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Signaling pathway for adiponectin expression in adipocytes by osteocalcin

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ABSTRACT

In addition to providing skeletal support, the bone is an endocrine organ that produces osteocalcin, whose uncarboxylated form (GluOC) increases insulin secretion either directly or indirectly by promoting incretin secretion. We have now investigated the signaling pathway by which GluOC increases expression of adiponectin in adipocytes. Activation of its putative receptor GPRC6A by GluOC induced the intracellular accumulation of cAMP and consequent activation of protein kinase A (PKA) in differentiated 3T3-L1 adipocytes. It also induced phosphorylation of CREB (cAMP response element binding protein), but this effect appeared to be mediated indirectly by extracellular signal-regulated kinase (ERK) rather than directly by PKA, given that it was attenuated by the ERK signaling inhibitor U0126. Activated PKA also induced activation of the tyrosine kinase Src, the small GTPase Rap1, an upstream of ERK and CREB phosphorylation. Activated CREB up-regulated the expression of peroxisome proliferator-activated receptor γ (PPARγ), which in turn led to induction of adiponectin expression. Finally, intermittent oral administration of GluOC in mice reduced the size of gonadal white adipocytes as well as increased the expression of PPARγ and adiponectin in these cells. Our results have thus revealed the signaling pathway by which GluOC induces adiponectin expression in adipocytes.

1. Introduction

Osteocalcin is a bone-derived hormone that, in the uncarboxylated (or undercarboxylated) form, plays an important role in glucose and energy metabolism, male fertility, and brain function [1–5]. Analysis of various genetically modified animals has revealed that an increase in the circulating osteocalcin concentration can prevent the development of obesity and glucose intolerance [1–3,6]. Examination of the therapeutic potential of osteocalcin in mice showed that continuous administration of uncarboxylated osteocalcin (GluOC) *via* a subcutaneous osmotic pump [7] or intermittent intraperitoneal injection of GluOC [8] lowered blood glucose concentration and increased pancreatic β-cell mass, insulin secretion, and insulin sensitivity [7,8]. Moreover, daily injection of GluOC for 14 weeks resulted in full recovery of the liver in mice with steatosis induced by a high-fat diet [8]. These effects of GluOC are likely mediated by its putative receptor GPRC6A [4,9] and achieved in part through stimulation of insulin secretion from [9,10] and the proliferation of [7] pancreatic β-cells, leading to an improvement in glucose homeostasis in muscle and adipose tissue [1,2].

We recently showed that oral administration of GluOC was as effective as intraperitoneal injection in increasing the serum concentration of insulin in mice. This effect of ingested GluOC was mediated in large part through stimulation of glucagon-like peptide-1 (GLP-1) secretion from intestinal endocrine cells, as revealed by its inhibition by the GLP-1 receptor antagonist exendin(9–39) [11]. We also found that orally administered GluOC is absorbed into the general circulation [11], suggesting that this method of application allows GluOC to trigger the secretion of GLP-1 by acting at both apical and basolateral membranes of intestinal endocrine cells. In addition, we showed that GPRC6A is localized to the apical and basolateral membranes of intestinal cells and that it is colocalized with GLP-1 in some of these cells in mice [12]. Intermittent or daily oral application of GluOC in mice for

Abbreviations: GluOC, uncarboxylated osteocalcin; GlaOC, γ-carboxylated osteocalcin; PKA, protein kinase A; ERK, extracellular signal-regulated kinase; PPAR, peroxisome proliferator-activated receptor; GLP-1, glucagon-like peptide-1; gWAT, gonadal white adipose tissue; DMEM, Dulbecco's modified Eagle's medium; IBMX, isobutylmethylxanthine; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; RNAi, RNA interference; siRNAs, small interfering RNAs; db-cAMP, dibutyryl-cAMP; CREB, cAMP response element binding protein; Epac, exchange protein directly activated by cAMP; GEF, GDP/GTP exchange factor; PGC-1α, PPARγ coactivator-1α; TNF-α, tumor necrosis factor-α

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13 or 4 weeks, respectively, was effective in lowering blood glucose concentration, increasing insulin secretion and β-cell mass, and promoting glucose tolerance [12].

On the other hand, information regarding the effects of GluOC on adipose tissue is limited. GluOC was previously shown to increase the amount of adiponectin mRNA in adipocytes in a concentration-dependent manner [8], and mice genetically deficient in *Esp* (a protein tyrosine phosphatase expressed in osteoblasts) were found to manifest an increased serum concentration of GluOC as well as increased adiponectin expression in fat tissue [1]. The signaling pathway underlying these observations has remained unexplored, however. With the use of differentiated 3T3-L1 cells, we have now examined the signaling pathway responsible for the up-regulation of adiponectin mRNA by GluOC in adipocytes. We have also examined the features of gonadal white adipose tissue (gWAT) in mice subjected to oral administration of GluOC.

2. Materials and methods

2.1. Cell culture and adipocyte differentiation

3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, VA) and were grown to confluence under a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% bovine calf serum (HyClone-Thermo Fisher Scientific, Waltham, MA), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Confluent cells were cultured first for 3 days in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen, Carlsbad, CA), 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich), 0.25 μM dexamethasone, 166 nM bovine insulin (Sigma-Aldrich), and 10 μM troglitazone (Sigma-Aldrich) and then for 2 days in DMEM supplemented with 10% FBS and 166 nM insulin. The medium was then replaced with DMEM supplemented with 10% FBS alone, which was replenished every 2 days. The differentiation of 3T3-L1 cells was evident from the accumulation of intracellular lipid droplets, and the efficiency of differentiation was consistently >90%. The cells were used for experiments after 10 to 20 passages.

2.2. Isolation of RNA and RT-PCR analysis

Total RNA was extracted from 3T3-L1 adipocytes with the use of an RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA concentration of each sample was determined from the absorbance at 260 nm. Isolated total RNA (2 μg) was subjected to reverse transcription (RT) and PCR analysis with the use of a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio, Shiga, Japan) and with the GPRC6A primers 5′-GCTTTACTCTCTGTGT CTC-3′ (forward) and 5′-GCTAGCTTTCAGTTTTGACCC-3′ (reverse).

2.3. Immunoblot analysis

3T3-L1 adipocytes were lysed in a solution containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 mM $Na_4P_2O_7$, 100 μM NaF, and 2 mM Na_3VO_4, whereas gWAT was lysed in a solution containing 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 10 mM $Na_4P_2O_7$, 100 μM NaF, and 2 mM Na_3VO_4. Both lysis solutions were also supplemented with protease inhibitors including pepstatin A (2.5 μg/ml), leupeptin (5 μg/ml), 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (25 μg/ml), and aprotinin (1.7 μg/ml). The lysates were centrifuged at 50,000 × *g* for 20 min at 4 °C, and the protein concentration of the resulting supernatants was determined with the use of a Protein Assay Rapid Kit (Wako, Osaka, Japan). Portions of the supernatants (2 to 15 μg of protein) were fractionated by polyacrylamide gel electrophoresis on 8, 10, or 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The separated proteins were transferred to a polyvinylidene difluoride membrane (Merck-Millipore), which was then exposed to 5% dried skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) before incubation with primary antibodies including those to PPARγ or to total or phosphorylated forms of CREB, ERK, or Src (Cell Signaling

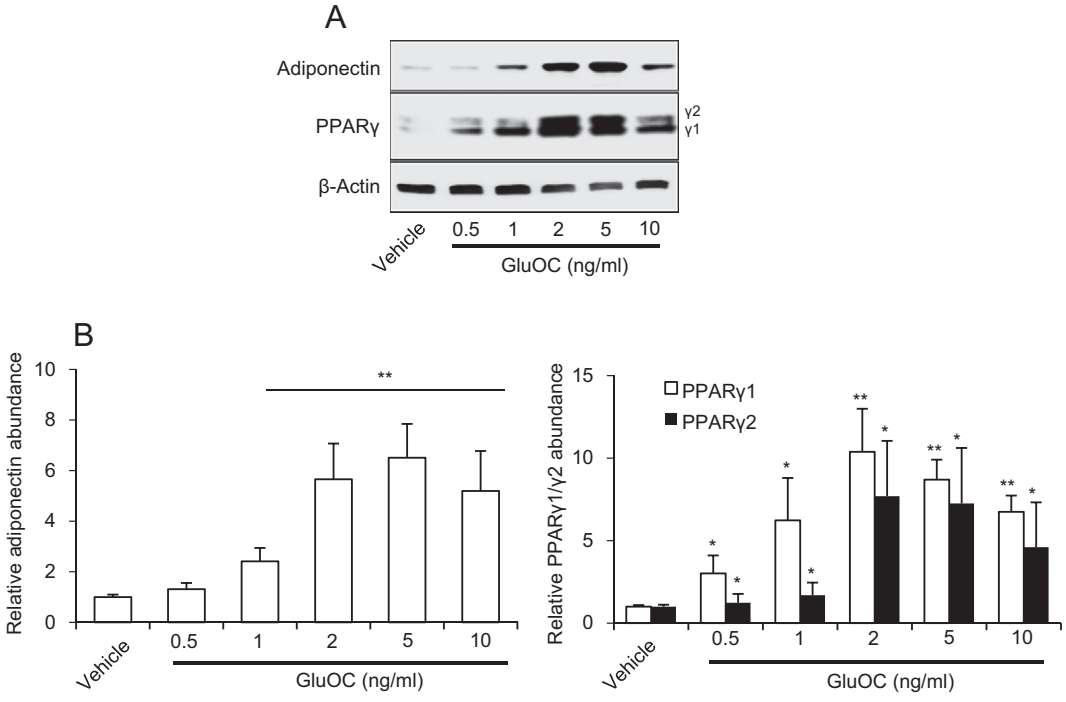

Fig. 1. Effects of GluOC on adiponectin and PPARγ expression in 3T3-L1 adipocytes. (A) Immunoblot analysis of adiponectin as well as of PPARγ1 and PPARγ2 in 3T3-L1 adipocytes incubated for 24 h in the presence of the indicated concentrations of GluOC. (B) Quantitation of adiponectin and PPARγ expression (normalized by the amount of β-actin) in immunoblots shown in (A). Data are expressed relative to the corresponding value for vehicle-treated (control) cells and are means ± SEM from five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* the corresponding control (Student's *t* test).