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Calcium sensing receptor mediated the excessive generation of β-amyloid peptide induced by hypoxia in vivo and in vitro

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ABSTRACT

Hypoxia played an important role in the pathogenesis of AD. Hypoxia increased Aβ formation, then caused Alzheimer's disease. Calcium sensing receptor (CaSR) was involved in the regulation of cell growth, differentiation, hormonal secretion and other physiological function. Increasing evidence supported CaSR might play a more prominent role in susceptibility to AD, but the role of CaSR in Aβ overproduction induced by hypoxia and its mechanisms remain unclear. To investigate whether CaSR mediated the overproduction of Aβ induced by hypoxia, immunoblot and immunochemistry were employed to determine the expression of CaSR and BACE1 in hippocampal neurons and tissue and Ca^{2+} image system was used to measure $[Ca^{2+}]_i$ in hippocampal neurons. The content of Aβ was detected with ELISA kits. Our research found that hypoxia increased the expression of CaSR in hippocampal neurons and tissue and $[Ca^{2+}]_i$ in hippocampal neurons. Calhex 231, a selective blocher of CaSR, inhibited the increase in $[Ca^{2+}]_i$ induced by hypoxia. Hypoxia or $GdCl_3$, an agonist of CaSR, increased the expression of BACE1 in hippocampal neurons and tissue, but Calhex 231 or Xesto C (a selective inhibitor of IP_3 receptor) partly prevented hypoxia-induced BACE1 overexpression. Hypoxia or $GdCl_3$ increased the content of $A\beta_{42}$ and $A\beta_{40}$ in hippocampal tissue, however Calhex 231 or Xesto C prevented hypoxia-induced the overproduction of $A\beta_{42}$ and $A\beta_{40}$ partly. Based on the above data, we suggested that hypoxia increased $[Ca^{2+}]_i$ by elevated CaSR expression to promote BACE1 expression, thereby resulting in the overproduction of $A\beta_{42}$ and $A\beta_{40}$.

1. Introduction

With the life expectancy increasing, Alzheimer's disease (AD) shows a trend of sharp rise in the incidence of its attack. The pathogenesis of AD is still unclear and there is not effective measure to treat AD, so it is particularly important to study the pathogenesis of AD. Senile plaque (SP), one of AD pathology feature, was formed by the deposition of β-amyloid peptide (Aβ) [1]. Amyloid precursor protein (APP) is cleaved by β-secretase (β site APP cleaving enzyme, BACE) and γ-secretase to generate Aβ [2]. A rise in the APP and/or BACE and γ-secretase activity increases the formation of Aβ.

Hypoxia is a frequent pathological process and plays an important role in the pathogenesis of AD. Studies showed that hypoxic injury, such as cerebral ischemia and stroke, significantly increased in the incidence of AD [3–5]. Hypoxia caused the increase of cytoplasm calcium concentration ($[Ca^{2+}]_i$) [6,7], and elevated $[Ca^{2+}]_i$ promoted the formation of Aβ [8]. However, the mechanisms of Aβ overproduction induced by hypoxia are still unclear.

Calcium sensing receptor (CaSR) was firstly separated and cloned from bovine parathyroid gland [9], and identified as the G protein coupled receptor. Brown et al. reported the activation of CaSR triggered a rise of $[Ca^{2+}]_i$ and related signal transduction pathways to inhibit the secretion of parathyroid hormone, therefor feeding back to regulate extracellular calcium concentration when extracellular calcium concentration was increased [9]. CaSR was expressed not only in parathyroid tissue but also in neural tissue [10,11] and its other functions, such as regulation of cell growth, differentiation, apoptosis, gene expression and cell secretion, were

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continuously found [12–14]. Studies demonstrated that CaSR might play a more prominent role in susceptibility to AD among individuals that lacked an APOE4 allele [15], but it is still unclear about the role and mechanisms of CaSR in Aβ overproduction induced by hypoxia.

The present study explored the effect of hypoxia on CaSR expression and the role of CaSR in the change of $[Ca^{2+}]_i$ and BACE1 expression induced by hypoxia, and researched the effects of the change of CaSR expression and $[Ca^{2+}]_i$ on hypoxia-induced Aβ overproduction.

2. Materials and methods

2.1. Materials

Poly-L-lysine, xestospongin C (Xesto C), gadolinium(III) chloride ($GdCl_3$) were purchased from Sigma. Calhex 231, anti-CaSR rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology. Anti-GAPDH rabbit monoclonal antibody was purchased from Cell Signaling Technology. Anti-BACE1 rabbit polyclonal antibody was purchased from Abcam. BCA kit, PMSF and RIPA were purchased from Beyotime Institute of Biotechnology (Haimen, China). Fura-2-acetoxymethyl ester (Fura-2/AM), trypsin and Neurobasal/B27 were purchased from Invitrogen. DNase I and Cytarabine were purchased from Roche. Fetal bovine serum (FBS) and horse serum were purchased from Hyclone.

2.2. Cell culture and hypoxic treatment

SD rats were purchased from the Experimental Animal Center of Medical College of Sun Yat-Sen University. Hippocampal neurons was cultured and cellular hypoxic model was replicated in reference to our previous description [7,16].

2.3. Lateral cerebral ventricle injection and hypoxic treatment in rats

According to our previous report [17], 30 μM Calhex 231 or 10 μM Xesto C or 6 mM $GdCl_3$ was injected into lateral cerebral ventricle every other day, then SD rats were treated in a hypoxic chamber at 8% O_2/92% N_2 for 10 h/day for a month. After treatment, rats were anesthetized intraperitoneally with 7% chloral hydrate and the brains were removed for subsequent experiments. The experiments were performed as approved by the institutional animal care and use committee of medical college of Jinggangshan University.

2.4. Western blot

After treatment, hippocampal neurons and hippocampus were lysated by RIPA buffer, respectively, then total protein was extracted and protein concentration was measured with BCA kits. After boiled protein extraction for 10 min in loading buffer containing Tris–HCl 62.5 mM, 20% glycerol, 2% sodium dodecyl sulfate, 4% β-mercaptoethanol, and 0.02% bromophenol, pH 7.4, the samples were separated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then protein were transferred to nitrocellulose membranes and probed with CaSR (1:200), BACE1 (1:1000) and GAPDH (1:1000) antibody, respectively. After blocking with 5% (w/v) nonfat milk solution, the blots were developed with HRP-conjugated anti-rabbit IgG and visualized by enhanced chemiluminescence substrate system (Thermo Fisher Scientific Inc., USA). The protein bands were quantitatively analyzed with Image J software (National Institutes of Health, USA).

2.5. Measurement of $[Ca^{2+}]_i$ in hippocampal neurons

Isolated hippocampal neurons were inoculated on the circular slides coated with 0.04 mg/ml poly-L-lysine. The circular slides inoculated with hippocampal neurons were taken out 7 days after culture and fixed in the stainless steel tank. $[Ca^{2+}]_i$ measurement in hippocampal neurons was performed according to our previous descriptions [7].

2.6. Immunohistochemistry

A month after hypoxic treatment, rats were anesthetized by intraperitoneal injection with 7% chloral hydrate. They were fast perfused in situ by 100 ml physiological saline, then fixed with 300 ml phosphate buffer (0.1 mol/L, pH7.4) containing 4% paraformaldehyde. The brain was removed from the skull of the fixed animals and sliced coronally into 20 μm that contained hippocampus. The sections were treated with 0.3% H_2O_2 in absolute methanol for 30 min, then were blocked with sheep serum for 30 min at room temperature, followed by incubation with BACE1 antibody (1:100) at 4 °C overnight. The sections were incubated with secondary antibody for 1 h at room temperature, and developed with biotinylated horseradish peroxidase and diaminobenzidine system. The intensity of BACE1 was quantitatively analyzed using Image-Pro Plus Version 6.0 (Media Cybernetics, Md, USA).

2.7. Aβ sandwich ELISA assay

Rat hippocampus lysates were applied to ELISA as described previously [18]. In short, protein inhibitors and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma) were added into standards and samples to prevent degradation of Aβ. The content of $Aβ_{42}$ and $Aβ_{40}$ were quantified using a colorimetric ELISA kit (Biosource International, Camarillo, CA), following the manufacturer's protocols.

3. Results

3.1. The effects of hypoxia on CaSR expression in cultured hippocampal neurons and tissue

The expression of CaSR protein in cultured neurons increased to 1.96 ± 0.5 times of control group 4 h after hypoxic treatment ($p < 0.05$, n = 3, Fig. 1A). However, 2 h after hypoxic treatment for cultured neurons, CaSR protein expression was no significant difference compared with control group ($p > 0.05$, n = 3). In rats, a month hypoxic-treatment increased CaSR expression to 2.03 ± 0.48 times of control group ($p < 0.01$, n = 7, Fig. 1B). These data suggested that hypoxia could increase the expression of CaSR protein in hippocampal neurons and tissue.

3.2. The role of calcium sensing receptor in $[Ca^{2+}]_i$ elevation induced by hypoxia in hippocampal neurons

In order to explore the effect of hypoxia on $[Ca^{2+}]_i$ in neurons, calcium ion fluorescent imaging system ($Cell^R$-MT20) and fluorescence probe Fura-2/AM were used to detect $[Ca^{2+}]_i$. To investigate the role of CaSR in the change of $[Ca^{2+}]_i$ induced by hypoxia, neurons were treated with 3 μM Calhex 231, a specific blocker of CaSR.

4 h hypoxic processing made 340/380 ratio value rise from 0.43 ± 0.05 to 0.71 ± 0.15 ($p < 0.01$), up (66 ± 35) %, however 340/380 ratio value in calhex 231 + hypoxia group was 0.53 ± 0.09, and it was decreased by (25 ± 12) % ($p < 0.01$) compared with hypoxia group. Compared with control group, it still increased by (24 ± 20)

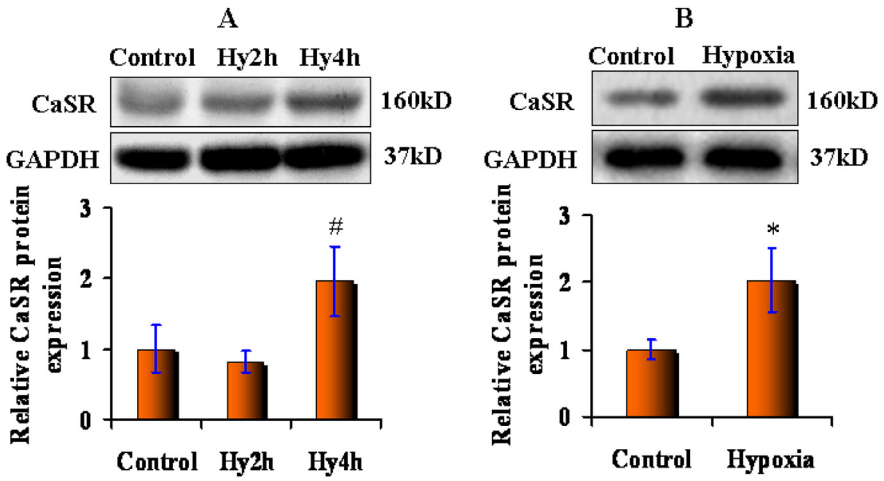

Fig. 1. The effects of hypoxia on CaSR protein expression in rat hippocampal neurons (A) and tissue (B). 7 d after rat hippocampal neurons being cultured, neurons were treated for 2 h (Hy2h) and 4 h (Hy4h) in hypoxic condition (2% O_2, 93% N_2, and 5% CO_2). SD rats (250 ± 20 g) were treated with 8% O_2/92% N_2 (hypoxia) for 10 h/day for a month. Western blot was employed to detect CaSR protein expression after treatment. Anti-CaSR rabbit polyclonal antibody (1:200) was used to specifically detect CaSR protein, and GAPDH was regarded as internal reference. #: $p < 0.05$, vs Control, n = 3. *: $p < 0.01$, vs Control, n = 7).

% ($p < 0.01$). These data indicated CaSR mediated the elevation of $[Ca^{2+}]_i$ induced by hypoxia. 340/380 Ratio value in Calhex 231 group was 0.45 ± 0.05 and there was no significant difference ($p > 0.05$) compared with control group (Fig. 2). Combining with the previous result, we suggested that hypoxia promoted CaSR expression to increase neuronal $[Ca^{2+}]_i$. Above mentioned results were from four independent experiment.

3.3. The effect of hypoxia on the expression of BACE1 in hippocampal neurons and the role of elevated CaSR expression and $[Ca^{2+}]_i$

The activation of CaSR increased IP_3 formation through phospholipase C (PLC) to promote endoplasmic reticulum Ca^{2+} release. To determine the effect of hypoxia on the expression of BACE1 in hippocampal neurons and the role of elevated CaSR expression and $[Ca^{2+}]_i$, Calhex 231 or Xesto C was employed to block CaSR or IP_3 receptor respectively, thereby inhibiting the increase in $[Ca^{2+}]_i$ induced by hypoxia and $GdCl_3$, an agonist of CaSR, was used to activate CaSR to increase $[Ca^{2+}]_i$. Western blot combining with anti-BACE1 antibidy was used to detect the expression of BACE1 in hippocampal neurons. The experiment was randomly divided into control, Calhex 231, Xesto C, hypoxia, $GdCl_3$, Calhex 231 + hypoxia and Xesto C + hypoxia seven group.

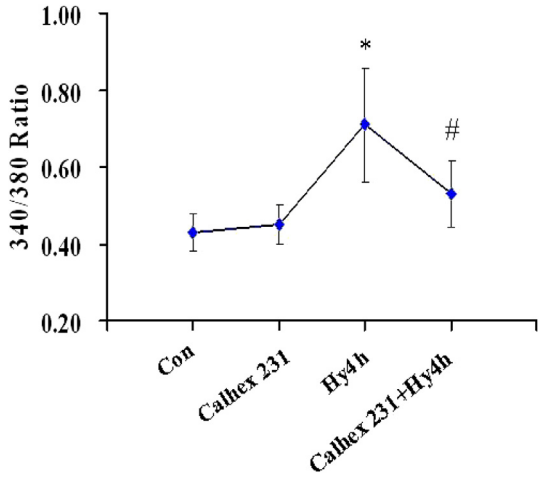

Fig. 2. The role of calcium sensing receptor in hypoxia-induced $[Ca^{2+}]_i$ elevation in hippocampal neurons. The circular slides inoculated with hippocampal neurons were taken out 7 days after neurons were cultured and fixed in stainless steel tank. After being washed with HBSS for 3 times, neurons were cultured with neurobasal/B27 culture media with 1μM Fura-2/AM for 50 min. Calcium ion imaging system was used to detect $[Ca^{2+}]_i$. *: $p < 0.01$, vs control, calhex 231 and calhex 231 + Hy4h; #: $p < 0.01$, vs control and calhex 231. Above results were from four independent experiment.

4 h after neurons were treated by hypoxia or 600 μM $GdCl_3$, the expression of BACE1 increased to 2.27 ± 0.29 and 2.36 ± 0.20 times ($p < 0.01$, n = 3) of control group, suggesting that hypoxia or $GdCl_3$ can promote BACE1 expression. But hypoxia made BACE1 expression increase to 1.59 ± 0.27 and 1.57 ± 0.18 times ($p < 0.01$, n = 3) of control group after treatment with 3 μM Calhex 231 or 1 μM Xesto C. Compared with hypoxia group, BACE1 expressions of Calhex 231 + hypoxia and Xesto C + hypoxia group were decreased significantly ($p < 0.01$, n = 3) (Fig. 3A). Immunoblotting data indicated that elevated CaSR expression and $[Ca^{2+}]_i$ mediated hypoxia-induced BACE1 overexpression.

3.4. The effect of hypoxia on the expression of BACE1 in hippocampus and the role of elevated CaSR expression and $[Ca^{2+}]_i$

In order to investigate the effect of hypoxia on the expression of BACE1 in hippocampus tissue, according to our previous report [17], immunohistochemistry was used to detect the expression of BACE1 in hippocampus. SD rats was assigned randomly to control, Calhex 231, Xesto C, hypoxia, Calhex 231 + hypoxia and Xesto C + hypoxia six group.

A month after hypoxia treatment for rats, optical density value with positive color was increased to 1.65 ± 0.25 times ($p < 0.01$, n = 8) of control group. This result suggested that hypoxia significantly increased the expression of BACE1. Calhex 231 or Xesto C reduced optical density values to 1.30 ± 0.18 ($p < 0.01$, n = 8) and 1.23 ± 0.15 times ($p < 0.05$, n = 8) of the control group, respectively. Compared with hypoxia group, they were significantly reduced ($p < 0.01$, n = 8) (Fig. 3B). These indicated that hypoxia elevated cytosolic calcium concentration through up-regulating the expression of CaSR, thereby promoting the expression of BACE1.

3.5. The effect of hypoxia on the content of Aβ in hippocampus and the role of elevated CaSR expression and $[Ca^{2+}]_i$

To determine the effect of hypoxia on the content of Aβ in hippocampus and the role of elevated CaSR expression and $[Ca^{2+}]_i$,

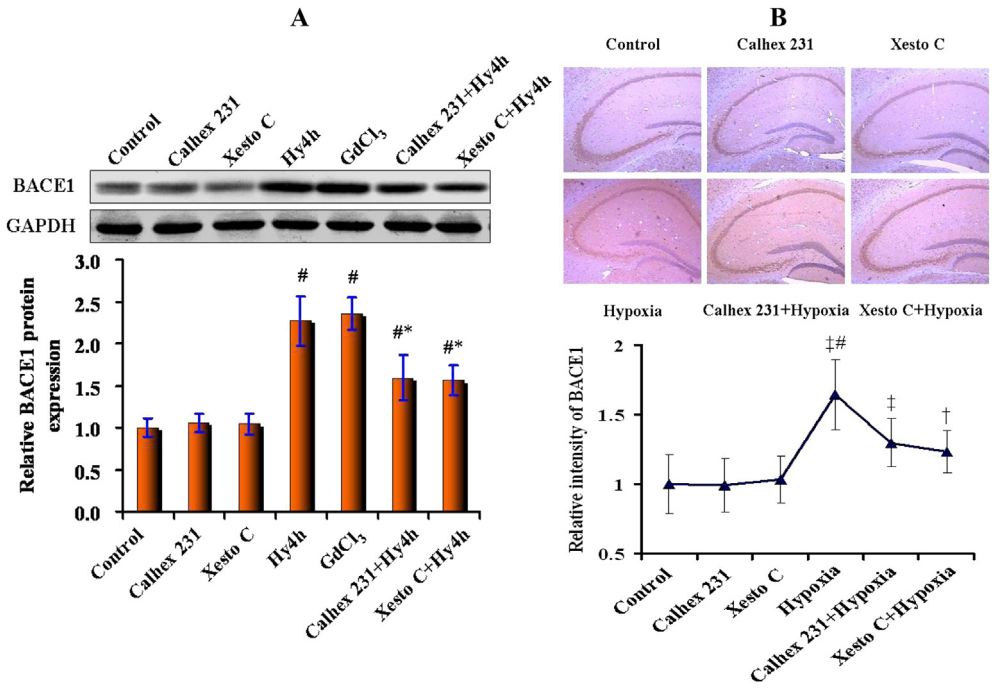

Fig. 3. The role of CaSR and $[Ca^{2+}]_i$ in the change of BACE1 expression induced by hypoxia. (A)Neurons were incubated for 4 h in hypoxic condition (2% O_2, 93% N_2, 5% CO_2, 37 °C) to establish hypoxic model after treatment with or without 3 μM Calhex 231 or 1 μM Xesto C, hypoxia, 600 μM $GdCl_3$. The expression of BACE1 was detected by western blot and anti-BACE1 rabbit polyclonal antibody (1:1000) and GAPDH was considered as internal reference. #: $p < 0.01$, *vs* control; *: $p < 0.01$, *vs* Hy4h, n = 3. (B)After having been treated with hypoxia for a month, rats were anesthetized and fixed with 4% paraformaldehyde. Then their brains were removed and fixed for 48 h again, then brains were sliced coronally into 20 μm that contained hippocampus. immunohistochemistry was used to detect the expression of BACE1. †: $p < 0.05$, *vs* control, ‡: $p < 0.01$, *vs* control, #: $p < 0.01$, *vs* Calhex 231 + hypoxia and Xesto C + hypoxia, n = 8, × 40.

Calhex 231 or Xesto C was employed to block CaSR and IP_3 receptor respectively, thereby inhibiting the increase in $[Ca^{2+}]_i$ induced by hypoxia and $GdCl_3$ was used to activate CaSR to increase $[Ca^{2+}]_i$. ELISA Kits were used to detect the content of $A\beta_{42}$ and $A\beta_{40}$ in hippocampus. SD rats was assigned randomly to control, Calhex 231, Xesto C, hypoxia, $GdCl_3$, Calhex 231 + hypoxia and Xesto C + hypoxia seven group.

A month after hypoxic treatment, the content of $A\beta_{42}$ increased to (222.2 ± 24.7) % ($p < 0.01$, n = 8) of control group, while that of Calhex 231 + hypoxia or Xesto C + hypoxia group were (173.4 ± 20.3)% and (166.0 ± 23.7)% ($p < 0.01$, n = 8) of control group, respectively, being significantly lower than that of hypoxia group ($p < 0.01$, n = 8). Treatment with $GdCl_3$ made the content of $A\beta_{42}$ increase to (206.1 ± 19.7)% ($p < 0.01$, n = 8) of control group, indicating that the activation of CaSR increased $A\beta_{42}$ generation. The content of $A\beta_{42}$ in Calhex 231 or Xesto C group had no significant change ($p > 0.05$, n = 8) compared with control (Fig. 4A).

As same as $A\beta_{42}$, a month after hypoxic treatment, the content of $A\beta_{40}$ increased to (226.9 ± 31.9) % ($p < 0.01$, n = 8) of control group, while that of Calhex 231 + hypoxia and Xesto C + hypoxia group were (167.1 ± 30.8)% and (172.2 ± 24.8)% ($p < 0.01$, n = 8) of control group, respectively, being significantly lower than that of hypoxia group ($p < 0.01$, n = 8). Treatment with $GdCl_3$ made the content of $A\beta_{40}$ increase to (232.7 ± 33.8)% ($p < 0.01$, n = 8) of control group, indicating that the activation of CaSR increased $A\beta_{40}$ generation. The content of $A\beta_{40}$ in Calhex 231 or Xesto C group had no significant change ($p > 0.05$, n = 8) compared with control (Fig. 4B). Combining with the above results, we suggested that hypoxia increased $[Ca^{2+}]_i$ by facilitating the expression of CaSR to promote BACE1 expression and Aβ formation.

4. Discussion

Extensive studies showed that hypoxia played a crucial role in the pathogenesis of AD [19–21]. Abnormal influx of calcium ion mediated axonal injuries in neurodegenerative diseases [22]. Ischemia and hypoxia could induce calcium overload, thereby leading to neuronal death [11]. In this and our prior studies, we incubated neurons in hypoxic gas mixtures to estabish hypoxic model and also found that hypoxia induced the increase of $[Ca^{2+}]_i$ [7].

The excessive production of Aβ aggregates was a key event in AD [23–26]. There were many factors to lead to Aβ generation and accumulation, such as genic mutation, environmental factor. Hypoxia was one of environmental factor. In vivo and in vitro data have demonstrated that hypoxia facilitated the generation and accumulation of Aβ. On one hand hypoxia up-regulated BACE1 gene expression by HIF-1a to increase β-secretase cleavage of APP [18,27]. On the other hand hypoxia dramatically increased anterior pharynx-defective-1A (APH-1A, a component of the γ-secretase complex) mRNA and protein expression through HIF-1a to promote γ-secretase cleavage of APP, at last facilitating the generation and accumulation of Aβ [28]. In this study, we also demonstrated that hypoxia increased the content of $A\beta_{42}$ and $A\beta_{40}$ through up-regulating BACE1 expression.

Imbalance in calcium homeostasis played an important role in abnormal generation and concentration of Aβ [29,30]. Treatment with calcium ionophore in cultured neurons increased cytoplasm calcium concentration and up-regulated BACE1 gene expression, resulting in Aβ overproduction, while calcium chelating agent inhibited the up-regulation of BACE1 gene expression [8]. But whether elevated cytoplasm calcium concentration mediates the

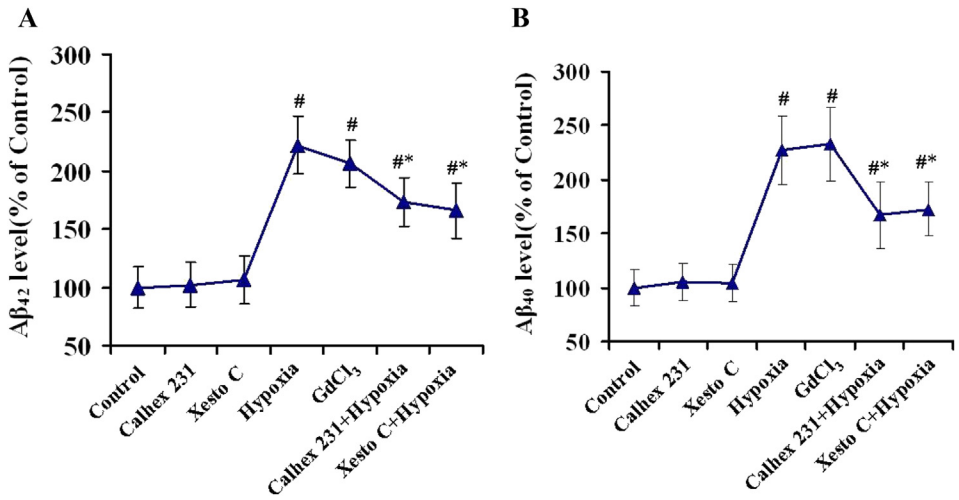

Fig. 4. The effect of hypoxia on the content of $A\beta_{42}$ and $A\beta_{40}$ in hippocampus and the role of elevated CaSR expression and $[Ca^{2+}]_i$. 30 μM Calhex 231 or 10 μM Xesto C or 6 mM $GdCl_3$ was injected into lateral cerebral ventricle every other day, then SD rats were treated with 8% O_2/92% N_2 (hypoxia) for 10 h/day for a month. Hippocampus isolated from brain was homogenated and centrifuged at 10000 g for 10 min and supernatant was used to detect $A\beta_{42}$ and $A\beta_{40}$ content with ELISA kits. #: $p < 0.01$, *vs* Control; *: $p < 0.01$, *vs* Hypoxia, n = 8.

up-regulation of BACE1 gene expression and Aβ overproduction, it remains unclear.

CaSR is widely distributed in the brain and believed to be involved in learning, memory, hormonal secretion and other physiological functions [14,31]. Research showed GaSR expression was up-regulated, but the inhibitory γ-aminobutyric acid-B receptor 1 (GABA-B-R1) was down-regulated, thereby to promote cell death when C57/BL6 mice were subjected to global cerebral ischemia [32]. However gene knockout for CaSR or blocking CaSR activation rendered robust neuroprotection and preserved learning and memory functions in ischemic mice [11]. In the present study, we observed that hypoxia promoted CaSR expression in hippocampal neurons and hippocampus and increased $[Ca^{2+}]_i$ in hippocampal neurons and the blocker of CaSR partly inhibited the increase of $[Ca^{2+}]_i$ induced by hypoxia, suggesting that hypoxia elevated $[Ca^{2+}]_i$ through promoting CaSR expression. At the same time, hypoxic treatment or CaSR activation increased BACE1 expression and the secretion of $A\beta_{42}$ and $A\beta_{40}$, however the inhibition of CaSR or IP_3 receptor partly prevented BACE1 overexpression and the overproduction of $A\beta_{42}$ and $A\beta_{40}$ induced by hypoxia. These results showed that hypoxia increased $[Ca^{2+}]_i$ by elevating CaSR expression to up-regulate BACE1 expression, resulting in the overproduction of $A\beta_{42}$ and $A\beta_{40}$, suggesting that CaSR might be a potential therapeutic target for AD.

Conflict of interest

None of the authors have any potential conflicts of interest or financial interests to disclose.

Acknowledgments

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