups at various time points were collected into different centrifugal tubes to measure the concentration of glutamate by high performance liquid chromatography (HPLC).

2.3 Double immunofluorescent staining All astrocytes were performed anti-glutamate/anti-GFAP double immunofluorescent staining as previously described^[18]. The main procedures were as following:

- (1) Astrocytes were incubated with blocking solution (0.1 mol/L PBS containing 5% NGS, 2% BSA, 0.1% Triton X-100, and 0.02% sodium azide) for 30 min at room temperature.
- (2) Astrocytes were incubated with mixed solution of rabbit anti-glutamate polyclonal antibody (1:4 000, Sigma) and mouse anti-GFAP monoclonal antibody (1:1 500, Sigma) overnight at 4°C.
- (3) After washing with PBS for six times (5 min each), the astrocytes were incubated with appropriate second fluorescent-conjugated antibodies for 2 h at room temperature.
- (4) Culture glass coverslips were rinsed with PBS, then coverslipped with mixed solution of PBS and glycerol (1:1), and examined under a laser confocal scanning microscope (Olympus IX-70, FV 1000, Japan).

2.4 HPLC The concentration of glutamate in the medium of five astrocyte groups was measured at various time points by HPLC as described previously^[8].

2.5 Flow cytometry (FCM) Glutamate level in cells was quantitatively measured by FCM using C6 cell line.

About 2×10^6 /mL of C6 cell suspension was prepared and washed twice with cold PBS containing 50% FACS permeabilizing solution. The C6 cells were divided into four groups. (1) IS group: C6 cells were incubated with the isotonic DMEM free of Ca²⁺ and Mg²⁺ and supplemented with 10% fetal bovine serum (FBS) for 1, 3, 5 and 10 min, respectively (5 dishes per time point). (2) HS group: C6 cells were removed from the isotonic FBS/DMEM to the hypertonic

(320 mosM) FBS/DMEM free of Ca^{2+} and Mg^{2+} ^[17] and incubated for 1, 3, 5 and 10 min, respectively. (3) CBX+IS group: C6 cells were pre-incubated with CBX (100 $\mu\text{mol/L}$) for 1 h at 37 °C in a 5% CO_2 / 95% atmosphere^[9], then removed to the isotonic FBS/DMEM free of Ca^{2+} and Mg^{2+} for 1, 3, 5 and 10 min, respectively. (4) CBX+HS group: C6 cells were pre-incubated with CBX (100 $\mu\text{mol/L}$) for 1 h at 37 °C in a 5% CO_2 / 95% atmosphere^[9], then removed to the hypertonic FBS/DMEM free of Ca^{2+} and Mg^{2+} for 1, 3, 5 and 10 min, respectively.

Then the C6 cells of all the four groups were incubated with rabbit anti-glutamate polyclonal antibody (1:4 000, Sigma) for 30 min at room temperature. After washing twice by the PBS containing 50% FACS permeabilizing solution, the cells were incubated with appropriate second fluorescent-conjugated antibodies for 30 min at room temperature. Finally, the cells were fixed by 4% paraformaldehyde.

The quantitative fluorescence intensity of glutamate in the C6 cells was detected by using Aria (BD), and it was analyzed by using the Multicycle Software Autofit Version2.50 (Phoenix Flow Systems, San Diego, CA, USA).

2.6 Statistical analysis The mean value at each time point is expressed as mean $\pm$ SEM and analyzed by one-way ANOVA, followed by Fisher's LSD test. The mean value of different time points in one group is expressed as mean $\pm$ SD. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Expression of GFAP immunofluorescent signal in astrocytes No obvious changes of anti-GFAP fluorescent signal expressed in astrocytes were found among the five time points within IS, HS, CBX+HS or CBX+HS groups, and no significant difference was detected among the four experimental groups ($n = 5$, $P > 0.05$, Fig. 1, Table 1).

3.2 Expression of glutamate immunofluorescent signal in astrocytes The expression and mean fluorescent intensity of glutamate immunofluorescent signal in the astrocytes of IS, HS, CBX+HS and CBX+HS groups are shown in Fig. 1, 2 and Table 2.

The glutamate signal intensity had no significant differences among 1, 3, 5, 10, and 15 min within IS group or CBX+IS group, and the mean values of the five points in the two groups were 755.3 $\pm$ 133 and 1038.0 $\pm$ 100 ($P < 0.01$), respectively.

Table 1 Mean GFAP fluorescent intensity of the five time points in different groups (mean $\pm$ SD)

	IS	HS	CBX+IS	CBX+HS
GFAP	1687.6 $\pm$ 200	1728.1 $\pm$ 250	1782.5 $\pm$ 250	1815.6 $\pm$ 250

Table 2 Mean fluorescent intensity of glutamate signal in different groups

	IS [†]	CBX+IS [†]	HS [‡]	CBX+HS [‡]
1 min ($n = 5$)			1144.9 $\pm$ 100	1328.1 $\pm$ 150 [#]
3 min ($n = 5$)			1154.1 $\pm$ 190	1219.4 $\pm$ 200 [#]
5 min ($n = 5$)	755.3 $\pm$ 133	1038.0 $\pm$ 100 [*]	1532.2 $\pm$ 300 ^{*§}	1363.6 $\pm$ 150 [#]
10 min ($n = 5$)			984.9 $\pm$ 300	1216.9 $\pm$ 150 [#]
15 min ($n = 5$)			696.6 $\pm$ 100	1391.5 $\pm$ 150 [#]

[†]Value is shown as mean $\pm$ SD of the five time points. [‡]Values are shown as mean $\pm$ SEM. ^{*} $P < 0.01$ vs IS group, [#] $P < 0.01$ vs CBX+IS group, [§] $P < 0.01$ vs other time points of the HS group.

The immunofluorescent intensity of glutamate signal in astrocytes changed with time after HS treatment. It increased significantly in response to HS stimulation and peaked at 5 min ($P < 0.01$ vs IS group and other time points of HS groups), and returned to the level of IS group at 15 min ($P < 0.01$ vs 5 min, $P > 0.05$ vs IS group). But the size and feature of astrocytes did not change (Fig. 1). It suggested that HS induced glutamate synthesis in astrocytes.

In the CBX+HS group, the glutamate signal intensities of the five different time points were all significantly higher as compared with CBX+IS group ($P < 0.01$). However, they had no marked difference among each other, *i.e.*, glutamate signal intensity was not detected to change with time, in contrary to the result of HS group. The results indicated that HS-induced time course of glutamate change was annulled by CBX.

3.3 Changes of glutamate concentration in astrocytic medium The changes of glutamate concentration in astrocytic medium of IS, HS, Ca^{2+} +HS, CBX+IS and CBX+HS groups are shown in Table 3 and Fig. 3.

In IS group and CBX+IS group, the concentration of glutamate in the medium had no significant difference among various time points within each group, and the mean values of the five points in the two groups were both 2.2 nmol/mL.

Under HS condition, the medium glutamate concentration changed with time. After treatment with HS for 1 min to 3

Fig. 1 The immunofluorescent staining of anti-glutamate (red) and anti-GFAP (green) in cultured astrocytes stimulated by IS, HS, CBX+IS or CBX+HS. After HS culturing, anti-glutamate immunofluorescent intensity in astrocytes increased and peaked at 5 min as compared with IS culturing, then decreased and returned to the IS level at 15 min. In the astrocytes pretreated with CBX, though the glutamate expression was significantly increased by HS, it did not decrease at 10 min and 15 min. The expressions of GFAP in the four groups had no marked change. Scale bar, 50 μ m.

min, the medium glutamate concentration had no change, the same as IS group (2.2 nmol/mL); while when the exposure time prolonged to 5 min to 15 min, it increased significantly ($P < 0.01$ vs 1 min or 3 min of HS group). The result suggested that HS induced glutamate release in astrocytic medium.

The mean concentrations of glutamate in the astrocytic medium of CBX+HS or Ca^{2+} +HS groups were significantly lower than those of HS group at the corresponding time points of 1, 3, 5, 10 and 15 min ($n = 5$, $P < 0.01$, one way ANOVA, Fig. 3, Table 3), suggesting that the release of glutamate was blocked by CBX.

Table 3 Changes of glutamate concentration in astrocytic medium (nmol/mL)

	IS [†]	CBX+IS [†]	HS [‡]	CBX+HS [‡]	Ca^{2+} +HS [‡]
1 min ($n = 5$)			2.2 $\pm$ 0.21	2.0 $\pm$ 0.34 [#]	2.0 $\pm$ 0.28 [#]
3 min ($n = 5$)			2.2 $\pm$ 0.18	1.7 $\pm$ 0.15 [#]	1.8 $\pm$ 0.11 [#]
5 min ($n = 5$)	2.2 $\pm$ 0.23	2.2 $\pm$ 0.28	3.1 $\pm$ 0.25 [*]	1.3 $\pm$ 0.21 [#]	1.6 $\pm$ 0.32 [#]
10 min ($n = 5$)			3.2 $\pm$ 0.31 [*]	1.4 $\pm$ 0.12 [#]	1.5 $\pm$ 0.31 [#]
15 min ($n = 5$)			3.5 $\pm$ 0.53 [*]	1.4 $\pm$ 0.19 [#]	1.5 $\pm$ 0.19 [#]

[†]Value is shown as mean $\pm$ SD of the five time points. [‡]Values are shown as mean $\pm$ SEM. ^{*} $P < 0.01$ vs the values of 1 and 3 min in HS group, [#] $P < 0.01$ vs the corresponding time point of HS group.

3.4 Changes of glutamate signal in C6 cells The quantitative changes of glutamate signal in the C6 cells of IS, HS, CBX+IS and CBX+HS groups were detected by FCM and the results are shown in Table 4.

The FCM results indicated that HS incubation significantly increased fluorescence intensity of glutamate in C6 cells from 1 min to 10 min ($n = 5$, $P < 0.01$ vs IS groups, one way ANOVA, Fig. 4, Table 4).

When pretreatment with CBX before HS exposure, the elevated glutamate fluorescence intensity induced by HS

Fig. 2 Time course of glutamate immunofluorescent intensity changes in cultured astrocytes treated by HS or CBX+HS. The glutamate expression was increased by HS stimulation at 1 min and 5 min, and peaked at 5 min as compared with that in the astrocytes cultured with IS medium ($*P < 0.01$). In the CBX+HS group, glutamate signal intensity was not detected to change with time.

Table 4 Changes of glutamate expression in C6 cells

	IS [†]	CBX+IS [†]	HS [‡]	CBX+HS [‡]
1 min ($n = 5$)			21.8±2.35 [*]	29.3±2.75 ^{#§}
3 min ($n = 5$)	1.1±0.17	1.1±0.19	18.3±2.13 [*]	27.5±2.68 ^{#§}
5 min ($n = 5$)			17.1±2.35 [*]	23.2±2.47 ^{#§}
10 min ($n = 5$)			15.6±2.50 [*]	47.8±3.25 ^{#§}

[†]Value is shown as mean±SD of the five time points. [‡]Values are shown as mean±SEM. ^{*} $P < 0.01$ vs IS group, [#] $P < 0.01$ vs the corresponding time point of HS group, [§] $P < 0.01$ vs CBX+IS group

Fig. 3 Astrocytic medium glutamate concentration measured by HPLC in the HS medium free of Ca^{2+} and Mg^{2+} , Ca^{2+} +HS medium, and the CBX+HS medium free of Ca^{2+} and Mg^{2+} . The concentration of glutamate was significantly increased by HS from 5 min to 15 min as compared with that in the IS astrocytic medium ($P < 0.01$). After pretreatment with Ca^{2+} or CBX, the elevated medium glutamate concentration induced by HS stimulation was decreased significantly ($n = 5$, $*P < 0.01$ vs HS group, one way ANOVA).

Fig. 4 Quantitative fluorescence intensity of glutamate detected by FCM in the C6 cells of IS and HS group. The expression of glutamate was increased by HS stimulation and peaked at 1 min ($P < 0.01$ vs IS group), and decreased at 3, 5 and 10 min ($P < 0.01$ vs 1 min), though still much higher than that in IS group ($P < 0.01$).

Fig. 5 Quantitative fluorescence intensity of glutamate detected by FCM in the C6 cells of CBX+IS and CBX+HS group. In C6 cells pretreated with CBX, the elevated fluorescent intensity of glutamate signal induced by HS was markedly increased from 1 min to 10 min.

stimulation further increased markedly from 1 min to 10 min ($n = 5$, $P < 0.01$ vs HS, one way ANOVA, Fig. 5, Table 4).

In the CBX+IS groups, the glutamate intensity in cells did not change with time, and the mean value of the four time points was same as that in IS.

4 Discussion

Glutamate is a main excitatory transmitter distributed extensively in the central nervous system. It is important for functions of the brain. Neurons synthesize the glutamate, which was stored in the synaptic vesicles and released through exocytosis when neuronal terminals were activated by some stimuli. Glutamate is released into the synaptic cleft and signals to the glutamate receptors on neighbor neurons. Simultaneously, it is transported into pre-synaptic part or neighbor astrocytes, and the effect of glutamate is immediately eliminated.

The present study showed that when the cultured hypothalamic astrocytes were removed from isotonic medium to hypertonic medium, the expression of glutamate in astrocytes increased and peaked at 5 min, then decreased and returned to IS level at 15 min. After treatment by HS medium for 1 or 3 min, the concentration of glutamate in medium did not change; while after treatment for 5, 10 and 15 min, medium glutamate concentration increased significantly as compared with IS group. The changes of glutamate signal

within astrocytes and the changes of glutamate concentration in medium from 1 min to 15 min formed a sharp contrast. After treatment by HS for 10 and 15 min, glutamate signal in astrocytes decreased markedly; on the contrary, the concentration of glutamate in medium increased significantly. It indicated that HS stimulation induced cultured astrocytes to synthesize glutamate firstly and next to release glutamate into the medium.

Our previous research suggested that cultured hypothalamic astrocytes could sensitively in response to the changes of osmotic pressure, that is, the level of Cx43 signal increased when cultured astrocytes were removed from isotonic medium to hypertonic medium, or from hypertonic medium to isotonic medium^[15]. Saez *et al.*^[11] reported that hypertonic stimulation induced Cx43 hemichannels opening. They indicated that Cx43 hemichannels were opened when the isomotic pressure between the internal and the external cells lost their balance; on the contrary, the hemichannels were closed when the isomotic pressure between the internal and the external cells reached balance^[20].

CBX is a gap junction blocker and could close gap junctions or hemichannels^[21]. The results from the present study showed that when the astrocytes were pre-treated with CBX, the glutamate expression increased markedly and much higher as compared with HS group, but the glutamate concentration in medium was significant lower as compared with HS group.

The results suggested that CBX did not disrupt the astrocytes to synthesize glutamate, but may block the release of glutamate via closing Cx43 hemichannels.

It is well known that astrocytes release glutamate via many means, such as P2X₇ receptor^[6], exocytosis^[7,8], and Cx43 hemichannels^[9]. The first evidence for Ca²⁺-dependent release of glutamate from cultured astrocytes was shown by using HPLC, which displayed that bradykinin-evoked intracellular Ca²⁺ elevation in cultured astrocytes resulted in glutamate release^[7,8]. However, some reports showed that cultured astrocytes may also release glutamate through Cx43 hemichannels and which could be blocked by multivalent cations (Ca²⁺, Ba²⁺, Sr²⁺, Mg²⁺, and La³⁺) and gap junction blocking agents (CBX, octanol, heptanol, flufenamic acid, and 18 α -glycyrrhetic acid)^[9].

The results of the present study showed that the medium glutamate level decreased, while intracellular glutamate increased under high extracellular Ca²⁺ (1 000 μ mol/L) or CBX, suggesting that Cx43 hemichannels were closed and thus blocked glutamate release from cultured astrocytes or C6 cells. It is in accordance with the results from Ye *et al.*^[9]. We considered that the two results mentioned above are not contradictory, the one indicated that the elevation of intracellular Ca²⁺ in cultured astrocytes resulted in the release of glutamate^[7,8]; the other one indicated that the elevation of extracellular Ca²⁺ resulted in cultured astrocytes to close Cx43 hemichannels and block the release of glutamate^[9].

About the difference of time course of glutamate expression in cultured astrocytes and C6 cells, we considered it because the cell types and experimental methods used are different.

Acknowledgments: This work was supported by the National Nature Science Foundation of China (No. 39770251), the Medical Foundation of the People's Liberation Army, China (No. 01Z082, 06MA234), and the Foundation of the Fourth Military Medical University (No. 05ZXJM001). The authors gratefully thank Hui GAO (State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai, China) for HPLC technical assistance.

References:

- [1] Hussy N, Deleuze C, Desarmenien MG, Moos FC. Osmotic regulation of neuronal activity: a new role for taurine and glial cells in a hypothalamic neuroendocrine structure. *Prog Neurobiol* 2000, 62: 113-134.
- [2] Duan L, Yuan H, Su CJ, Rao ZR. Ultrastructure of junction areas between neurons and astrocytes in rat supraoptic nuclei. *World J Gastroenterol* 2004, 10: 117-121.
- [3] Ludwig M, Johnstone LE, Neumann I, Landgraf R, Russell JA. Direct hypertonic stimulation of the rat supraoptic nucleus increases *c-fos* expression in glial cells rather than magnocellular neurons. *Cell Tissue Res* 1997, 287: 79-90.
- [4] Yuan H, Duan L, Qiu Y, Gao LZ, Zhang P, Cao R, *et al.* Response of son astrocytes and neurons to hyperosmotic stimulation after carbenoxolone injection into the lateral ventricle. *Acta Anatom Sin* 2004, 35: 127-131. (Chinese, English abstract)
- [5] Parpura V, Basarsky TA, Liu F, Jęftinija, Jęftinija K, Jęftinija S, *et al.* Glutamate-mediated astrocyte-neuron signaling. *Nature* 1994, 369: 744-747.
- [6] Duan S, Anderson CM, Keung EC, Chen Y, Chen Y, Swanson RA. P2X₇ receptor-mediated release of excitatory amino acids from astrocytes. *J Neurosci* 2003, 23: 1320-1328.
- [7] Parpura V, Scemes E, Spray DC. Mechanisms of glutamate release from astrocytes: gap junction "hemichannels", purinergic receptors and exocytotic release. *Neurochem Int* 2004, 45: 259-264.
- [8] Montana V, Ni Y, Sunjara V, Hua X, Paupura V. Vesicular glutamate transporter-dependent glutamate release from astrocytes. *J Neurosci* 2004, 24: 2633-2642.
- [9] Ye ZC, Wyeth MS, Baltan-Tekkok S, Ransom BR. Functional hemichannels in astrocytes: a novel mechanism of glutamate release. *J Neurosci* 2003, 23: 3588-3596.
- [10] Bennett MV, Contreras JE, Bukauskas FF, Sáez JC. New roles for astrocytes: Gap junction hemichannels have something to communicate. *Trends Neurosci* 2003, 26: 610-617.
- [11] Saez JC, Contreras JE, Bukauskas FF, Retamal MA, Bennett MV. Gap junction hemichannels in astrocytes of the CNS. *Acta Physiol Scand* 2003, 179: 9-22.
- [12] Thimm J, Mechler A, Lin H, Rhee S, Lal R. Calcium-dependent open/closed conformations and interfacial energy maps of re-constituted hemichannels. *J Biol Chem* 2005, 280: 10646-10654.
- [13] Christ GJ, Day NS, Day M, Zhao W, Persson K, Pandita RK, *et al.* Increased connexin43-mediated intercellular communication in a rat model of bladder overactivity *in vivo*. *Am J Physiol Regul Integr Comp Physiol* 2003, 284: R1241-R1248.
- [14] Laskawi R, Rohlmann A, Landgrebe M, Wolff JR. Rapid astroglial reactions in the motor cortex of adult rats following peripheral facial nerve lesions. *Eur Arch Otorhinolaryngol* 1997, 254: 81-85.
- [15] Cao R, Lan L, Duan L, Zhang P, Xiong YF, Gao B, *et al.* The effects of osmotic pressure changing on connexins expression in cultured rat hypothalamic astrocytes and neurons. *Acta Anatom Sin* 2006, 37: 371-375. (Chinese, English abstract)
- [16] Qu WD, Peng XD, Wu DS, Xiao B. New method for the isolation of astroglial cells and oligodendrocytes. *J Hyg Res* 1999, 28:

- 263-266. (Chinese, English abstract)
- [17] Strange K, Emma F, Paredes A, Morrison R. Osmoregulatory changes in myo-inositol content and Na^+ /myo-inositol cotransport in rat cortical astrocytes. *Glia* 1993, 12: 35-43.
- [18] Qin M, Wang JJ, Cao R, Zhang H, Duan L, Gao B, *et al.* The lumbar spinal cord glial cells actively modulate subcutaneous formalin induced hyperalgesia in the rat. *Neurosci Res* 2006, 55: 442-450.
- [19] Saez JC, Retamal MA, Basilio D, Retamal MA, Bennett MV. Connexin-based gap junction hemichannels: gating mechanisms. *Biochim Biophys Acta* 2005, 1711: 215-224.
- [20] John SA, Kondo R, Wang SY, Goldhaber JJ, Weiss JN. Connexin-43 hemichannels opened by metabolic inhibition. *J Biol Chem* 1999, 274: 236-240.
- [21] Jellinck PH, Monder C, McEwen BS, Sakai RR. Differential inhibition of 11 β -hydroxysteroid dehydrogenase by carbenoxolone in rat brain regions and peripheral tissues. *J Steroid Biochem Mol Biol* 1993, 46: 209-213.

高渗刺激引起培养的大鼠下丘脑星形胶质细胞和 C6 细胞合成并释放谷氨酸

曹荣, 江山, 段丽, 熊鹰飞, 高蓓, 饶志仁

第四军医大学神经科学研究所, 西安 710032

摘要: **目的** 观察高渗刺激对培养的大鼠下丘脑星形胶质细胞或 C6 细胞合成、释放谷氨酸的影响。**方法** 获取一日龄 SD 大鼠下丘脑组织, 进行星形胶质细胞培养、纯化和鉴定。培养细胞随机分五组: (1)等渗组: 用新鲜等渗培养液置换原先的培养液, 将细胞分成 5 个亚组, 分别孵育 1、3、5、10 和 15 min。(2)高渗组: 用 320 mOsm NaCl 高渗溶液置换原先的培养液, 将细胞分成 5 个亚组, 分别孵育 1、3、5、10 和 15 min。(3)甘伯酸(carbenoxolone, CBX, 一种缝隙连接阻断剂)+等渗组 和(4)CBX+高渗组: 用含有 CBX(终浓度为 100 mmol/L)的等渗培养液作用 1 h, 后分别用等渗或高渗培养液置换出原培养液, 将细胞分成 5 个亚组, 分别孵育 1、3、5、10 和 15 min。(5) Ca^{2+} 高渗组: 用含有 Ca^{2+} (1 000 $\mu\text{mol/L}$)的等渗培养液作用 1 h, 后用高渗培养液置换出原培养液, 将细胞分成 5 个亚组, 分别孵育 1、3、5、10 和 15 min。每个亚组 5 个培养皿。收集各组的细胞, 进行抗谷氨酸和抗胶质原纤维酸性蛋白(Glial fibrillary acidic protein, GFAP)的双重免疫荧光染色, Confocal 显微镜观察。用反相高效液相色谱荧光测定法测定细胞培养液中谷氨酸的含量。培养的 C6 细胞分为四组: 等渗组, 高渗组, CBX+ 等渗组和 CBX+高渗组, 用于流式细胞仪(flow cytometry, FCM)定量检测细胞内谷氨酸的含量。**结果** 星形胶质细胞内抗 GFAP 染色的荧光强度在五组间无明显差异。星形胶质细胞内抗谷氨酸染色的荧光强度在等渗组中各时间点无明显变化, 高渗组中在刺激 1 min 后表达增加, 5 min 达到高峰, 15 min 恢复到正常。CBX+ 高渗组的抗谷氨酸染色荧光强度明显高于 CBX+ 等渗组和等渗组, 一直维持到 15 min 不下降。培养基中的谷氨酸浓度在等渗组各时间点无明显变化, 高渗组中谷氨酸浓度从 5 min 到 15 min 明显增加。 Ca^{2+} 高渗组和 CBX+ 高渗组中, 培养基中谷氨酸浓度明显低于高渗组, 各时间点之间无明显变化。FCM 测定的结果表明高渗或 CBX+ 高渗刺激引起 C6 细胞内谷氨酸的水平明显上调。**结论** 高渗刺激可以激发培养的大鼠下丘脑星形胶质细胞合成、释放谷氨酸, CBX 不能抑制其合成, 但可阻断其释放。

关键词: 星形胶质细胞; 高渗刺激; 谷氨酸; 免疫荧光染色; 反相高效液相色谱荧光测定法