Chemico-Biological Interactions 259 (2016) 295–300

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Flavonoids induce the expression of acetylcholinesterase in cultured osteoblasts

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ARTICLE INFO

Article history:
Received 16 January 2016
Received in revised form
16 March 2016
Accepted 22 March 2016
Available online 26 March 2016

Keywords:
AChE
PRiMA
Flavonoid
Estrogen
Osteoporosis

ABSTRACT

Flavonoids, a group of natural compounds mainly derived from plants, are known to possess osteogenic effects in bone cells. Here, we aimed to test if flavonoid could induce a cholinergic enzyme, acetylcholinesterase (AChE), as well as bone differentiation. In cultured rat osteoblasts, twenty flavonoids, deriving from Chinese herbs and having known induction of alkaline phosphatase (ALP[1]) expression, were tested for its induction activity on AChE expression. Eleven flavonoids showed the induction, and five of them had robust activation of AChE expression, including baicalin, calycosin, genistin, hyperin and pratensein: the induction of AChE included the levels of mRNA, protein and enzymatic activity. Moreover, the flavonoid-induced AChE expression in cultured osteoblast was in proline-rich membrane anchor (PRiMA)-linked tetrameric globular form (G_4) only. In parallel, the expression of PRiMA was also induced by the application of flavonoids. The flavonoid-induced AChE in the cultures was not affected by estrogen receptor blocker, ICI 182,780. Taken together, the induction of PRiMA-linked AChE in osteoblast should be independent to classical estrogen signaling pathway.

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1. Introduction

Bone remodeling is a lifelong process involving the active and dynamic balance between bone resorption by osteoclasts and the formation by osteoblasts. The two processes need to be tightly coupled to maintain bone strength and integrity [1]. Osteoporosis, the most common bone remodeling disease that leads to an increased risk of fracture, is the result of an increased osteoclast function and/or a reduced osteoblast activity: this bone problem most frequently observed in post-menopausal women [2]. Current treatments for osteoporosis are mostly based on an anti-resorptive approach, e.g. estrogen replacement therapy (ERT[2]) is widely used in clinic to alleviate post-menopausal osteoporosis. However, the side effects of long term usage of estrogen (e.g. stroke, blood clots, gallstones, dementia, ovarian and breast cancer) hinder the use of such therapy [3]. Other anti-osteoporosis drugs, e.g. bisphosphonates and calcitonin, are acting as inhibitors to the bone resorption, but they show limitation in increasing or recovering bone mass. Teriparatide is currently the only osteoporosis medicine approved by the FDA that rebuilds bone. Therefore, there is a medical need for alternative therapies based on the stimulating of anabolic pathways in bone.

Flavonoids, a group of naturally occurring compounds mainly derived from plants, are well known to possess diverse biological effects. Structurally, flavonoids resemble estrogen, and some of them are known as phytoestrogen [4]. In menopause, the disturbance in estrogen level induces various symptoms, e.g., osteoporosis, hyperlipidemia and depression. ERT is widely used to alleviate menopausal syndrome [5]. Flavonoids, having the estrogenic effect, became very attractive as alternative therapies today to replace the use of estrogen. Several lines of evidence suggest that a number of flavonoids can activate estrogen receptor (ER) and stimulate ER-dependent transcriptional response in different cell models [6,7]. As a result of lacking estrogen, bone resorption increases while deposition of new bone material decreases. As an alternative of estrogen, flavonoids could be regarded as candidates for drug discovery against osteoporosis.

Acetylcholinesterase (AChE) is an enzyme to hydrolyze acetylcholine in synapses. By alternative splicing, three variants are being generated: "read-through ($AChE_R$)," "hydrophobic ($AChE_H$)," and "tailed ($AChE_T$)" [8]. $AChE_T$ exists in all vertebrates and is largely

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[1] ALP: alkaline phosphatase.
[2] ERT: estrogen replacement therapy.

http://dx.doi.org/10.1016/j.cbi.2016.03.025
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predominant in the brain and muscle, generating different oligomers. The function of $AChE_T$ is determined by localization and oligomerization of the enzyme, which essentially depends on a tight interaction between its C-terminal peptide with anchoring proteins like proline-rich membrane anchor (PRiMA). PRiMA-linked AChE exists in mammalian brain and muscle as amphiphilic tetrameric globular forms (detergent-interacting G_4 components) [9,10]. Starting from the last decade, the non-cholinergic functions of AChE have been proposed. This notion is strongly supported by the existence of AChE in tissues that have no cholinergic transmission, e.g. in bone [11]. However, the exact functional roles of AChE in bone cells are yet to be revealed. Here, twenty common flavonoids having osteogenic role in osteoblast were screened on their abilities to induce the expression of AChE in cultured osteoblasts [12]. Five of the tested flavonoids showed robust activation on AChE expression, as well as its anchor protein PRiMA; however, this activation was independent of estrogen receptor.

2. Materials and methods

2.1. Chemicals and flavonoids

Apigenin, kaempferol, baicalin, genistein, genistin and puerarin were purchased from Wakojunyaku (Osaka, Japan). Galangin, 4′,7-$OCOCH_3$ quercetin and calycosin were obtained from the School of Pharmaceutical Science, Peking University (Beijing, China). All the other flavonoids were purchased from National Institute of the Control of Pharmaceutical Biological Products (NICPBP) (Beijing, China). The purities of all the chemicals were over 98%, and all chemicals were dissolved in dimethyl sulfoxide (DMSO) to give a stock solution at concentration of 50–100 mM. Chemical structures of these flavonoids were given in supplementary figure (Supplementary Figure).

2.2. Cell culture

Rat primary osteoblasts were cultured and prepared by the method previously described [13] with minor modifications [14]. In brief, postnatal day 1 rats were decapitated to collect calvarias. Tissues were sequentially digested by 1% trypsin for 10 min, 0.2% collagenase for 20 min, and 0.2% collagenase for another 40 min. After the digestion, the supernatant was collected and centrifuged for 3 min at 1500 rpm. Osteoblastic cells were re-suspended and maintained in a water-saturated atmosphere at 37 °C in 5% CO_2/95% air, in modified Eagle's medium α (MEMα) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Osteoblastic differentiation was induced by the treatment of vitamin C (Vit. C[3]) (250 μM) and dexamethasone (Dex.[4]) (20 nM). All reagents for cell cultures were purchased from Invitrogen (Carlsbad, CA).

2.3. Alkaline phosphatase assay

In drug-treated cultured osteoblasts, the cultures were collected by lysis buffer containing 0.2% Triton X-100, 1 mM dithiothreitol, and 100 mM potassium phosphate buffer (pH 7.8). ALP[1] activity was measured by mixing the cell extract with 5 mM *p*-nitrophenyl phosphate (Sigma) in a buffer containing 0.1 M glycine (pH 10.4), 1 mM $MgCl_2$, and 1 mM $ZnCl_2$ at 37 °C, and absorbance was measured at 405 nm.

[3] Vit. C: vitamin C.
[4] Dex.: dexamethasone.

2.4. Real time quantitative PCR

Total RNA from cultured osteoblasts was isolated by RNAzol® RT reagent (Molecular Research Center, Cincinnati, OH), and 5 μg of RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (MMLV) (Invitrogen), according to the manufacturer's instructions. Real time PCRs of AChE, PRiMA and 18S rRNA transcripts were performed on equal amounts of reverse-transcribed products, using KAPA SYBR®FAST qPCR kits, according to the manufacturer's instruction (Kapa Biosystems, South Africa). Primers employed in RT-PCR were as follows: AChE catalytic subunit (5′-AAT CGA GTT CAT CTT TGG GCT CCC CC-3′ and 5′-CCA GTG CAC CAT GTA GGA GCT CCA-3′; NM_015831); PRiMA (5′-TCT GAC TGT CCT GGT CAT CAT TTG CTA C-3′ and 5′-TCA CAC CAC CGC AGC GTT CAC-3′; NM_178023); and 18S rRNA (5′-GAC TGT TAT GGT CAA GGT GAA-3′ and 5′-GAT AGT CAA GTT CGA CCG TC-3′; NR_003286). The SYBR Green signal was detected by Mx3000p™ multiplex quantitative PCR machine (Stratagene, La Jolla, CA). The relative levels of transcript expression were quantified by using $2^{-}\Delta\Delta Ct$ method [15]. The calculation was done by using the *Ct* value of 18S rRNA to normalize the *Ct* value of target gene in each sample to obtain the Δ*Ct* value, which was then used to compare among different samples. The PCR products were analyzed by gel electrophoresis, and the specificity of amplification was confirmed by a melting curve.

2.5. DNA constructions and transfection

Three repeats of estrogen responsive elements (ERE) (5′-GGT CAC AGT GAC C-3′) were synthesized as described previously [16,17] and then subcloned into a promoter-reporter vector pTAL-Luc (Clontech) that has a downstream reporter of firefly *luciferase* gene; this DNA construct was named as pERE-Luc. The DNAs (~2.2 kb) encompassing the human AChE promoter and human PRiMA promoter were subcloned into pGL3 vector (BD Biosciences Clontech, Palo Alto, CA) upstream of a *luciferase* gene, designated as pAChE-Luc and pPRiMA-Luc, respectively [18,19]. Transient transfection of osteoblasts with the cDNA constructs was performed with a Lipofectamine Plus reagent (Invitrogen), according to the manufacturer's instruction. The transfection efficiency was

Table 1
Potential flavonoids induce ALP[1] and AChE activities in cultured osteoblasts. Data are in means, $n = 5$, each with triplicate samples. + to +++ indicate the ranking of ALP[1] or AChE activity. – indicates no effect, i.e. below 10% of the increase in the tested activities. The working concentrations of positive control Dex.[4] plus Vit. C[3] were 20 nM and 250 μM, respectively. For the tested flavonoids, three concentrations (0.5, 5, and 50 μM) were tested. The ALP[1] activity data was used as a reference.

I: **ALP activity**
\+ > 10%
++ > 30%
+++ > 50%

II: **AChE activity**
\+ > 10%
++ > 50%
+++ > 100%

Flavonoids	ALP	AChE	Flavonoids	ALP	AChE
Alpinetin	++	+	Pratensein	++	++
Farrerol	+	–	4',7-OCOCH3 puerain	+	–
Naringenin	+	–	Tectorigenin	+	+
Neohesperidin	+	+	Galangin	+	–
Baicalin	+++	++	Hyperin	+++	+++
Luteolin	+	–	Icariin	+	+
Calycosin	+	++	Kaempferol	++	+
Daidzein	+	–	Kaempferol-3-O-glucoside	+	–
Daidzin	+	–	Quercetin	+	+
Genistein	+	–			
Genistin	++	++	Dex + Vit. C	++	+

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