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Lactate enhanced the effect of parathyroid hormone on osteoblast differentiation via GPR81-PKC-Akt signaling

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ABSTRACT

Osteoblast uses aerobic glycolysis to meet the metabolic needs in differentiation process. Lactate, the end product of glycolysis, presents in the environment with elevated PTH and osteoblast differentiation. Although previous findings showed that lactate promoted osteoblast differentiation, whether lactate affects PTH—mediated osteoblast differentiation is unclear. To investigate this, pre-osteoblast cell line MC3T3-E1 was treated PTH with or without physiological dose of lactate. Lactate increases ALP positive cell formation, increases ALP activity and expression of differentiation related markers, enriches the CREB transcriptional factor target genes in PTH treated cells. Using inhibitors for MCT-1 reveales that lactate effects are MCT-1 independent. Lactate selectively increases Akt and p38 activation but not Erk1/2 and β-Catenin activation. The inhibitors for Akt and p38 inhibit lactate effects on PTH mediated osteoblast differentiation. Using inhibitors for Gαi signaling of GPR81 further increases Alp mRNA levels in lactate and PTH co-treatment cells. However, with the inhibitors for Gβγ-PLC-PKC signaling, the effect of lactate on PTH mediated osteoblast differentiation is inhibited. Our data demonstrate that lactate activates GPR81-Gβγ-PLC-PKC-Akt signaling to regulate osteoblast differentiation that mediated by PTH treatment.

1. Introduction

During bone development and bone remodeling process, osteoblast differentiation is tightly regulated for its specific metabolic needs [1]. Recently, it has been shown that mature calvarial osteoblastic cells relied more on aerobic glycolysis than oxidative phosphorylation during differentiation process [2,3]. In hypoxic bone marrow environment, the direct activation of glycolysis via the expression of oxygen-stable form of hypoxia inducible factor 1α (Hif-1α) or by deleting von Hippel-Lindau (VHL) stimulates bone formation [4,5]. Multiple bone inducing factors, as WNT proteins and PTH, stimulate aerobic glycolysis to control osteoblast differentiation [2,6]. WNT3A promotes osteoblast differentiation and bone formation through activating glycolysis via a LRP5 dependent but β-Catenin independent signaling [6]. PTH stimulates aerobic glycolysis via Igf-mTORC2 signaling in cultures of osteoblast-lineage cells that contributes to bone anabolism effect of PTH [2].

Lactate (LA), the end product of aerobic glycolysis, has been long neglected and seen as a byproduct until recently the findings for its important role in global gene transcription, cancer progression, functional polarisation of immune cells and the sustenance of stem cells [7–9]. Lactate transfers its signal into the cell via its receptor or transporter mediated signal transduction [8]. For example, its transporter MCT-1 mediated lactate transportation into macrophages promotes polarisation via Hif-1α stabilization and the resulting increased production of vascular endothelial growth factor (VEGF), which further enhances tumor growth [7,8]. LA also binds with its receptor GPR81, a Gαi protein coupled receptor, then mediates the insulin-induced reduction of lipolysis and regulates the lipid metabolism homeostasis [10–13]. There is evidence that LA alters cellular responses in inflammatory environments, for example, LA suppresses the effect of IL-33 on primary human mast

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cells in an MCT-1 dependent manner [14]. In our previous study, we found that lactate was transported into cell by MCT-1 and plays a role in osteoblast differentiation via the stabilization of Hif-1α [15]. Whether LA affects osteoblast differentiation that induced by bone formation inducing factors is not clear.

Here we report that LA acutely stimulated osteoblast differentiation that mediated by PTH. Distinct from our previous findings, the regulation described here is independent of LA-MCT-1 signaling, but requires the regulation on its receptor GPR81 and the downstream Gβγ-PKC −Akt signaling. Thus, LA signaling reprograms osteoblalst differentiation that mediated by PTH through a novel GPR81 dependent but MCT-1 independent mechanism.

2. Materials and methods

2.1. Reagents and antibodies

Fetal bovine serum (FBS), L-glutamine, antibiotics, alpha-modified essential medium (α-MEM) and trypsin/EDTA were obtained from Gibco (Life Technologies, Grand Island, NY, USA). L-(+)-lactic acid (L1750) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). PTH1-34 was purchased from BACHEM (Bachem AG, Bubendorf, Switzerland). The inhibitors for Erk1/2 (SCH772984), p38 (SB203580), Akt (MK2206), β-Catenin (XAV939), PKA (H89-2HCl), PKC (Sotrastaurin) and MCT1 (α-CCA and AZD3965) were purchase from Selleck (Selleckchem, Houston, TX, USA). The inhibitor for PLC (U73122) was from TORCRIS (Tocris Bioscience, Bio-Techne China Co. Ltd., Shanghai, China). The inhibitor for Gβγ protein (Guanosine 5′-O-(2-Thiodiphosphate) trilithium salt, GDPβs) was from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The antibodies for p-Erk1/2, p-Akt-S473, p-p38, p-β-Catenin-S647, p38, Akt and β-Catenin were from Cell Signaling Technology (Cell Signaling Technology, Inc., Danvers, MA, USA). Antibodies for β-actin and Erk1/2 were from Proteintech (Wuhan Sanying, Wuhan, Hubei, China).

2.2. Cell culture

The pre-osteoblast cell line MC3T3-E1 was purchased from the cell bank of Chinese Academy Science and maintained in α-MEM contained 10% FBS, 2 mM L-glutamine and antibiotics. For LA treatments, medium without LA was used as a control as indicated previously [7].

2.3. Real-time PCR

mRNA extracted from cells with RNAiso Plus (TaKaRa Bio Inc., Japan) was firstly reverse transcribed to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied biosystems, Carlsbad, CA, USA), and then analyzed on Roche Lightcycler 480II detection system with SYBR Premix Ex Taq Mix (TaKaRa Bio Inc., Japan) for the expression of Alkaline Phosphatase (Alp), Runx2 and β-actin. The primers for these genes were retrieved from PrimerBank [16]. All detections were in triplicate for each sample and data were normalized to β-actin levels (ΔΔcT).

2.4. Western blotting

For Western blotting, total cytoplasmic protein was isolated with RIPA lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing protease and phosphatase inhibitors (Pierce Protease and Phosphatase Inhibitor Mini Tables) and run on 6–9% SDS-PAGE gels. After blotting to PVDF membrane (Life Technologies, Grand Island, NY, USA), membranes were blocked for 60 min at room temperature and incubated overnight at 4 °C in buffer (TBS with 0.1% Tween 20 and 5% w/v BSA) containing diluted antibodies. Detection of primary antibodies was performed with HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, INC., West Grove, PA, USA). Immunoreactive bands were visualized with Chemiluminescent substrate (Clarity™ Western ECL Substrate, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Densitometric analysis was performed using Image J software.

2.5. Alp staining and Alp activity analysis

The analysis of osteoblast differentiation was done as previously indicated [15,17]. Briefly, cells were received PTH (500 ng/ml) with or without LA (5 mM) treatments for indicated time with regular medium changing. Then alkaline phosphatase staining positive cells were detected with TRACP & ALP double-staining Kit (TaKaRa Bio Inc., Japan). For alkaline phosphatase activity analysis, cells were firstly lysed with RIPA buffer that without the inhibitors (Beyotime Institute of Biotechnology, Nantong, China), then the centrifuged lystaes were measure by Alkaline Phosphatase Assay Kit (Beyotime Institute of Biotechnology, Nantong, China) followed the protocol provided. The activity was further normalized by the protein concentration of corresponded lysate.

2.6. Luciferase activity analysis

pGL3-basic, pRL-Tk (thymidine kinase promoter driven *Renilla* luciferase) and dual luciferase assay kit were bought from Promega (Promega Corporation, Madison, WI, USA). MC3T3-E1 cells were firstly co-transfected with CREB-Luc (YEASEN Biology, Shanghai, China) or their control vectors and pRL-Tk for 48 h then received PTH with or without LA treatments for 24 h. The cells were lysed using 0.1 ml of passive lysis buffer (Promega, Madison, WI, USA). The luciferase activity was measured by dual luciferase assay kit (Promega, Madison, WI, USA) according to manufacturer's manuals.

2.7. Microarray analysis and gene set enrichment analysis

For Affymetrix based microarray, RNA was extracted from the MC3T3-E1 cells that received PTH with or without LA treatment for 24 h. All subsequent technical procedures and quality controls were performed at CapitalBio (CapitalBio Corporation, Beijng, China). Briefly, an aliquot of 2 μg total RNA as the start RNA was used for cDNA synthesis, then cRNA labeled with biotin was synthesized using all of the above 12 μl synthesized cDNA as template and applying the Message Amp TM II aRNA Amplification Kit. Biotin-labeled cRNA was randomly fragmented to strands of 35–200 bases in length according to Affymetrix's protocol. The Gene set enrichment analysis was done followed the previous study [18].

2.8. Statistical analyses

All data were expressed as mean ± SEM. Statistical significance was identified by one-way analysis of variance (ANOVA) where appropriate, with probability $P<0.05$ being considered significant.

3. Results

We have revealed that lactate (LA) in culture environment induced osteoblast differentiation [15]. Whether LA had any effect on osteoblast differentiation that induced by PTH was still not clear. In pre-osteoblast cell MC3T3-E1, PTH treatment stimulated the formation of ALP positive cells, which further increased in cells co-treated with LA (Fig. 1A). In consistence, the ALP activity (Fig. 1B) and mRNA level of Alp (Fig. 1C), Runx2 (Fig. 1D) also increased in cells with LA and PTH co-treatment. The intake of LA into the cell

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