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Compressive force inhibits adipogenesis through COX-2-mediated down-regulation of PPARγ2 and C/EBPα

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Various mechanical stimuli affect differentiation of mesoderm-derived cells such as osteoblasts or myoblasts, suggesting that adipogenesis may also be influenced by mechanical stimulation. However, effects of mechanical stimuli on adipogenesis are scarcely known. Compressive force was applied to a human preadipocyte cell line, SGBS. Levels of gene expression were estimated by real-time reverse transcription–polymerase chain reaction. The accumulation of lipids was evaluated by Sudan III or Oil Red O staining. In SGBS cells subjected to a compressive force of 226 Pa for 12 h before adipogenic induction, adipogenesis was inhibited. Compressive force immediately after adipogenic induction did not affect the adipogenesis. The expression of peroxisome proliferator-activated receptor (PPAR) γ2 and CCAAT/enhancer binding protein (C/EBP) α mRNA during adipogenesis was inhibited by compressive force, whereas C/EBPβ and C/EBPδ mRNA levels were unaffected. In preadipocytes, compressive force increased mRNA levels of Krüppel-like factor 2, preadipocyte factor 1, WNT10b, and cyclooxygenase-2 (COX-2) which are known as negative regulators for the PPARγ2 and C/EBPα genes. Furthermore, a COX-2 inhibitor completely reversed the inhibition of adipogenesis by compressive force. In conclusion, compressive force inhibited adipogenesis by suppressing expression of PPARγ2 and C/EBPα in a COX-2-dependent manner.

[**Key words:** Compressive force; Preadipocytes; Adipogenesis; COX-2; PPARγ2; C/EBPα]

Obesity is considered a risk factor for atherosclerosis as well as diabetes, hypertension, and dyslipidemia (1). Although normal adipose tissue secretes adiponectin and leptin which are known to increase insulin sensitivity (2), obese adipose tissue secrete free fatty acids and inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin-6, resistin, plasminogen activator inhibitor-1, and angiotensinogen (3). These factors induce angiopathy or insulin resistance, resulting in diseases such as hypertension, diabetes, and dyslipidemia. Thus, obesity is widely recognized as a major public health problem owing to its rising prevalence and deleterious impact on many chronic diseases.

Obesity is characterized by an increased mass of adipose tissue (4). Hypertrophy of adipocytes is an apparent cause of obese adipose tissue, but an increase in the number of adipocytes is also observed in obese adipose tissue (4). Three mechanisms for the increase in the number of adipocytes have been proposed (5, 6): i) differentiation from preadipocytes, ii) cell division of normal adipocytes and then an increase in the size of divided cells, and iii) cell division of adipocytes with large lipid droplets. Although which mechanism actually functions *in vivo* is unknown, adipogenic induction of preadipocytes is considered to be one cause of obesity.

Treatment for obesity is aimed at achieving and maintaining a healthier weight. The mainstay of treatment is an energy-limited diet and increased exercise (1). Weight loss by exercise may result from not only an increase in caloric expenditure but also the influence of mechanical stimulation. Mechanical stimuli such as stretching, rubbing, pressing of fat through gymnastic exercises or massage, and whole body vibration are believed to decrease or prevent obesity (7, 8). There are many reports investigating the effects of mechanical stimuli on differentiation in cell lineages derived from mesenchymal stem cells (MSCs) (9–11), but reports concerning effects of mechanical stimuli on adipogenesis are rare. MSCs can differentiate into adipose tissue as well as bone, muscle, cartilage, and tendon (12), indicating that mechanical stimulation may affect adipogenesis. In the present study, the effect of mechanical stimulation by compressive force on adipogenesis in preadipocytes was investigated.

MATERIALS AND METHODS

Materials Insulin, cortisol, and dexamethasone were purchased from Wako (Osaka, Japan). Biotin, pantothenic acid, 3-isobutyl-1-methylxanthine (IBMX), troglitazone, triiodothyronine (T_3), 4′,6-diamidino-2-phenylindole (DAPI), Sudan III, and Oil Red O were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Transferrin was

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purchased from Calbiochem (Darmstadt, Germany). NS-398, a COX-2 inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture and adipocyte differentiation SGBS (Simpson–Golabi–Behmel syndrome) cells, a preadipocyte cell line derived from human adipose tissue, were obtained from Dr. Wabitsch (13). SGBS cells were propagated on 10-cm dishes with Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) medium (Wako) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 33 μM biotin, and 17 μM pantothenic acid in a humidified 5% CO_2 incubator at 37 °C. The cells were passaged on 6-well plates and incubated to confluence. Adipogenic differentiation was induced with an induction medium composed of FBS-free DMEM/Ham's F-12 (1:1) medium supplemented with 20 nM insulin, 100 nM cortisol, 200 pM T_3, 0.01 mg/ml transferrin, 2 μM troglitazone, 500 μM IBMX, and 25 nM dexamethasone for 4 days. Then the medium was changed to a maintenance medium composed of FBS-free DMEM/Ham's F-12 (1:1) medium supplemented with 20 nM insulin, 100 nM cortisol, 200 pM T_3, and 0.01 mg/ml transferrin. The maintenance medium was changed every 3 days.

Application of compressive force The SGBS cells were compressed continuously using a uniform compression method as described by Kanzaki et al. (14). Briefly, confluent cells were subjected to a static compressive force by placing a glass cylinder with lead weights onto the cells of a 6-well plate. The intensity of the force was controlled by the number of lead granules placed in the cylinder. A cell area in contact with the cylinder was beforehand marked on the bottom of wells. Then, cells exclusively in the area were used for the experiments as cells subjected to compressive force. Cells cultured on the same area of plates without any compressive force served as controls. To determine the optimal conditions for compression, a force of 0, 128, 177, 226, 275, 324, or 426 Pa was applied to the cells for 3, 6, 12, 24, 36, or 48 h. The reactivity of cells to the compressive force was evaluated by measuring cyclooxygenase-2 (COX-2) mRNA levels, because the COX-2 gene is a mechanical stress-responsive gene (15). A compressive force of 226 Pa for 12 h was judged as optimum for SGBS cells and used throughout the experiments.

Evaluation of adipogenesis At 13 days after the differentiation was first induced, Oil Red O staining or staining with DAPI and Sudan III was performed. Stained Oil Red O in the area of cells subjected to compressive force or the same area of control cells was dissolved in 1 ml/well of 2-propanol with holding a hollow cylinder to the marked area and the optical density (OD) at 510 nm was measured by a spectrophotometer (Ultrospec 6300 *pro*; Amersham Biosciences, Piscataway, NJ). The cells stained by DAPI and Sudan III were observed under a fluorescence microscope (TE-2000; Nikon, Tokyo, Japan). Differentiation rate was estimated by determining the ratio of Sudan III-positive cells to the DAPI-stained cells for total cell number in 8 randomly selected low-power fields (×40).

Isolation of RNA and real-time reverse transcription–polymerase chain reaction (RT–PCR) Each total RNA from the cells subjected to compressive force or control cells was extracted using ISOGEN (Nippongene, Toyama, Japan) with holding the hollow cylinder to the marked area. cDNA was synthesized from total RNA (500 ng) using the Prime Script™ RT Reagent Kit (TaKaRa, Kyoto, Japan). Real-time RT–PCR analyses were performed at least in triplicate using the Applied Biosystems Prism 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The nucleotide sequences of the primers used in this study are listed in Table 1. The final volume of 20 μl contained 25 ng of cDNA, 1 × Power SYBR Green PCR Master Mix (Applied Biosystems), and 1 μM of each primer. cDNAs were amplified at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 sec, and annealing and extension at 60 °C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was simultaneously quantified as an endogenous control and the gene expression of the target in each sample was normalized to that of GAPDH. The specificity of the PCR was determined from a dissociation curve analysis. The data were analyzed using 7300 System Sequence Detection Software (version 3.1; Applied Biosystems) to determine the relative quantitative gene expression.

TABLE 1. Nucleotide sequences of primers used for real-time quantitative RT–PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
Adiponectin	GTGATGGCAGAGATGGCAC	ACACTGAATGCTGAGCGGTA
aP2	CCTGGTACATGTGCAGAAAT	AGAGTTCAATGCGAACTTCA
C/EBPα	AAGAAGTCGGTGGACAAGAACAG	GCAGGCGGTCATTGTCACT
C/EBPβ	CTGGAGACGCAGCACAAG	ACAGCTGCTCCACCTTCTTC
C/EBPδ	GGTGCCCGCTGCAGTTT	CTCGCAGTTTAGTGGTGGTAAGTC
COX-2	GTGCGCGGTCCTGGCGCTCAG	CCTGTCCGGGTACAATCGCAC
DGAT2	CTCTTCTCCTCCGACACCTG	TGGTCTTGTGCTTGTCGAAG
FASN	CTGGCTCAGCACCTCTATCC	CAGGTTGTCCCTGTGATCCT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
KLF2	CTACACCAAGAGTTCGCATCTG	CCGTGTGCTTTCGGTAGTG
KLF3	ATGCTCATGTTTGACCCAGTTC	ACCCCATACTTGTTAGGCTTCAT
KLF5	CCTGGTCCAGACAAGATGTGA	GAACTGGTCTACGACTGAGGC
KLF15	TTCCAGTCCCGACTCTCAAG	GGCAAGCAGAAATGCTCCC
PPARγ2	AGCAAACCCCTATTCCATGCT	ATCAGTGAAGGAATCGCTTTCTG
Pref-1	GTCTGCCCAACGGCTATGG	AGGAAGACGATACCCACAGTG
WNT10b	TGTGCAGTCGGGCTCTAAG	GGTGCTGACACTCGTGGAC

Statistical analysis The data are expressed as means ± SEM. Statistical analyses were performed using Student's *t*-test. Differences were considered to be significant when the *P*-value was less than 0.05.

RESULTS

Effect of compressive force on adipogenesis The effect of compressive force on adipogenesis in SGBS cells was investigated at two time points, 12 h before and 12 h after the start of adipogenic induction as indicated in Fig. 1. At 13 days after adipogenic induction, adipogenesis was evaluated by Oil Red O staining. Quantitation of the extracted Oil Red O revealed that significantly less triglyceride had accumulated in the cells subjected to compressive force before induction but not immediately after the induction (Fig. 1A). Moreover, the effect of compressive force on adipogenesis was evaluated by measuring mRNA levels of an adipocyte fatty acid-binding protein, aP2, an adipocyte-specific marker. aP2 mRNA levels were significantly lower in the cells subjected to compressive force before induction, but unaffected by compressive force after the start of induction (Fig. 1B). Based on these results, compressive force was applied before the adipogenic induction in subsequent experiments.

Next, we investigated effects of compressive force on differentiation into adipocytes and maturation of adipocytes. The percentage of adipogenic-differentiated cells in cells subjected to compressive force was significantly lower than that in control cells (Fig. 1C). Furthermore, the size of lipid droplets in adipocytes differentiated from cells subjected to compressive force was smaller than that in control cells (Fig. 1D). These results suggested that compressive force decreases differentiation rate from preadipocytes into adipocytes and attenuates adipocyte maturation.

mRNA levels of various adipocyte-predominant proteins such as adiponectin, fatty acid synthase (FASN), diacylglycerol *O*-acyltransferase 2 (DGAT2), and mitochondrial glycerol-3-phosphate acyltransferase (GPAM) were determined by real-time RT–PCR at 13 days after adipogenic induction. As shown in Fig. 1E, mRNA levels of adiponectin, FASN, DGAT2, and GPAM were significantly lower in the differentiated cells subjected to compressive force. These results also confirmed that compressive force in preadipocytes inhibited adipogenesis.

Effects of compressive force on induction of peroxisome proliferator-activated receptor γ2 (PPARγ2) and CCAAT/enhancer binding protein (C/EBP) family mRNA PPARγ2 and the C/EBP family comprising C/EBPα, C/EBPβ, and C/EBPδ play important roles in the regulation of adipocyte differentiation and are therefore considered key regulators of adipogenesis. C/EBPβ and C/EBPδ positively regulate PPARγ2 and C/EBPα (16). The effect of compressive force on the expression of these key regulators was investigated by real-time RT–PCR. mRNA expression of C/EBPα and PPARγ2 was induced at 48 h and 96 h after the induction, respectively (Figs. 2A and B). Their mRNA levels were significantly lower in the cells subjected to compressive force. mRNA expression of C/EBPβ and C/EBPδ was rapidly induced at about 12 h after the start of adipogenic induction (Figs. 2C and D) but was not affected by compressive force. These results suggested that compressive force in preadipocytes inhibited the expression of PPARγ2 and C/EBPα mRNA independently of C/EBPβ and C/EBPδ.

Effects of compressive force on mRNA levels of regulatory genes for PPARγ2 and C/EBPα During adipogenesis, a number of factors such as Krüppel-like factor (KLF) 2 (17), KLF3 (18), preadipocyte factor 1 (Pref-1) (19), WNT10b (20), COX-2 (21), and TNF-α (22) are known to suppress the expression of PPARγ2 and C/EBPα independently of C/EBPβ and C/EBPδ. Effects of compressive force on mRNA levels of these negative regulators in SGBS cells were investigated as indicated in Fig. 3. mRNA levels of KLF2, Pref-1,