Prostaglandins, Leukotrienes and Essential Fatty Acids 101 (2015) 23–29

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

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

PGD_2 stimulates osteoprotegerin synthesis via AMP-activated protein kinase in osteoblasts: Regulation of ERK and SAPK/JNK

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ARTICLE INFO

Article history:
Received 26 May 2015
Received in revised form
17 August 2015
Accepted 24 August 2015

Keywords:
AMP-activated protein kinase
PGD_2
Osteoprotegerin
Osteoblast

ABSTRACT

AMP-activated protein kinase (AMPK), a key enzyme sensing cellular energy metabolism, is currently known to regulate multiple metabolic pathways. Osteoprotegerin plays a pivotal role in the regulation of bone metabolism by inhibiting osteoclast activation. We have previously reported that prostaglandin D_2 (PGD_2) stimulates the synthesis of osteoprotegerin through the activation of p38 mitogen-activated protein (MAP) kinase, p44/p42 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. On the basis of these findings, we herein investigated the implication of AMPK in PGD_2-stimulated osteoprotegerin synthesis in these cells. PGD_2 induced the phosphorylation of AMPKα (Thr-172) and AMPKβ (Ser-108), and the phosphorylation of acetyl-coenzyme A carboxylase, a direct AMPK substrate. Compound C, an AMPK inhibitor, which suppressed the phosphorylation of acetyl-coenzyme A carboxylase, significantly attenuated both the release and the mRNA levels of osteoprotegerin stimulated by PGD_2. The PGD_2-induced phosphorylation of p44/p42 MAP kinase and SAPK/JNK but not p38 MAP kinase were markedly inhibited by compound C. These results strongly suggest that AMPK regulates the PGD_2-stimulated osteoprotegerin synthesis at a point upstream of p44/p42 MAP kinase and SAPK/JNK in osteoblasts.

1. Introduction

AMP-activated protein kinase (AMPK) is one of the central regulators of cellular energy metabolism in eukaryotes [1,2]. AMPK has been identified as a mammalian protein kinase which is allosterically activated by AMP [1]. It is generally known that AMPK exists as a heterotrimeric complex consisting of three subunits, α, β and γ. The α-subunit is recognized as the catalytic site, while β- and γ-subunits are as regulatory sites. The phosphorylation of Thr-172 in the α-subunit of AMPK is reportedly essential for its activation [3]. Although AMPK was firstly considered as an emergency response enzyme activated only during severe metabolic stress such as low nutrients or prolonged exercise causing the increase of intracellular AMP/ATP ratio, it is currently established that AMPK plays a pivotal role in the regulation of metabolic homeostasis also in the whole body [1]. Several essential enzymes implicated in the synthesis of lipid or carbohydrate such as acetyl coenzyme A (acetyl-CoA) carboxylase are known as targets of AMPK [2,4]. It has been shown that the activation of AMPK suppresses glucose-induced insulin secretion and pancreatic-β-cell survival [4], but increases the glucose transporter type 4 (Glut4) translocation from intracellular compartment to the plasma membrane in skeletal muscle, resulting in the stimulation of glucose uptake [5]. In addition, the inhibition of fatty acid synthase reportedly activates AMPK causing cytotoxicity in ovarian cancer cells [6]. Therefore, evidence is accumulating that AMPK might be a new therapeutic target for diabetes, obesity, and cancer.

It is firmly established that bone metabolism is highly coordinated processes performed mainly by two types of functional cells, osteoblasts and osteoclasts [7,8]. The former are responsible for the formation of new bone and the latter are for the removal of old bone. Bone resorption and bone formation are coupling processes called bone remodeling, which are strictly regulated, and bone mass and its quality are maintained by the fine-tuning process. The disorders of bone remodeling cause metabolic bone diseases such as osteoporosis. As for the roles of AMPK in bone metabolism, metformin, an activator of AMPK used as anti-hyperglycemic pharmaceutics, reportedly increases

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http://dx.doi.org/10.1016/j.plefa.2015.08.003
0952-3278/

collagen-1 and osteocalcin mRNA expression, stimulates alkaline phosphatase activity, and enhances cell mineralization in osteoblast-like MC3T3-E1 cells [9]. It has recently been shown that AMPK activation up-regulates bone mass using AMPKα knockout mice [10]. We have previously shown that AMPK positively regulates basic fibroblast growth factor-induced vascular endothelial growth factor synthesis and platelet-derived growth factor-BB-stimulated interleukin-6 synthesis in MC3T3-E1 cells [11,12]. However, the precise role of AMPK in osteoblasts remains to be unclear.

Osteoprotegerin, which is synthesized by osteoblasts, is a member of the tumor necrosis factor receptor family along with receptor activator of nuclear factor-κB (RANK) [13]. Osteoprotegerin binds to RANK ligand (RANKL) in osteoblasts as a decoy receptor, and prevents RANKL from binding to RANK in osteoclasts, resulting in the suppression of osteoclastogenesis [13]. It has been shown that RANKL knockout mice suffer from severe osteopetrosis [14], suggesting that osteoblasts regulate osteoclast functions. Thus, RANK–RANKL–osteoprotegerin axis is currently recognized as a major regulatory system for osteoclast activity [15].

In bone metabolism, prostaglandins (PGs) are known to act as autocrine/paracrine modulators [16]. With regard to PGD_2, PGD_2 reportedly induces collagen synthesis during calcification of osteoblasts [17]. In addition, it has recently been demonstrated that PGD_2 modulates osteoblast function and bone anabolism [18]. In our previous study [19], we have shown that PGD_2 stimulates osteoprotegerin synthesis via the activation of p38 mitogen-activated protein (MAP) kinase, p44/p42 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. However, the exact mechanism underlying PGD_2-stimulated osteoprotegerin synthesis in osteoblasts has not yet been clarified.

In the present study, we investigated whether AMPK is implicated in the synthesis of osteoprotegerin induced by PGD_2 in osteoblast-like MC3T3-E1 cells. We herein show that AMPK positively regulates the PGD_2-stimulated osteoprotegerin synthesis at a point upstream of p44/p42 MAP kinase or SAPK/JNK in osteoblasts.

2. Materials and methods

2.1. Materials

PGD_2 was obtained from Sigma Chemical Co. (St Louis, MO). Mouse osteoprotegerin enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, Inc. (Minneapolis, MN). Compound C was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific AMPKα (Thr-172) antibodies, phospho-specific AMPKβ (Ser-108) antibodies, phospho-specific acetyl-CoA carboxylase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An ECL Western blotting detection system was purchased from GE Healthcare UK Ltd. (Buchinghamshire, UK). Other materials and chemicals were obtained from commercial sources. PGD_2 was dissolved in ethanol. Compound C was dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect either the assay for osteoprotegerin or the Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells, which have been derived from newborn mouse calvaria [20], were maintained as described previously [21]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes (5×10^4 cells/dish) or 90-mm diameter dishes (2×10^5 cells/dish) in α-MEM containing 10% FBS. After five days, the medium was exchanged for α-MEM containing 0.3% FBS. The cells were then used for experiments after 48 h.

2.3. Assay for osteoprotegerin

The cultured cells were pretreated with various concentrations of compound C for 60 min, then stimulated by 10 μM of PGD_2 or vehicle in 1 ml of α-MEM containing 0.3% FBS, and incubated for the indicated periods. The conditioned medium was collected, and the osteoprotegerin concentration in the medium was measured by the mouse osteoprotegerin ELISA kit according to the manufacturer's protocol.

2.4. Real-time RT-PCR

The cultured cells were pretreated with 10 μM of compound C or vehicle for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle in α-MEM containing 0.3% FBS for 3 h. Total RNA was isolated and transcribed into complementary DNA using Trizol reagent (Invitrogen Co., Carlsbad, CA) and Omniscript Reverse Transcriptase kit (QIAGEN Inc., Valencia, CA), respectively. Real-time RT-PCR was performed using a Light Cycler system in capillaries and the Fast Start DNA Master SYBR Green I provided with the kit (Roche Diagnostics, Basel, Switzerland). Sense and antisense primers for mouse osteoprotegerin or GAPDH mRNA were purchased from Takara Bio Inc., (Tokyo, Japan; primer set ID: MA026526). The amplified products were determined using a melting curve analysis and agarose electrophoresis. The osteoprotegerin mRNA levels were normalized to those of GAPDH mRNA.

2.5. Western blot analysis

The cultured cells were pretreated with various concentrations of compound C for 60 min or vehicle, and then stimulated by 10 μM of PGD_2 or vehicle for the indicated periods. When indicated, the cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCL, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method described by Laemmli [22] in 10% polyacrylamide gels. The protein was fractionated and transferred onto an Immun-Blot ploy vinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). Western blot analysis as described previously [23] was performed using the indicated primary antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG as secondary antibodies. Phospho-specific AMPKα antibodies, phospho-specific AMPKβ antibodies, phospho-specific acetyl-CoA carboxylase antibodies, GAPDH antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies were used as primary antibodies. The peroxidase activity on the PVDF membranes was visualized on X-ray film by means of the ECL Western blotting detection system.