Oroxylin A Induces BDNF Expression on Cortical Neurons through Adenosine A2<sub>A</sub> Receptor Stimulation: A Possible Role in Neuroprotection

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Abstract

Oroxylin A is a flavone isolated from a medicinal herb reported to be effective in reducing the inflammatory and oxidative stresses. It also modulates the production of brain derived neurotrophic factor (BDNF) in cortical neurons by the transactivation of cAMP response element-binding protein (CREB). As a neurotrophin, BDNF plays roles in neuronal development, differentiation, synaptogenesis, and neural protection from the harmful stimuli. Adenosine A2<sub>A</sub> receptor colocalized with BDNF in brain and the functional interaction between A2<sub>A</sub> receptor stimulation and BDNF action has been suggested. In this study, we investigated the possibility that oroxylin A modulates BDNF production in cortical neuron through the regulation of A2<sub>A</sub> receptor system. As expected, CGS21680 (A2<sub>A</sub> receptor agonist) induced BDNF expression and release, however, an antagonist, ZM241385, prevented oroxylin A-induced increase in BDNF production. Oroxylin A activated the PI3K-Akt-GSK-3β signaling pathway, which is inhibited by ZM241385 and the blockade of the signaling pathway abolished the increase in BDNF production. The physiological roles of oroxylin A-induced BDNF production were demonstrated by the increased neurite extension as well as synapse formation from neurons. Overall, oroxylin A might regulate BDNF production in cortical neuron through A2<sub>A</sub> receptor stimulation, which promotes cellular survival, synapse formation and neurite extension.

Key Words: Oroxylin A, BDNF, CREB, Adenosine A2<sub>A</sub> receptor, CGS21680, ZM241385

INTRODUCTION

Oroxylin A (5,7-dihidrixy-6-methoxyflavone) is a flavonoid originated from the root of Scutellaria baicalensis Georgi, which acts as a γ-aminobutyric acid (GABA<sub>A</sub>) receptor antagonist (Huen et al., 2003). Oroxylin A ameliorated memory dysfunction induced by scopolamine (Kim et al., 2006), which might be associated with the neuroprotective effects of oroxylin A in cultured rat primary neuron (Jeon et al., 2011). BDNF is a member of the neurotrophin family (Lewin, 1996) which plays important roles in central nervous system (CNS) such as protection of neuronal degeneration (Lindholm et al., 1993), differentiation of hippocampal and cortical neurons (Ip et al., 1993; Croll et al., 1994; Nawa et al., 1994; Marty et al., 1996) and synaptogenesis (Shen et al., 2006). It is also well known that BDNF is involved in the regulation of neurite out-
growth as well as directional movement via a variety of different mechanisms (Bartrup et al., 1997; Winckler, 2007; Sasaki et al., 2010).

Adenosine is a purine nucleoside, which transmits its physiological signal through adenosine receptors. Four subtypes of adenosine receptors have been described to date, A1, A2α, A2β, and A3 subtypes (Tucker and Linden, 1993), which are classified as 2 categories by functions; A1 and A3 which are negatively coupled to adenylate cyclase via G proteins and A2α and A2β which are positively coupled to the same effectors (Proll et al., 1988; Moser et al., 1991). Among them, A2β receptor modulates tonic expression of BDNF as well as synaptic actions of BDNF on hippocampal neurons (Diégenes et al., 2004; Tebano et al., 2008). A2β receptor also activates one of BDNF receptors, tropomyosin-related kinase B (TrkB), and Akt signaling molecule, which promotes motor neuron survival (Wiese et al., 2007) and modulates neurite outgrowth in several different cell types (Cheng et al., 2002; Canals et al., 2005; O’Driscoll and Gorman, 2005). Greengard group showed that A2β receptor-mediated modulation of PC12 cell differentiation and neurite extension in collaboration with FGF receptors (Flajolet et al., 2008). These strong trophic actions of A2β receptor activation make it one of the promising targets for several psychiatric and neurodegenerative diseases (Cunha et al., 2008).

The positive relationship of A2β receptor activation and increased BDNF action as well as our preliminary results suggesting that oroxylin A may bind to A2β receptor (our unpublished results) prompted us to investigate whether oroxylin A might regulate BDNF production via modulating A2β receptor on cultured rat primary neurons using a pharmacological agonist and antagonist. We also investigated the possible intracellular signaling pathway mediating the increased BDNF production by oroxylin A as well as the role of oroxylin A on neurite extension.

MATERIALS AND METHODS

Materials

Neurobasal medium was purchased from Gibco BRL (NY, USA) and B-27 supplements was obtained from Invitrogen (CA, USA). The BDNF ELISA kit was from Promega (Madison, WI). Specific primary antibody against BDNF was purchased from Santa Cruz Biotechnology Inc. (sc-546, Santa Cruz, CA), and TuJ-1 was from Covance (Richmond, CA). Other phospho- and total-forms of antibodies (CREB, ERK, Akt, and GSK-3β) were obtained from Cell Signaling Technology (Beverly, MA, USA). Oxyrin A was obtained from Korea food & drug administration. U0126 and wortmannin were obtained from Calbiochem (San Diego, CA). CGS21680 and ZM241385 were purchased from Tocris Bioscience (Bristol, UK), and all other chemical reagents were purchased from Sigma (St. Louis, MO, USA).

Methods

Cell culture: Primary cortical neurons were isolated from embryonic cerebral cortex of Sprague-Dawley rat (SD rat) as previously described (Jeon et al., 2011). Cerebral cortex obtained from E16 pups was digested and re-suspended in NBM containing B-27 and then cells were placed on poly-D-lysine (PDL) pre-coated plates. The cultures were kept in a humidified 10% CO₂ atmosphere at 37°C for 10 days and media were half-replaced with fresh media every 3 days.

Drug treatment: Inhibitors were pre-treated 1 hr before the oroxylin A treatment. As an ERK1/2 phosphorylation inhibitor, U0126 was used at 10 μM and wortmannin was used as an Akt inhibitor at 100 nM. To modulate the activity of adenosine A2β receptor, an antagonist (50 nM of ZM241385) and an agonist (20 nM of CGS21680) was used.

Western blot: Treated cells were lysed with 2× sample buffer (4% w/v SDS, 20% glycerol, 200 mM DTT, 0.1 M Tris-HCl, pH 6.8, and 0.02% bromophenol blue) and the samples were fractionated by 8-12% SDS-PAGE and electrotransferred to nitrocellulose (NC) membrane. The NC membrane was blocked with 1 μg/ml polyvinyl alcohol (PVA) for 0.5 hr at room temperature (RT) and incubated overnight at 4°C with the appropriate primary antibodies which were diluted at 1:5000 in 5% skim milk (Roth, Germany). After washing with Tris-buffered saline containing 0.1% Tween20 (TBS-T), NC membranes were incubated with peroxidase conjugated secondary antibody for 2 hr at RT. Following three times of washings with TBS-T, blots were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Reverse transcription polymerase chain reaction (RT-PCR): Cellular total RNA was extracted from primary neurons using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 2 μg of them was converted to cDNA (Maxime RT Premix Kit, INtRON Biotechnology, Seoul) according to the manufacturer’s protocol. The PCR amplification was performed using Maxime PCR premix Kit (INtRON Biotechnology, Seoul) and was consisted of 26 cycles with the oligonucleotide primers for BDNF (accession number EF125679.1, Tm=60°C, (Kobayashi et al., 2008)), GABA R2 (accession number NM_00135779.1, Tm=60°C), GABA R5 (accession number NM_017295.1, Tm=55°C), A2R (accession number L08102, Tm=55°C), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession number M17701, Tm=60°C). The following primers were used for amplification reactions:

| Primer Position | Forward Primer | Reverse Primer |
|----------------|----------------|----------------|
| BDNF | 5'-ATA GGA GAC CCT CCG CAA CT-3' | 5'-CTG CCA TGC ATG AAA CAC TT-3' |
| for BDNF | 5'-AGA TCC ACA ACG GAT AAC TT-3' |
| for GAPDH | 5'-TCC CTC AAG ATT GTC AGC AA-3' | 5'-AGA TCC ACA ACG GAT AAC TT-3' |
| for GABA R2 | 5'-GGG CGT AGT TGG CAA CGG CT-3' | 5'-GCC CGG AAT TCG CTG CCC AA-3' | 5'-GTC CGG CCT GGA AGC TGC TC-3' |
| for GABA R5 | 5'-CGG TCG CAG CGA GAA CTG TGT-3' | 5'-GCC CGG AAT TCG CTG CCC AA-3' | 5'-GTC CGG CCT GGA AGC TGC TC-3' |
| for A2β R | 5'-CCA TGC TTG GCT GAC ACA-3' | 5'-GAA GGG GCA GTA ACA CGA-3' |

The amplified products were analyzed on 1% agarose gel and stained with EtBr. The expected size of the amplified DNA fragments was 280 base pairs for BDNF, 297 base pairs for GABA R alpha subunit 2, 275 base pairs for GABA R alpha subunit 2, and stained with EtBr.
subunit 5, and 308 base pairs for GAPDH.

**ELISA assay:** The amount of released BDNF was quantified from medium of treated neuron using the Emax ImmunoAssay system (Promega, Madison, WI). BDNF enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's manual. Briefly, 96 well plates were pre-coated with anti-BDNF mAb diluted with carbonate coating buffer at 4°C for 24 hr. After 1 hr blockade with supplied Blocking buffer, the plates were incubated with standard and culture medium sample for 2 hr at RT followed by incubation with anti-human BDNF pAb. After 1 hr incubation of Anti-IgY HRP conjugate, the reaction was developed with tetramethylbenzidine (TMB One Solution) and the absorbance was read at 450 nm with a microplate reader (Tecan Trading AG, Switzerland) after stopping the reaction with 1N HCl.

**Immunocytochemistry:** DIV 2 primary cortical neurons plat-

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**Fig. 1.** Oroxylin A induced BDNF production in cortical neurons through adenosine A2A receptor. (A) Oroxylin A (20 μM), CGS21680 (20 nM), and ZM241385 (50 nM) was treated to primary cortical neurons for 24 hr and cells were lysed and analyzed by RT-PCR. (B) In case of BDNF protein level, samples were analyzed by Western blot. (C) Released BDNF was quantified by ELISA assay. (D) Cortical neurons were collected at DIV2, and DIV10 to confirm the expression of A2AR and GABAAR, respectively. Each graph represents quantification of RT-PCR and Western blot band intensity, respectively. Data represent Mean ± S.E.M. **Significantly different as compared with control and ##significantly different as compared with oroxylin A or CGS21680 stimulation (p<0.01, n=4).
ed on the PDL pre-coated cover glasses (Fisher Scientific, PA) were treated with 20 &mu;M of oroxylin A for 24 hr. Glasses were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 0.5 hr. After three times of washing, samples were permeabilized using 0.3% Triton X-100 solution for 15 min at RT and blocked by blocking buffer (1% BSA, 5% FBS in PBS) for 30 min at RT. Samples were incubated overnight at 4°C with the primary antibody against neuron (Tuj-1, 1:500 in blocking

Fig. 2. Oxylin A induced the activation of Akt, which was necessary for the phosphorylation of ERK1/2-CREB. (A) Rat primary cortical neurons were treated with o oxylin A (1, 5, 10, 20 &mu;M) for 3 hr and analyzed by Western blot. Phospho/total- Akt and GSK-3j ratio was determined by densitometric quantification of Western blot. (B) A PI3K activation inhibitor, wortmannin, was used to investigate the role of Akt and GSK-3j signaling on BDNF production. After 1 hr treatment of wortmannin (100 nM), cells were treated with o oxylin A for 24 hr and the level of BDNF protein expression was measured by Western blot. (C) ERK1/2 and CREB phosphorylation was determined after wortmannin and o oxylin A treatment. (D) The level of BDNF release was measured using ELISA assay from U0126-, wortmannin-, or o oxylin A-treated cell culture supernatants. Detailed protocol was described in Materials and Methods. Each graph represents quantification of Western blot band intensity. Data represent Mean ± S.E.M. **Significantly different as compared with control and ##significantly different as compared with oroxylin A or CGS21680 stimulation (p<0.01, n=4).
buffer). Next day, after washing, samples were incubated for 2 hr with secondary antibodies conjugated with TMRE (diluted at 1:500 in blocking buffer). Then samples were washed and mounted using Vectashield (Vector laboratories, Burlingame, CA, USA). Cellular images were observed by fluorescence microscope (motorized research microscope bx61, Olympus, Japan) and analyzed by Image J software (NIH, USA).

Statistics
Data are expressed as the mean ± standard error of mean (S.E.M.) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Newman-Keuls test as a post hoc test and a p value<0.05 was considered significant.

RESULTS
Oroxylin A stimulated BDNF expression and release in rat primary neurons through Adenosine A2A receptor activation
Treatment of oroxylin A or an A2A receptor agonist, CGS21680 increased BDNF mRNA level 6.24 ± 0.44 and 4.65 ± 0.26 folds compared with control, respectively (Fig. 1A). Co-treatment with an A2A antagonist ZM241385 inhibited the increased BDNF mRNA expression induced by oroxylin A or CGS21680. Similar pattern of changes was also observed for BDNF protein level (Fig. 1B) as well as BDNF release (Fig. 1C), which was determined by Western blot and ELISA, respectively. Interestingly, while A2A receptor antagonist ZM241385 completely prevented the increased expression of BDNF induced by A2A receptor agonist CGS21680, ZM241385 only partially inhibited oroxylin A-mediated increase in BDNF expression. To investigate the expression of A2A receptor in rat primary neuron, we performed RT-PCR analysis. The expression of A2A receptor was confirmed at DIV 2 neuron, which was increased in DIV 10. Similar pattern was also observed with GABAA receptor (Fig. 1D). These results suggest that oroxylin A may induce BDNF expression by A2A receptor activation as well as other intracellular signaling pathways.

Oroxylin A-induced BDNF production was mediated by Akt and GSK-3β phosphorylation in rat primary neurons
We previously reported that oroxylin A-induced BDNF production was mediated by CREB and ERK1/2 phosphorylation (Kim et al., 2008; Jeon et al., 2011). Because Akt-GSK3β pathway is a downstream pathway of A2A receptor activation and is also involved in the modulation of BDNF production (Mai et al., 2002), we next investigated the activation of Akt-GSK3β pathway by oroxylin A (Fig. 2A). Oroxylin A induced phosphorylation of Akt and its downstream target GSK-3β in a concentration dependent manner (Fig. 2A). At the highest concentration of oroxylin A (20 μM), phosphorylation of Akt and GSK-3β reached 319.07 ± 36% and 431.20 ± 28.31% of control level, respectively (Fig. 2A). Oroxylin A-induced BDNF protein expression determined by Western blot was inhibited by pretreatment of an Akt inhibitor, wortmannin (Fig. 2B) and in this condition, ERK1/2 and CREB phosphorylation, a whole mark of transcriptional activation of oroxylin A-induced BDNF expression, was also inhibited as reported previously (Fig. 2C) (Jeon et al., 2011). We next measured the release of BDNF using ELISA assay and either Akt inhibitor wortmannin or

![Fig. 3. Adenosine A2A receptor mediates oroxylin A-induced Akt and ERK1/2 phosphorylation and BDNF production. To identify the signaling pathway involved in the Adenosine A2A receptor-mediated stimulation of BDNF production, phosphorylation level of Akt and ERK1/2 were analyzed using Western blot. (A, B) Cells were treated with ZM241385 (50 nM) for 1 hr before the treatment of oroxylin A (20 μM). After 1 hr, cells were harvested to analyze the level of phosphoAkt-GSK-3β (A) and ERK1/2-CREB (B) by Western blot. Each graph represents quantification of Western blot band intensity. Data represent the mean ± S.E.M. **Significantly different as compared with control and ***significantly different as compared with oroxylin A stimulation (p<0.01, n=4).}
ERK1/2 inhibitor, U0126 treatment (Fig. 2D) inhibited oroxylin-induced BDNF release. These results suggest that oroxylin A induces phosphorylation of Akt-GSK3β pathway which may modulate, at least in part, the phosphorylation of ERK1/2 and CREB pathways.

Oroxylin A activates Akt-GSK-3β pathways through adenosine A2a receptor

Next, we investigated whether adenosine A2a receptor is involved in the activation of Akt pathway induced by oroxylin A (Fig. 3). Pretreatment of adenosine A2a receptor antagonist, ZM241385, inhibited oroxylin A-induced phosphorylation of Akt and GSK-3β (Fig. 3A) and in this condition, ERK1/2-CREB phosphorylation was also significantly inhibited (Fig. 3B). Taken together with the decreased oroxylin-induced BDNF production by ZM241385 (Fig. 1B), these results suggest that oroxylin A induces phosphorylation of Akt-GSK3β and downstream pathways leading to the BDNF production by modulating the activation of A2a receptor.

Oroxylin A induces neurite outgrowth in cultured rat primary neuron

Considering the roles of BDNF in neurons, we finally investigated the role of oroxylin A on the neurite outgrowth in immature rat primary cortical neuron. We previously reported the neuroprotective effect of oroxylin A against glutamate, presumably via the increased production of BDNF, in rat primary cortical neurons (Jeon et al., 2011). DIV 2 rat primary cortical neurons were treated with oroxylin A and visualized by a neuronal marker, Tuj-1 immunostaining (Fig. 4). Oroxylin A increased the average number of neurite branches extending from a single neuron as well as the length of individual neurite. Interestingly, pre-incubation of ZM241385 prevented oroxylin-induced neurite outgrowth, again suggesting the essential role of A2a receptor activation in this process (Fig. 4). The increased neurite extension by oroxylin A was also inhibited by the addition of anti-BDNF antibody (Fig. 4D). Overall, these results suggest that oroxylin A enhances neurite outgrowth by regulating BDNF expression and release, at least in part, through A2a receptor.

Fig. 4. Oroxylin A-induced up-regulation of BDNF facilitated differentiation of rat primary cortical neuron. Neurons were treated with oroxylin A and were immunostained against neuronal marker Tuj-1 (A). The number of neurite extending from each neuron was analyzed by Image J (B) as described in materials and methods. The length of neurite (C) was also analyzed by Image J analysis of immunostaining data. (D) At DIV2, cortical neurons were treated with oroxylin A (5, 20 µM) and immunostained with Tuj-1 antibody to visualize the neurite extension. Anti-BDNF antibody (2 µg/ml) was used to block BDNF action from oroxylin A treated neurons. Data represent the mean ± S.E.M. Scale bar represents 50 µm. **Significantly different as compared with control and ## significantly different as compared with oroxylin A treated group (p<0.01, n=4).
DISCUSSION

In this study, we provided evidences that oroxylin A increased BDNF production and neurite outgrowth at least in part by A$_2$A receptor stimulation followed by activation of Akt-GSK3β pathway. In our previous reports, oroxylin A improved working memory in scopolamine treated animals (Kim et al., 2007) as well as in transiently bilateral common carotid artery occluded animals (Kim et al., 2006), which may also be related to the increased BDNF production and neurogenesis in brain. In addition, we observed increased synaptogenesis by oroxylin A as evidenced by increased expression of synaptic marker proteins in immature rat primary cortical neuron (Jeon et al., 2011). Gasiorowski et al. recently showed oroxylin A dramatically increased cognition and memory from aged animals, which was consistent with our data (Gasiorowski et al., 2011). However they did not elucidate the exact mechanism of neuroprotective effects in their report. Nevertheless, these results suggest that using the flavonoids like oroxylin A, as a potential neuroprotective agent may be a potential neuroprotective booster (food additive, drug) target. Our data suggesting the possible role of oroxylin A in neuroprotection, neurogenesis and neural differentiation via modulation of BDNF expression by mechanism involving regulation of A$_2$A activation may support and strengthen this view.

Previously, oroxylin A is reported to suppress nitric oxide generation (Jiwajinda et al., 2002) and inhibit LPS induced iNOS and COX-2 expression by modulating NF-κB activation (Chen et al., 2000; Chen et al., 2001). The effect of oroxylin A on the prevention of uterine contraction was also suggested. Oxyrilocil A may inhibit uterine contractions by opening calcium dependent potassium channels or adenosine triphosphate dependent potassium channel (Shih et al., 2009), which suggest that oroxylin A may modulate cellular membrane channels or receptors, in our cases, A$_2$A adenosine receptor.

Adenosine receptors control essential brain functions like synaptic plasticity, neurotransmitter transport, and astroglisis (Sebastião and Ribeiro, 2009) by receptor dimerization. Especially, A$_2$A receptor-dopamine D2 receptor heterodimers may exist in the striatal GABA pathways, where activation of A$_2$A receptors inhibits D2 receptor action. As a result of the A$_2$A receptor-induced reduction of D2 receptor signaling, the activity of GABA neurotransmission is increased, which may provide novel tools to treat Parkinson’s disease, schizophrenia (Angelucci et al., 2005) as well as neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and amyotrophic lateral sclerosis (Pezet and Malcangio, 2004; Pardon, 2010). Considering inefficient transport of BDNF through BBB, one possible approach to take advantage of BDNF as a therapeutic target is to use small molecules to boost endogenous level of BDNF. Interestingly, daily administration of A$_2$A agonist CGS21680 ameliorates the symptoms of Huntington’s disease animal models (Chou et al., 2005). Whether oroxylin A may provide clinical efficacy against neurological diseases such as mood disorder and neurodegenerative diseases by inducing BDNF expression and release through its action on A$_2$A receptor should be further investigated in future studies.

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Jeon et al.  Oroxylin Induces BDNF in Neurons via Adenosine A2A Receptor

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