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Sustained expression of lipocalin-type prostaglandin D synthase in the antisense direction positively regulates adipogenesis in cloned cultured preadipocytes

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ABSTRACT

Adipocytes express preferentially lipocalin-type prostaglandin (PG)D synthase (L-PGDS) that is responsible for the biosynthesis of PGD_2 and other related prostanoids with pro-adipogenic or anti-adipogenic effects. To evaluate the role of L-PGDS in cultured adipocytes and the precursor cells, we attempted to interfere the intracellular expression of L-PGDS in cultured 3T3-L1 preadipocytes by stable transfection with a mammalian expression vector having the full-length cDNA of L-PGDS oriented in the antisense direction. The cloned transfectants with antisense L-PGDS exhibited the reduction in the transcript and protein levels of L-PGDS, resulting in the significant inhibition of the PGD_2 synthesis from exogenous and endogenous arachidonic acid. By contrast, the synthesis of PGE_2 was not influenced appreciably, indicating no interfering effects on cyclooxygenases and PGE synthases. The stable transfection with antisense L-PGDS induced markedly the stimulation of fat storage in cultured adipocytes during the maturation phase. In addition, the spontaneous accumulation of fats occurred in the transfectants with antisense L-PGDS without undergoing the stimulation with inducing factors. The gene expression studies revealed the enhanced expression of adipocyte-specific markers in the transfectants with antisense L-PGDS, indicating the up-regulation of adipogenesis program. The stimulated adipogenesis was significantly reversed by anti-adipogenic prostanoids including PGE_2 and $PGF_{2\alpha}$, while the storage of fats was additionally enhanced by pro-adipogenic 15-deoxy- $\Delta^{12,14}$-prostaglandin J_2. These results suggest that the stably reduced expression levels of L-PGDS regulates positively adipogenesis program in a cellular mechanism independent of pro-adipogenic action of PGJ_2 series.

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1. Introduction

Arachidonate cyclooxygenase (COX) pathway generates prostaglandin D_2 (PGD_2) with versatile effects, through the coordinated actions of the isoformic enzymes of COX and prostaglandin D synthases (PGDSs) [1]. PGD_2 exerts its effects via cell-surface receptors including DP1 and CRTH2 [2,3]. Moreover, PGD_2 is non-enzymatically converted to PGJ_2 series, such as 15-deoxy- $\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and Δ^{12}-PGJ_2 [4]. Of these, 15d-PGJ_2 is the most potent natural ligand for the nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ) [5,6]. Alternatively, 15d-PGJ_2 serves as a negative regulator of prostanoid synthesis and inflammation [7].

White adipose tissue is specialized for the storage and mobilization of fats as fuel molecules. As well, adipocytes serve as signaling cells to secrete bioactive factors like adipocytokines and prostanoids with opposite effects at different life stages. During the differentiation and maturation of adipocytes, PPARγ is expressed abundantly and acts as a master regulator of a series of the gene expression leading to adipogenesis [8,9]. Indeed, exogenous PGD_2 and PGJ_2 series have been demonstrated to promote adipogenesis in PPARγ-expressing cultured adipocytes [5,6,10,11]. For the study on the roles of prostanoids in adipocytes and the precursor cells, we have been making use of cultured preadipogenic 3T3-L1 cells [10–13]. Our recent studies have described the contribution of endogenous PGJ_2 series to the up-regulation of adipogenesis [10,11]. Earlier, cultured 3T3-L1 cells have been shown to express specifically lipocalin-type PGDS (L-PGDS) during the progress of

Abbreviations: PG, prostaglandin; L-PGDS, lipocalin-type PGD synthase; COX, cyclooxygenase; 15d-PGJ_2, 15-deoxy- $\Delta^{12,14}$-prostaglandin J_2; PPARγ, peroxisome proliferator-activated receptor γ; G418, geneticin; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; aP2, adipocyte protein 2; GLUT-4, glucose transporter-4; LPL, lipoprotein lipase; ELISA, enzyme-linked immunosorbent assay.

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adipogenesis associated with co-expression of PPARγ [10,12,14]. Nevertheless, the role of intracellular L-PGDS is still complicated because PGD_2 and the related metabolites or derivatives exert other different effects on the function of adipocytes. For example, PGD_2 can be metabolized to 9α, 11β-$PGF_{2\alpha}$, which acts as an anti-adipogenic factor in cultured adipocytes [15]. In addition, 15d-PGJ_2 has been implicated in the inhibition of inflammatory response associated with inducible synthesis of COX-2 [7]. Moreover, the action of PGD_2 through the cell-surface receptors with different subtypes may not be excluded. Interestingly, active form of L-PGDS has been shown to inhibit the phosphorylation of Akt, a serine/threonine kinase [16]. The phosphorylated Akt has been previously implicated in spontaneous differentiation into adipocytes in a PG-independent manner [17]. Thus, the functions of L-PGDS in adipocytes at different life stages still remain to be determined. Here, to determine the specific role for L-PGDS in the control of adipogenesis in cultured adipocytes, we attempted to suppress L-PGDS in cultured preadipocytes transfected stably with the mammalian expression vector having the cDNA insert oriented in the antisense direction.

2. Materials and methods

2.1. Materials

Authentic prostanoids, arachidonic acid, and rabbit polyclonal antibody for L-PGDS were obtained from Cayman Chemical (Ann Arbor, MI, USA). Geneticin (G418) and Triglyceride E-Test Kit were supplied by Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from MP Biomedicals (Solon, OH, USA). SuperFect Transfection Reagent was provided by Qiagen (Valencia, CA, USA). Other materials were obtained as described earlier [12,13,18]. All other chemicals were of reagent or tissue culture grade.

2.2. Cell culture of 3T3-L1 cells for the differentiation and maturation of adipocytes

Preadipogenic mouse 3T3-L1 cells were plated at 5×10^4 cells/ml in the growth medium and cultured until confluence. The monolayer cells were exposed to the differentiation medium for 45 h and followed by the continued cultures in the maturation medium by changing every 2 days to promote the accumulation of fats during the maturation phase under the established cultured conditions [10–13,19–21]. Exogenous prostanoids and arachidonic acid used for the cell cultures were dissolved in ethanol as a vehicle while A23187 was added to the culture medium after dissolving in dimethyl sulfoxide. The volume of the vehicle was adjusted to 0.2%.

2.3. Construction of L-PGDS expression vector oriented in the antisense direction

Total RNA was extracted from brain of BALB/c mouse and used for the amplification of cDNA insert encoding the full-length open reading frame by reverse transcriptase-polymerase chain reaction (RT-PCR) with 5′-ACTTGAATTCCAAATGGCTGCT-3′ as a 5′-primer having the *Eco*RI site and 5′-TGCAAAGCTTGCGTTTACTCTT-3′ as a 3′-primer containing the *Hind*III site. The amplified cDNA fragment was cut doubly with the restriction enzymes, purified, and ligated to the sites of *Hind*III and *Eco*RI of the mammalian expression vector, pcDNA3.1(+) with neomycin-resistant gene (Invitrogen, Carlsbad, CA, USA) in the antisense direction. The recombinant DNA was extracted from *Escherichia coli* DH5α that had been transformed with the ligation product, and subjected to the determination of the DNA sequence as describe before [13,20,21].

2.4. Stable transfection and cloning of transfectants

Parent 3T3-L1 cells were transfected with the mammalian expression vector, pcDNA3.1(+) having the cDNA insert of mouse L-PGDS oriented in the antisense direction or the vector only as a control using the SuperFect Transfection Reagent according to the manufacture's instructions. The cultured cells were grown in the growth medium supplemented with G418 as described previously [20,21]. The growing cells were employed for the cloning of single cells. The isolated cloned cells were further propagated and subjected to the gene expression analysis of the mRNA and protein levels of L-PGDS as described earlier [10,12].

2.5. Analysis of expression levels of mRNA and protein

Total RNA was extracted from cultured 3T3-L1 cells at the indicated stages of adipocytes as described before [22]. For the specific detection of the expressed genes, the resulting total RNA was used for the analysis by RT-PCR as reported previously [10,20,21]. The amplification of the target gene was conducted using pairs of 5′- and 3′-primers specific for each of L-PGDS, adipocyte protein 2 (aP2), leptin, PPARγ, glucose transporter-4 (GLUT-4), adiponectin, lipoprotein lipase (LPL), and β-actin as listed in our previous reports [10,12,13,20,21]. The amplified DNA fragments were separated by 1.5% agarose electrophoresis and the DNA sequences were confirmed as described previously [13,20,21]. Cultured 3T3-L1 cells were cultured to confluence in the growth phase. The resulting cells were harvested for the analysis of protein expression levels of L-PGDS by Western blot analysis with a rabbit polyclonal antibody for mouse L-PGDS according to our previous method [13,20].

2.6. Enzyme-linked immunosorbent assay (ELISA) for prostanoids

The culture medium was collected and applied to our immobilized ELISA specific for PGD_2 [23], PGE_2 [13,18,20,21], and Δ^{12}-PGJ_2 [11] as reported previously. For the assay, the conjugate of each PG and bovine γ-globulin was used as an immobilized antigen in 96-well EISA plate. The immobilized antigen was allowed to react competitively with a specific antibody for each prostanoid species in the presence of standards or samples to be tested. The resulting immunocomplex was detected spectrophotometrically as described earlier [18].

2.7. Other methods

The storage of triacylglycerols after the maturation phase of adipocytes was measured using Triglyceride E-Test Kit as described before [12,13]. Cultured cells were harvested and used for the determination of the cellular proteins after the precipitation of proteins with cold trichloroacetic acid [24]. The accumulation of lipid droplets in adipocytes was monitored by staining with Oil Red O [12,25]. All of the quantified data were represented as the mean ± S.E.M. of three or more experiments. Student's *t* test was used for evaluating statistical significance, and the difference was considered to be significant when $p < 0.05$.

3. Results

3.1. Preparation of cloned stable transfectants with L-PGDS oriented in the antisense direction

Cultured 3T3-L1 cells were transfected stably with L-PGDS oriented in the antisense direction. Finally, we isolated several

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