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

Running Title: Compressive force inhibits adipogenesis in preadipocytes

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Abstract

Various mechanical stimuli affect differentiation of mesoderm-derived cells such as osteoblasts or myoblasts, suggesting that adipogenesis may be also 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

1 inhibited adipogenesis by suppressing expression of PPARγ2 and C/EBPα in a

2 COX-2-dependent manner.

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Introduction

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 becomes to 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 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 spectrophotometer (Ultrospec 6300 *pro*; Amersham Biosciences, Piscataway, NJ). The cells stained by DAPI and Sudan III were observed under a fluorescence microscopy (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 (x 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^{TM} 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 x Power SYBR Green PCR Master Mix (Applied Biosystems), and 1 μM of each primer. cDNAs were amplified at 50^{o}C for 2 min and 95^{o}C for 10 min followed by 40 cycles of denaturation at 95^{o}C for 15 sec, and annealing and extension at 60^{o}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.

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 2B). 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 2D), 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, WNT10b, and COX-2, but not TNF-α and KLF3, were increased significantly by compressive force (Fig. 3), suggesting that compressive force may suppress PPARγ2 and C/EBPα mRNA expression by up-regulating KLF2, Pref-1,

WNT10b, and/or COX-2 mRNA expression in preadipocytes. On the other hand, KLF5 and KLF15 positively regulate the expression of PPARγ2 and C/EBPα (23, 24). No significant effects of compressive force on the levels of KLF5 and KLF15 mRNA were observed in SGBS cells (data not shown).

Effect of a COX-2 inhibitor on inhibition of adipogenesis by compressive force

To demonstrate the involvement of COX-2 in the inhibition of adipogenesis by compressive force, the effects of a COX-2 inhibitor on compressive force-induced inhibition of adipogenesis were investigated. NS-398, a selective COX-2 inhibitor, was added to the medium as indicated in Fig. 4. NS-398 did not affect COX-2 mRNA levels induced by compressive force in preadipocytes (Fig. 4A). The inhibition of triglyceride accumulation by compressive force was completely reversed by the NS-398 treatment (Fig. 4B, C), indicating that COX-2 plays a key role in the inhibition of adipogenesis by compressive force. Induction of WNT10b, KLF2, and Pref-1 mRNA expression by compressive force was not affected in the presence of NS-398 (data not shown), suggesting that WNT10b, KLF2, and Pref-1 act upstream of the COX-2 gene or do not play major roles in the inhibition of adipogenesis by compressive force.

Effect of compressive force before or after adipogenic induction on COX-2 mRNA levels in SGBS cells

To clarify why compressive force after adipogenic induction did not affect the adipogenesis, COX-2 mRNA levels after adipogenic induction were investigated. As shown in Fig. 5, COX-2 mRNA expression was not up-regulated by compressive force after adipogenic induction. During adipogenic induction, mRNA levels of COX-2 were reduced in cells with and without compressive force compared to the cells before the induction. The absence of effects of compressive force after adipogenic induction on the inhibition of adipogenesis may be caused by the interruption of COX-2 expression during adipogenesis.

Discussion

Mechanical forces modulate various cellular functions such as proliferation and differentiation, which are crucial for development, growth, and the regeneration of various tissues in mammals (25). Effects of mechanical stimuli on differentiation in mesoderm-derived cells have been particularly studied (9-11). Regarding the effect of mechanical stress on adipogenesis, adipogenic differentiation was attenuated by cyclic stretching in 3T3-L1 cells and C3H10T1/21 cells (26-28). Recently, low-magnitude mechanical signals were reported to suppress the differentiation of stem cells into adipocytes in mice (8). Thus, mechanical stress is likely to inhibit adipogenesis. In this study, inhibition of adipogenesis by compressive force was demonstrated in a human preadipocyte cell line, SGBS.

It is now established that C/EBPβ and C/EBPδ play important roles in the expression of PPARγ2 and C/EBPα (16). During adipogenesis, the expression of PPARγ2 and C/EBPα, but not C/EBPβ or C/EBPδ, was inhibited by compressive force in SGBS cells. These results suggest that the inhibition of PPARγ2 and C/EBPα mRNA expression by compressive force is regulated by factors other than C/EBPβ and C/EBPδ. For example, KLF5 and KLF15 accelerate adipogenesis by inducing expression of PPARγ2 in 3T3-L1 cells (23, 24). In contrast, KLF2 inhibits the differentiation of 3T3-L1 cells by

attenuating the expression of PPARγ2 and C/EBPα without affecting C/EBPβ and C/EBPδ (17) . TNF-α, WNT10b, COX-2, and Pref-1 are also involved in the inhibition of adipocyte differentiation by preventing both PPARγ2 and C/EBPα expression (19-22). In the present study, mRNA levels of Pref-1, WNT10b, KLF2, and COX-2, but not TNF-α, KLF5, and KLF15, were increased in the SGBS cells subjected to compressive force. The COX-2 gene is recognized as a mechanical stress-responsive gene in various cell types (15). Moreover, expression of WNT10b and KLF2 was up-regulated by mechanical stimulation in myoblasts and human umbilical vein endothelial cells, respectively (29, 30). In SGBS cells, KLF2, Pref-1, WNT10b, and COX-2 mRNA expression was also induced by compressive force, suggesting that these factors may potentially be used to inhibit adipogenesis by suppressing the expression of PPARγ2 and C/EBPα mRNA.

The compressive force-induced blockade of adipogenesis was completely reversed by a COX-2 inhibitor, suggesting that the COX-2 gene is a key compressive force-induced gene for the inhibition of adipogenesis. Various factors regulate COX-2 expression through signaling pathways. For example, the induction of COX-2 expression by TNF-α (31) and lysophosphatidic acid (32) is mediated through NF-κB and extracellular signal-regulated protein kinase (ERK)1/2, respectively. Indeed, Yan et al. (21) reported

that TNF-α–induced COX-2 expression was followed by inhibition of adipogenesis in 3T3-L1 cells. However, TNF-α expression was unaffected by compressive force in SGBS cells. Levels of KLF2, Pref-1, and WNT10b mRNA were not affected by the COX-2 inhibitor suggesting that these factors act upstream of COX-2. It is necessary to investigate whether WNT10b, KLF2, and Pref-1 regulate the COX-2 gene in preadipocytes.

Compressive force applied after adipogenic induction did not affect adipogenesis. The expression of the Pref-1 and COX-2 genes was reportedly inhibited by dexamethasone included in the adipogenic differentiation medium (21, 33). In this study, mRNA levels of COX-2 and Pref-1 (data not shown) were reduced during adipogenesis in SGBS cells. Suppression of COX-2 transcription by dexamethasone may overcome the induction of COX-2 gene expression by compressive force, and COX-2 protein may not reach levels high enough to inhibit PPARγ2 and C/EBPα mRNA expression. Other papers reported that cyclic stretching even after adipogenic induction could inhibit adipogenesis in 3T3-L1 cells and C3H10T1/21 cells (26-28). This difference may be due to the conditions and type of mechanical stimulus cyclic stretching versus compressive force. Indeed, Liu et al. (34) reported that cyclic tensile stretching

induced ERK and c-fos gene expression in a human osteoblast-like cell line, MG-63, more markedly and quickly than did compressive force.

In conclusion, compressive force inhibits adipogenesis by suppressing expression of PPARγ2 and C/EBPα in a COX-2-dependent manner.

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Figure legends

Fig. 1. Effect of compressive force on adipogenesis in SGBS cells. The time schedule of these experiments is shown. A, Quantitation of lipid accumulation. Stained Oil Red O was dissolved in 2-propanol and the OD at 510 nm was measured. OD levels are shown relative to control values. B, aP2 mRNA levels in the cells subjected to compressive force. C, Effect of compressive force on the differentiation rate of preadipocytes. The cells differentiated from preadipocytes with or without compressive force were stained by DAPI (blue) and Sudan III (red). Graph represents average of the percentage of Sudan III-positive cells in DAPI-stained cells per microscopic low-power field (x 40) (n=8). D, Representative bright field images of living cells differentiated from preadipocytes with or without compressive force in high-power field (x 400). E, Effects of compressive force on mRNA levels of adipokines and markers of lipogenesis. SGBS cells were subjected to compressive force for 12 h before adipogenic induction. The expression of each gene was normalized to that of GAPDH mRNA. The normalized mRNA level of each gene is shown relative to the control value. Graphs are representative of three independent experiments. Open bars, control; solid bars, under compression (compressed). Values are expressed as the mean ± SEM; n = 3. n.s., not significant; *, $P < 0.05$ as compared with the control.

Fig. 2. Effects of compressive force on gene expression of key regulators of adipogenesis. At 0, 3, 12, 24, 48, 96, and 120 h after adipogenic induction, total RNA was extracted from cells with or without compressive force. mRNA levels of C/EBPα (A), PPARγ2 (B), C/EBPβ (C), and C/EBPδ (D) at each indicated time in the cells with or without compressive force were measured by real-time RT-PCR. The expression of each gene was normalized to that of GAPDH mRNA. Graphs are representative of three independent experiments. Values are expressed as the mean ± SEM; n = 3. n.s., not significant; *, $P < 0.05$ as compared with the control.

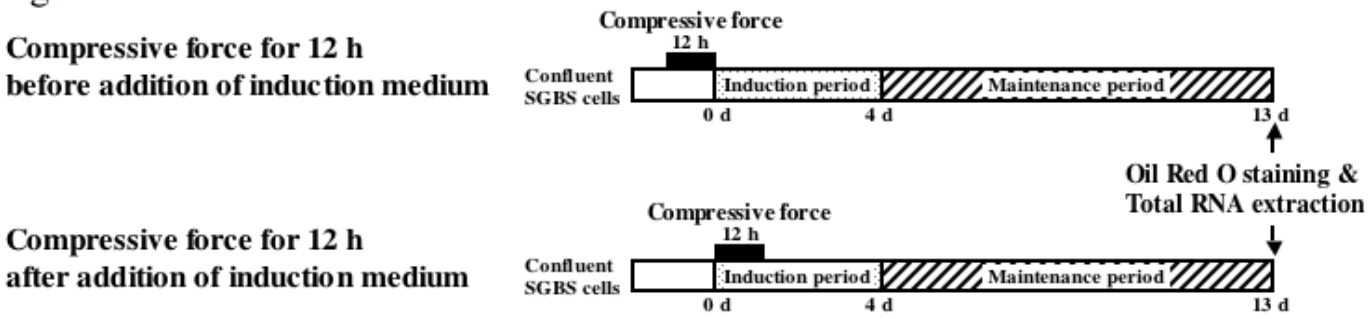
Fig. 3. Effects of compressive force on gene expression of negative regulators for PPARγ2 and C/EBPα in SGBS cells. Total RNA was extracted from the cells with or without compressive force. mRNA levels of negative regulatory genes in SGBS cells were determined by real-time RT-PCR. The expression of each gene was normalized to that of GAPDH mRNA. The normalized mRNA level of each gene is shown relative to the control value. Open bars, control; solid bars, compressed. Graphs are representative of three independent experiments. Values are expressed as the mean ± SEM; n = 3. n.s., not significant; *, $P < 0.05$ as compared with the control.

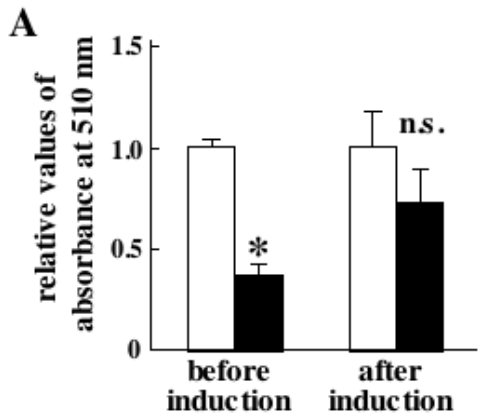
Fig. 4. Effect of NS-398 on compressive force-induced inhibition of adipogenesis. NS-398 was applied as shown. A, Effect of NS-398 on COX mRNA induction by compressive force. COX-2 mRNA levels were measured in SGBS cells subjected to 12 h of compressive force in the presence or absence of NS-398. B, Representative Oil Red O staining of SGBS cells subjected to compressive force in the presence or absence of NS-398. Oil Red O staining was performed at 13 days after the induction of differentiation and was observed under microscope. SGBS cells were subjected to compressive force without (top) and with (bottom) NS-398. C, Quantitation of lipid accumulation. Stained Oil Red O was dissolved in 2-propanol and the OD at 510 nm was measured. OD levels are shown relative to control values. Open bars, control; solid bars, compressed. The graph is representative of three independent experiments. Values are expressed as the mean ± SEM; n = 3. n.s., not significant; *, $P < 0.05$ as compared with the control.

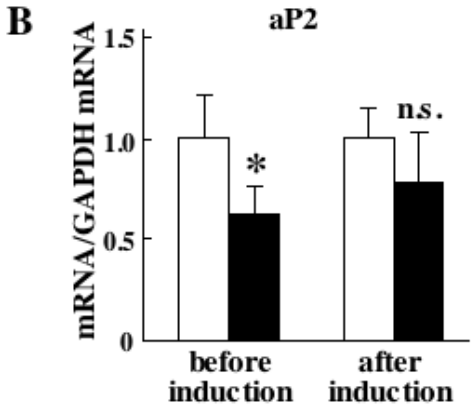
Fig. 5. Effect of compressive force before or after adipogenic induction on COX-2 mRNA levels in SGBS cells. Compressive force was applied for 12 h before or after adipogenic induction. After the removal of compressive force, total RNA was extracted.

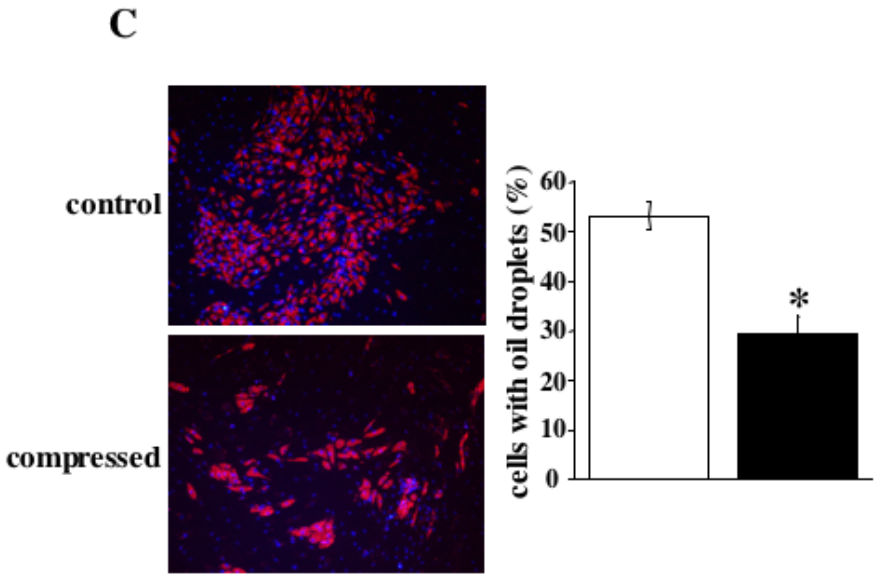
mRNA levels were measured by real-time RT-PCR. Expression levels of COX-2 were normalized to those of GAPDH. Open bars, control; solid bars, compressed. The graph is representative of three independent experiments. Values are expressed as the mean ± SEM; n = 3. n.s., not significant; *, $P < 0.05$ as compared with the control.

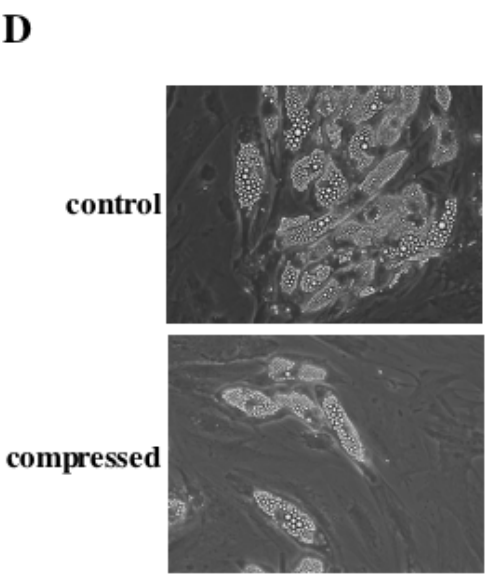
Fig. 1


Compressive force for 12 h
before addition of induction medium
Compressive force
12 h
Confluent SGBS cells
Induction period
Maintenance period
0 d
4 d
13 d
Oil Red O staining &
Total RNA extraction
Compressive force for 12 h
after addition of induction medium
Compressive force
12 h
Confluent SGBS cells
Induction period
Maintenance period
0 d
4 d
13 d


A
relative values of absorbance at 510 nm
1.5
1.0
0.5
0
*
n.s.
before induction
after induction


B
aP2
mRNA/GAPDH mRNA
1.5
1.0
0.5
0
*
n.s.
before induction
after induction


C
control
compressed
cells with oil droplets (%)
60
50
40
30
20
10
0
*


D
control
compressed

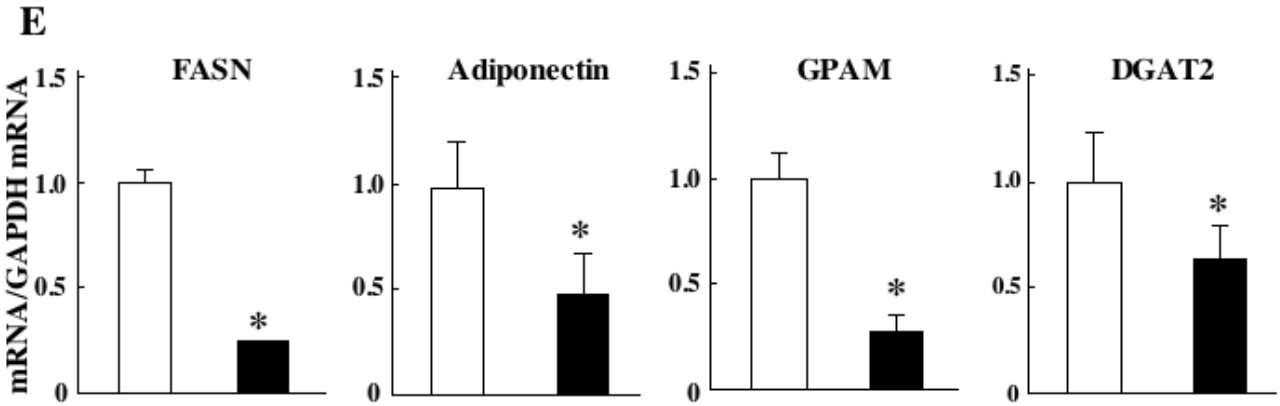

E
mRNA/GAPDH mRNA
FASN
Adiponectin
GPAM
DGAT2
1.5
1.0
0.5
0
*

Fig. 2

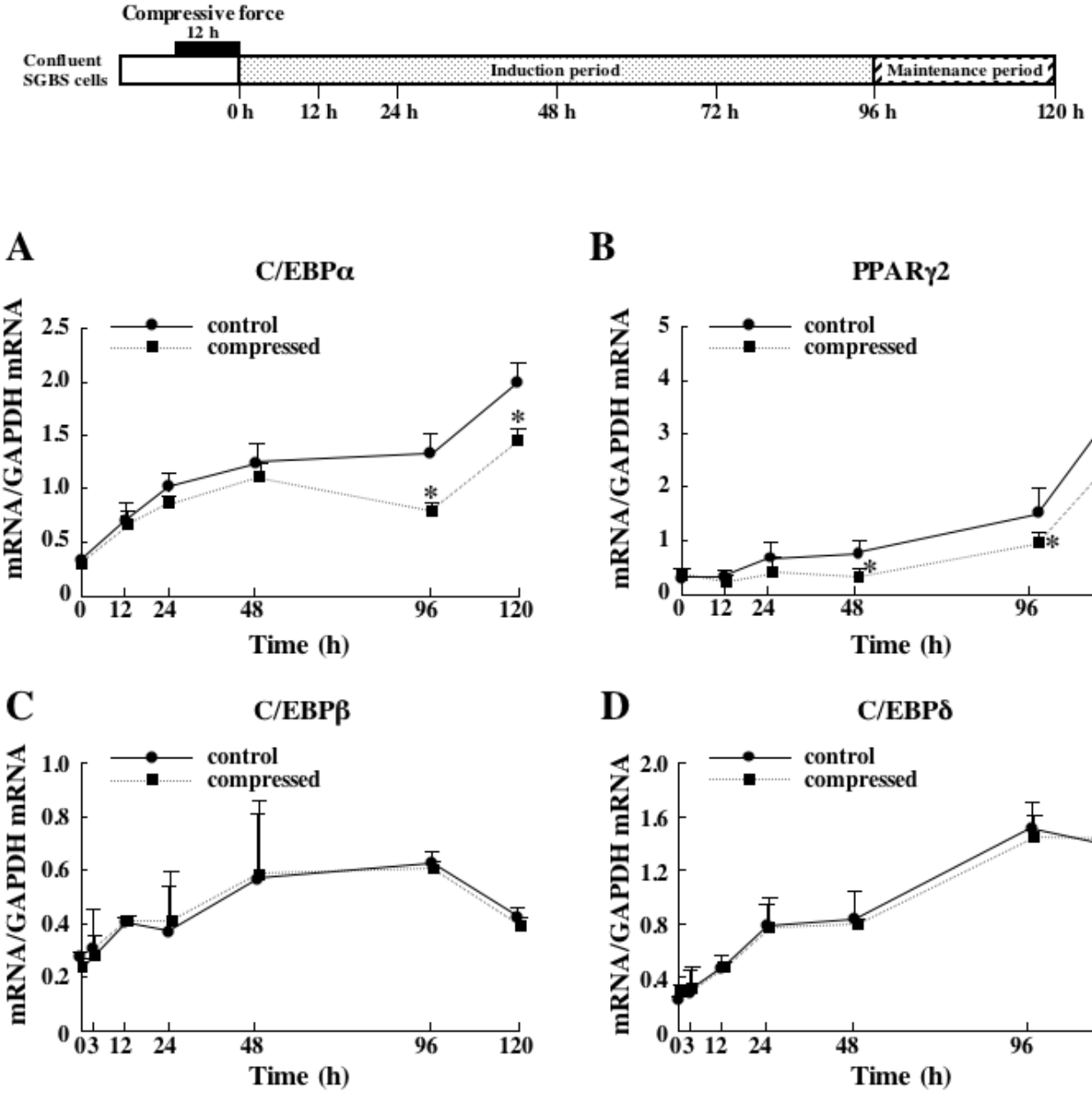

Compressive force
12 h
Confluent
SGBS cells
Induction period
Maintenance period
0 h
12 h
24 h
48 h
72 h
96 h
120 h
A
C/EBPα
B
PPARγ2
C
C/EBPβ
D
C/EBPδ
control
compressed
mRNA/GAPDH mRNA
Time (h)

Fig. 3

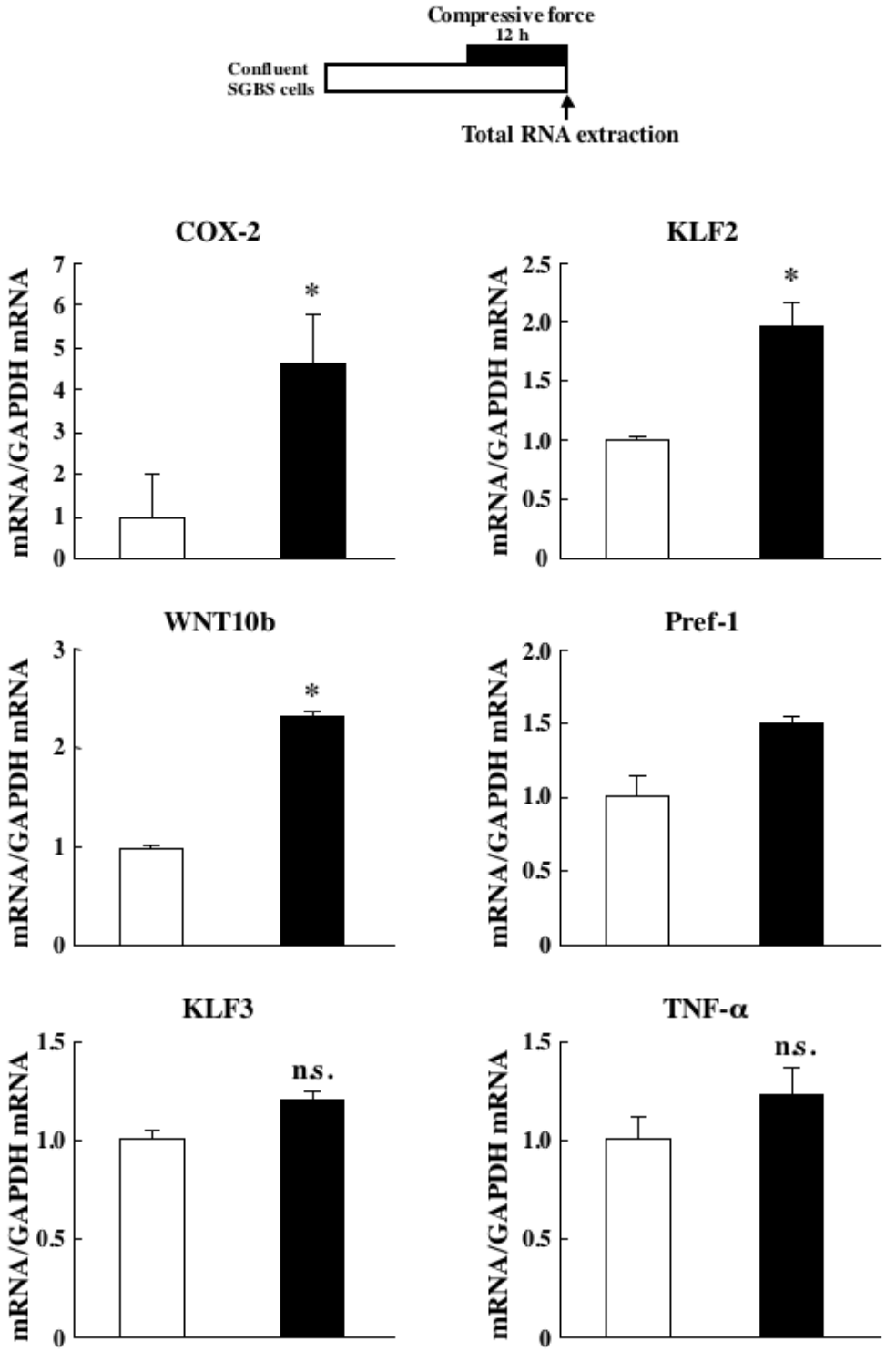
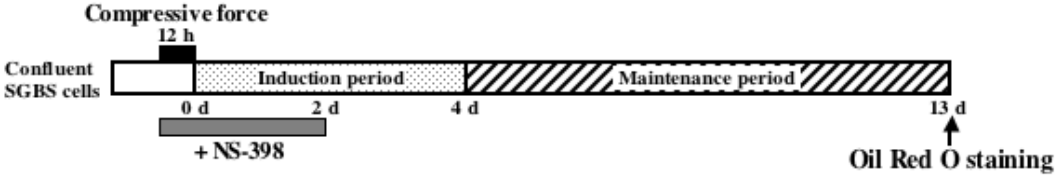
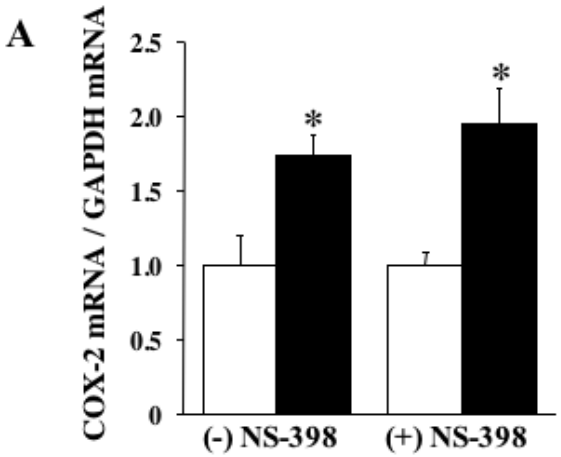
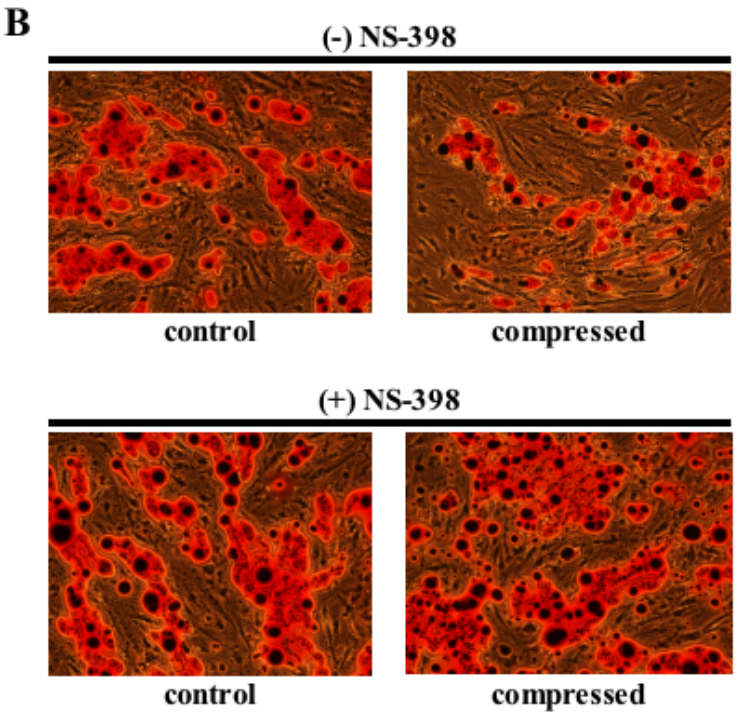

Compressive force
12 h
Confluent
SGBS cells
Total RNA extraction
COX-2
KLF2
WNT10b
Pref-1
KLF3
TNF-α
mRNA/GAPDH mRNA
*
n.s.

Fig. 4


Compressive force
12 h
Confluent
SGBS cells
Induction period
Maintenance period
0 d
2 d
4 d
13 d
+ NS-398
Oil Red O staining


A
COX-2 mRNA / GAPDH mRNA
2.5
2.0
1.5
1.0
0.5
0
*
*
(-) NS-398
(+) NS-398


B
(-) NS-398
control
compressed
(+) NS-398
control
compressed

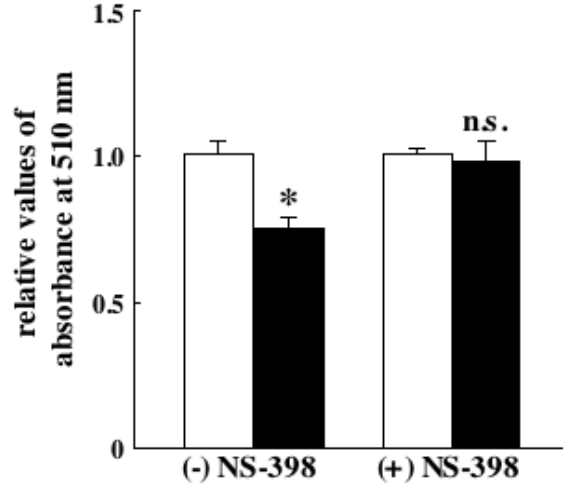
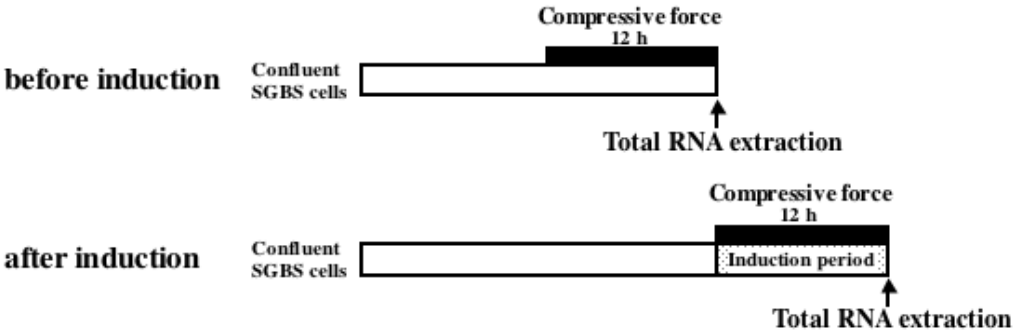
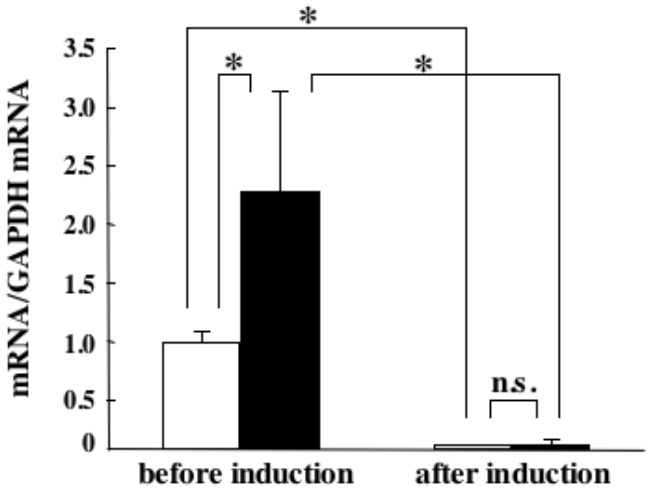

C
relative values of
absorbance at 510 nm
1.5
1.0
0.5
0
*
n.s.
(-) NS-398
(+) NS-398

Fig. 5


Compressive force
12 h
before induction
Confluent
SGBS cells
Total RNA extraction
Compressive force
12 h
after induction
Confluent
SGBS cells
Induction period
Total RNA extraction


mRNA/GAPDH mRNA
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
*
n.s.
before induction
after induction