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Suppression of adipogenesis program in cultured preadipocytes transfected stably with cyclooxygenase isoforms

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ABSTRACT

Prostaglandins (PGs) are known to play a variety of roles in adipocytes and precursor cells, which have the arachidonate cyclooxygenase (COX) pathway to generate several series of PGs at different stages of life cycle of adipocytes. To gain a unique insight into the specific roles of the COX isoforms during the life cycle of adipocytes, 3T3-L1 preadipocytes were stably transfected with a mammalian expression vector harboring either cDNA coding for murine COX-1 or COX-2. The cloned stable transfectants with COX-1 or COX-2 exhibited higher expression levels of their corresponding mRNA and proteins, and greater production of PGE_2 upon stimulation with free arachidonic acid or A23187 than the parent cells and the transfectants with vector only. However, either type of transfectants brought about the marked reduction in the accumulation of triacylglycerols after the standard adipogenesis program. Unexpectedly, aspirin or other COX inhibitors at different phases of life cycle of adipocytes failed to reverse the reduced storage of fats. The transfectants with COX-2 were sensitive to exogenous 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and troglitazone as peroxisome proliferator-activated receptor γ (PPARγ) agonists during the maturation phase for restoring the adipogenesis. By contrast, the transfectants with COX-1 were much less sensitive, which was reflected by much lower gene expression levels of PPARγ and the related adipocyte-specific markers. Taken together, the results suggest that the sustained overexpression of either COX-1 or COX-2 resulted in the interference of adipogenesis program through a PG-independent mechanism with a different mode of action of COX isoforms.

1. Introduction

Adipose tissues are important in the control of energy homeostasis by storing the extra energy as neutral fats following excess food intake as well as by mobilizing the fuel molecules as lipolytic products during the deficiency. Alternatively, adipose tissues serve as endocrine cells to secrete a number of endocrine hormones referred to as adipocytokines, such as leptin, adiponectin, and others, which affect the metabolism of fuel molecules in the other organs [1]. Furthermore, increased generation of inflammatory factors in adipose tissues of obese subjects is more likely to underlie the development of life-style disorders [2]. As well, adipocytes can produce prostanoids as local hormones by sensing external signaling molecules, and their adipogenesis undergoes the regulation by endogenous or exogenous prostanoids in an autocrine or paracrine manner [3]. The action of prostanoids on the process of adipogenesis in adipocytes is complex due to the opposite effects of different types of prostanoids. For example, prostaglandin (PG)I_2 [4] and PGJ_2 derivatives [5–7] have been reported to promote the differentiation of adipocytes. By contrast, PGE_2 and $PGF_2\alpha$ have been shown to be anti-adipogenic factors [8]. The existence of multiple receptors, such as those for PGE_2 with different signaling systems, also complicated the understanding of the action of endogenous and exogenous prostanoids in the adipocytes at different stages [3,8].

Interest in the nuclear action of some prostanoids on adipocytes has been greatly enhanced by recent recognition of the important action of the nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ), in the modulation of adipogenesis

Abbreviations: PG, prostaglandin; COX, cyclooxygenase; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; PPARγ, peroxisome proliferator-activated receptor γ; L-PGDS, lipocalin-type PGD synthase; DME–HEPES, Dulbecco's modified Eagle's medium with 25 mM HEPES; FBS, fetal bovine serum; RT, reverse transcriptase; PCR, polymerase chain reaction; IBMX, 3-isobutyl-1-methylxanthine; G418, geneticin disulfate; ELISA, enzyme-linked immunosorbent assay; GM, growth medium; DM, differentiation medium; MM, maturation medium; GLUT-4, glucose transporter-4; LPL, lipoprotein lipase; C/EBP, CCAAT/enhancer binding protein; aP2, adipocyte protein 2; PBS (−), phosphate-buffered saline without Ca^{2+} and Mg^{2+}; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; AA, arachidonic acid

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program and insulin sensitivity [9]. Initially, PPAR-related nuclear receptors were found to be activated by a class of antidiabetic drugs like thiazolidinediones. Following these findings of exogenous action of ligands, the naturally occurring lipophilic molecules have been shown to act as active ligands for PPARγ and are regarded as the candidates of endogenous ligands. Of these natural ligands, 15-Deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), one of J_2 series of prostanoids, have been shown to be the most potent ligand for the activation of PPARγ [5,6]. The PGJ_2 derivatives can be formed through the non-enzymatic dehydration of PGD_2 that is synthesized in the arachidonate cyclooxygenase (COX) pathway [10,11]. Earlier, we [7,12] and other groups [13,14] have revealed the specific gene expression of lipocalin-type PGD synthase (L-PGDS) in cultured 3T3-L1 adipocytes. More recently, we have provided the evidence for the endogenous synthesis of 15d-PGJ_2 by adipocytes during the maturation phase and its contribution to the up-regulation of fat storage [7]. For the synthesis of PGD_2 and PGJ_2 series, phospholipase A_2, COXs, and L-PGDS should be linked functionally in mature adipocytes. However, the control mechanism for these actions remains still elusive.

Other types of prostanoids including PGE_2 and $PGF_{2\alpha}$ exert their effects by binding to the specific cell-surface membrane receptors. For PGE_2, four types of receptor subtypes are involved, such as EP1, EP2, EP3, and EP4. They are differently coupled with their specific signal transduction systems [15]. Recent studies have described the involvement of the EP4 receptor in the suppression of adipocyte differentiation through a cAMP-dependent signaling [8,16]. On the other hand, $PGF_{2\alpha}$ interacts specifically with the FP receptor which activates phospholipase C to raise the intracellular levels of Ca^{2+}. $PGF_{2\alpha}$ have also been shown to attenuate the adipogenesis in adipocytes in a mechanism different from PGE_2 [3,8]. Recently, we have shown that cultured 3T3-L1 preadipocytes have the ability to promote the delayed synthesis of PGE_2 and $PGF_{2\alpha}$ in response to external stimuli [3]. The observation indicates the induced expression of COX-2 in the formation of anti-adipogenic prostanoids in preadipocytes. In addition, we have demonstrated that the sensitivity of cultured adipocytes to PGE_2 and $PGF_{2\alpha}$ is different depending on the life stage of adipocytes [3].

Prostanoids are synthesized by the arachidonate COX pathway in which the formation of PGH_2 from free arachidonic acid through the COX reaction is the rate-limiting step. The COX enzyme occurs as two types of isoforms, the constitutive COX-1 and the inducible COX-2. Both COX isoforms have distinct physiological and pathological roles [17,18]. For example, COX-1 is involved in the control of platelet aggregation and vascular homeostasis, whereas the expression of COX-2 can be induced in inflammatory sites in response to pro-inflammatory factors [18]. Moreover, overexpression of COX-2 has been shown to be associated with colorectal cancer [17,19]. Therefore, the selective inhibitors for COX-2 have been developed for the use of anti-inflammatory and anti-tumor drugs [18]. On the other hand, little is known about the specific roles of COX isoforms in adipocytes and precursor cells.

Here, we attempted to manipulate the expression of either COX isoform in cultured 3T3-L1 preadipocytes [3,7,8,12] in order to evaluate phenotypic and biochemical changes associated with stable overexpression of either COX-1 or COX-2. We provide evidence that stable overexpression of either COX isoform in 3T3-L1 preadipocytes blocks the adipogenesis program through a novel PG-independent mechanism.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium with 25 mM HEPES (DME–HEPES), penicillin G potassium salt, streptomycin sulfate, dexamethasone, calcium ionophore A23187, and recombinant human insulin were purchased from Sigma (St. Louis, USA). Fetal bovine serum (FBS) was supplied by Biological Industries (Kibbutz Beit Haemek, Israel). Plasmid Mini Kit and SuperFect Transfection Reagent were obtained from Qiagen (Valencia, CA, USA). Rabbit polyclonal antibodies against murine COX-1 and COX-2, authentic PGs, aspirin, and troglitazone were purchased from Cayman Chemical (Ann Arbor, MI, USA). M-MLV reverse transcriptase (RT) (ribonuclease H minus) and Polymerase Chain Reaction (PCR) MasterMix were supplied by Promega (Madison, WI, USA). Triglyceride E-Test Kit, 3-isobutyl-1-methylxanthine (IBMX), and geneticin disulfate (G418) were obtained from Wako (Osaka, Japan), and oligonucleotides for PCR were from Kurabo Biomedical (Osaka, Japan) or Sigma Genosys Japan (Ishikari, Japan). Other materials used for cell culture, RT-PCR, and enzyme-linked immunosorbent assay (ELISA) for PGE_2 were obtained as described previously [3,12,20]. All other chemicals used were of reagent or tissue culture grade.

2.2. Cell culture and adipocyte differentiation

The preadipogenic mouse 3T3-L1 cells (JCRB9014) were plated at 5×10^4 cells/ml in the growth medium (GM) containing DME–HEPES, 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 200 μM ascorbic acid, and cultured at 37 °C under 7% CO_2. After the cells become confluent, the monolayer cells were exposed to the differentiation medium (DM) corresponding to GM supplemented with 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin for 45 h. Then, the culture medium was replaced with the maturation medium (MM) consisting of GM with 5 μg/ml insulin, and refed every 2 days to promote the maturation of adipocytes as described before [3,12]. For evaluating the effect of a COX inhibitor at different phases on the amounts of triacylglycerols accumulated after 6 days of the maturation phase in parent cells and the transfectants, the cultured cells were pretreated with 500 μM aspirin either in GM from 80% to 100% confluence for 2 days, in both GM for 2 days and DM for 45 h, or in all of GM for 2 days, DM for 45 h, and MM for 6 days. To check the effect of PPARγ agonists on adipogenesis in parent cells and the transfectants, the cultured cells after the differentiation phase were treated with 1 μM 15d-PGJ_2 or 1 μM troglitazone in the presence of 500 μM aspirin by replacing with the fresh corresponding culture medium every 2 days. The compounds to be tested were dissolved in ethanol as a vehicle and added to the culture medium. The volume of ethanol was adjusted to 0.2%.

2.3. Construction of expression plasmids

To construct the mammalian expression vectors for murine COX-1 and COX-2, cDNA coding for COX-1 or COX-2 was prepared using total RNA from cultured mouse MC3T3-E1 cells. Total RNA was used for the amplification of the desired cDNA by RT-PCR with oligonucleotide primers to which the Xba I recognition site was added. Briefly, cDNA for COX-1 was amplified using 5′-GCTCTAGATGAGTCGAAGGAGTC-TCTC-3′ as a 5′-primer and 5′-GCTCTAGATTAGAGCTCAGTGGAGCGT-3′ as a 3′-primer. In addition, cDNA for COX-2 was amplified using 5′-GCTCTAGATGCTCTTCCGAGCTGTG-3′ as a 5′-primer and 5′-ACATCTA-GACTTTTACAGCTCAGTTG-3′ as a 3′-primer. The amplified cDNA fragments were excised, cut with Xba I, purified, and ligated individually to the Xba I site of pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA). For the preparation of the recombinant DNA, *E. coli* DH5α strain was transformed with either of the above recombinant plasmids in the presence of ampicillin. From the resulting cultures, the desired DNA was extracted by employing the Plasmid Mini Kit. DNA sequences of the inserts of mouse COX-1 and COX-2 were confirmed using the ABI Prism 3100 Genetic Analyzer and BigDye Terminator v. 1.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA).