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ORIGINAL PAPER

Heat stimulation reduces early adipogenesis in 3T3-L1 preadipocytes

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Abstract In this study, we used 3T3-L1 preadipocytes as a model to investigate the effects of heat stimulation on adipogenesis, which is a key process in the development of obesity. Heat stimulation at 43°C for 60 min significantly reduced lipid accumulation, as measured by Oil Red-O assay. In the early phase of adipogenesis, heat stimulation almost completely blocked the increase of CCAAT/enhancer binding protein delta (C/EBPdelta) gene expression and delayed the onset of the increase of C/EBPbeta gene expression. The expression of proliferator-activated receptor gamma (PPARgamma), which is regulated by these factors, was also reduced. In the later phase of adipogenesis, the induction of adipocyte-specific genes, such as C/EBPalpha, adipocyte protein 2 (aP2), lipoprotein lipase (LPL), adiponectin, and glucose transporter 4 (Glut4), which are regulated by PPARgamma, was reduced. However, adipogenesis was not significantly reduced if heat stimulation was carried out after the early phase of adipogenesis. These results suggest that heat stimulation reduces adipogenesis by decreasing the expression of adipogenesis-related transcriptional factors during early adipogenesis.

Keywords Adipocyte · Adipogenesis · 3T3-L1 · Heat stimulation · Differentiation

Introduction

Obesity is a significant risk factor for various metabolic diseases, such as hypertension, cardiovascular disease, and type 2 diabetes [1]. Obesity is characterized by an increase in the size and number of adipocytes differentiated from fibroblastic preadipocytes in adipose tissue [2]. Thus, controlling adipogenesis might be effective to decrease obesity [3].

To study adipogenesis, 3T3-L1 preadipocytes have been frequently used in vitro. This cell is derived from cell line of 3T3-Swiss albino by Green et al. [4], and differentiates into adipocyte under the culture condition existing 1-methyl-3-isobutylxanthine (IBMX), dexamethasone, and insulin. Using this cell, molecular mechanisms of adipogenesis have been widely investigated, and has been shown to involve sequential activation of various transcriptional factors such as CCAAT/enhancer binding proteins (C/EBPs) and proliferator-activated receptor gamma (PPARgamma) [5, 6].

Heat stimulation has a variety of effects on cells and is used for the therapy to prevent inflammation and to treat various types of tissue trauma [7, 8]. Heat stimulation regulates T cell proliferation and activation, promotes the priming and activation of dendritic cells, and enhances the migration of Langerhans cells from the epidermis to lymph nodes [9]. It also increases matrix production at cartilage [10].

Chung et al. [11] reported that heat stimulation decreases obesity-induced insulin resistance in mice by inducing heat shock protein 72 (HSP72) in skeletal muscle. However, they did not examine the effect of heat stimulation on the development of obesity or its direct effect on adipose tissue. Morino et al. [12] reported that heat stimulation with mild electrical stimulation decreases insulin resistance, improves glucose homeostasis, and decreases subcutaneous and visceral fat mass in obese mice, although the effect on adipocytes was not examined. Collectively, these findings suggest that heat stimulation may affect the adipose tissue directly, and may be useful to decrease obesity. Therefore, in this study, we examined the effect of heat stimulation on

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3T3-L1 preadipocytes and found that heat treatment suppresses adipogenesis by inhibiting the expression of adipogenesis-related transcriptional factors during early adipogenesis.

Materials and methods

Materials

3T3-L1 preadipocytes were purchased from Japanese Collection of Research Bioresources (JCRB, Tokyo, Japan). IBMX and dexamethasone were purchased from Sigma (St. Louis, MO). Superscript II, calf serum (CS), and DMEM were from GIBCO/BRL (Carlsbad, CA). Fetal bovine serum (FBS) was from Biowest (Nuailles, France). Oil Red-O was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Cell culture flasks and dishes were from Falcon (Becton Dickinson, Franklin Lakes, NJ). Lactate dehydrogenase (LDH) kit was from Boehringer Mannheim (Indianapolis, IN). DNase and RNeasy Protect Kit were purchased from Qiagen (Valencia, CA).

Cell culture

3T3-L1 preadipocytes were cultured in flasks using Dulbecco's modified Eagle's medium containing 10% (w/v) CS in a humidified atmosphere of 5% CO_2 at 37°C. When cells became subconfluent, they were harvested with 0.025% trypsin and 0.01% EDTA, and plated at 7,500 cells/cm^2 density in 24-well dishes (for Oil Red-O staining) and 6-well dishes (for gene expression analysis). Culture was continued for an additional 2 days after the cell reached confluence. Cells were heated by immersion of the plates in a water bath at 42 or 43°C for 30 or 60 min according to the common method [13–15]. After the heat stimulation, adipogenesis were induced according to the methods reported previously [16, 17]. Briefly, cells were cultured in DMEM with 10% FBS containing 150 μM IBMX, 1 μg/ml insulin, and 0.3 μM dexamethasone at 37°C for 2 days, and cultured in DMEM containing 10% FBS and 1 μg/ml insulin for additional 1 day (Fig. 1e). Lipids were stained with Oil Red-O, the dye was extracted with isopropyl alcohol, and its absorbance was measured at 540 nm [18]. Cytotoxicity of heat stimulation was measured in terms of LDH release from the heat-treated cells. LDH was quantitated with a colorimetric cytotoxicity kit according to the instructions of the manufacturer [19].

Quantitative real-time PCR

Extraction of RNA was performed using RNeasy Protect Kit followed by DNase treatment according to the manufacturers' instructions. Then RNA was translated to cDNA using Superscript II according to the manufacturers' instructions. The quality of RNA was confirmed using the criterion that the quantitative ratio of 28S and 18S ribosomal RNA should be greater than 1.5 after electrophoresis of RNA on an Agilent-2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The 28S rRNA was quantified for internal control by real-time PCR using a Lightcycler (Roche, Indianapolis, IN). Reaction mixture included 1X FastStart DNA Master HybProbe master Mix (Roche), 2 mM $MgCl_2$, 500 nM primer, 500 nM fluorescein-labeled probe, 500 nM LC-red-labeled probe, and the 1 μl of cDNA (equivalent to 1 ng of RNA). Cycling conditions were 95°C for 10 min, followed by 30 cycles at 95°C for 10 s, at 60°C for 15 s, and at 72°C for 12 s. The products were continuously detected by fluorescence measurement. Quantification of the cDNA for each selected gene was conducted by real-time PCR amplification using a LightCycler. C/EBPdelta, C/EBPbeta, and PPARgamma were measured for early adipogenetic transcription factors, C/EBPalpha, adipocyte protein 2 (aP2), lipoprotein lipase (LPL), adiponectin, and glucose transporter 4 (Glut 4) were measured as adipocyte marker genes. Reaction mixture included 1X FastStart DNA Master SYBR Green master Mix (Roche), 1–4 mM $MgCl_2$, 500 nM primer, and the 1 μl of cDNA (equivalent to 1 ng of RNA). Cycling conditions were 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, at 60°C for 5 s, and at 72°C for 4–8 s. The products were continuously detected by fluorescence measurement. The cycle threshold values (*C*t values) calculated by Lightcycler software 3.5 were normalized to 28S rRNA for each sample. Expression level of each target gene is shown as the relative amount with respect to the non-treated preadipocyte. The standard curves of each gene plotted *C*t value against cDNA concentration were confirmed that $r > 0.99$. These analyses were performed in duplicate for each sample from three different cell cultures, and each experiment were performed 2–4 times. Primers and probes used in this study are shown in Table 1. Amplification of the target genes was confirmed by sequence analysis of the PCR products.

Statistical analysis

All data were expressed as means ± S.E.M. Difference between groups were examined for statistical significance using the ANOVA (Fig. 1) or repeated measures ANOVA (Figs. 2, 3, 4) followed by Fisher's protected least significant difference (Fisher's PLSD) procedure as a multiple comparison test performed by Microsoft Excel. A *P* value of less than 0.05 was considered to indicate a significant difference.

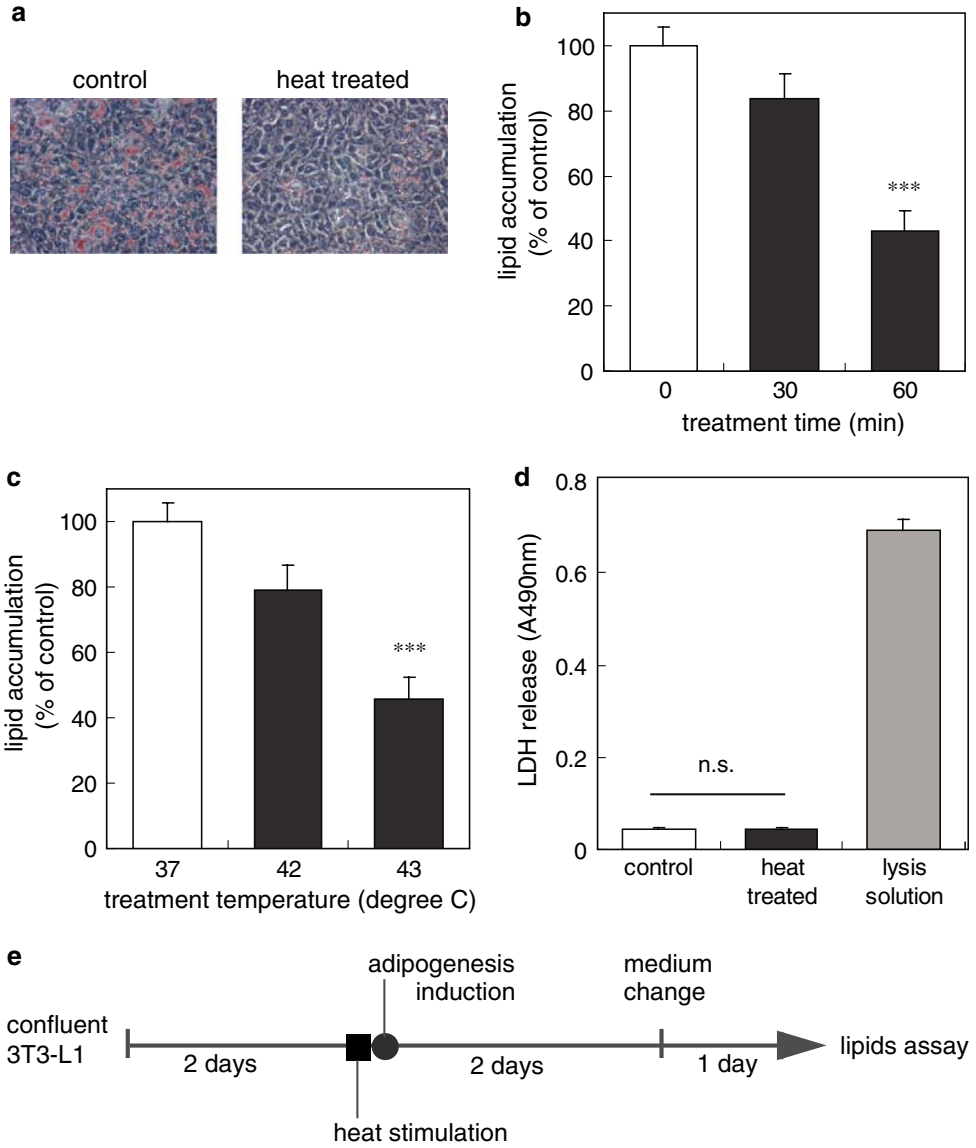

Fig. 1 Heat stimulation reduces adipogenesis-induced lipids accumulation in 3T3-L1 preadipocytes. Cells were heat treated or not, and induced adipogenesis. After 3 days, accumulated lipids were stained with Oil Red-O and photographed (**a**). Time and temperature dependency of inhibitory effect. Cells were treated at 43°C for the indicated time (**b**) or treated for 60 min at the indicated temperature (**c**), and the amount of accumulated lipid was measured in terms of the absorbance of Oil Red-O extracted from stained cells. Results are expressed as means ± S.E.M. of 4 wells in each group. Statistical significance of differences was determined by ANOVA following by a Fisher's PLSD as a multiple comparison test. $***P < 0.001$ compared with non-treated cells. **d** Heat stimulation has no cytotoxic effect. Cells were heat treated at 43°C for 60 min or not, and release of LDH into the medium was measured as a marker of cytotoxicity after 48 h. Cell lysate was prepared as a control for cytotoxicity. Results are expressed as means ± S.E.M. of 3 wells in each group. Statistical significance of differences was determined by ANOVA following by a Fisher's PLSD as a multiple comparison test. *n.s.* not significant compared with non-treated cells. **e** Scheme of adipogenesis induction and heat stimulation

Results

Heat stimulation reduces adipogenesis-induced lipids accumulation in 3T3-L1 preadipocytes

To examine the effect of heat stimulation on adipogenesis, 3T3-L1 preadipocytes were treated at 43°C for 60 min and induced adipogenesis. In non-treated control cells, lipid droplets, which were stained with Oil red-O, were visible at 3 days after the induction (Fig. 1a). However, heat treatment reduced the accumulation of lipid droplets. The amount of accumulated lipids, measured in terms of the absorbance of Oil red-O extracted from stained cells, was significantly reduced by 43.1 ± 6.4% ($P < 0.001$) by treatment at 43°C for 60 min (Fig. 1b). This inhibitory effect was dependent on both time and temperature

Table 1 Polymerase chain reaction primers and predicted size of PCR products

Gene name	Orientation	Sequences	Size (bp)	Accession No.
Adiponectin	Forward	AAAGGAGAGCCTGGAGAAGC	169	NM_009605
	Reverse	GTCCCGGAATGTTGCAGTAG		
aP2	Forward	TCACCTGGAAGACAGCTCCT	205	NM_024406
	Reverse	ACTCTCTGACCGGATGGTGA		
C/EBPalpha	Forward	GTGGACAAGAACAGCAACGA	123	NM_007678
	Reverse	GGTCAACTCCAGCACCTTCT		
C/EBPbeta	Forward	GACTTCCTCTCCGACCTCTTC	80	NM_009883
	Reverse	AGGCTCACGTAACCGTAGTCG		
C/EBPdelta	Forward	ACTCCTGCCATGTACGACGA	131	NM_007679
	Reverse	GCTTTGTGGTTGCTGTTGAAG		
LPL	Forward	GACTGAGGATGGCAAGCAAC	154	NM_008509
	Reverse	CAGTTCTCCGATGTCCACCT		
Glut 4	Forward	CAACAGCTCTCAGGCATCAA	131	NM_009204
	Reverse	ACCGAGACCAACGTGAAGAC		
PPARgamma2	Forward	CTGCCTATGAGCACTTCACAAG	98	NM_011146
	Reverse	ATCACGGAGAGGTCCACAGA		
28S rRNA	Forward	GGTAAACGGCGGGAGTAACT	200	X00525
	Reverse	TCACCGTGCCAGACTAGAGT		
	Fluorescein probe	GGATGAACGAGATTCCCACTGTCCCT		
	LC-red probe	CCTACTATCCAGCGAAACCACAGCCA		

aP2 adipocyte protein 2; *C/EBP* CCAAT/enhancer binding protein; *LPL* lipoprotein lipase; *Glut4* glucose transporter 4; *PPARgamma* proliferator-activated receptor gamma

(Fig. 1b, c). Furthermore, heat stimulation at 43°C for 60 min was not cytotoxic, since LDH release were not induced at 12 h and 48 h (Fig. 1d). These results suggested that heat stimulation reduced the early stage of adipogenesis from preadipocytes.

Heat stimulation reduces gene expression of adipogenic transcription factors in early adipogenesis

In order to further explore the anti-adipogenesis effects of heat stimulation, the gene expression of several adipogenic transcription factors in the early phase of adipogenesis was examined. After the induction of adipogenesis, C/EBPdelta mRNA abundance was transiently increased, reached a maximum level at 1 h (Fig. 2a). Heat stimulation significantly and almost completely inhibited this increase of C/EBPdelta mRNA expression from 15 min to 3 h (Fig. 2a). C/EBPbeta mRNA abundance was also transiently increased, and reached a maximum level at 3 h after induction (Fig. 2b). Heat stimulation significantly reduced this increase of C/EBPbeta mRNA expression at 15 min and 1 h after induction (Fig. 2b), suggesting that heat stimulation inhibits the induction of C/EBPdelta mRNA expression and delays the increase of C/EBPbeta mRNA expression in early adipogenesis.

Since these factors are known to up-regulate PPARgamma, a master regulator of adipogenesis, we next examined PPARgamma gene expression. In control cells, PPARgamma started to increase at 3 h after induction of adipogenesis, following the up regulation of C/EBPdelta and beta reached their maximal levels of expression. Heat stimulation significantly reduced the increase of PPARgamma expression at 3 and 8 h (Fig. 2c), suggesting the reduced PPARgamma induction may be involved in the reduction of adipogenesis by heat treatment. In order to confirm this, we next investigated the change in C/EBPalpha mRNA abundance as it is a downstream target of PPARgamma. In control cells, C/EBPalpha was increased from 24 h after adipogenesis induction, and heat stimulation significantly reduced the increase after 24 and 48 h of induction. Since C/EBPalpha is another master regulator of adipogenesis, the mRNA abundance of its target genes, which include ap2, LPL, adiponectin, and Glut4 gene were next examined. In control cells, the expression levels of ap2, LPL, adiponectin, and Glut4 gene were increased from 48 h after adipogenesis induction, and these increases were significantly reduced by the heat treatment at 48 and 72 h after induction. In order to confirm that heat treatment affects only the early stage of adipogenesis, heat treatment was also carried out at 8 h after adipogenesis induction, at which time

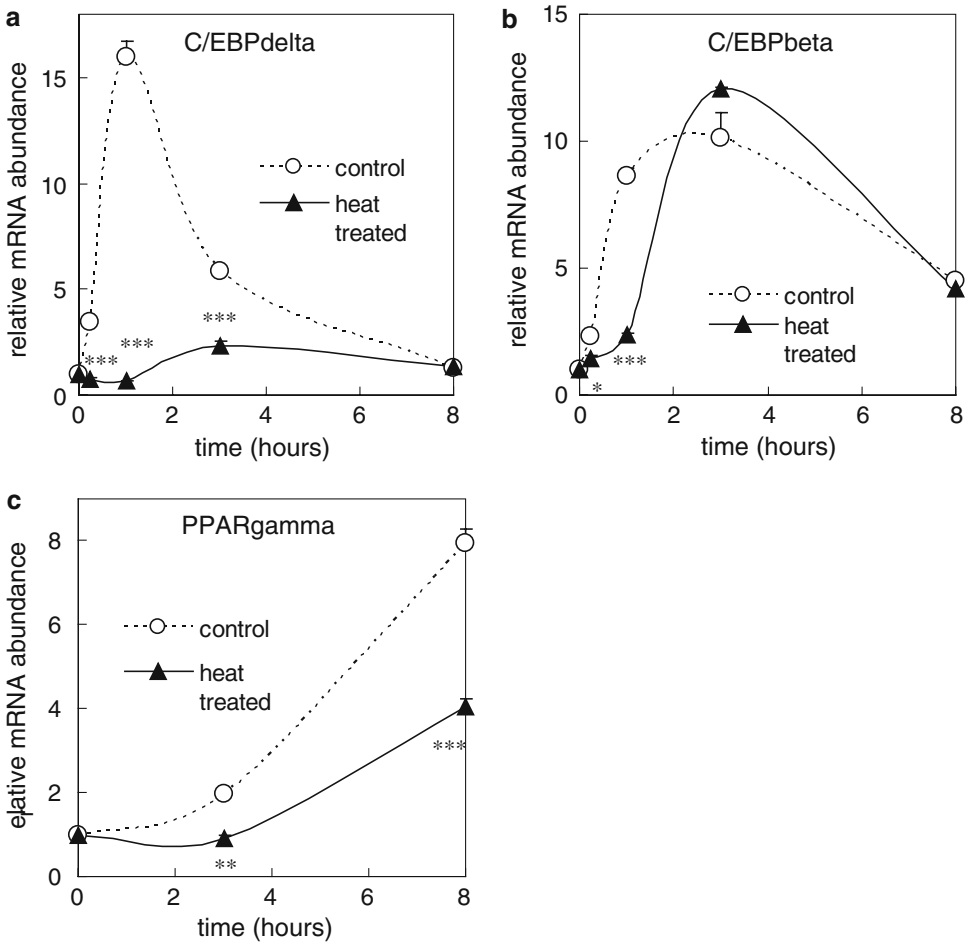

Fig. 2 Heat stimulation reduces adipogenesis-induced gene expression of early-phase adipogenic transcription factors in 3T3-L1 preadipocytes. Cells were treated for 43°C for 60 min or not, and adipogenesis was induced. After the indicated time, mRNA expression levels of C/EBPdelta (**a**), C/EBPbeta (**b**), and PPARgamma (**c**) were measured by real-time PCR. Results are expressed as means ± S.E.M. of 3 wells in each group. Statistical significance of differences was determined by ANOVA following by a Fisher's PLSD as a multiple comparison test. $*P < 0.05$, $**P < 0.05$, $***P < 0.001$ compared with non-treated cells

the expression of C/EBPdelta had returned to the control level after the transient increase. Heat stimulation at 8 h after the induction of adipogenesis had no inhibitory effect on lipid accumulation (Fig. 4), supporting the idea that heat stimulation reduces adipogenesis by decreasing the expression of early adipogenic transcription factors.

Discussion

In the present study, we investigated the effect of heat stimulation on adipogenesis using 3T3-L1 preadipocytes as a model. We found that heat stimulation reduced adipogenesis temperature- and time dependently. Heat stimulation reduced the gene expression of early adipogenesis-related transcriptional factors (C/EBPdelta, C/EBPbeta, and PPARgamma) and this was followed by reduction of adipocyte-specific gene expression (C/EBPalpha, ap2, LPL, adiponectin, and Glut4). Since heat stimulation had no effect on adipogenesis when applied after the end of the early phase of adipogenesis (e.g., at 8 h), we hypothesized that it could act through a down regulation of expression of transcriptional factors involved in the early phase of adipogenesis induction.

The mechanism of adipogenesis has been widely investigated, using adipogenic cell lines, and has been shown to involve sequential activation of various transcriptional factors [5, 6]. C/EBPbeta [20] and delta [21] are the earliest transcription factors induced upon adipogenic stimuli [22], and they transcriptionally activate the PPARgamma and C/EBPalpha genes through C/EBP regulatory elements in their proximal promoters [23]. PPARgamma and C/EBPalpha serve as pleiotropic transcriptional activators that coordinately promote expression of adipocyte-specific genes. Subsequently, the differentiated cells express markers characteristic of the adipocyte phenotype, such as aP2, LPL, adiponectin, and Glut4 [24]. We found that heat stimulation almost completely inhibited the increase of C/EBPdelta mRNA expression and delayed C/EBPbeta mRNA expression upon induction of adipogenesis in 3T3-L1 preadipocytes, leading to a reduction in the expressions of PPARgamma and C/EBPalpha. Furthermore, mRNA expressions of aP2, LPL, adiponectin, and Glut4 which are regulated by PPARgamma and C/EBPalpha were also reduced by heat stimulation. These results suggest that heat stimulation reduced adipogenesis by decreasing the gene expression of early adipogenesis-

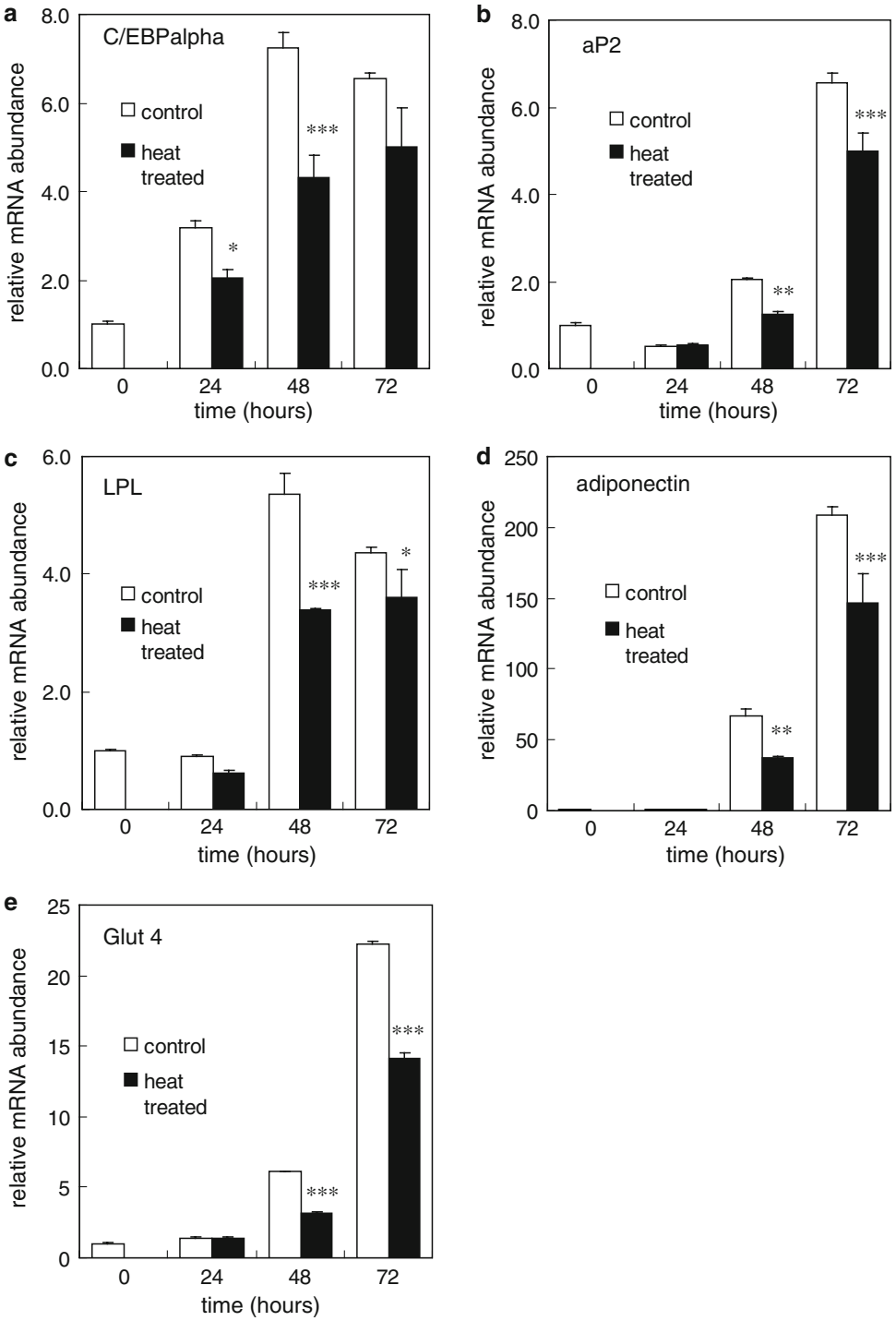

Fig. 3 Heat stimulation reduces adipocyte-specific gene expression in adipogenesis-induced 3T3-L1 preadipocytes. Cells were heat treated at 43°C for 60 min or not, and adipogenesis was induced. After the indicated time, mRNA expression levels of C/EBPalpha (**a**), aP2 (**b**), LPL (**c**), adiponectin (**d**), and Glut4 (**e**) were measured by real-time PCR. Results are expressed as means ± S.E.M. of 3 wells in each group. Statistical significance of differences was determined by ANOVA following by a Fisher's PLSD as a multiple comparison test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with non-treated cells

inducing transcriptional factors, such as C/EBPbeta and delta. This was supported by the finding that heat stimulation conducted after the early phase failed to inhibit adipogenesis. Heat stimulation is known to activate components of the mitogen-activated protein kinase (MAPK) signaling pathway, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38MAPK in various cells [25–28], and p38MAPK activation blocks adipogenesis via inhibition of C/EBPbeta phosphorylation and PPARgamma expression [29]. Therefore, it is possible that heat stimulation reduces early adipogenic transcription factors via activation of these pathways, although further study is needed to confirm this.

In conclusion, we have shown that heat stimulation reduces adipogenesis, and this reduction appears to be due to a decrease in the expression of early adipogenic transcriptional factors.

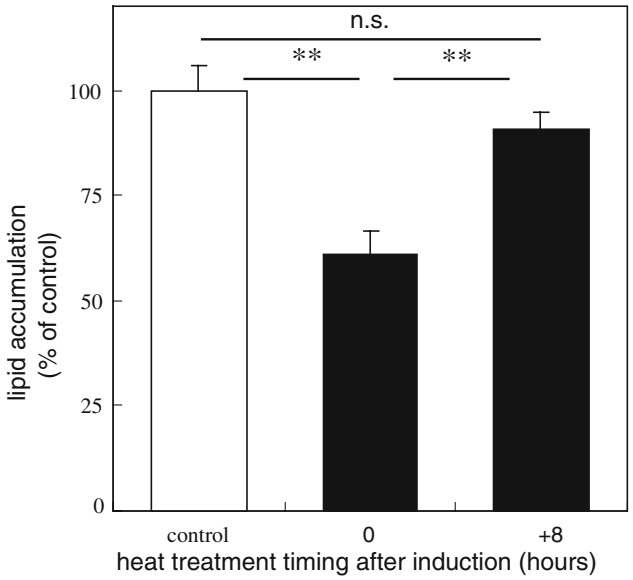

Fig. 4 Heat stimulation performed after the early phase of adipogenesis cannot reduce adipogenesis. Heat stimulation were done before (pre) or at 8 h after (post) induction of adipogenesis at 43°C for 60 min. After 3 days, lipid accumulated in cells was stained with Oil Red-O. Results are expressed as means ± S.E.M. of 4 wells in each group. Statistical significance of differences was determined by ANOVA following by a Fisher's PLSD as a multiple comparison test. $*P < 0.05$, $**P < 0.01$, *n.s.* not significant compared with the indicated groups

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