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Functional role of acetylcholine and the expression of cholinergic receptors and components in osteoblasts

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ABSTRACT

Recent studies have indicated that acetylcholine (ACh) plays a vital role in various tissues, while the role of ACh in bone metabolism remains unclear. Here we demonstrated that ACh induced cell proliferation and reduced alkaline phosphatase (ALP) activity via nicotinic (nAChRs) and muscarinic acetylcholine receptors (mAChRs) in osteoblasts. We detected mRNA expression of several nAChRs and mAChRs. Furthermore, we showed that cholinergic components were up-regulated and subunits/subtypes of acetylcholine receptors altered during osteoblast differentiation. To our knowledge, this is the first report demonstrating that osteoblasts express specific acetylcholine receptors and cholinergic components and that ACh plays a possible role in regulating the proliferation and differentiation of osteoblasts.

1. Introduction

A growing body of literature has demonstrated that neuronal factors such as vasoactive intestinal peptide (VIP), calcitonin gene-related peptide, and substance P directly affect the differentiation of both osteoblasts and osteoclasts and that the sympathetic nervous system regulates bone remodeling [1–3].

Increasing evidence indicates that acetylcholine (ACh), a well-known neurotransmitter, is involved in the regulation of basic functions such as proliferation, differentiation, cell–cell contact, immune functions, secretion, and absorption in non-neuronal cells [4,5]. Immune cell function is regulated by its own cholinergic system. For example, muscarinic ACh receptors (mAChRs) play a crucial role in the cytokine production and the differentiation of T cells [6]. In lung, all components needed for an autocrine cholinergic signaling pathway are expressed in airway bronchial epithelial cells, and ACh acts as an autocrine growth factor in small cell lung carcinoma [7,8]. ACh attenuates macrophage activation and decreases the production of proinflammatory mediators by macrophages stimulated with endotoxin [9]. In rodent models of endotoxemia and hemorrhagic shock, stimulation of the efferent vagus nerve dampens macrophage activation [5]. In addition, many non-neuronal cells, including keratinocytes, lymphocytes, placental trophoblasts, embryonic stem cells, epithelial cells, and endothelial cells, can synthesize ACh and release autocrine or paracrine hormones [10–12]. Non-neuronal cells that possess cholinergic components uptake choline by means of the high affinity choline transporter (CHT1) and then synthesize ACh by choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (acetyl-CoA). ACh is translocated into small synaptic vesicles by vesicular ACh transporter (VAChT) and is released via exocytosis. Once released, ACh exerts its cellular functions via mAChRs, including 5 subtypes (M1, M2, M3, M4 and M5), and nicotinic ACh receptors (nAChRs), including 16 subunits (α1, α2, α3, α4, α5, α6, α7, α9,

Abbreviations: ACh, acetylcholine; Nic, nicotine; Mus, muscarine; CCh, carbachol; Atr, atropine; Mec, mecamylamine; nAChRs, nicotinic acetylcholine receptors; mAChRs, muscarinic acetylcholine receptors; VAChT, vesicular acetylcholine transporter; ChAT, choline acetyltransferase; CHT1, high affinity choline transporter; AChE, acetylcholinesterase; VIP, vasoactive intestinal peptide; BrdU, 5-bromo-2′-deoxyuridine; ALP, alkaline phosphatase; pOB, primary osteoblasts

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α10, β1, β2, β3, β4, γ, δ and ε). Finally, ACh is rapidly degraded into choline and acetate by acetylcholinesterase (AChE) [12].

Periosteum, which consists of an outer layer of dense connective tissue with resident fibroblasts and an inner cellular layer containing immature osteoblasts and osteochondral progenitors, receives two classes of sympathetic innervation: noradrenergic fibers associated with the vasculature and non-adrenergic VAChT- and VIP-immunoreactive fibers associated with the parenchyma [13–15]. Asmus et al. have demonstrated that axons decrease their expression of catecholaminergic properties and acquire VAChT- and VIP-immunoreactivity on contact with the periosteum during development in vivo and after transplantation and that osteoblasts induce cholinergic function in cultured sympathetic neurons [16,17].

It is conceivable that VIP and ACh derived from VAChT- and VIP-immunoreactive fibers affect osteoblasts in the periosteum. Previous studies have demonstrated that osteoblasts express VIP receptors and that VIP stimulates the activity and mRNA expression of alkaline phosphatase (ALP) [18–20]. However, it remains unknown how ACh acts on osteoblasts and whether osteoblasts have cholinergic receptors and components. We hypothesized that ACh plays a functional role in bone metabolism. In the present study, we investigated the action of ACh during osteoblastic proliferation and differentiation and the mRNA expression of cholinergic receptors and components for ACh synthesis and release in osteoblasts.

2. Materials and methods

2.1. Reagents and antibodies

2-Acetoxy-*N*,*N*,*N*-trimethylethanaminium chloride (ACh), (−)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt (nicotine; Nic), (2*S*,4*R*,5*S*)-(4-hydroxy-5-methyl-tetrahydrofuran-2-ylmethyl)-trimethyl-ammonium chloride (muscarine, Mus), 2-carbamoyloxy-ethyl-trimethyl-azanium chloride (carbachol, CCh), (8-methyl-8-azabicyclo[3.2.1]oct-3-yl) 3-hydroxy-2-phenylpropanoate (atropine, Atr), and *N*,2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride (mecamylamine, Mec) were obtained from Sigma–Aldrich. Antibodies against Cdk4 (C-22), Cdk6 (DCS-83), cyclin D1 (HD11) and β-actin (C-4) were obtained from Santa Cruz Biotechnologies (California, USA).

2.2. Cell culture

Murine primary osteoblasts (pOB) were isolated from calvarie taken from newborn ddY mice (SLC, Shizuoka, Japan). The experiments were conducted according to the institutional ethical guidelines for animal experiments. Neonatal mouse calvaria were dissected free of adherent soft tissue, washed in PBS, and sequentially digested with 0.2% dispase and 0.1% collagenase. pOB and the mouse pre-osteoblastic cell line MC3T3-E1 were maintained in growth medium, consisting of α-modified minimum essential medium (α-MEM) (WAKO, Osaka, Japan) with 10% fetal bovine serum (FBS) (BioWest, Nuaillé, France). For osteoblastic differentiation assay, cells were cultured in growth medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) (conditioned medium). The growth medium and the conditioned medium were changed every three days. All cultures were maintained at 37 °C in humidified air including 5% CO_2 and were passaged every seven days.

2.3. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

DNA synthesis was assessed with a Cell Proliferation Biotrack ELISA System (GE Healthcare Life Sciences, Buckinghamshire, UK) according to manufacturer's recommendations. This assay measures BrdU incorporation during DNA synthesis by proliferating cells. Briefly, cells were plated to a density of 5×10^3 cells/well in 96-well plates and allowed to proliferate for 24 h. Next, their growth was arrested by incubation for 24 h in serum-free medium. Cells were then treated with stimulants (ACh, CCh, Mus, and Nic) at the indicated doses in growth medium for 60 h. To assess the effects of inhibitors (Mec and Atr), growth-arrested cells were treated with growth medium for 60 h plus stimulants in the presence or absence of inhibitors at the indicated doses, which were added 1 h before treatment with stimulants. The absorbance was measured at 450 nm using a Model 680 XR plate reader (BIO RAD, California, USA). Measurements are represented by the means of at least three independent experiments, with each data point based on six replicates.

2.4. Reverse-transcriptase polymerase chain reaction (RT-PCR)

After reaching confluence, cells were incubated in conditioned medium in the presence or absence of ACh (1nM) for 14 days. Total RNA (2 μg) was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) and was subjected to RT-PCR using a SuperScript One-Step RT-PCR kit (Invitrogen, California, USA) according to manufacturer's instructions. The gene-specific primer pairs used are shown in Table 1. For RT-PCR analysis, cDNA synthesis was performed for 30 min at 45 °C, and the products were denatured for 2 min at 94 °C. PCR amplification was carried out for 38 cycles (denaturation for 60 s at 94 °C, followed by primer annealing for 90 s at 55 °C, and extension for 120 s at 72 °C). GAPDH was used as a loading control.

2.5. Quantitative real time RT-PCR

To validate gene expression changes, quantitative real time RT-PCR analysis was performed with an Applied Biosystems Prism 7900HT Sequence Detection System according to manufacturer's instructions (Applied Biosystems Inc., California, USA) for runx2 and osterix. TaqMan Gene Expression Assays for runx2 (assay identification number Mm00501580_m1) and osterix (assay identification number Mm00504574_m1) were inventoried products (Applied Biosystems Inc.). Mouse GAPDH gene was used as endogenous control (assay identification number Mm03302249_g1). After reaching confluence, cells were incubated in conditioned medium for 14 days. Total RNA (2 μg) was extracted from cells using ISOGEN (Nippon Gene). Reverse-transcriptase reaction was performed with the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems Inc.). The thermal cycler conditions were as follows: step 1 for 60 min at 37 °C and step 2 for 5 min at 95 °C. PCR amplification with real-time detection was performed with TaqMan Gene Expression Master Mix (Applied Biosystems Inc.) and total RNA of 100 ng/μl. Thermal cycling conditions comprised an initial UNG incubation at 50 °C for 2 min, AmpliTaq Gold DNA polymerase activation at 95 °C for 10 min, 40 cycles of denaturing at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. All samples were performed in triplicate. Amplification data were analyzed with an Applied Biosystems Prism Sequence Detection Software version 2.1 (Applied Biosystems Inc.). To normalize the relative expression of the genes of interest to the GAPDH control, standard curves were prepared for each gene mentioned above and the GAPDH in each experiment.

2.6. Western blot analysis

To detect cyclin D1, cdk4, and cdk6, cells were plated at a density of 5×10^5 cells in a 100-mm-diameter dish and allowed to proliferate for 24 h. Their growth was then arrested by incubation for 48 h in α-MEM containing 0.5% FBS. Growth-arrested cells were