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Nicotinic receptor-dependent and -independent effects of galantamine, an acetylcholinesterase inhibitor, on the non-neuronal acetylcholine system in C2C12 cells

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ABSTRACT

We previously reported that satellite cells possess the ability to produce angiogenic factors, including fibroblast growth factor (FGF)-2 and vascular endothelial growth factor (VEGF) in vivo. However, whether C2C12 cells possess a non-neuronal cholinergic system (NNCS) or non-neuronal ACh (NNA) remains to be studied; therefore, we investigated the system using C2C12 cells and its regulatory mechanisms. C2C12 cells synthesized ACh, the level of which was comparable with that of cardiomyocytes, and the synthesis was augmented by the acetylcholinesterase inhibitor galantamine. The ChAT promoter activity was upregulated by nicotine or galantamine, partly through nicotinic receptors for both agents as well as through a non-nicotinic receptor pathway for galantamine. Further, VEGF secretion by C2C12 cells was also increased by nicotine or galantamine through nicotinic receptors as well as partly through non-nicotinic pathways in the case of galantamine. These results suggest that C2C12 cells are equipped with NNCS or NNA, which is positively regulated through nicotinic or non-nicotinic pathways, particularly in the case of galantamine. These results provide a novel concept that myogenic cells expressing NNA can be a therapeutic target for regulating angiogenic factor synthesis.

1. Introduction

We previously reported that donepezil, an anti-Alzheimer's drug, can accelerate angiogenesis by targeting endothelial cells [1]; further research disclosed that this acceleration of angiogenesis by donepezil is executed by both endothelial and satellite cells, i.e., myogenic stem cells, because satellite cells play powerful roles in producing angiogenic factors when they are activated from quiescent states [2,3]. Moreover, we elucidated the detailed molecular mechanisms using C2C12 cells, a murine myoblast cell line, which exhibited increased expression of angiogenic factors through nicotinic receptors [3]. Altogether with these results, it is suggested that cholinergic intervention may be involved in both angiogenic and myogenic reactions.

In the last decade, a concept of a non-neuronal cholinergic system (NNCS) or non-neuronal ACh (NNA) has been gradually developed and known [4,5], and evidence supporting their existence has been accumulated. This has resulted in establishing a novel system that should be considered crucial in the pathophysiology of diseases [6–8]. Based on previous studies, it has been reported that NNA is found in many cell types, including immune, endothelial and epithelial cells [6,7]. In addition, cardiomyocytes are reported to possess NNA, indicating that the heart is equipped with NNA as the intrinsic cholinergic system [9–12].

In contrast, excluding cardiomyocytes, whether myogenic cells possess NNA, particularly satellite like cells (C2C12 cells), and NNA in skeletal muscle-derived cells can be pharmacologically modulated, like cardiomyocytes, remains to be elucidated. Therefore, the current study focused on whether NNA is expressed by C2C12 cells.

2. Materials and methods

2.1. C2C12 cells and reagents

C2C12 cells were obtained from the National Institute of Biomedical Innovation (Osaka, Japan). C2C12 cells were established from murine skeletal myoblasts. Specifically, they are more differentiated and not identical to satellite cells in terms of differentiation. However, a satellite cell line was not available, and the isolation of murine primary satellite cells was time-consuming; therefore, the current study used C2C12 cells instead of primary satellite cells. Nicotine hemisulfate salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich Co Ltd. (St. Louis, Missouri, USA), and galantamine hydrobromide was obtained from Tocris Bioscience (Bristol, UK).

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2.2. Cell culture of C2C12 cells

As previously reported [3], C2C12 cells were cultured in DMEM including antibiotics and 10% FBS, i.e., a culture medium for undifferentiated cells. Cells, transfected with the ChAT promoter assay vectors, were incubated with 100 μM galantamine hydrobromide or 200 nM nicotine hemisulfate for 24 h, followed by sampling.

2.3. Measurement of ACh in C2C12 cells

ACh levels in C2C12 cells, not supernatants, were measured as previously reported in our study [9]. For the measurement, C2C12 cells were cultured in a multiple culture plate and a cell number in each well was clarified to be identical. After 24 h treatment, culture medium was removed and cells were washed three times with PBS. Cells were scraped and homogenated in a cell lysis buffer (1 mL) including 0.1 M perchloric acid and an internal standard isopropylhomocholine chloride (10^{-8} M). As reported by us [9], to measure an intracellular ACh content, C2C12 cells were treated for 24 h with 0.1 mM physostigmine and these experiments were regarded as control. In separate experiments, the test substances including 100 μM galantamine and 200 nM nicotine were additionally present. At the end of the 24 h culture period, supernatant was removed and cells were washed with PBS. Thereafter, cells were lysed to measure the ACh content of the cells. Then, the ACh cell content was compared between both groups of experiments. The difference reflects possible effects of the test substances on ACh synthesis independent of acetylcholinesterase inhibition, because galantamine has been known as an acetylcholinesterase inhibitor and this study was performed to evaluate whether galantamine could upregulate ACh synthesis or not.

Analysis of ACh contents in C2C12 cells was performed by HPLC. As previously reported, the current system almost followed the previous one [9]. Isopropylhomocholine chloride (IPHC; 1×10^{-8} M) was used as an internal standard. The sample (10 μL) was injected into the system (HTEC-500, Eicom, Kyoto, Japan) including both a pump and electrochemical detector with a platinum electrode. In this system, a guard column (CH-Gel) and immobilized enzyme column (AC-Enzymepack II) were placed before and after a separation column (AC-Gel), respectively and a degasser (DG-100) was connected. The mobile phase was the buffer containing 50 mM $KHCO_3$, 300 mg/L sodium 1-decanesulfonate and 50 mg/L EDTA. The pump flow rate was 0.15 ml/min. Peak data were recorded and analyzed with a computer. In our system, the detection limit of an intracellular ACh level was 10^{-10} M.

2.4. ChAT promoter assay

C2C12 cells were transfected with attractene according to a commercially available protocol (QIAGEN). At 24 h after transfection, C2C12 cells were treated with 100 μM galantamine hydrochloride or 200 nM nicotine hemisulfate for 24 h and were then lysed and sampled for measurement with a luminometer (GloMax® 20/20 Luminometer, Promega).

2.5. Measurement of VEGF in culture medium

According to our previous study [3], VEGF levels in culture medium were measured using a Mouse VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota, USA).

2.6. Statistical analysis

Data were expressed as the mean ± standard error. A nonparametric Mann–Whitney U-test was used to compare the two groups.

3. Results

3.1. C2C12 cells produce ACh

To evaluate whether C2C12 cells can produce ACh, i.e., a non-neuronal ACh system (NNA) exists in C2C12 cells, we measured ACh levels in C2C12 cells. ACh could be detected in C2C12 cells at levels comparable with those in cardiomyocytes ($1.2 \pm 0.1 \times 10^{-9}$ M; $n = 17$), suggesting that they produce ACh through NNA, (Fig. 1A).

Furthermore, ACh synthesis by C2C12 cells was upregulated by 24-h treatment with 100 μM galantamine chloride ($4.1 \pm 0.3 \times 10^{-9}$ M; $P < 0.01$; $n = 18$), compared with that of 24-h non-treated cells. Galantamine chloride is an ACh esterase inhibitor that functions as an allosteric potentiating ligand (APL); this means that galantamine also functions as a nicotinic receptor agonist [13–15]. The fact that ACh levels in C2C12 cells were increased indicated that NNA in C2C12 cells was activated by galantamine through either nicotine receptor-dependent or -independent mechanisms. Supportively, C2C12 cells treated with 200 nM nicotine for 24 h upregulated intracellular ACh levels even with physostigmine, compared with non-treated cells but with physostigmine ($3.4 \pm 0.2 \times 10^{-9}$ M vs. $1.9 \pm 0.1 \times 10^{-9}$ M; $P < 0.05$; $n = 6–7$) (Fig. 1B).

3.2. Activation of ACh synthesis by galantamine through nicotinic receptor and non-nicotinic receptor pathways

To delineate the mechanisms by which galantamine regulated ACh synthesis, a reporter assay was performed using the specific ChAT promoter region subcloned into a luciferase expression vector, i.e., a ChAT reporter vector, as previously reported [9]. Fig. 2A demonstrated that luciferase activity was increased by 100 μM galantamine to 114.8 ± 2.8% of control ($P < 0.01$; $n = 9$), suggesting that galantamine increased ChAT transcription. However, this increase in activity was not completely abolished by the nicotinic receptor antagonist mecamylamine, even at higher doses of 15 and 30 μM (123.6 ± 3.8% and 115.0 ± 4.6%, respectively). This suggests that galantamine-induced ChAT transcriptional activation was not necessarily mediated by the nicotinic receptor alone, but instead was mediated by an as yet unidentified galantamine-specific and non-nicotinic receptor-mediated pathway. In comparison with the galantamine's effect of upregulating a ChAT promoter activity, donepezil also augmented the activity in C2C12 cells (133.3 ± 0.4%, $P < 0.05$, $n = 6$), suggesting that both acetylcholinesterase inhibitors with different molecular structures commonly upregulate the ChAT promoter activity.

As shown in Fig. 2B, in contrast to mecamylamine failing to suppress the galantamine's ChAT promoter activity upregulating effect (133.4 ± 6.3%, $n = 6$), 1 μM atropine did not blunt the activity (122.6 ± 7.1%, $n = 9$), suggesting that a galantamine's role may be mediated by non-muscarinic receptors.

3.3. Nicotine activates NNA through a nicotinic receptor

In contrast to galantamine, 200 nM nicotine definitely activated ChAT promoter activity within 24 h (142.7 ± 3.3%; $P < 0.01$; $n = 9$), and the activation was completely blunted by mecamylamine. Mecamylamine concentration-dependently decreased ChAT promoter activity (Fig. 3). This suggests that the nicotinic receptor plays a role in conferring the signal to activate the NNA system in C2C12 cells. As revealed in Fig. 1B, nicotine upregulated ACh levels in C2C12 cells, supporting the concept that nicotine increases ACh synthesis in C2C12 cells. These results using nicotine greatly contrasted that obtained for galantamine; therefore, it is suggested that galantamine plays a role in activating the NNA system through both nicotinic and non-nicotinic receptor-mediated pathways.

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