Neuroscience Letters 444 (2008) 264–269

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Baicalein suppresses hypoxia-induced HIF-1α protein accumulation and activation through inhibition of reactive oxygen species and PI 3-kinase/Akt pathway in BV2 murine microglial cells

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ARTICLE INFO

Article history:
Received 3 July 2008
Received in revised form 19 August 2008
Accepted 21 August 2008

Keywords:
Baicalein
Hypoxia
BV2 microglia
HIF-1α
PI 3-kinase
ROS

ABSTRACT

Hypoxia induces an inflammatory activation of microglia during cerebral ischemia. The transcription factor of hypoxia-inducible genes hypoxia-inducible factor-1 (HIF-1) is known to be involved in inflammation and immune response. Although baicalein (BE), a flavonoid, is shown to have anti-inflammatory effects and attenuate ischemic injury, its action mechanism is not understood well. Thus, we examined effect of BE on hypoxia-induced HIF-1 activation and its signaling mechanism in BV2 microglial cells. BE inhibited hypoxia-induced HIF-1α protein accumulation and HIF-1 transcriptional activation. Consistently, BE suppressed hypoxia-induced expression of hypoxia responsive genes, iNOS, COX-2, and VEGF. We then showed that BE inhibited hypoxia-induced phosphorylation of Akt but not that of ERK and p38. Moreover, BE inhibited hypoxia-induced PI 3-kinase activation. Finally, we showed that BE inhibited hypoxia-induced ROS generation, and an antioxidant *N*-acetylcysteine reduced hypoxia-induced HIF-1α and iNOS protein expression and PI 3-kinase/Akt activation in BV2 microglia. Taken together, these results suggest that BE suppresses hypoxia-induced HIF-1α protein and activation as well as expression of hypoxia responsive genes by inhibiting ROS and PI 3-kinase/Akt pathway in BV2 microglia.

Hypoxia is associated with a variety of pathophysiological states such as ischemia and tumorigenesis [19]. The hypoxia-inducible factor-1 (HIF-1), a master regulator of oxygen homeostasis is a heterodimeric transcription factor composed of HIF-1α and HIF-1β [24]. In normoxia HIF-1α protein is kept at a low or undetectable level by continuous degradation via the 26S proteasome involving E3 ubiquitin ligase von Hippel–Lindau protein, while HIF-1β protein is constitutively present. [7]. Stabilization and accumulation of HIF-1α protein under hypoxia promote active HIF-1 complex formation. Activated HIF-1 increases transcription of hypoxia-inducible genes involved in angiogenesis, vasodilation, and cell survival [1]. Furthermore, an important role of HIF-1 in inflammation and activation of immune response has also been reported [6].

During cerebral ischemia, hypoxia may not only cause neuronal cell injury, but also induces microglial activation. Activated microglia exert cytotoxic effects by releasing inflammatory mediators, such as nitric oxide (NO), prostaglandins, and reactive oxygen species (ROS). Overactivation of microglial cells may cause severe brain tissue damage in various neurodegenerative diseases [9]. Multiple signaling molecules, including extracellular signal-regulated kinase (ERK), p38 MAPK (p38), and phosphatidylinositol (PI) 3-kinase/Akt are activated in response to hypoxia and mediate stabilization or activation of HIF-1α [10,15,18]. Some of these pathways are also involved in the induction of proinflammatory mediators during microglial activation [17]. It is recently demonstrated that hypoxia induces iNOS expression through PI 3-kinase/Akt pathway and HIF-1α activation in microglia [14].

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is one of the major flavonoids of *Scutellaria baicalensis*, which has

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doi:10.1016/j.neulet.2008.08.057

long been used in Chinese herbal medicine. Among its biological activities, baicalein (BE) has been reported to exhibit several beneficial effects, including antioxidant, anti-apoptosis, and anti-inflammatory activities [13,20,21,25]. Anti-inflammatory effects of BE on iNOS and COX-2 are shown to be mediated through inhibition of transcription factor C/EBPβ or NF-IL6 and NF-κB activation in LPS-stimulated macrophages or microglia [3,21,26].

Although BE is shown to protect cells from ischemic brain damage [11,23], its action mechanism is not understood in hypoxic microglia. Thus, in this study, we examined effects of BE on hypoxia-induced HIF-1 activation and its signaling mechanism involved in BV2 microglia. The results show that BE inhibits hypoxia-induced HIF-1α protein accumulation and activation as well as expression of iNOS gene through inhibition of ROS generation and PI 3-kinase/Akt activation in BV2 microglia.

BV2 mouse microglial cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics mixture (Life Technologies) in a humidified atmosphere of 5% CO_2 at 37 °C. Primary microglial cells were obtained from cerebral cortices of 1-day-old rat pups as previously described [16]. All animal use procedures conformed to the animal care guidelines of the Korean Academy of Medical Sciences. For the hypoxic condition, cells were washed three times with deoxygenated serum free DMEM, and placed into hypoxic chamber (0.1% O_2) InViVo2000 (Ruskin, UK).

Baicalein, cycloheximide (CHX), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), diphenyliodonium (DPI), *N*-acetylcysteine (NAC), and anti-phosphotyrosine agarose beads were purchased from Sigma–Aldrich (St. Louis, MO). Apocynin and LY294002 were purchased from Calbiochem (La Jolla, CA).

Immunoblot analysis was carried out as follows. The cell lysates were separated by 8–10% SDS-PAGE and transferred onto nitrocellulose membrane. After transfer, The membrane was blocked with 5% skim milk and incubated for 1 h with primary antibodies specific to HIF-1α, phospho-ERK, phospho-p38, and phospho-Akt (Cell Signaling, Beverly, MA), iNOS (BD Bioscience, San Jose, CA) or ERK, p38, Akt, and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a secondary antibody. The blots were detected with ECL kit.

To determine mRNA levels of HIF-1α, iNOS, COX-2, and VEGF, total RNA was isolated and RT-PCR was performed as described [16]. The primers used for PCR are as follows: HIF-1α (5′-CTTGCTCACAGTTGCCACTT-3′; 5′-GCCATTTCTGTGTGTAAGCAT-3′), iNOS (5′-ATGTGGTACTCAGCGTGCTCCAC-3′; 5′-CCACAATAGTACAATACTACTTGG-3′), COX-2 (5′-GAACATTGTGAACAACATCCCC-3′; 5′-GGTGGCATACATCATCAGACC-3′), VEGF (5′-ACATCTCAAGCCGTCCTGTGTGC-3′; 5′-AAATGGCGAATCCAGTCCCACGAG-3′),

Luciferase assay was performed as described [16]. The cells in six-well plates were co-transfected with 2 μg plasmid of HIF-1 luciferase reporter (HRE-Luc) (Dr. F. Bunn; Harvard Medical School).

NO production was measure as described [17]. BV2 cells (3×10^4 cells in 200 μl/well) were exposed to hypoxia (8 h)–reoxygenation (24 h) and nitrite levels in culture medium were assayed with Griess reagent. The absorbance at 550 nm was measured on a microplate reader.

PI 3-kinase activity was measured as described previously [12]. The cell lysates (500 μg) were immunoprecipitated with the anti-phospho-tyrosine antibody coupled to agarose beads. After washing, the immunoprecipitated samples were incubated in kinase assay buffer containing 10 μCi [γ-^{32}P]ATP. The reaction was stopped and products were then separated by TLC plate (Merck) and visualized by autoradiography.

ROS were measured as described [25]. Cells were incubated with 10 μM DCFH-DA for 30 min, and fluorescence intensity was measured by flow cytometry.

All data are expressed as the mean ± S.E. of more than three independent experiments. Statistical analysis was performed using Student's *t*-test and an analysis of variance (one-way ANOVA). The accepted level of significance was $P < 0.05$.

To investigate the effect of BE on hypoxia-induced HIF-1α protein accumulation and HIF-1 activation in BV2 microglial cells, we treated cells with BE (50 μM) for 30 min before stimulating cells with hypoxia (0.1% O_2) for 4 h. The result showed that BE strongly inhibited hypoxia-induced HIF-1α protein accumulation but not HIF-1α mRNA levels (Fig. 1A). In agreement with a previous report [14], PI 3-kinase inhibitors LY294002 (20 μM) and wortmannin (150 nM) also blocked HIF-1α protein accumulation in BV2 cells (Fig. 1A). Although other recent reports show that BE alone induces HIF-1α protein in different cell types [4,22], BE at 50 μM did not significantly induce HIF-1α protein expression in BV2 microglial cells (Fig. 1B). Consistent with HIF-1α protein level, BE as well as PI 3-kinase inhibitors significantly blocked hypoxia-induced HRE luciferase transcriptional activity (Fig. 1C). In addition, LPS-induced HIF-1 transactivation was also inhibited by BE as well as PI 3-kinase inhibitors in these cells (Fig. 1C). As hypoxia is known to induce HIF-1α protein stability, we examined whether BE affects hypoxia-induced HIF-1α protein stabilization using protein synthesis inhibitor cycloheximide. After exposure of BV2 cells to hypoxia for 4 h, cycloheximide (50 μM) was added to the cells, and cells were further incubated for additional 60 min. The HIF-1α in hypoxia-induced cells appeared to be relatively stable, whereas HIF-1α protein in BE-treated cells rapidly degraded when protein synthesis was blocked by cycloheximide (Fig. 1D). The results suggest that BE blocks HIF-1α protein accumulation by inhibiting hypoxia-induced HIF-1α protein stabilization in BV2 microglia.

Next, to examine the effect of BE on the expression of hypoxia-induced genes, we treated BV2 cells with different concentrations of BE for 30 min before stimulating cells with hypoxia for 6 h. The results showed that BE blocked expression of hypoxia-induced genes including iNOS, COX-2, and VEGF mRNA, albeit to a different potency (Fig. 2A). Similarly, BE at 50 or 100 μM concentration inhibited expression of iNOS and COX-2 protein under hypoxia condition (Fig. 2B). Consistently, it significantly inhibited NO production in hypoxic BV2 cells (Fig. 2C). As shown in Fig. 2D, in rat primary microglia, BE (50 μM) also significantly inhibited hypoxia-induced HIF-1α protein and iNOS protein expression (Fig. 2D).

Next, to investigate the signaling mechanism by which BE inhibits HIF-1 activation, we examined effects of BE on phosphorylation of ERK, p38, and Akt in hypoxia-exposed BV2 cells. Previously, ERK, p38, and PI 3-kinase/Akt are known to be involved in HIF-1α protein stabilization and HIF-1 activation in various cell types [10,15,18]. Similar to the previous reports [17,26], BE alone induced phosphorylation of ERK and p38, while hypoxia induced phosphorylation of p38 and Akt, and BE did not inhibit phosphorylation of ERK and p38 in hypoxic BV2 cells (Fig. 3A). In contrast, BE markedly inhibited hypoxia-induced Akt phosphorylation (Fig. 3A). Consistent with its effect on Akt, BE effectively inhibited hypoxia-induced PI 3-kinase activation in BV2 microglia (Fig. 3B).

Next, to understand the upstream signaling component of PI 3-kinase/Akt that is involved in BE action and HIF-1 activation in hypoxic BV2 cells, we examined effect of BE on hypoxia-induced ROS levels and the role of ROS in HIF-1 activation. ROS are reported to be increased or decreased during hypoxic conditions and are suggested to play a critical role in HIF-1α expression [8]. ROS are known to be produced from mitochondria and NADPH oxidase [2,5]. As shown in Fig. 4A, hypoxia as well as BE alone induced