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Inhibition of 3T3-L1 adipocyte differentiation by 6-ethoxyzolamide: repressed peroxisome proliferator-activated receptor γ mRNA and enhanced CCAAT/enhancer binding protein β mRNA levels

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Abstract

The effects of 6-ethoxyzolamide (ETZ), a carbonic anhydrase (CA) inhibitor, on differentiation of the mouse preadipocyte cell line 3T3-L1 were examined by quantitative image analysis of intracellular fat storage. For adipocyte differentiation, postconfluent cells were treated with a ligand of the peroxisome proliferator-activated receptor γ (PPARγ), troglitazone (TRG), and dexamethasone for 2 days. Differentiated cells showed weak fat staining at day 4 which increased thereafter, correlating with CAIII expression. ETZ treatment for 10 days at a 200 μM concentration reduced both the percentage of differentiated adipocytes storing fat and the amount of fat stored in individual cells. These findings were also supported by the results of fluorescence activated cell sorting analysis. Despite their light fat staining, however, CAIII was not expressed in ETZ-treated cells. Furthermore, ETZ delayed the clonal expansion of cells, an early event preceding differentiation. Northern blot analysis revealed a high CCAAT/enhancer binding protein (C/EBP) β mRNA level and low PPARγ mRNA in ETZ-treated cells. Thus, increased C/EBPβ mRNA did not lead to enhanced *PPARγ* expression in this case. Another CA inhibitor acetazolamide did not inhibit adipocyte differentiation, although the drug exhibited a similar inhibition pattern for CA activity as ETZ. These results suggested that inhibitory effects of ETZ on adipocyte differentiation were not due to inhibition of CA activity but rather due to altered levels of the transcription factors.

Keywords: Adipocyte differentiation; Carbonic anhydrase; 6-Ethoxyzolamide; CCAT/enhancer binding protein β; Peroxisome proliferator-activated receptor γ

1. Introduction

Adipocytes play central roles in the synthesis, storage, and hydrolysis of nutritional energy in the form of triacylglycerols. Abnormal regulation of adipocyte differentiation and lipogenesis is linked to pathological conditions such as obesity and diabetes mellitus [1]. Adipocytes are generally considered to be derived from undetermined mesenchymal cells [2] and cell culture systems employing preadipose cell lines such as 3T3-L1 have been extensively used to study adipocyte differentiation [3]. Many hormones and growth factors have been shown to modulate differentiation processes in either a positive or negative manner, resulting in dramatic changes in cell morphology and gene expression [4]: insulin, glucocorticoids, and growth hormone exert promotion effects [5,6]. PPARγ, a member of the nuclear hormone receptor family, has been demonstrated to be one of key transcription factors to induce adipocyte differentiation [7,8]. It has also been demonstrated that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and synthetic antidiabetic compounds, thiazolidinediones, are direct ligands of the receptor which induce adipogenesis [8–10]. The expression of *PPARγ* is known to be stimulated by C/EBPβ [7–10].

The CA (EC 4.2.1.1) family comprises at least nine isoforms [11,12] that catalyze the interconversion of bicarbonate (HCO_3^-) and H^+ with CO_2 and H_2O. Four of the isoforms are cytosolic (CAI, CAII, CAIII and CAVII) and one is mitochondrial (CAV). Among them, CAIII has a

Abbreviations: CA, carbonic anhydrase; DMEM, Dulbecco's modified Eagle's medium; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TRG, troglitazone; DEX, dexamethasone; ETZ, 6-ethoxyzolamide; ACZ, acetazolamide; FACS, fluorescence activated cell sorting; PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/enhancer binding protein

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number of unique properties: its CO_2 hydration action is only 1% of that of CAII [11], but it exhibits phosphatase activity [13], and is relatively insensitive to inhibition by sulfonamide inhibitors, like ACZ and ETZ [14]. CAII, CAIII and CAV are expressed in adipose tissues and also in 3T3 adipocytes in a differentiation-dependent fashion [15,16]. CAIII constitutes about 10% of the total protein in fat cells and has been considered as a marker for differentiated adipocytes [15]. Analysis employing inhibitors has suggested that CA may be involved in lipogenesis by providing bicarbonate, a substrate for lipogenic pyruvate carboxylase and acetyl-CoA carboxylase [16,17]. However, such involvement is not supported by the finding that CAIII and lipogenic enzymes are not coordinately regulated in obesity [18]. Thus, the functional relationship between CA and adipocyte differentiation remains to be clarified.

In the present study, to examine the effects of ETZ on adipocyte differentiation, intracellular fat storage was quantitatively evaluated. ETZ treatment reduced both the percentage of differentiated adipocytes storing fat and the amount of fat stored in individual cells, as well as delaying their clonal expansion. This treatment also resulted in decreased PPARγ and increased C/EBPβ mRNA levels. Another CA inhibitor ACZ, however, did not inhibit adipocyte differentiation. Inhibitory effects of ETZ on adipocyte differentiation were not entirely due to inhibition of CA activity but rather due to altered levels of the transcription factors.

2. Materials and methods

2.1. Materials

Nitrocellulose membranes were purchased from Schleicher and Schuell Inc. for Western blotting and from Micron Separation Inc. for Northern blotting; goat anti-rabbit IgG-horseradish peroxidase conjugate was from Bio-Rad Laboratories. Mouse preadipocyte cell line, 3T3-L1 (passage 7), was provided by the Japanese Collection of Research Bioresources. Fetal bovine serum was from JRH Biosciences; calf serum from HyClone; DMEM from Nissui Pharmaceutical Co., Ltd.; penicillin–streptomycin and trypsin from Gibco-BRL; ETZ, ACZ, DEX and TPCK-treated trypsin from Sigma; L-glutamine from ICN Biomedicals Inc. TRG was kindly provided by Sankyo Co., Ltd. All other chemicals were of the highest grades commercially available.

2.2. Cell culture and differentiation induction

3T3-L1 cells were cultured to confluence in DMEM with 10% calf serum and penicillin–streptomycin (100 units/ml each) under humidified air containing 5% CO_2 at 37 °C. For adipocyte differentiation, postconfluent cells were placed in DMEM, containing 10% fetal bovine serum, 10 μM of TRG, 1 μM of DEX and 10 μg/ml of insulin (TRG group) [19]. TRG had been dissolved in dimethylsulfoxide at 1000-fold the final concentration and DEX in ethanol. After 48 h, the medium was removed and 10% fetal bovine serum-DMEM, containing 10 μg/ml of insulin, was added. Cells were further maintained for 8 days, with medium changed every 3 days. To examine the effects of ETZ or ACZ on adipocyte differentiation, respective drug dissolved in dimethylsulfoxide was added to the culture medium at a final concentration of 200 μM on day 0 and this treatment was continued throughout the experimental period (TRG + ETZ or TRG + ACZ groups). In some experiments, cells were treated with ETZ for the first 2 days or from day 3 to day 5 after induction of differentiation by TRG and DEX. As a control group, TRG and DEX were omitted from the medium but insulin was added. In the ETZ alone or ACZ alone groups, cells without differentiation induction were treated with 200 μM ETZ or ACZ for 10 days.

2.3. Fat staining and image analysis of 3T3-L1 cells

3T3-L1 cells cultured in 12-well plates were stained for fat with Oil red O by the method of Green and Kehinde [20]. The intensity of fat staining was measured with the aid of a film scanner (Polascan 35 ultra, Polaroid) interfaced with a computer using the NIH Image software 1.61. Red color was converted to a gray scale and average gray values for a unit area (40 μm × 40 μm, a pixel) were scored in the range of 0 (white) to 255 (black). In each case a total of 150,000 pixels were used for quantitative analysis.

2.4. FACS analysis and cell count

For measurement of fat storage in individual cells by FACS, cultured cells were trypsinized and stained with Nile Red according to the method of Smyth and Wharton [21]. Aliquots of 10,000 stained cells suspended in phosphate buffered saline (consisting of 8 mM disodium phosphate, 2 mM monopotassium phosphate, 3 mM KCl, and 0.14 M NaCl, pH 7.4) were analyzed by flow cytometry (FACScan, Becton-Dickinson). Staining intensity was evaluated in the range of 1–1000. For measurement of DNA content, cells fixed with 70% ethanol were stained with propidium iodide and applied to a flow cytometer [22].

For cell count, cells in 12-well plates were trypsinized and suspended with 1 ml of phosphate buffered saline as described above, containing 5 mM EDTA, 2% bovine serum albumin and 25 mM glucose, and counted with improved Neubauer hematocytometer by triplicate assay.

2.5. Northern blot analysis

Total RNA was extracted from 3T3-L1 cells using an RNeasy mini kit (Qiagen), and samples (12 μg) were electrophoresed through denaturing formaldehyde–agarose gels. Equal loading was checked by observing the

amounts of 18S and 28S ribosomal RNA stained with ethidium bromide. Then RNAs were transferred to nitrocellulose membranes and hybridized with specific cDNA probes labeled with [^{32}P]-dCTP [22]. Results of autoradiography were quantified with an image scanner interfaced with a computer and the values expressed relative to the ribosomal RNA level. C/EBPβ cDNA [23] was kindly provided by Dr. M. Imagawa (Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan) and PPARγ cDNA [24] was a generous gift from Dr. M. Sakai (Hokkaido University School of Medicine, Department of Biochemistry, Sapporo, Japan).

2.6. Western blot analysis

SDS–PAGE was carried out with 12.5% acrylamide gels by the method of Laemmli [25]. Western blotting was performed using an anti-rat CAIII antibody by the method of Towbin et al. [26]. The antibody was raised in a rabbit by immunizing with purified rat CAIII as described below.

2.7. Enzyme assays

CO_2 hydratase activity of CA was assayed by a colorimetric method as described by Wilbur and Anderson [27] with slight modifications. The color change of bromothymol blue, a pH indicator, was monitored at 615 nm with a spectrophotometer (Beckman DU-640). The assay mixture consisted of 1.4 ml of 22 mM sodium barbiturate buffer, pH 8.15, containing 0.0025% (w/v) of bromothymol blue, 0.5 ml of CO_2-saturated cold water, and 0.1 ml of enzyme solution. CO_2 hydratase activity was calculated by the method of Maren et al. [28]. Phosphatase activity of CAIII was assayed according to the method of Koester et al. [29]. Protein amounts were measured by the method of Bradford [30].

2.8. Purification of CAIII from rat adipose tissue

Retroperitoneal adipose tissues were excised from Sprague–Dawley rats (body weight about 300 g) after ether anesthesia and decapitation and aliquots (80 g) were homogenized on ice with 4 vol. of 20 mM Tris–HCl, pH 7.4, and centrifuged at 105,000 × *g* for 60 min. For purification of CAIII, supernatants were applied to DEAE-cellulose and subsequently a Sephadex G-75 column, essentially according to the method of Armstrong et al. [31]. The final preparation with a specific activity value of 4.6×10^3 units/mg protein exhibited only a 29-kDa band on SDS–PAGE. Protein was stained with Coomassie brilliant blue R-250. This preparation also showed a significant phosphatase activity.

2.9. Amino acid sequencing

An aliquot (8 nmol) of the purified CAIII dissolved in 20 mM Tris–HCl, pH 8.0, was incubated with 160 pmol of TPCK-treated trypsin at 35 °C overnight. The digest was fractionated by reverse-phase high performance liquid chromatography on a TSK-gel ODS-80TS column (4.6 mm × 250 mm, Tosoh) with a gradient of 3.2–80% acetonitrile in aqueous 0.1% trifluoroacetic acid. Major peaks resolved were applied to a protein sequencer (Applied Biosystems, model 492) and amino acid sequencing was carried out as reported previously [32]. The peptide sequence was identical to that reported for CAIII [11].

3. Results

3.1. Effects of ETZ on fat storage in 3T3-L1 cells

For differentiation induction, postconfluent 3T3-L1 cells were treated with TRG and DEX for 2 days and maintained in the presence of insulin for further 8 days (TRG group).

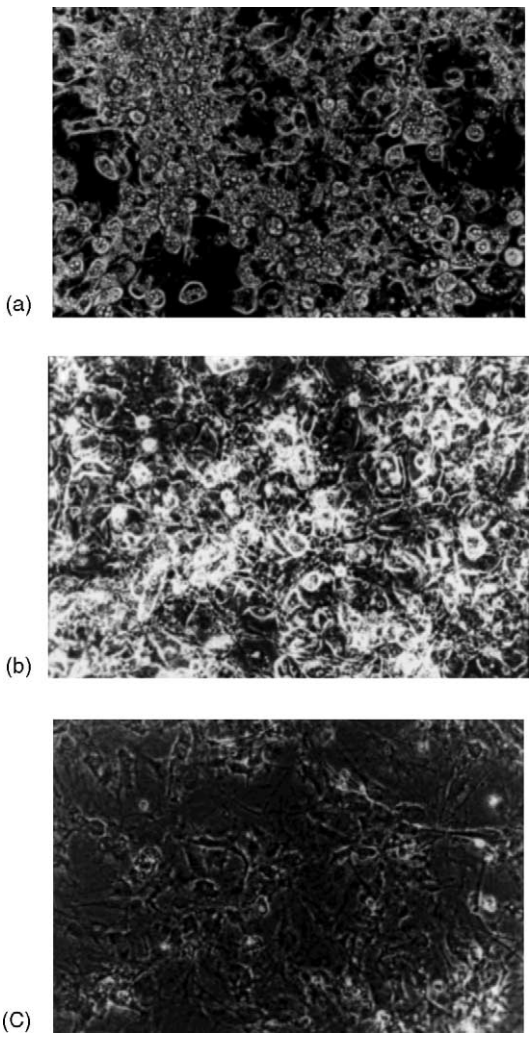

Fig. 1. Effects of ETZ treatment on morphological change in 3T3-L1 cells induced by TRG. (a) Micrograph of 3T3-L1 cells of the TRG group on day 10, (b) 3T3-L1 cells of the TRG + ETZ group, and (c) cells of the control group. Original magnification: 200×.

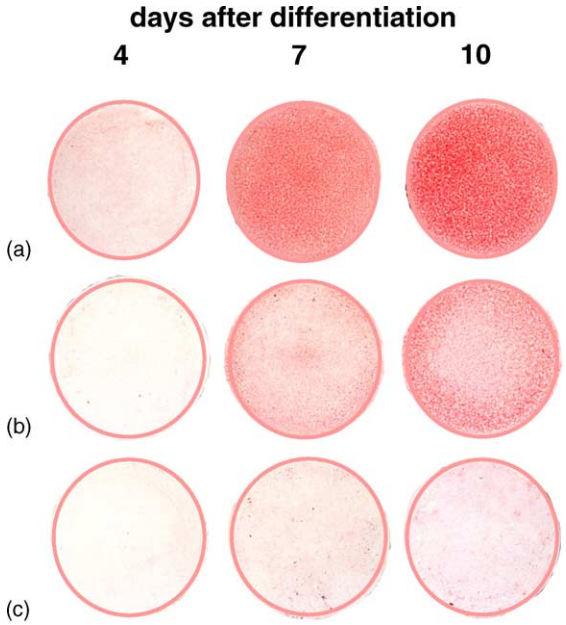

Fig. 2. Time course of fat storage in 3T3-L1 cells. (a) Fat staining with Oil red O of 3T3-L1 cells of the TRG group on days 4, 7 and 10, (b) 3T3-L1 cells of the TRG + ETZ group, and (c) cells of the control group.

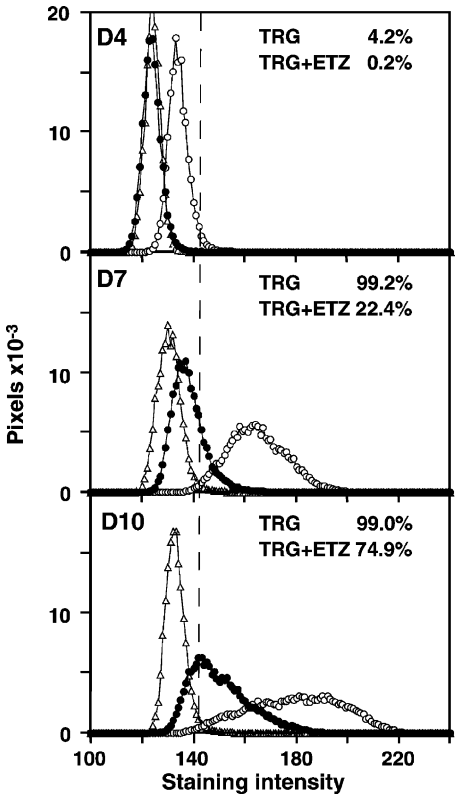

Fig. 3. Quantitative analysis of fat staining. Oil red O staining of 3T3-L1 cells, shown in Fig. 2, was scanned with a film scanner interfaced with a computer using NIH Image software 1.61. The red color of stained fat was converted to a gray scale and staining intensity was expressed as the average for a unit area (40 μm × 40 μm, a pixel), scored in the range of 0 (white) to 255 (black), only the 100–240 being depicted. A total of 150,000 pixels were evaluated for quantitative analysis. The ordinate is the number of pixels. Individual panels denote results obtained on days 4, 7 and 10 for 3T3-L1 cells of the TRG group (○), TRG + ETZ group (●) and control group (△). The percent values of pixels for the TRG and TRG + ETZ groups that exceeded 142, the highest staining intensity value of the control, are also shown in the figure.

On microscopic examination, most cells exhibited a round shape and many bright fat droplets in the cytoplasm on day 10 (Fig. 1a). Cells in the control group were spindle-shaped and had no visible fat droplets (Fig. 1c). In the TRG + ETZ group, some cells exhibited round shape and fat droplets, while others retained the spindle-shape without fat (Fig. 1b). Thus, ETZ partly inhibited morphological changes of 3T3-L1 cells induced by TRG and DEX. ETZ treatment alone did not affect the cell shape (data not shown).

Oil red O staining was performed to evaluate intracellular fat storage during the process of adipocyte differentiation. A weak positive result was obtained on day 4 and strong positivity thereafter for the TRG group (Fig. 2a). Staining intensities of the TRG + ETZ group were less than those for the TRG group on days 7 and 10 (Fig. 2b), with more heterogeneity. Cells of the control group were very faintly stained on days 7 and 10, with their staining intensities being much lower than those for TRG + ETZ group (Fig. 2c). Fat staining results were quantitatively evaluated from intensity data per unit area, 40 μm × 40 μm, the value being close to the area of a single differentiated adipocyte with a diameter of 30–40 μm. Staining intensity in the TRG group was 135 ± 10 on day 4, and increased to 180 ± 40 on day 10 (Fig. 3), apparently due to increased fat stores in individual cells. The values of the control group were hardly changed, at 122–130 on day 4 to day 10. Addition of ETZ at a final 200 μM concentration reduced the staining intensity, distributed in the range of 130–180, with a peak at 140 on day 10. The percentages of pixels that exceeded 142, the highest staining intensity value of the control group, were 22.4 and 74.9% on days 7 and 10, respectively, for the TRG + ETZ group, in contrast to values more than 99% for the TRG group. The results suggested that ETZ decreased the number of cells storing fat on day 7 and also the amounts of fat in individual cells on day 10. Addition of ACZ, another CA inhibitor, even at a final 200 μM concentration, however, did not affect increased fat stores due to TRG on days 7 and 10 (Fig. 4).

For precise measurement of fat storage in individual cells, 10,000 cells stained for fat were analyzed by flow cytometry. The staining intensities for the TRG group on day 10 were distributed in the range of 1–1000, with a small peak at 10 and a major peak at 100 (Fig. 5a), indicating variation in the amount of fat in individual cells. Cells of the TRG + ETZ group were distributed in the range of 4–40, with a peak at 10, but none at 100 (Fig. 5b), indicating suppression of fat storage. Cells of the control group exhibited a peak at 4 (Fig. 5c). At a concentration of 20 μM, ETZ did not influence fat staining in response to TRG (Fig. 5d).

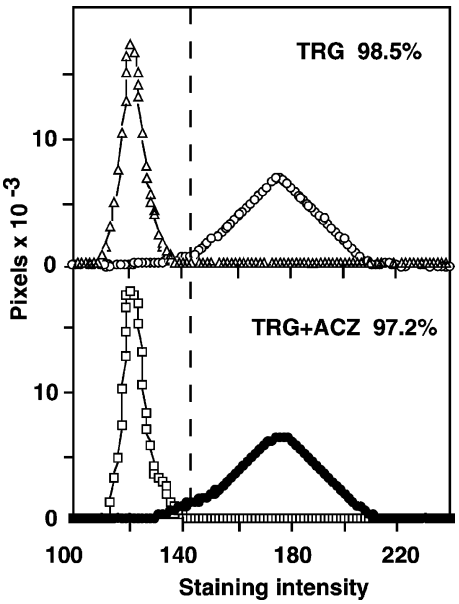

Fig. 4. The effect of ACZ on fat staining of 3T3-L1 cells treated with TRG. Quantitative analysis of fat staining was performed as described in Fig. 3. The upper panel denotes results obtained on day 10 for 3T3-L1 cells of the TRG group (○) and control group (△). The lower panel does those of the TRG + ACZ group (●) and the ACZ alone group (□). The percent values of pixels for the TRG and TRG + ACZ groups that exceed 142 are also shown in the figure.

3.2. Effects of ETZ on clonal expansion

Many studies on preadipose cell lines, including 3T3-L1, revealed that growth-arrested cells undergo at least one round of DNA replication and cell doubling before subsequent differentiation [33,34]. The effect of ETZ treatment on DNA replication induced by TRG and DEX was studied by cell number and flow cytometry. Cell number of the TRG group was doubled on day 2 and was not significantly increased thereafter (Fig. 6). On the other hand, that of the TRG + ETZ group was gradually increased and doubled on day 6. FACS analysis revealed that cells in S phase comprised 8.6% before differentiation and increased to 53.2% at 12 h in the TRG group (Table 1). ETZ treatment delayed the increase of S phase cells at 24–48 h (TRG + ETZ group).

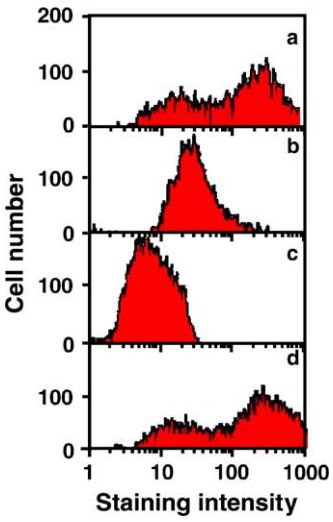

Fig. 5. Estimation of fat stores in individual 3T3-L1 cells by fluorescence activated cell sorter. 3T3-L1 cells of the TRG (a), TRG + ETZ (b), and control (c) groups at day 10 were trypsinized, stained with Nile Red for fat, and analyzed by flow cytometry. Staining intensity was evaluated in the range of 1–1000, expressed in logarithmic scale. In the TRG + ETZ group, ETZ was used at a 200 μM concentration. In (d), ETZ was used at a 20 μM concentration in the presence of TRG and DEX.

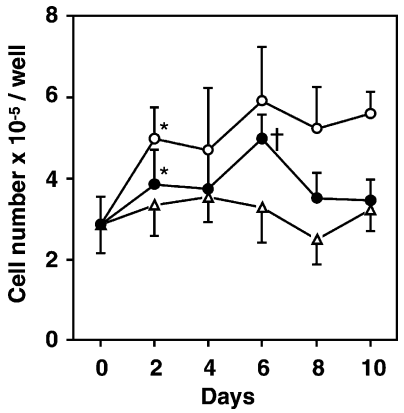

Fig. 6. Changes of cell number during 3T3-L1 differentiation. 3T3-L1 cells in the TRG (○), TRG + ETZ (●) and control (△) groups were cultured on 12-well plates for indicated days. After trypsinization, cell number was counted with an improved Neubauer hematocytometer. Data were obtained from three independent experiments and triplicate assay was performed in each experiment. Bars indicate standard deviation. *: $P < 0.05$ vs. value on day 0; †: $P < 0.05$ vs. value on day 2. P value was calculated by Student's t-test.

3.3. Effects of ETZ on CAIII expression

The effects of ETZ treatment on CAIII expression during adipocyte differentiation were examined by Western blotting (Fig. 7). A CAIII band was not detected before differentiation but was present on day 4 and increased thereafter in the TRG group. The degree of CAIII expression was proportional to the fat staining and the time courses of increase also coincided. However, ETZ treatment completely repressed CAIII expression even on day 10, despite the light fat staining observed. Less but significant CAIII band was detected on day 10 in the control group, although fat staining was almost negative.

The contribution of CAIII to total CA activity of adipocytes was evaluated by the addition of ETZ or ACZ, CA activity being assayed by employing CO_2 as a substrate. As compared with purified CAIII, the supernatant of differentiated 3T3-L1 cells was more sensitive to ETZ, but inhibition patterns were almost parallel for the two enzyme preparations (Fig. 8a). The ETZ concentration giving a 50% inhibition of purified CAIII was 1 μM, close to the values reported by other investigators [14,16]. At a 0.3 nM ETZ concentration, about 30% of the CA activity was lost in the supernatant while purified CAIII was hardly affected. ACZ exhibited similar inhibition patterns for both

Table 1
Effects of ETZ on clonal expansion of 3T3-L1

Group	Stage of cell cycle	Cell percentage			
		Time after addition of differentiation inducers			
		0 h	12 h	24 h	48 h
TRG	G1	71.0	33.4	57.2	69.8
	S	8.6	53.2	15.7	10.9
	G2/M	20.4	13.4	27.1	19.3
TRG + ETZ	G1		67.1	36.1	46.4
	S		10.6	41.1	31.6
	G2/M		22.3	22.8	22.0

3T3-L1 cells of the TRG and TRG + ETZ groups were harvested at 0, 12, 24 and 48 h after addition of differentiation inducers. DNA was stained with propidium iodide and applied to a flow cytometer for DNA replication analysis. The data shown are from one experiment and are similar to results obtained in two other experiments.

enzyme preparations and almost completely inhibited their activity at a 300 μM concentration (Fig. 8b). These results suggest that CAIII accounts for about 70% of the total CA activity of supernatant. This estimate was in line with the finding of a similar extent of inhibition by anti-CAIII antibody (data not shown).

3.4. Effects of ETZ on C/EBPβ and PPARγ expression

Since two transcription factors, C/EBPβ and PPARγ, are known to be induced during adipocyte differentiation [34,35], effects of ETZ on their expression were examined by Northern blotting. This analysis revealed PPARγ mRNA level to be marginal on day 2 but markedly increased on day 4, attaining a peak by day 6 (six- to eight-fold of the value at day 0), in cells differentiated by TRG and DEX (Fig. 9A). A significant amount of C/EBPβ mRNA was detected before differentiation and subsequent increase was not prominent (less than two-fold). Treatment with ETZ at a final 200 μM concentration, however, resulted in decreased PPARγ mRNA and enhanced C/EBPβ mRNA levels (three- to four-fold). The latter

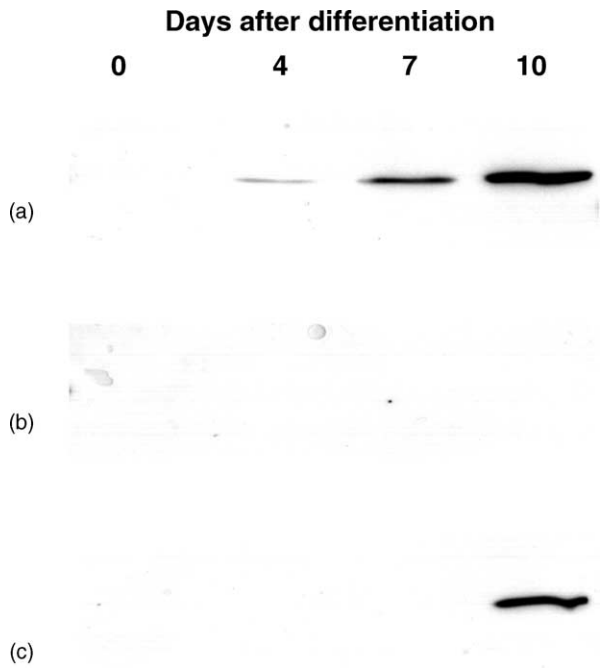

Fig. 7. Western blot analysis of CAIII expression in 3T3-L1 cells. SDS–PAGE was carried out with 12.5% acrylamide gels and supernatant samples (100 μg protein each) obtained from 3T3-L1 cells of the TRG (a), TRG + ETZ (b), and control (c) groups on days 0, 4, 7 and 10, applied to the individual lanes. Western blotting was performed using anti-rat CAIII antibody.

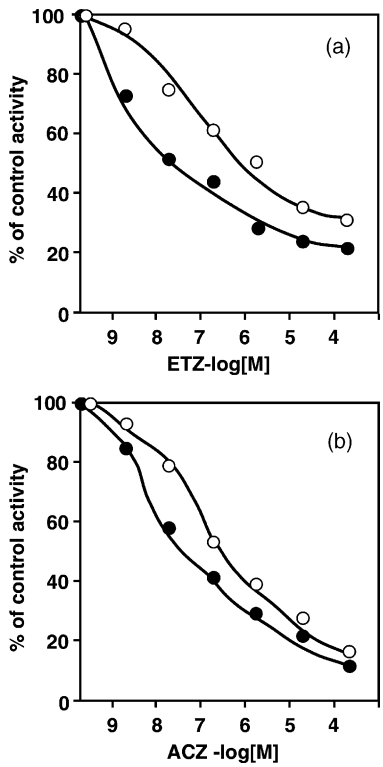

Fig. 8. Inhibition of activities of purified CAIII and adipocyte supernatant CA by ETZ (a) and ACZ (b). An 1.4 ml aliquot of 22 mM sodium barbiturate buffer, pH 8.15, containing 0.0025% bromothymol blue and 0.3 nM–0.3 mM ETZ or 0.3 nM–0.3 mM ACZ was mixed with 0.5 ml of CO_2-saturated water and 0.1 ml (7000 units/ml) of purified CAIII solution (○) or adipocyte supernatant (●). Supernatant was prepared from 3T3-L1 cells of the TRG group cultured for 10 days. After incubation at 10 °C for 2 min, the CA activity was assayed. Purification of CAIII from rat adipose tissue was carried out as described in Section 2.

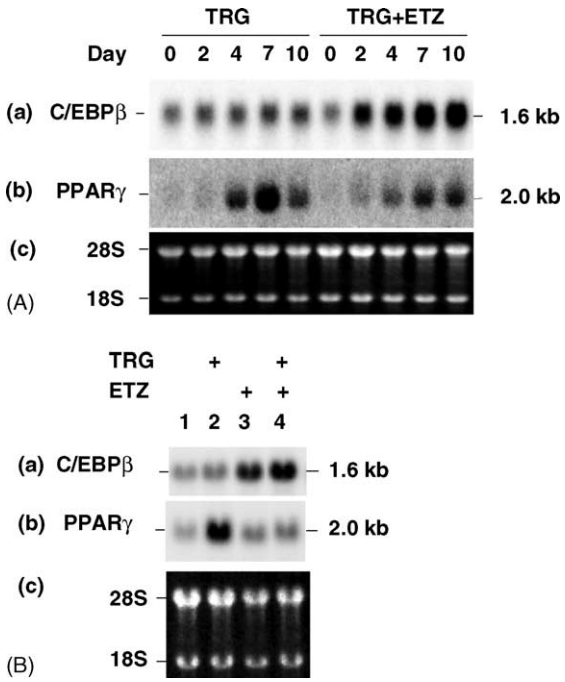

Fig. 9. C/EBPβ and PPARγ mRNA levels in 3T3-L1 cells. (A) Individual RNA samples (12 μg each) extracted from 3T3-L1 cells of the TRG and TRG + ETZ groups at the indicated time points were electrophoresed through agarose gels and transferred to nitrocellulose membranes. In the TRG + ETZ group, ETZ was used at a 200 μM concentration. (B) Individual RNA samples from 3T3-L1 cells of the control (lane 1), TRG (lane 2), 200 μM ETZ alone (lane 3) and TRG + ETZ (lane 4) groups on day 10, were similarly treated and transferred to membranes. These membranes were hybridized with C/EBPβ cDNA (a) and PPARγ cDNA (b) labeled with ^{32}P. (c) 28S and 18S ribosomal RNA were stained with ethidium bromide.

was evident on day 2, before the alteration of PPARγ mRNA. Difference in PPARγ mRNA level between the TRG group and the TRG + ETZ group was apparent on days 4 and 7. When ETZ was added at 20 μM, no appreciable effects on PPARγ or C/EBPβ mRNA levels were evident (data not shown). In the ETZ alone group, C/EBPβ mRNA level was increased but PPARγ mRNA was not, as compared with that in the control group (Fig. 9B).

4. Discussion

In the present study, ETZ partly inhibited morphological changes of 3T3-L1 cells induced by TRG and DEX (Fig. 1), depressing not only the amount of fat stored in individual adipocytes but also the percentage of differentiated cells (Figs. 2, 3 and 5). ETZ also exerted an inhibitory effect on fat storage when methylisobutylxanthine [36], instead of TRG, was used to induce differentiation (data not shown). Since CA is thought to be involved in lipogenesis, such inhibition of fat storage by ETZ has been considered to be due to inhibition of its activity [16,17]. The dominant CA isoform in the supernatant of differentiated 3T3-L1 cells has been identified as CAIII, known to be less sensitive to CA inhibitors than other CA isoforms [37]. In in vitro experiment, 1 μM ETZ inhibited 70 and 50% of CA activities of adipocyte supernatant and purified CAIII, respectively (Fig. 8a). However, ETZ at a concentration of 200 μM completely repressed CAIII expression, and its treatment for the first 2 days delayed the clonal expansion of 3T3-L1 cells (Table 1) before significant amount of CAIII was expressed. Furthermore, ACZ at 30–300 μM concentrations almost completely inhibited CA activities of adipocyte supernatant and purified CAIII (Fig. 8b) but did not affect adipocyte differentiation (Fig. 4). These findings suggested that the inhibitory effect of ETZ on adipocyte differentiation is not primarily due to inhibition of CA activity. Decrease in PPARγ mRNA and enhanced C/EBPβ mRNA levels by ETZ seemed to be involved in decreased fat storage. ETZ also exerted inhibitory effect when added from day 3 to day 5 after differentiation induction by TRG and DEX for 2 days. Since clonal expansion is completed by day 2 in the TRG group (Fig. 6), inhibitory effect of ETZ in this case is not due to a delay in clonal expansion, but due to inhibition of fat storage in differentiated adipocytes. CAIII expression and fat store became apparent on day 4 in the TRG group. Twenty micromolars ETZ, 20-fold higher than a concentration to inhibit CA activity, did not block fat storage or repress C/EBPβ or PPARγ mRNA levels. CAIII is a major protein of fat cells and has been considered as a marker for differentiated adipocytes [15]. Although CAIII expression is reported to be repressed by insulin [38], significant amounts were found in cells of the control group that contained few fat droplets (Fig. 7). In ETZ-treated cells significant fat storage was detected, but CAIII expression was completely repressed. Thus, its expression is not proportional to the degree of fat accumulation in this case.

C/EBPβ and C/EBPα expression is known to be transiently increased at an early step in adipocyte differentiation [33], potentiating the expression of PPARγ, a key transcription factor for adipocyte-specific genes [7–10,35,39], rapidly upregulated after induction of differentiation [40,41]. TRG treatment resulted in increased *C/EBPβ* and *PPARγ* expression while ETZ repressed TRG-induced *PPARγ* expression (Fig. 9). Treatment with ETZ alone induced *C/EBPβ* expression but its expression did not lead to enhanced *PPARγ* expression. Phosphorylation of C/EBPβ is known to promote its transcriptional activity [42,43], while phosphorylation of PPARγ by mitogen-activated protein kinase blocks its function [44,45]. Among possible mechanisms for inverse relationship between C/EBPβ and PPARγ mRNAs in terms of ETZ influence, modulation of phosphorylation or dephosphorylation of these transcription factors may deserve consideration since ETZ inhibits phosphatase activity of CAIII [13]. As described above, CAIII does not seem a target of ETZ and phosphatases to be modulated are not identified yet. In addition to the full-length C/EBPβ protein, there is a shorter form, which lacks a DNA-binding domain and acts as an inhibitor of transcription [46]. The appearance of

these two forms is demonstrated to be due to differential use of two translation initiation sites within the same transcript. The question of which of the two is mainly expressed by ETZ treatment remains to be clarified. *C/EBPβ* gene knockout mice showed hypoglycemia and impaired hepatic glucose production [47], suggesting that the encoding product is responsible for the expression of enzymes involved in glucose production. PPARγ promotes fat storage in adipocytes by facilitating synthesis of triacylglycerol from glucose through pathways for glycolysis and fatty acid synthesis [48].

In conclusion, the present study indicates that inhibitory effect of ETZ on adipocyte differentiation is not due to inhibition of CA activity but rather due to altered levels of PPARγ and C/EBPβ mRNA.

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