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Endogenous acetylcholine regulates neuronal and astrocytic vascular endothelial growth factor expression levels via different acetylcholine receptor mechanisms

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ABSTRACT

Vascular endothelial growth factor (VEGF), a signaling molecule involved in angiogenesis, plays an important role in neuroprotection and neurogenesis. In the present study, we aimed to elucidate the mechanisms underlying endogenous acetylcholine (ACh)-induced VEGF expression in neurons and astrocytes, and identify the neuronal cells contributing to its expression in the medial septal area, a nuclear origin of cholinergic neurons mainly projecting to the hippocampus. The mRNA expression and secretion of VEGF were measured by RT-PCR and ELISA using mouse primary cultured cortical neurons and astrocytes. VEGF expression in the medial septal area was assessed by RT-PCR and immunostaining using mice treated with tacrine [9-amino-1,2,3,4-tetrahydroacridine HCl (THA); 2.5 mg/kg, i.p.] once daily for 7 days. The THA treatment increased VEGF mRNA expression in neurons in a manner that was reversed by mecamylamine, a nicotinic ACh receptor (AChR) antagonist, whereas in mouse primary cultured astrocytes, carbachol, but not THA dose-dependently increased VEGF mRNA expression and secretion in a manner that was inhibited by scopolamine, a muscarinic AChR inhibitor. In *in vivo* studies, the administration of THA significantly increased the expression of VEGF in medial septal cholinergic neurons and the effects of THA were significantly blocked by mecamylamine. THA also significantly increased the expression levels of a phosphorylated form of VEGF receptor 2 (p-VEGFR2), an activated form of VEGFR2. The present results suggest that endogenous ACh plays an up-regulatory role for VEGF expression in neurons and astrocytes via different mechanisms. Moreover, endogenous ACh-induced increases in VEGF levels appear to activate VEGFR2 on medial septal cholinergic neurons via an autocrine mechanism.

1. Introduction

Vascular endothelial growth factor (VEGF) is involved in angiogenesis and vasculogenesis and plays an important role in pathological and physiological phenomena such as wound healing, the menstrual cycle, and permeability of the blood brain barrier (Adini et al., 2017; Detmar et al., 1995; Ferrara, 2004; Greenberg and Jin, 2005; Shifren et al., 1996; Zhang et al., 2000). VEGF is also a key mediator of angiogenesis and neovascularization, which are required for cancer cell proliferation. However, recent evidence indicates that VEGF plays a critical role in neuroprotection and neurogenesis (Greenberg and Jin, 2005). VEGF was also shown to protect HN33 cells, an immortalized hippocampal neuronal cell line, from hypoxia and glucose deprivation, suggesting that its neuroprotective effects are due to the stimulation of VEGF receptor 2 (VEGFR2), which was led by the phosphorylation of the receptor (Jin et al., 2000). In addition, VEGF from astrocytes enhanced cell survival signaling in neurons through the activation of VEGFR2 after oxygen and glucose deprivation (Zhang et al., 2017).

The acetylcholinesterase (AChE) inhibitor, tacrine (THA), exerts neuroprotective effects in time- and does-dependent manners against glutamate neurotoxicity (Takada-Takatori et al., 2006a, 2006b) and amyloid-β protein toxicity in PC12, a pheochromocytoma line (Wang et al., 2002; Xiao et al., 2000). We previously reported that cognitive deficits in *db/db* mice, an animal model of type 2 diabetes, were accompanied by decreases in the number of medial septal cholinergic neurons and the expression levels of VEGF in the hippocampus and that

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; AraC, cytosine β-D-arabinofuranoside hydrochloride; CCh, carbachol; ChAT, choline acetyltransferase; GFAP, Glial fibrillary acidic protein; HIF-1α, hypoxia inducible factor-1α; MCM, mecamylamine hydrochloride; NMDA, N-methyl-D-aspartic acid; OBX, olfactory bulbectomy; PBS, phosphate-buffered saline; PKC, protein kinase C; Ro, Ro-318220 methanesulfonate; SCP, scopolamine; THA, 9-amino-1,2,3,4-tetrahydro-acridine HCl (tacrine); TBS-T, Tris-buffered saline; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2

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the administration of THA reversed these behavioral and pathological alterations. These findings suggested that the VEGF/VEGFR2 loop is involved in the amelioration of cognitive and emotional deficits (Zhao et al., 2012). Moreover, in *in vitro* studies using organotypic hippocampal slice cultures, we found that a treatment with THA rescued hippocampal neurons from excitotoxicity-induced long-lasting hippocampal cell damage via not only the endogenous acetylcholine (ACh) stimulation of a muscarinic M1 receptor subtype (Inada et al., 2013, 2014), but also via paracrine VEGF signaling between astrocytes and hippocampal neurons or autocrine VEGF signaling in hippocampal neurons (Inada et al., 2014). Our findings suggested a link between cholinergic systems and VEGF signaling systems in the brain.

In the present study, we investigated the cholinergic mechanisms regulating VEGF expression and VEGF signaling in primary cultured cortical neurons and astrocytes. We also elucidated these mechanisms in the medial septum of the mouse brain for the following reasons. The medial septum is a major nucleus in which cholinergic neurons are located and project their nerve terminals to the hippocampus, thereby playing an important role in learning and memory (Chen et al., 2017; Jeong et al., 2014; Khakpai et al., 2013). Furthermore, the degeneration of septal cholinergic neurons and cognitive deficits in olfactory bulbectomized (OBX) animals, a model of dementia including AD, were found to be attenuated by the administration of THA (Le et al., 2013).

In the present study, we demonstrated that endogenous ACh regulated neuronal and astrocytic VEGF expression levels via different AChR mechanisms and that endogenous ACh-induced elevations in VEGF levels appeared to activate VEGFR2 expressed on cholinergic neurons by an autocrine signaling loop.

2. Materials and methods

2.1. Animals

Female ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and embryos at E15 and P0 mice were used in primary neuron cultures and astrocyte cultures, respectively. Male ddY mice (Japan SLC Inc., Shizuoka, Japan) were obtained at 7 weeks old and housed with a 12-h light-dark cycle (lights on: 07:30–19:30) at 24 ± 1 °C with constant humidity (65%). Animals were habituated to the laboratory animal room for at least 1 week before the drug treatment. Food and water were available *ad libitum*. The present animal studies were conducted in accordance with the Guiding Principles (NIH publication #85–23, revised in 1985) for the Care and Use of Animals and were approved by the Institutional Animal Use and Care Committee in the University of Toyama.

2.2. Primary culture for cerebral cortical neurons

Embryos at E15 were obtained from pregnant ICR mice after cervical dislocation, and cerebral cortices were dissected from embryos. After carefully removing the meninges, primary cortical neuronal cells were prepared using Neuron Dissociation Solutions (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions. Cortical neuronal cells were suspended in culture medium consisting of Neurobasal Medium (Gibco-Life Technologies, Carlsbad, CA, USA) with B-27 Serum-Free Supplement (Gibco-Life Technologies, Carlsbad, CA, USA), 0.5 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 20 U/each penicillin-streptomycin solution (Wako, Osaka, Japan), and plated on poly-D-lysine (Corning, NY, USA)-coated 6-well plates at a density of 1×10^6 cells per well for a quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting, or a 96-well plate at a density of 1×10^4 cells for ELISA assay. After 24 h, medium was replaced with fresh medium. The following day, half of the medium was changed to fresh medium with 10 μM cytosine β-D-arabinofuranoside hydrochloride (AraC) (Sigma-Aldrich Japan, Tokyo, Japan). Neurons were incubated at 37 °C under 95% moist air/5% CO_2 before experiments. Culture medium was changed every few days.

2.3. Primary culture for cerebral cortical astrocytes

Cortical neuronal cells were prepared from postnatal day 1 according to the protocol described in the previous section. Cells were suspended in culture medium consisting of Dulbecco's Modified Eagle Medium (Gibco-Life Technologies, Carlsbad, CA, USA) with 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), and penicillin-streptomycin solution (25,000 units/each) and plated on a culture flask (250 ml, 75 cm^2). Culture medium was changed every few days. When cells grew to confluence, the flask was shaken on a rotary shaker at 100 rpm at 37 °C for 24 h in order to detach contaminants such as microglia. The attached astrocytes were subsequently detached using trypsin-EDTA and plated on 6-well plates at a density of 1×10^5 cells per well for qRT-PCR, ELISA and Western blotting.

2.4. Drug treatment

In *in vitro* experiments, THA (9-amino-1,2,3,4-tetrahydro-acridine HCl, Sigma-Aldrich Japan, Tokyo, Japan) was employed to induce an increase in endogenous ACh in primary cultured neurons, while carbachol (CCh; Wako, Osaka, Japan), a muscarinic AChR agonist and nicotinic AChR agonist (Kiss et al., 2014), was treated to stimulate AChR in primary cultured astrocytes. In order to pharmacologically elucidate the mechanisms by which cholinergic drugs alter the expression levels of VEGF, mecamylamine hydrochloride (MCM; a nicotinic AChR inhibitor, Sigma, St. Louis, MO, USA), scopolamine hydrobromide n-hydrate (SCP; a muscarinic AChR inhibitor, Wako, Osaka, Japan), and Ro-318220 methanesulfonate (Ro; a conventional PKC and PKCε inhibitor, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were applied to cells 30 min before the treatment with THA or CCh.

In *in vivo* experiments, animals were administered THA daily at a dose of 2.5 mg/kg (i.p.) or vehicle saline for 7 days. Animals were decapitated 24 h after the last injection. In order to pharmacologically examine the mechanism by which THA elevates the expression levels of VEGF *in vivo*, animals were injected with MCM (3.0 mg/kg, i.p.) 30 min before the last treatment with THA and then decapitated 24 h later.

2.5. qRT-PCR

qRT-PCR was performed as previously described to measure the mRNA expression levels of the target genes (Zhao et al., 2007, 2011). Briefly, total RNA was extracted by the phenol-chloroform extraction method from cultured primary neurons and astrocytes 24 h after starting the test drug treatment using TRIsureTM (BIOLINE, London, UK) for the *in vitro* study and from the medial septal area using SepazolR (Nacalai Tesque, Kyoto, Japan) for the *in vivo* study according to the manufacturer's instructions. First-stand cDNA was synthesized with oligo (dT) primers and M-MLV reverse transcriptase (Invitrogen-Life Technologies, Carlsbad, CA USA). qRT-PCR was conducted using the Fast SYBR Green Master Mix and Step One Real-time PCR System (Applied Biosystems-Life Technologies, Carlsbad, CA, USA). The primers used for the PCR of each gene were synthesized by Nippon Gene Material Co. (Toyama, Japan) (Table 1). Standard curves of the log concentration vs. cycle threshold were plotted to prove negative linear correlations. The correlation coefficients for the standard curves of the target genes were 0.967–0.998.

2.6. ELISA

VEGF secreted from neurons and astrocytes was assessed by ELISA. Medium was collected from neuronal and astrocytic culture plates 36 and 24 h after starting the test drug treatment, respectively. The