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ORIGINAL PAPER

Expression of Hypoxia Inducible Factor 1alpha Is Protein Kinase A-dependent in Primary Cortical Astrocytes Exposed to Severe Hypoxia

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Abstract
The hypoxia inducible factor 1 (HIF-1) and the cyclic AMP-responsive element binding protein (CREB) are two transcription factors that have been studied in the context of neuronal survival and neurodegeneration. HIF-1 upregulation and CREB activation have been observed not only in neurons but also in astrocytes under conditions of hypoxia. We hypothesized that activation of CREB regulate HIF-1α expression in the nucleus of cortical astrocytes under in vitro ischemic condition. To test the hypothesis, we determined the effects of inhibiting the CREB activation pathway on the expression of HIF-1α protein in astrocytes exposed to $CoCl_2$ and severe hypoxia (near anoxia, 0.1% O_2). The results demonstrated that inhibition of CaMKII and CaMKIV had no effect on both HIF-1α and pCREB expression in cortical astrocytes exposed to $CoCl_2$ and anoxia. In contrast, PKA inhibition lowered the expression of HIF-1α and pCREB expression. Furthermore, the inhibition of PKA but not CaMKII or CaMKIV increased cell death of astrocytes exposed to near anoxia. The results suggest that PKA plays an important role in the cell survival signaling pathways in astrocytes.

Keywords Brain ischemia · CREB · HIF-1 · PKA · CaMK

Introduction

Astrocytes are a significant component of the brain and furnish important support for brain functions [1]. They provide trophic support for neurons and maintain metabolite and electrolyte homeostasis. They release neuroactive substances including ATP, glutamine, neuropeptides, and growth factors. Glutathione homeostasis of neurons depends on astrocytes [2], and its depletion in astrocytes correlates with an increase in neuronal death. Moreover, astrocytes are an integral component of the neurovascular unit and regulate the blood–brain barrier function. Under pathological conditions, astrocytes are a critical player in protecting neurons from stress insults. Astrocyte activation is a common manifestation of ischemic brain injury and is vital in ischemic brain tissue protection and recovery [3, 4]. Astrocytes are known to be more resistant to ischemia than neurons. For example, Thoren et al. demonstrated that, after temporary ischemia, a portion of astrocytes within the ischemic core remains metabolically active in the early phases after reperfusion [5]. Most recently, Morizawa et al. demonstrated that astrocytes are transformed into a phagocytic phenotype as a result of increase in the ABCA1 pathway activity and contribute to remodeling of damaged tissues and penumbral networks [6]. Astrocytes have been suggested as a possible target for regenerative and rehabilitative strategies [7]. Results from an animal model of MCAO reported by Chen et al. showed recently that physical activity increased astrocyte proliferation and BDNF expression, which may contribute to the improved neurological function of animals subjected to stroke [8].

Hypoxic/ischemic insults cause activation of adaptive mechanisms and alteration of gene expression within the injured areas to combat the progression of pathological events. Hypoxia inducible factor 1 (HIF-1) is a transcriptional factor that enables rapid adaptation to altered oxygen tensions and has been suggested as a target for potential stroke treatment. There are several members in the HIF

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family, and HIF-1 has been suggested the most effective for neuronal protection by regulating many growth factors such as erythropoietin [9]. Expression of HIF-1α, the regulatable subunit of HIF-1, in neurons has been the focus of study in ischemic stroke [10–12]. Recently, Hirayama et al. demonstrated that HIF-1α was increased in not only neurons but also astrocytes after preconditioning and that astrocytic HIF-1α was essential for the induction of ischemic tolerance [13, 14]. Another study also demonstrated that HIF-1 inhibition decreased reactive astrocyte formation and was a major regulatory gene that controlled multiple downstream genes [e.g. vascular endothelial growth factor (VEGF), nestin, and glial fibrillary acidic protein (GFAP)] associated with the formation of astrogliosis [15]. However, there is a lack of study on factors that affect HIF-1 expression in astrocytes.

The transcription factor cAMP-responsive element binding protein (CREB) is a component of intracellular signaling pathways that regulate a wide range of biological functions such as neuronal plasticity and cell survival. CREB is a phosphorylation-dependent transcription factor and is activated by many kinases such as cAMP-dependent PKA and Ca^{2+}/calmodulin-dependent protein kinases (CAMK) on the Serine 133 [16]. CREB activation represents an important pathway of cellular defense against ischemic and oxidative stress. It was reported that cerebral ischemia induced the expression of pCREB in vulnerable neurons of the hippocampal CA1 sector and penumbral region [17, 18], supporting a role for transcriptional activation by CREB in neuronal survival following ischemia. Recently, Guirao et al. reported that pharmacological agents that increased pCREB levels in non-GABAergic neurons after in vitro ischemia produced long-lasting neuronal survival [19]. Sharma et al. demonstrated that an increase in pCREB resulted in a better performance in Morris Water Maze Task of hypoxic animals [20]. The activation of CREB is not confined to neurons. Astrocytes are among the cells demonstrating CRE-mediated gene expression in an ischemic brain [18, 21]. Interestingly, HIF-1α and CREB seem to interact with each other to turn on gene expression for cellular protection. For example, both CREB and HIF-1α interact with CREB-binding protein (CBP)/p300, and CBP/p300 acts like a physical bridge between CREB and HIF-1α [22]. Activated CREB (pCREB) also binds to hypoxia response element and initiates transactivation [23]. Furthermore, it was shown that high glucose-induced HIF-1α and VEGF expression in cultured rat retinal Müller cells depended on phosphorylation of CREB [24]. The interaction between HIF-1α and pCREB has been implicated in inducing VEGF expression in prostate cancer bone metastasis and transactivating murine neuroblastic glucose transporter [25, 26]. To identify factors that regulate astrocyte adaptation to ischemia, we investigated the effect of several CREB activation pathways on the expression of HIF-1α in primary astrocytes.

Materials and Methods

Primary Astrocyte Culture

Primary astrocytes were cultured from brains of Sprague–Dawley rats [postnatal day 1 (P1) to P3] as described previously [27]. Briefly, cortical tissues were treated with trypsin (Sigma type XI) for 50 min at 37 °C and dispersed by trituration. Cells were plated into tissue culture flasks and maintained at 37 °C in culture medium (DMEM/10% FBS) in a humidified 5% CO_2/95% air atmosphere. Once cultures became confluent (7–10 days), the flasks were shaken at 300 rpm at 37 °C in air, initially for 2 h (h) and then after a complete exchange of medium for 18–20 h (repeated three times). The remaining cells were detached using trypsin/EDTA and plated on coverslips coated with poly-D-lysine. Purified astrocytes were used 10–15 days in vitro (DIV). This procedure eliminated weakly adhesive cells including neurons and microglia. Staining with an anti-GFAP antibody (see below) indicated that 85% of the cells were intensely GFAP immunoreactive (GFAP-ir). The lack of neurons in these cultures was confirmed by the absence of immunoreactivity of microtubule-associated protein (MAP), a neuronal dendritic marker. As a positive control, we confirmed that the MAP antibody stained dendritic processes in glial–neuronal co-cultures (data not shown).

Near Anoxia (n-Anoxia) and $CoCl_2$ Treatments

After culture medium was replaced to fresh serum free DMEM, astrocytes were exposed to 0.1% oxygen in a hypoxia glove box (Coy) at 37 °C for 3 h. For normoxic treatments, astrocytes were exposed to 21% O_2 at 37 °C for 3 h. For $CoCl_2$ treatments, astrocytes were incubated with 0.3 mM $CoCl_2$ at 37 °C for 3 h under normoxic condition [28]. For pharmacological treatments, before n-anoxia or $CoCl_2$ treatments astrocytes were treated with H89 (5 μM, PKA inhibitor, Tocris), K93 (20 μM CaMKII inhibitor, Tocris), or STO-609 (2 μg/ml, CaMKIV inhibitor, Tocris) in DMSO (< 0.15%) for 30 min.

In Vitro Immunocytochemistry

After a brief wash with phosphate buffered saline (PBS), cells were fixed with 4% paraformaldehyde in PBS at room temperature (RT) for 20 min and permeabilized with 0.3% Triton X-100 in PBS for 15 min. To block nonspecific binding, cells were exposed to blocking solution (PBS containing 0.05% Triton X-100 and 0.25% BSA) for 30 min and incubated with primary antibody overnight at 4 °C. Then, cells were washed in blocking solution (four times for 15 min) and incubated with a secondary antibody for 90 min at RT. After washing with blocking solution and PBS, the coverslips were mounted with Vectashield (Vector Laboratories).

Primary antibodies used were mouse anti-GFAP (1:600; MAB3402; Millipore Bioscience), goat anti-HIF-1α (1–100; sc-8711; Santa Cruz), mouse anti-MAP2 (1:200; MAB378; Millipore Bioscience), rabbit anti-pCREB (Cell Signaling Tech.), and mouse anti-GFAP (1–200; Millipore Bioscience). Secondary antibodies were donkey anti-goat Alexa 488 (1–100; Molecular Probes), donkey anti-mouse TRITC (1–50; Jackson ImmunoResearch) and donkey anti-rabbit daylight 549 (1–2500; Rockland). For double-staining experiments, antibodies were applied sequentially or together. For antibody specificity, goat anti-HIF-1α antibody was incubated with HIF-1α blocking peptide (1–100; Santa Cruz) for 2 h at RT before staining the neurons for HIF-1α. Control experiments showed there was no significant bleed-through of the fluorescent labels or cross-reactivity between antibodies. Images were obtained with a Leica DMI4000 microscope with a ×40 and a Leica DFC340 FX Digital camera using Leica LAS AF software. For quantification, the intensity of HIF-1α and pCREB in the nucleus was randomly counted using GFAP positive cells. Image-Pro Plus 5.1 (Media Cybernetics), OriginPro7, and Excel were used for data analysis.

Immunoblotting

Cells were washed with ice cold PBS and lysed using 200 µl of RIPA lysis buffer (RadioImmunoPrecipitation, Thermo Scientific, Meridian, IL) with a cocktail of protease inhibitors. Cell lysates were sonicated for 60 s and centrifuged at 12,000 rpm and 4 °C for 10 min. Supernatants were carefully discarded, and then pellets were re-suspended in 100 µl lysis buffer. The protein concentration was measured by the Bio-Rad DC protein assay reagent (Bio-Rad, Hercules, CA). An equal volume of protein was resolved on SDS–polyacrylamide gel (6% for HIF-1α or 10% for GFAP, pCREB and CREB) using electrophoresis over 60 min at a constant voltage of 100 V. The samples were then electrophoretically transferred to a nitrocellulose membrane (Type NC, 0.45-µm pore size) using a blotting apparatus. Nonspecific binding sites were blocked overnight with wash buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM sodium azide, and 20% Tween 20, TBST) containing 5% (w/v) fat-free dry milk powder at 4 °C. Primary antibody [mouse anti-HIF-1α (610959, BD Biosciences, San Jose, CA), mouse anti-GFAP (MAB 3402, Millipore), rabbit anti-CREB (86B10, Cell Signaling), rabbit anti-pCREB (S133, Cell Signaling), and rabbit anti-ß-actin (sc-1616, Santa Cruz)] was dissolved in TBST, incubated for 4 h at RT, and then washed with TBST containing 5% non-fat dry milk for three times with 20 min interval. Secondary antibody (see details in previous paragraph) was added and incubated for 90 min at RT. The signal development was carried out with an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). The intensity of immunoreactive bands was quantified using Image-J. All the bands were normalized to ß-actin.

Cell Death Analysis

Astrocyte viability was analyzed by using two methods. (1) The release of lactate dehydrogenase (LDH) was measured by using the Takara Cytotoxicity Detection Kit. (2) Hoechst and propidium iodide (PI) were used to detect cell death by imaging the penetration of PI into the cells and binding to the nucleus.

Statistical Analysis

One-way ANOVA and student's *t* test were used for overall significance. Data were presented as mean ± SD from minimum n = 3 separate experiments. Difference was considered significant at $p < 0.05$.

Results and Discussion

Inhibition of PKA Activity Attenuates the Expression of HIF-1α Induced by Cobalt

First, we carried out experiments to determine if $CoCl_2$ can increase expression of the protein in primary astrocytes. $CoCl_2$ is well known for HIF-1α protein stabilization and translocation to the nucleus. Figure 1 shows that HIF-1α expression was upregulated and co-localized with astrocytes (GFAP-ir) in a primary cortical astrocyte culture after $CoCl_2$ treatment. Quantification analysis showed that there was 4.85-folds increase in HIF-1α in the astrocytes treated with $CoCl_2$ when compared to control.

Next, we addressed the major question by inhibiting the pathways that activate CREB in $CoCl_2$-treated astrocytes. Many pathways have been revealed to phosphorylate CREB, including the PKC/MEK, Ca^{2+}/CaMKII, Ca^{2+}/CaMKIV, and cAMP/PKA. Excitotoxicity is a hallmark of the pathogenesis of ischemic stroke. Elevated Ca^{2+} levels would activate CaMKII and CaMKIV. PKA has been shown to be involved in the phosphorylation of HIF-lα in endothelial cells under intermittent hypoxia [29]. Moreover, Kvietikova et al. reported that the PKA, but not the PKC signaling pathway is involved in oxygen sensing [30] and that the PKA-signaling pathway enhances oxygen-dependent gene expression via the HRE [31]. Thus, we focused on the PKA and CaMK pathways in this study. The CaMKII, CaMKIV, and PKA pathways were inhibited by K93, STO-609, and H89, respectively. Astrocytes were pretreated with the inhibitors and incubated for 3 h in the presence of $CoCl_2$ under normoxia at 37 °C. The cultures were immunostained for HIF-1α and GFAP. The CaMKII and CaMKIV inhibitors, K93 and STO-609, showed little effect on the level of nuclear HIF-1α protein expression, compared to $CoCl_2$ treatment alone (Fig. 2a). In contrast,

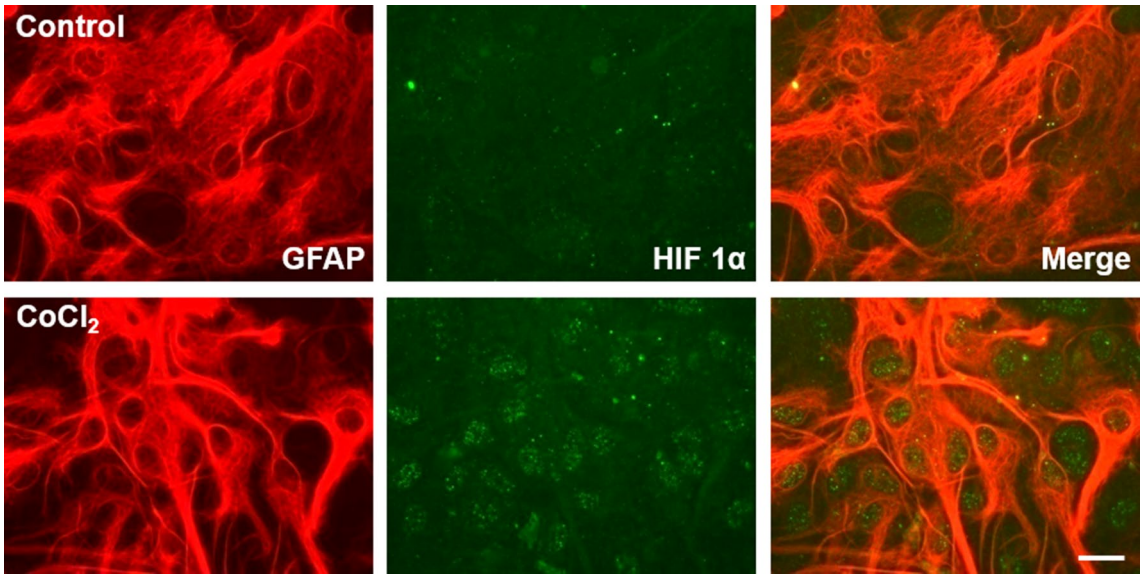


Fig. 1 Astrocytes expressed HIF-1α protein after $CoCl_2$ treatment. Astrocytes were treated with and without $CoCl_2$ for 3 h under normoxia. Immunocytostaining analysis was carried out to determine HIF-1α protein expression (green) in GFAP (red, astrocyte marker) positive cells. Scale bars 20 µm. (Color figure online)

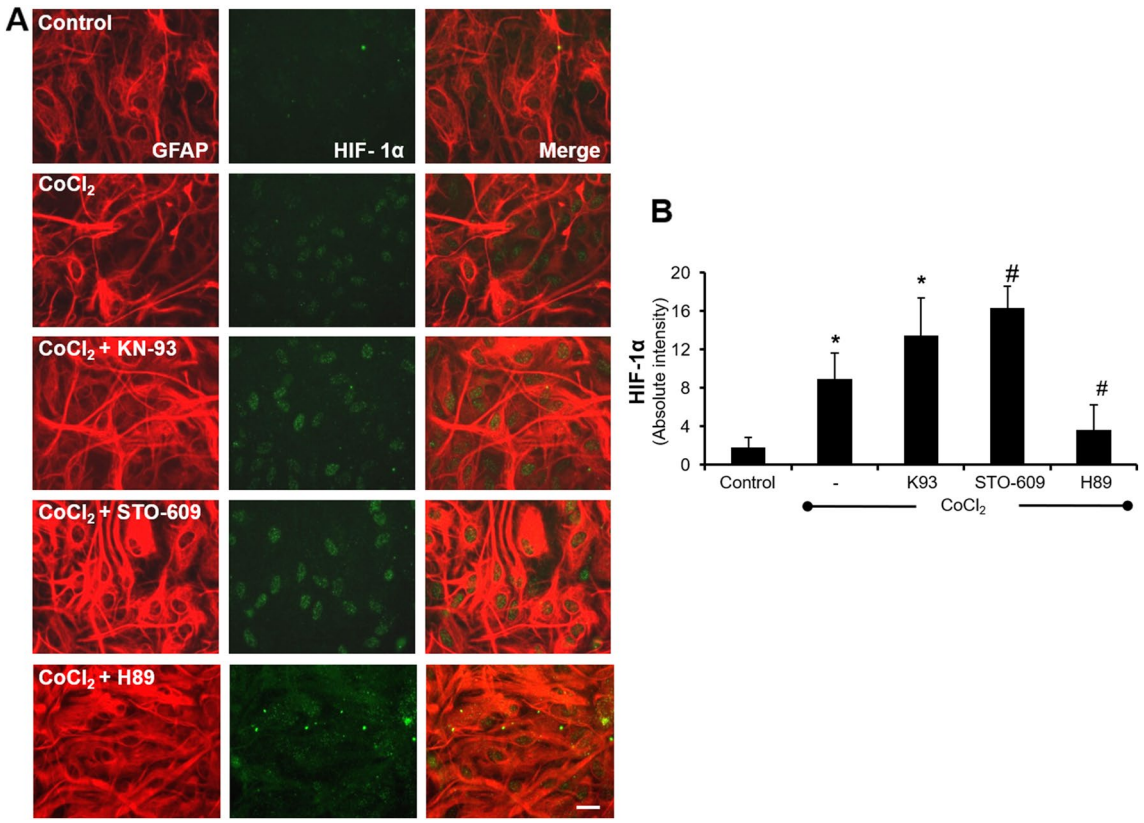


Fig. 2 PKA inhibitor attenuates HIF-1α expression in primary cortical astrocytes exposed to $CoCl_2$. Astrocytes were treated with K-93 (CaMKII inhibitor), STO-609 (CaMKIV inhibitor), or H89 (PKA inhibitor) for 1 h prior to $CoCl_2$ incubation for 3 h. **a** Representative images of expression of HIF-α protein and GFAP. **b** Quantification data of the staining intensity of HIF-1α. Values are mean ± SD (n = 3 separate experiments). $^{*}p < 0.05$ versus normoxia; $^{\#}p < 0.05$ versus n-anoxia. Scale bars 20 µm

the PKA inhibitor, H89, decreased HIF-1α expression in the nuclei of astrocytes, compared with $CoCl_2$, K93, or STO-609. In addition, we also tested effects of the inhibitors on HIF-1α expression in the absence of $CoCl_2$. We didn't observe any difference in the level of HIF-1α expression among all the treatments, compared to control cells (without any treatment).

In addition, as CREB protein needs to be phosphorylated to initiate transcriptional activities, we investigated the effect of the three inhibitors on the level of pCREB. As shown in Fig. 3, expression of pCREB co-localized with HIF-1α in astrocytes treated with $CoCl_2$. When astrocytes were pre-treated with K93 and STO-609, similar to the above results of HIF-1α expression, there was little effect on the level of pCREB protein. Furthermore, similar to the effect of H89 on the level of HIF-1α expression, pre-treatment of H89 caused a significant decrease in pCREB. This result is evident that the PKA pathway is involved in HIF-1α expression and CREB activation in astrocytes treated with $CoCl_2$.

Inhibition of PKA Decreases HIF-1α Expression in Astrocytes Exposed to Oxygen Deprivation

In hypoxic conditions, it is known that astrocytes activate various signaling pathways for their own protection and to offer protection for neurons. During such conditions, it is still not known what signaling mechanism is required for HIF-1 activation in astrocytes. Based on the above results, we hypothesized that activation of the PKA/CREB might be involved in the upregulation of HIF-1α expression. To test the hypothesis, we treated astrocytes with 0.1% O_2 (n-anoxia) and analyzed the expression of HIF-1α and pCREB using immunocytochemistry. We observed that n-anoxia dramatically increased the level of expression of HIF-1α in astrocytic nuclei, compared to 21% O_2. No changes were observed in HIF-1α expression under n-anoxia in the presence of K93 and STO-609, compared to n-anoxia. In contrast, H89 significantly decreased HIF-1α expression in the astrocytes exposed to n-anoxia (Fig. 4a). Quantification analysis demonstrated that H89 almost reduced the level of HIF-1α protein expression to that at normoxia (Fig. 4b).

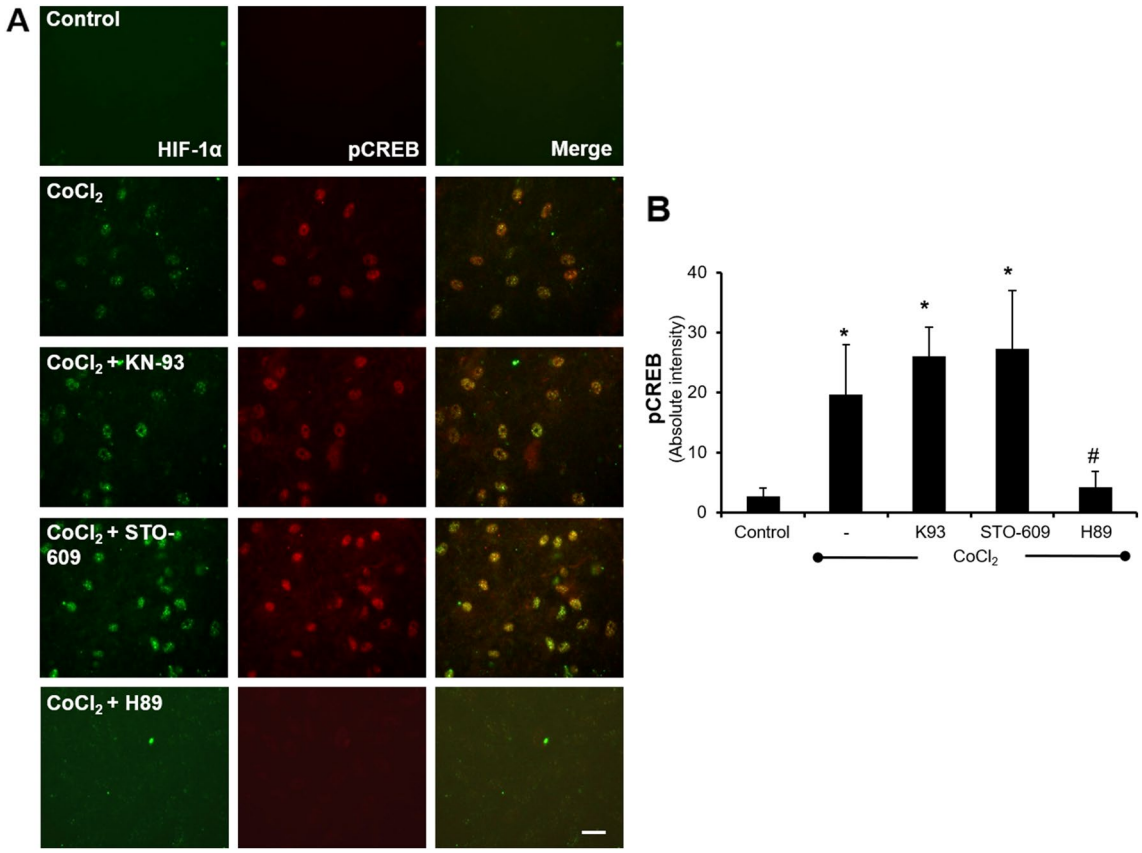


Fig. 3 Effect of PKA inhibition on the expression of pCREB and HIF-1α in astrocytes exposed to $CoCl_2$. Astrocytes were treated with K-93 (CaMKII inhibitor), STO-609 (CaMKIV inhibitor), or H89 (PKA inhibitor) for 1 h prior to $CoCl_2$ incubation for 3 h. **a** Representative images of expression of HIF-α protein and GFAP. **b** Quantification data of the staining intensity of pCREB. Values are mean ± SD (n = 3 separate experiments). *p < 0.05 versus normoxia; #p < 0.05 versus n-anoxia. Scale bars 20 μm

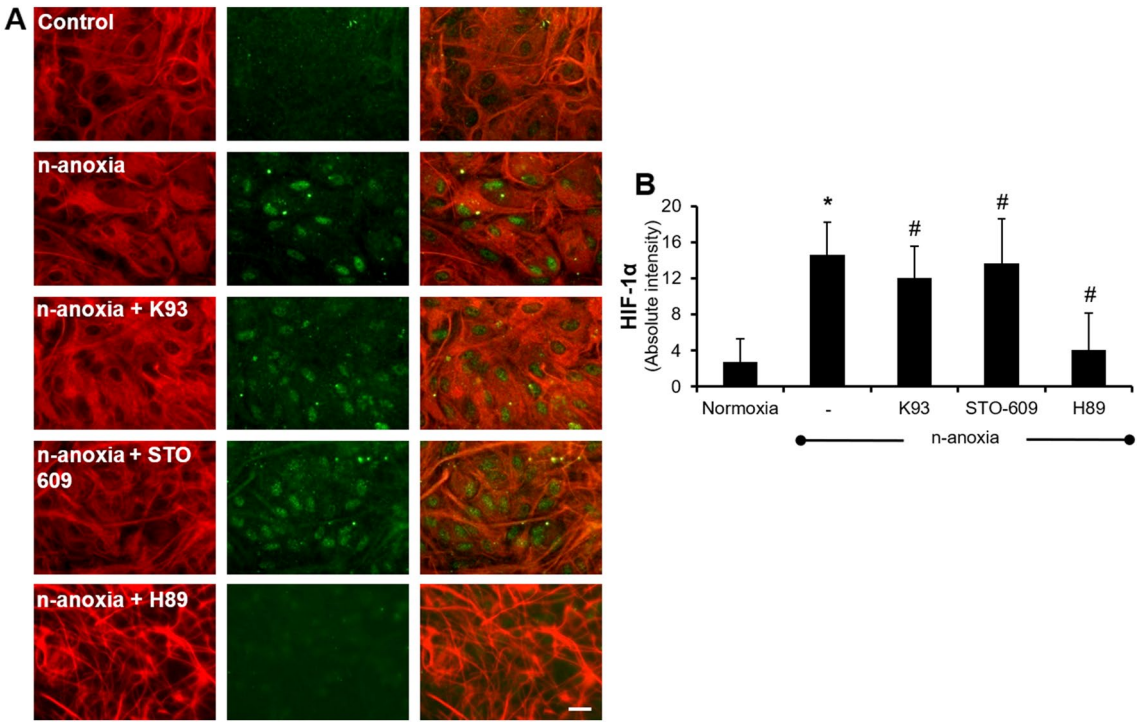


Fig. 4 Inhibition of PKA decreased the expression of HIF-1α protein under n-anoxia. Astrocytes were treated for 1 h in the presence of K-93 (CaMKII inhibitor), STO-609 (CaMKIV inhibitor), or H89 (PKA inhibitor) prior to 3 h of n-anoxia (0.1% O_2). **a** Representative images of immunostaining of HIF-α protein and GFAP. **b** Quantification data of the staining intensity of HIF-1α. Values are mean ± SD (n = 3 separate experiments). *p < 0.05 versus normoxia; #p < 0.05 versus n-anoxia. Scale bars 20 µm

Next, we determined if pCREB co-localized with HIF-1α in the presence of K93, STO-609, and H89 under n-anoxia. Figure 5a shows that both pCREB and HIF-1α expressions were observed in the same astrocytic nuclei under n-anoxia. Both K93 and STO-609 had no effect on the pCREB expression in the astrocytes exposed to n-anoxia. Quantification analyses of the immunostaining confirmed the significant increase in pCREB-ir under n-anoxia, K93 + n-anoxia and STO-609 + n-anoxia, compared to normoxia. In contrast, decrease in pCREB-ir was observed in H89 treatment under n-anoxia (Fig. 5b). These data suggest that the expression of HIF-1α under n-anoxia required PKA-dependent pathway, not CaMKII or CaMKIV.

For further confirmation, we performed immunoblotting to analyze the protein levels of HIF-1α, pCREB, CREB, and GFAP. Figure 6a shows that there is a significant increase in HIF-1α band intensity under n-anoxia and further increase was observed when the cells were treated with K93 and STO-609. In contrast, increase in the HIF-1α band intensity was attenuated by H89 treatment under n-anoxia. Activation of CREB (pCREB) also showed increase in n-anoxia. This activity was inhibited by H89 treatment but not by K93 or STO-609. Next, we wanted to know whether this increase in pCREB activity was due to increase in CREB protein under n-anoxia treatment. The results (Fig. 6c) showed that the level of CREB protein did not show any significant changes under n-anoxia or any of the drug treatments, compared to normoxia.

The results from these studies demonstrated that PKA facilitated HIF-1α expression and CREB activation but not CaMKII and CaMKIV (Scheme 1). The current study can't confirm if PKA increases HIF-1α and pCREB parallely or serially. HIF-1 has two subunits, HIF-1α and HIF-1β. Both subunits are basic-helix-loop-helix proteins of the PAS family. Different from HIF-1β that is relatively stable and is not affected by oxygen levels, HIF-1α protein expression is oxygen dependent. In normal physiological condition, HIF-1α is hydroxylated by prolyl-4-hydroxylase domains (PHD), ubiquitinated, and rapidly degraded by the 26S proteosomal degradation pathway [32]. Under low oxygen conditions, PHD activity is inhibited. As a result, HIF-1α is stabilized, is translocated to the nucleus, binds to HIF-1ß, and initiates the transcriptional process to increase gene expression for cell survival. Besides oxygen, there are other factors that can reduce HIF-1α protein degradation. A recent study has shown that PKA phosphorylated Thr63 and Ser692 on HIF-1α and enhanced HIF transcriptional activity in HeLa cells and rat cardiomyocytes [33]. PKA

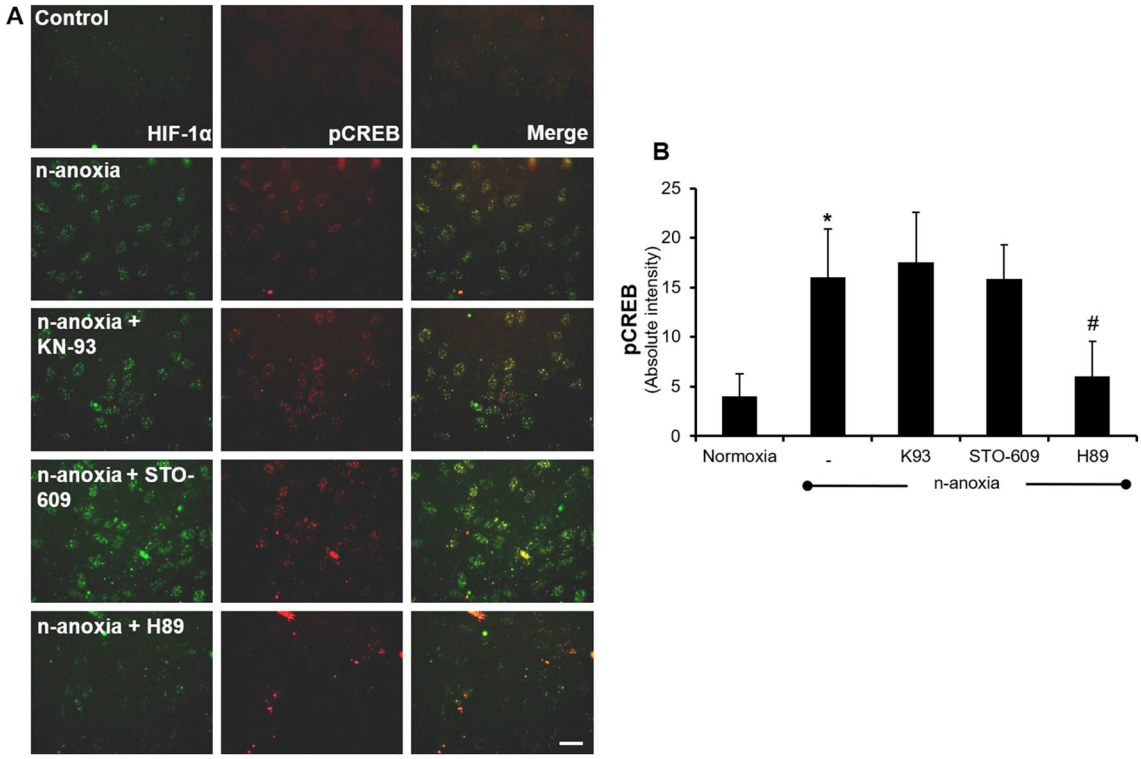


Fig. 5 Co-localization of HIF-1α and pCREB in astrocytes under n-anoxia. Astrocytes were treated for 1 h in the presence of K-93 (CaMKII inhibitor), STO-609 (CaMKIV inhibitor), or H89 (PKA inhibitor) prior to 3 h of n-anoxia (0.1% O_2). **a** Representative images of immunostaining of HIF-α protein and pCREB. **b** Quantification data of the staining intensity of pCREB. Values are mean ± SD (n = 3 separate experiments). *$p < 0.05$ versus normoxia; #$p < 0.05$ versus n-anoxia. Scale bars 20 μm

could inhibit the proteasomal degradation of HIF-1α in an O_2-independent manner that required the phosphorylation of Thr63 and Ser692. PKA may also be able to counteract the inhibitory effect of asparaginyl hydroxylation on the association of p300 with HIF-1α and thus enhance their binding. Moreover, it has been shown that PKA may act in the cytoplasm and not via CREB to promote survival of spin ganglion neurons [34]. Thus, PKA may upregulate HIF-1α protein levels in the astrocytes, independent from pCREB. In addition, it has been reported that unlike neurons, astrocytes may not depend on PHD activity inhibition to increase in HIF-1α. Instead, a P2 × 7 receptor-mediated pathway may allow astrocytes to cause long-lasting HIF-1α expression [14].

In addition, we determined effects of the three inhibitors on cell survival of astrocytes exposed to n-anoxia as both HIF-1 and pCREB initiate gene expressions for cell survival. For example, neuronal deficiency of PHD2 in mice improves ischemic stroke recovery in an HIF dependent manner [11, 12]. Activation of CREB regulates the expression of several important neuroprotective genes, such as BDNF, c-*fos*, and Bcl-2. Those genes are upregulated in the penumbra after cerebral ischemia [21]. Activation of CREB rescued vulnerable CA1 pyramidal neurons [35]. We first examined the changes in GFAP protein during n-anoxia treatment. There is no significant change in the GFAP protein under n-anoxia or K93, STO-609, or H89 treatment. We then examined cell death by using PI staining. Example images shows no significant difference in cell death in the presence K93 and STO-609 (Fig. 7). In contrast, significant amount of PI staining was observed when the cells were treated with H89 prior to n-anoxia. Similar results were observed with LDH assay. The results confirmed that PKA inhibition caused cell death by downregulating HIF-1α and pCREB expression.

The focus of the current research was on the molecular pathway of CREB phosphorylation and the HIF-1α expression in astrocytes exposed to low oxygen. The mechanisms of CREB activation in ischemic neurons and brain endothelial cells may be different from that in the astrocytes. For example, CaMK II–IV is involved in CREB phosphorylation and protective response in neurons after metabolic stress [36], which is different from our observation in astrocytes exposed to near anoxia. In addition, VEGF was reported to be able to activate CREB in brain vascular

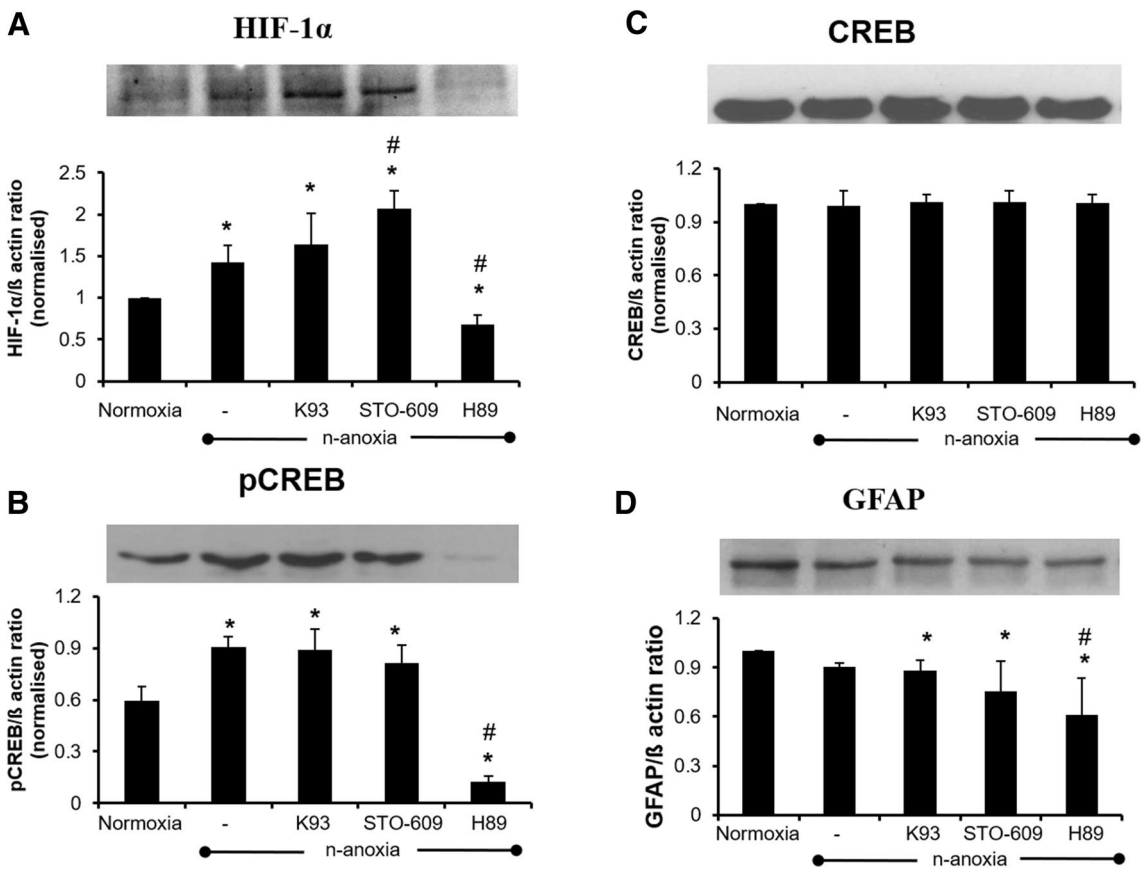
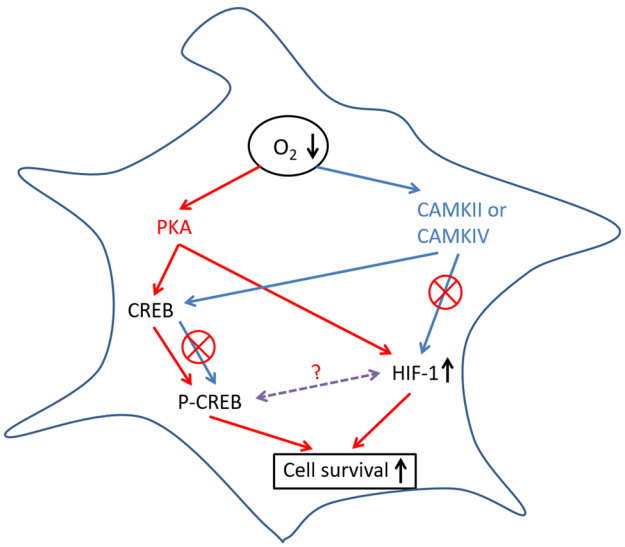


Fig. 6 Effect of pCREB inhibition on the expression of HIF-1α and pCREB in astrocytes. Astrocytes were treated for 1 h in the presence of K-93 (CaMKII inhibitor), STO-609 (CaMKIV inhibitor), or H89 (PKA inhibitor) prior to 3 h of n-anoxia (0.1% O_2). Protein levels were determined by western blotting. **a** The expression of HIF-α protein. **b** The expression of pCREB protein. **c** The expression of CREB protein. **d** The expression of GFAP protein. Values are mean ± SD (n = 3 separate experiments). *p < 0.05 versus normoxia; #p < 0.05 versus n-anoxia

endothelial cells exposed to hypoxia [37]. It warrants further investigation whether the PKA-dependent activations of HIF-1α and pCREB are specifically presented in the astrocytes or also existed in neurons or brain microvascular endothelial cells.

Furthermore, activation of the PKA-pCREB pathway has been demonstrated to be highly protective in animal models of hypoxia and stroke. Lee et al. reported that rolipram that promotes PKA-pCREB pathway protected rat pups from ischemic brain injury at behavioral and pathological levels by sustained increased CREB phosphorylation [37]. Qu et al. suggested that enhanced structural plasticity and the behavioral recovery promoted by post-stroke forced limb-use was mediated through the cAMP/PKA/CREB signal transduction pathway [38]. Liu et al. found that the cAMP/PKA/pCREB pathway contributed to Gadd45b-stimulated recovery after stroke [39]. In addition, Tanaka demonstrated that elevated binding activity of PKA to cAMP and CREB phosphorylation in the peri-ischemia area during the post-ischemic recirculation were associated with neuronal survival [40]. In those



Scheme 1 Proposed molecular regulations of the activation of HIF-1 and pCREB in ischemic astrocytes. The proposed mechanism is based on the effects of PKA, CaMKII, and CaMKIV inhibitors on CREB phosphorylation and the protein level of HIF-1α in astrocytes exposed to near anoxia

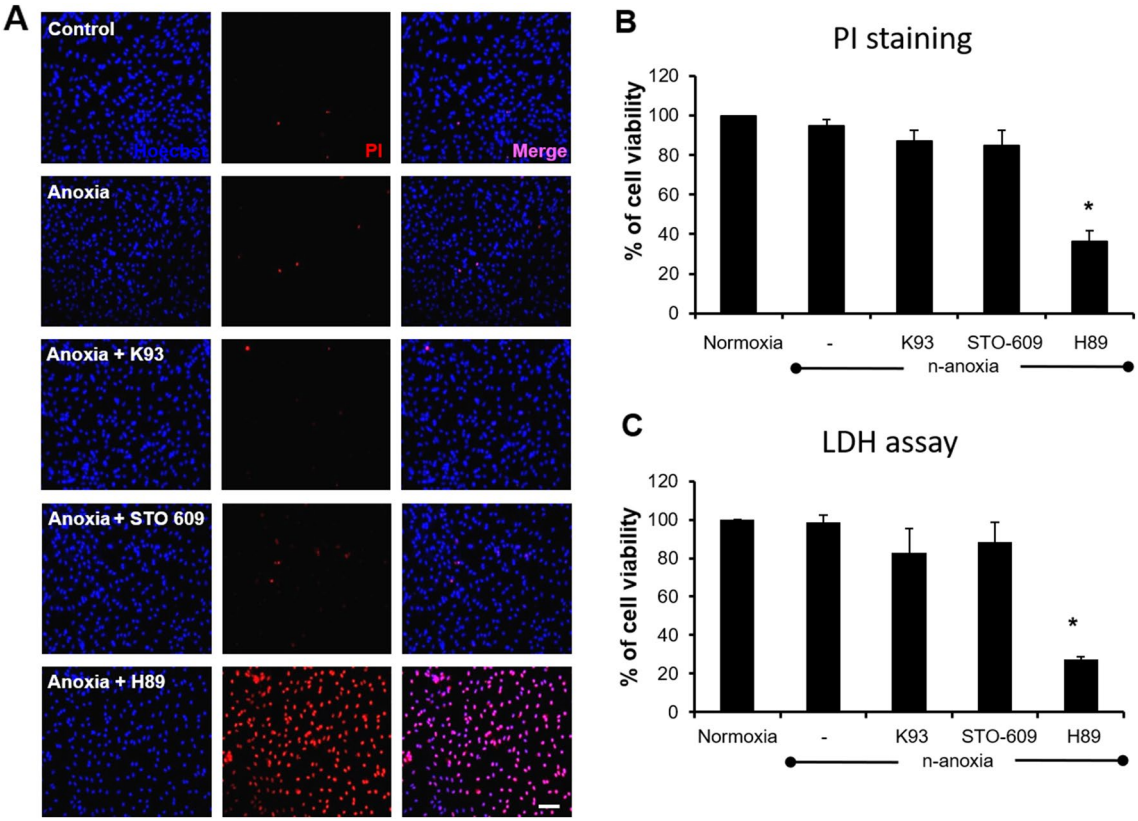


Fig. 7 Inhibition of PKA increases cell death under n-anoxia. Astrocytes were treated for 1 h in the presence of K-93 (CaMKII inhibitor), STO-609 (CaMKIV inhibitor), or H89 (PKA inhibitor) prior to 3 h of n-anoxia (0.1% O_2). **a** Representative images of propidium iodide (red) immunostaining with Hoechst (blue). **b** Summarized data of the immunostaining data. **c** Cell viability assessed by LDH assay. Values are mean ± SD (n = 3 separate experiments). *p < 0.05 versus normoxia. Scale bars 20 µm. (Color figure online)

studies, the activation of PKA/CREB signal pathway was not distinguished in various cell types in the brain. Our results clearly demonstrate, for the first time, that the PKA/pCREB pathway is activated and provide protection astrocytes, a major components of the brain and the BBB. The pathway's activation and function in other brain cells such as neurons and brain endothelial cells need to be determined for a complete understanding of its contribution to cellular signaling modulations of the BBB and brain functions in ischemic stroke.

In summary, we investigated the role of CREB activation on the expression of HIF-1α protein in cortical astrocytes exposed to $CoCl_2$ and anoxia. Inhibition of CaMKII and CaMKIV had no effect on HIF-1α and pCREB expression in cortical astrocytes exposed to either $CoCl_2$ or n-anoxia. In contrast, PKA inhibition lowered the expression of HIF-1α and pCREB expression. Furthermore, the inhibition of PKA but not CaMKII or CaMKIV increased cell death of astrocytes exposed to near anoxia. The results suggest that PKA plays an important role in the cell survival signaling pathways in astrocytes.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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