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Control of Life Cycle of Mouse Adipogenic 3T3-L1 Cells by Dietary Lipids and Metabolic Factors

Kohji Nishimura,*[,1] Yoko Hatano,[1] Tsutomu Setoyama,[1] Hirohumi Tsumagari,[1] Kazuo Miyashita,[2] Shan Lu,[1] Mitsuo Jisaka,[1] Tsutomu Nagaya,[1] and Kazushige Yokota[1]

[1]Department of Life Science and Biotechnology, Shimane University, Matsue, Shimane 690-8504, Japan, E-mail: knishimu@life.shimane-u.ac.jp; and [2]Division of Marine Bioscience, Graduate School of Fisheries Science, Hokkaido University, Hakodate 041-8611, Japan

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Abstract

Adipocytes function not only as in the storage and mobilization of lipids but also as endocrine cells by secreting tumor necrosis factor-α (TNF-α), free fatty acids, and other cytokines. To study the effects of dietary lipids and metabolic factors on the control of the life cycle of adipocytes, we utilized mouse 3T3-L1 preadipocytes that could be induced to differentiate into adipocytes. To evaluate the role of endogenous prostaglandins (PGs) in the adipogenic changes, we examined the effect of specific inhibitors of cyclooxygenase (COX). SC-560, a specific COX-1 inhibitor, suppressed adipogenesis dose dependently, suggesting a role of constitutive COX-1 in the endogenous synthesis of PGs, including PGJ_2 derivatives formed by mature adipocytes with the ability to promote adipogenesis. NS-398, a COX-2 inhibitor, had little influence on the maturation processes. Both COX inhibitors were effective in stimulating apoptosis of preadipocytes induced by TNF-α, indicating that both PGE_2 and $PGF_{2\alpha}$ produced by preadipocytes through the action of both COX isoforms serve as survival factors. However, the effect of both inhibitors was negligible for the proliferation of preadipocytes. Moreover, conjugated linolenic acid from bitter gourd at lower concentrations that was without effects by itself synergistically stimulated TNF-α-induced apoptosis. Therefore, dietary lipid factors are capable of controlling the life cycle of adipocytes together with metabolic factors.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Adipocyte; apoptosis; arachidonate cascade; conjugated linolenic acid; cyclooxygenase; lipoxygenase; obesity; peroxisome proliferator-activated receptor γ.

Introduction

Arachidonic acid is an essential fatty acid that can be derived from exogenous dietary lipids. In mammalian cells, arachidonic acid can be converted into eicosanoids, potent bioactive molecules, by the biosynthetic pathways called the arachidonate cascade including cyclooxygenase (COX) and lipoxygenase (LOX) pathways *(1,2)*. The arachidonate cascade is involved in various biologic events such as cell proliferation, differentiation, T-cell differentiation, inflammation, and apoptosis *(3–6)*. Concerning differentiation processes, the arachidonate cascade is known to participate in various types of cells. For example, during oocyte maturation in cattle, the expression of COX-2 was found to markedly increase with the elevated production of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ *(7)*. Alternatively, the expression of COX-2 has been shown to contribute to the production of thromboxane A_2 during dimethyl sulfoxide (DMSO)–induced granulocytic differentiation in HL-60 cells *(8)*. Moreover, both COX and LOX metabolites are suggested to function not only as cell survival factors but also as apoptosis inducers in various types of cells *(9–11)*. For example, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15 d-PGJ_2) has been shown to induce apoptosis in the human breast cancer cell line, MCF-7 *(12)*. By contrast, specific COX inhibitors were reported to stimulate apoptosis of HT-29, cultured human colon carcinoma cells, indicating the involvement of COX metabolites in cell survival *(13)*. As for the LOX pathways, their products have also been shown to play contrasting roles in the control of cell survival and apoptosis *(14,15)*.

Obesity is a risk factor for severe diseases including noninsulin-dependent diabetes, atherosclerosis, and certain cancers *(16,17)*. The biologic events leading to obesity include changes in the cell properties of adipocytes characterized by an increase in the number or size, or both *(18)*. Adipocytes play a critical role in the storage and mobilization of lipids. Moreover, recent advances have shown that adipocytes can function as endocrine cells by secreting TNF-α, free fatty acids, and other cytokines *(19)*.

The life cycle of adipocytes includes cell proliferation, induced differentiation, maturation, cell death, and changes in insulin sensitivity. Some kinds of eicosanoids are known as inducers of adipocyte differentiation in cultured cells. For example, recent studies provided evidence that exogenous 15-d-PGJ_2 *(20)* and 8-HETE *(21)* have potent activities that induce adipogenesis in NIH-3T3 cells by the forced expression of peroxisome proliferator-activated receptors (PPARγ). More recently, 15-d-PGJ_2 was shown to bind directly to PPARγ *(22)*. In addition, a PPAR-response element was identified in the regulatory gene of COX-2 mRNA *(23)*. Moreover, a recent study provided evidence that COX-2 might be involved in body fat regulation *(24)*. Heterozygous COX-2-deficient mice revealed

increased body weight and pad weight compared with those from wild-type and COX-1-deficient mice. Although such PGs are likely to be involved in the regulation of adipogenesis, the roles of endogenous PGs in the life cycle of adipocytes still seem controversial.

To determine the effects of dietary lipids and metabolic factors on the control of the life cycle of adipocytes, we utilized mouse 3T3-L1 preadipocytes that could be induced to differentiate into adipocytes after exposure to a standard mixture of dexamethasone, 1-methyl-3-isobutylxanthine, and insulin *(25)*. Mouse preadipogenic 3T3-L1 cells produced several types of PGs during adipogenesis *(26)*. Exogenous 15-d-PGJ_2 was shown to induce adipocyte differentiation in certain cultured adipocytes *(22)*. On the other hand, $PGF_{2\alpha}$ had the effect of inhibiting adipogenesis of 3T3-L1 cells *(27)*.

Conjugated linoleic acid (CLA) has been reported to have beneficial and biologic effects of suppressing obesity and tumorigenesis. Tsuboyama-Kasaoka et al. *(28)* showed that CLA was more effective in reducing body weight as well as in promoting apoptosis of adipose tissues in mice fed high-fat diets. CLA has also been shown to induce apoptosis mainly in cancer cells *(29)*. Although CLA has been implicated in the activation of PPARs as an endogenous ligand *(30)*, the involvement of CLA in the regulation of the arachidonate cascade in adipocytes has only recently been clarified. Suzuki et al. *(31)* identified the high content of a unique conjugated linolenic acid (CLN) in the flesh of bitter gourd. Fatty acids from some kinds of seed oils containing CLNs were reported to have cytotoxity against human monocytic leukemia cells. The mechanism to show this cytotoxity remains unclear. One possible mechanism may involve lipid peroxidation *(32)*. In terms of the potential use of food therapy in regulating obesity, we tested the effect of CLN from bitter gourd on mouse preadipogenic 3T3-L1 cells.

The present study was undertaken to evaluate the role of the arachidonate cascade in the different stages of the life cycle of mouse adipogenic 3T3-L1 cells by the use of specific COX inhibitors. Furthermore, we investigated the control of the life cycle of adipocytes by CLN from bitter gourd as a dietary factor in terms of the potential use of CLN from bittergourd for antiobese functions.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium with 25 m*M* HEPES (DMEM-HEPES), glutaraldehyde, and Hoechst 33342 were obtained from Sigma (St. Louis, MO). Aspirin, pronase from *Streptomyces griseus*, and ascorbic acid were purchased from Wako (Osaka, Japan). Fetal calf serum (FCS) was supplied by Biological Industries (Kibbutz Beth Haemek, Israel). Acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (DEVD-pNa) was obtained from Cosmo-Bio (Tokyo, Japan), and a Cell Counting Kit-8 was from Dojindo (Kumamoto,

Japan). Authentic free fatty acids, SC-560, NS-398, and PGs were purchased from Cayman (Ann Arbor, MI). 9c,11t,13t-Octadecatrienoic acid (9c,11t,13t-18:3) (CLN) was extracted and purified from seeds of bitter gourd as described previously *(31)*. This CLN is included as the major component of seeds of bitter gourd at a level of 60%. All other chemicals were of reagent or tissue culture grade.

Cell Culture

A mouse preadipogenic 3T3-L1 (JCRB9014) cell line was obtained from Japanese Cancer's Research Resources Bank (JCRB) (Tokyo, Japan). 3T3-L1 cells were grown in a growth medium of DMEM-HEPES supplemented with 10% FCS and 200 μ*M* ascorbic acid at 37°C under 7% CO_2. For determination of cell proliferation, 3T3-L1 cells were grown in the growth medium. When the cells were grown to about 80% confluence, they were plated at a concentration of 5×10^3 cells/well in a 96-well microplate and cultured in 50 μL of growth medium in each well for 24 h. Then, the culture was mixed with 50 μL of twofold concentrated solution of COX inhibitors in the culture medium. Inhibitors were added to give the desired final concentrations using DMSO as a vehicle. The final concentration of DMSO was <1%. After 24 h, the cells were analyzed for the cell proliferation assay as described in the next section. For adipocyte differentiation, 3T3-L1 cells were grown to confluence in growth medium in a 60-mm dish, and then the cells were further incubated for 45 h. The culture medium was replaced with differentiation medium consisting of the growth medium supplemented with 1 μ*M* dexamethasone, 0.5 m*M* 1-methyl-3-isobutylxanthine, and 10 μg/mL of insulin, and the cells were incubated for 42 h. Then, the culture medium was replaced with the maturation medium prepared from the growth medium with 5 μg/mL of insulin every 2 d for a total of 6 d. For estimation of the effect of COX inhibitors on adipogenesis, SC-560 was added to both induction and maturation media at a concentration of either 0.1, 1, or 10 μ*M*. To determine the effect of COX inhibitors or CLN on TNF-α-induced apoptosis, 3T3-L1 cells were grown until 70–80% confluence in a 100-mm dish containing 10 mL of growth medium. 3T3-L1 cells were pretreated for 1 h with 10 μ*M* SC-560 or 10 μ*M* NS-398 before being stimulated with 40 ng/mL of TNF-α for 24 h. In the case of CLN, the culture medium was replaced with fresh growth medium containing 10 μ*M* vitamin E to prevent autooxidation of CLN. The cultured cells were also pretreated for 1 h with 30 μ*M* CLN from bitter gourd before being stimulated with 40 ng/mL of TNF-α for 24 h. After 3T3-L1 cells were treated with various reagents for the indicated periods, cell viability, adipocyte differentiation, and apoptosis were analyzed as described subsequently.

Determination of Cell Proliferation

Quantification of cell proliferation was performed according to the WST-8 assay on the basis of 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT) assay *(33)*. 3T3-L1 cells were grown as described in the previous section and then mixed with 10 µL of the Cell Counting Kit-8 solution, and the plate was incubated at 37°C for a further 4 h. Cell proliferation was determined by monitoring the increase in the absorbance of the product 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophynyl)-2H-tetrazolium at 450 nm and was represented as the percentage of viability obtained in control cultures without treatment of COX inhibitors.

Assay of Glycerol-3-Phosphate Dehydrogenase Activity

Cellular enzymatic activity of glycerol-3-phosphate dehydrogenase (GPDH) was used as an index of adipocyte differentiation. 3T3-L1 cells were grown as described in Cell Culture, and then GPDH activity was determined according to the method of Wise and Green *(34)*.

Oil Red O Staining

The accumulation of lipid droplets in 3T3-L1 adipocytes was visualized by staining with Oil Red O *(35)*.

Caspase Assay

Caspase assay was done essentially according to the modified method of a CaspACE™ Assay System kit (Promega, Madison, WI). DEVD-pNa was used for measurement of caspase-3 activity. After treatment with or without apoptotic inducers in 60-mm dishes, cells were scraped with a rubber policeman and harvested by centrifuging at 10,000*g* for 10 min at 4°C. The collected cells were rinsed with ice-cold phosphate-buffered saline (–) and suspended in 200 µL of lysis buffer containing 10 m*M* Tris-HCl buffer (pH 7.4) with 150 m*M* NaCl and 1% (w/v) Triton X-100. After the suspension was kept on ice for 10 min, the supernatant was obtained by centrifugation and subjected to caspase assay. The enzyme solution (20 µL) was mixed with an equal volume of reaction buffer consisting of 200 m*M* HEPES buffer (pH 7.4), 20% sucrose, 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 20 m*M* dithiothreitol, 4% DMSO, and 0.4 m*M* DEVD-pNa. After incubating at 37°C for 2 h, the reaction mixture was diluted fivefold with distilled water. The caspase activity was determined by monitoring the increase in the absorbance of the reaction product pNa at 405 nm from DEVD-pNa as a substrate. Caspase activity was expressed as $\Delta(A_{405} - A_{660})/2$ h/mg of protein and represented as the percentage of the maximum value.

Nuclear Condensation Analysis

Nuclear condensation was used as an index of apoptosis *(36)*. Observation of nuclear condensation was performed as described previously by Nishimura et al. *(37)*.

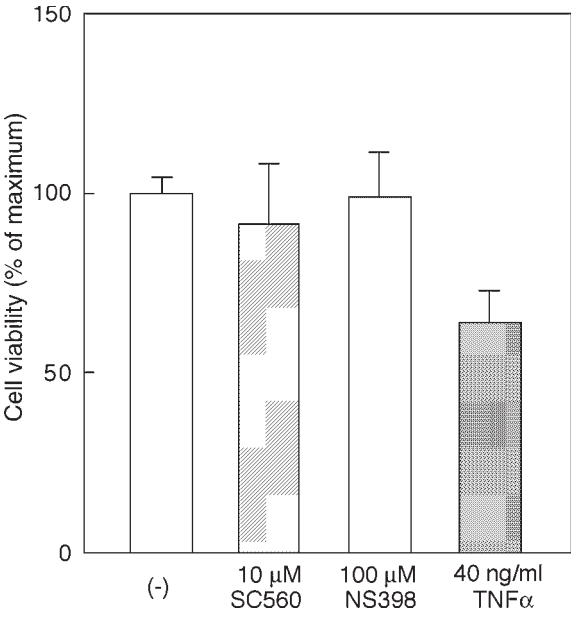

Fig. 1. Effect of inhibitors of COX-1 and COX-2 on cell proliferation of mouse 3T3-L1 preadipocytes. The cells were plated at 5×10^3 cells/mL in a 96-well microplate containing growth medium supplemented with 10% FCS. The cells were preincubated for 24 h before being stimulated with either 10 μ*M* SC-560, 100 μ*M* NS-398, or 40 ng/mL of TNF-α for a further 24 h. Cell viability was determined as described in Materials and Methods. Values are the results from at least three independent experiments. Data represent the mean ± SD.

Results and Discussion

Effect of COX Inhibitors on Proliferation of Mouse Preadipogenic 3T3-L1 Cells

We examined the effect of COX inhibitors on proliferation of 3T3-L1 preadipocytes. As shown in Fig. 1, the exposure of cultured cells to 10 μ*M* SC-560 for 24 h as well as 100 μ*M* NS-398 had little effect on cell proliferation, which was in contrast to the decrease with 40 ng/mL of TNF-α. Therefore, both COX enzymatic activities would not be critical for cell proliferation in mouse preadipogenic 3T3-L1 cells.

Attenuation of Adipogenesis by COX-1 Inhibitor in 3T3-L1 Adipocytes

We recently reported that the gene expression of COX-2 isoform was enhanced transiently during the induction of adipocyte differentiation or maturation process, whereas the COX-1 gene was constitutively expressed *(38)*. These findings implicated the involvement of COX metabolites in the maturation process of adipocytes. Therefore, we investigated the effects of COX inhibitors on adipocyte differentiation. The confluent cells were refed

with maturation medium including the COX-1 inhibitor SC-560 every 2 d until 4 d following 2 d of treatment with differentiation medium with the same inhibitor (Fig. 2A,B). SC-560 decreased cellular GPDH activity in a dose-dependent manner. In particular, culture with 10 μ*M* SC-560 significantly reduced GPDH activity to a level of 70% of that without the inhibitor. We confirmed the inhibitory effect of 0.1 μ*M* SC-560 on the accumulation of lipid droplets by differential interference microscopy (Fig. 2C). As shown in Fig. 2B, the COX-2 inhibitor NS-398 slightly reduced GPDH activity at 1 μ*M*, whereas NS-398 at a higher concentration of 10 μ*M* rather stimulated GPDH activity. Therefore, NS-398 appeared to have totally little effects on the enzymatic activity of GPDH. Since NS-398 at higher concentrations was reported to bind and activate PPARγ *(39)*, the stimulatory effect of NS-398 seems to be caused by the activation of PPARγ. Thus, constitutive COX-1 activity is mainly responsible for stimulation of adipocyte differentiation. Further studies of the effect of COX-2 will be required.

Alternatively, gene expression of the biosynthetic enzyme for PGD_2 was recently shown to increase during adipocyte differentiation of 3T3-L1 cells *(38)*, suggesting the involvement of PGD_2 in adipocyte differentiation. Moreover, the synthesis of PGE_2 occurred during the changes in the life cycle of adipocytes. Taken together, PGD_2 and the related PGJ_2 as well as PGE_2 would be responsible for maintenance of the maturation process.

Recently, the treatment of 3T3-L1 cells with either SC-560 or NS-398 was reported to enhance slightly adipocyte differentiation as monitored by GPDH activity *(40)*. The result of this report appeared to be different from ours. However, we cannot make a direct comparison because the experimental conditions were different in terms of the period of treatment with COX inhibitors, medium components, and concentrations of inhibitors. More recently, we obtained evidence that COX metabolites such as PGD_2 and PGJ_2 derivatives produced by mature adipocytes can promote adipocyte differentiation (data not shown).

Induction of Apoptosis by TNF-α and Further Stimulation With COX Inhibitors in 3T3-L1 Preadipocytes

TNF-α has been reported to induce apoptosis as well as block adipocyte differentiation, depending on the stages of the life cycle *(41)*. Exposure of 3T3-L1 cells to TNF-α suppressed the expression of adipogenesis-related genes such as PPARγ and aP2 during adipocyte differentiation *(42)*. Moreover, TNF-α has been implicated to induce apoptosis in human adipocytes *(43)*. To determine whether the COX pathway plays a significant role in TNF-α-induced apoptosis, we examined the effect of COX inhibitors on this type of apoptosis. First, we investigated the time course and dose dependence of TNF-α-induced apoptosis in 3T3-L1 preadipocytes. As shown in Fig. 3A, the stimulatory effect of TNF-α was evident at a concentration of 40 ng/mL in the presence of 10% FCS. Incubation for 24 h was found to be more effective than 48 h in detecting the potency of TNF-α. As shown in Fig. 3B, caspase-3 activity was stimulated with TNF-α

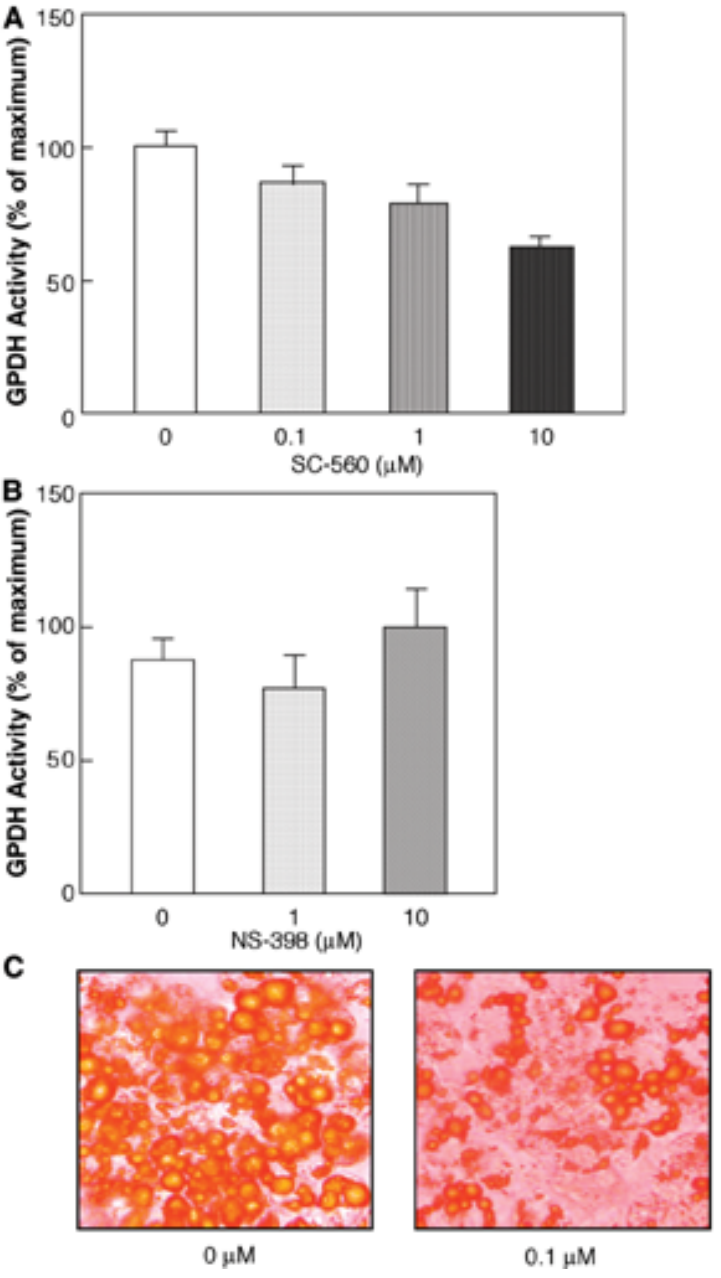

Fig. 2. Effect of SC-560 on differentiation of 3T3-L1 adipocytes. 3T3-L1 cells were plated and grown in growth medium until reaching 70–80% confluence. The resulting cells were cultured for 45 h in differentiation medium containing an increasing concentration of either SC-560 or NS-398. In addition, the culture medium was replaced with the maturation medium containing the indicated inhibitor every 2 d until 4 d. The cells were harvested in order to determine GPDH activity, and for Oil Red O staining. **(A)** Effect of SC-560 on GPDH activity. **(B)** Effect of NS-398 on GPDH activity. Values of GPDH activities are the results from at least three independent experiments. Data represent the mean ± SD. **(C)** Microscopic images of 3T3-L1 adipocytes using a differential interference contrast microscope (×100 magnification). The cells were stained with Oil Red O. The cells containing lipid droplets are visualized as orange cells.

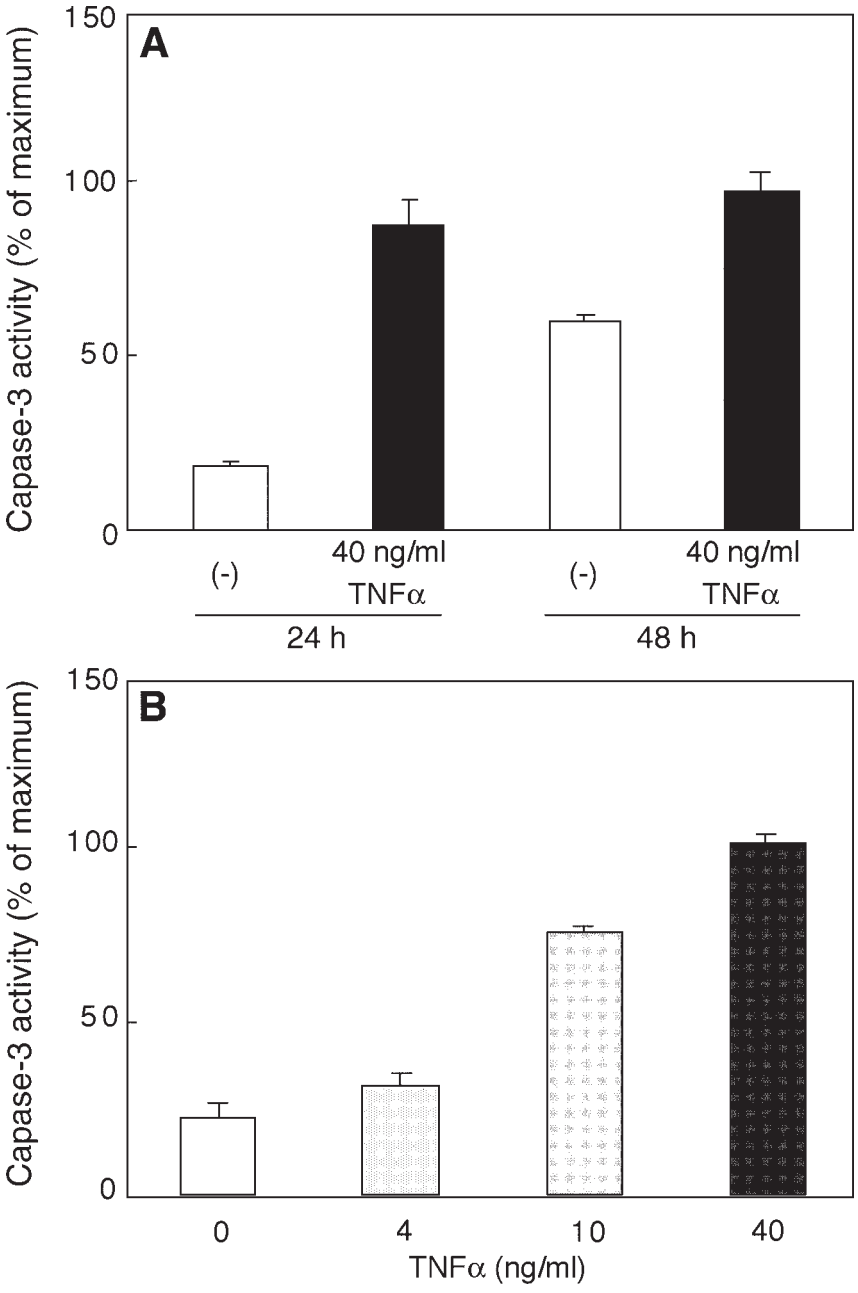

Fig. 3. Induction of apoptosis by TNF-α in 3T3-L1 preadipocytes. 3T3-L1 cells were grown to 70–80% confluence in growth medium, and then the culture medium was replaced with fresh growth medium supplemented with TNF-α. Cells were harvested to determine caspase-3 activity. **(A)** Time course for TNF-α-induced apoptosis. The cells were treated with 40 ng/mL of TNF-α for 24 or 48 h. **(B)** Dose dependence of apoptosis on TNF-α. The cells were treated with the indicated concentration of TNF-α for 24 h. Values are the results from at least three independent experiments. Data represent the mean ± SD.

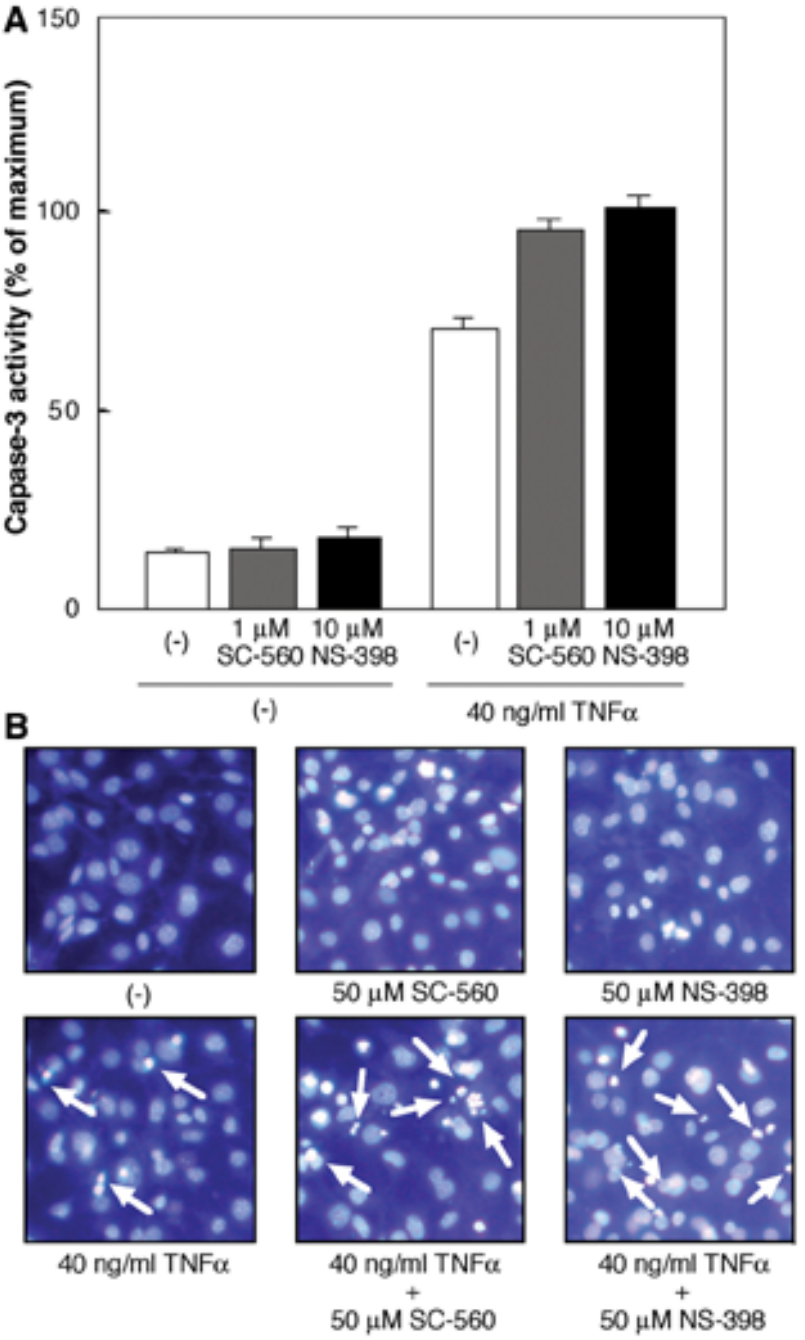

Fig. 4. Effects of COX inhibitors on TNF-α-induced apoptosis in mouse preadipogenic 3T3-L1 cells. 3T3-L1 cells were grown to 70–80% confluence in growth medium, and then the culture medium was replaced with fresh growth medium. 3T3-L1 cells were pretreated for 1 h with SC-560 or NS-398, followed by incubation for 24 h with 40 ng/mL of TNF-α. **(A)** Stimulation of TNF-α-induced apoptosis by COX inhibitors in 3T3-L1 cells. 3T3-L1 cells were harvested to determine caspase-3 activity. Values are the results from at least three independent experiments. Data represent the mean ± SD. **(B)** Fluorescence microscopic images of nuclear condensation (×100 magnification). The cells were stained with Hoechst 33342. Arrows indicate typical apoptotic cells.

in a dose-dependent manner. In sharp contrast, neither the activation of caspase-3 activities nor chromatin condensation was found with a higher concentration of TNF-α (100 ng/mL) and serum depletion in mature 3T3-L1 adipocytes (data not shown), indicating resistance against the action of TNF-α in the mature adipocytes. In agreement with this finding, the resistance of mature adipocytes has been reported elsewhere *(44)*. One conceivable explanation for the mechanism of resistance against TNF-α-induced apoptosis in 3T3-L1 adipocytes is the decrease in deoxyribonucleic activity and the increased levels of antiapoptotic protein, Bcl-2, in mature adipocytes, compared with 3T3-L1 preadipocytes *(44)*.

Second, we examined the effects of COX inhibitors on TNF-α-induced apoptosis in 3T3-L1 preadipocytes. As shown in Fig. 4A, treatment of the cells with 1 μ*M* SC-560 or 10 μ*M* NS-398 for 25 h enhanced caspase-3 activity by 1.5-fold. The chromatin condensation induced by TNF-α was also synergistically stimulated with both SC-560 and NS-398 (Fig. 4B), suggesting that prostanoids generated from 3T3-L1 preadipocytes by the action of both COX-1 and COX-2 isoforms might contribute to inhibition of TNF-α-induced apoptosis. Since PGE_2 and $PGF_{2\alpha}$ are produced in mouse 3T3-L1 preadipocytes during cell proliferation *(26)*, these COX metabolites should function as cell survival factors in mouse 3T3-L1 preadipocytes.

Induction of Apoptosis by a Variety of Dietary Unsaturated Fatty Acids in 3T3-L1 Preadipocytes

Because CLN from some seed oils was found to attenuate cell proliferation in some types of tumor cells *(32)*, such conjugated fatty acids were expected to promote apoptosis in adipocytes. In terms of the potential use of food therapy to regulate obesity, we tested the effect of CLN from bitter gourd on the enzymatic activity of caspase-3, a downstream protease in the apoptotic pathway, in preadipogenic 3T3-L1 cells. As shown in Fig. 5, exposure of cells to CLN alone at a concentration of 0–30 μ*M* CLN for 24 h had little effect on caspase-3 activity, but CLN alone at a higher concentration of 100 μ*M* enhanced caspase-3 activity. To elucidate the structural specificity of CLN in the activation of caspase-3 activity, we investigated the effect of structurally related C18 polyunsaturated fatty acids on caspase-3 activity. As shown in Fig. 6A, 9c,11t,13t-CLN at 100 μ*M* potently enhanced caspase-3 activity. A similar potent effect was seen with oleic acid. This apoptosis was confirmed by monitoring the increased chromatin condensation (Fig. 6B). Different from other fatty acids, linoleic acid had the negligible capability of inducing apoptosis (Fig. 6A,B). Linoleic acid can be converted into arachidonic acid and further metabolized by a series of enzymes in the arachidonate cascade. Hence, eicosanoids derived from linoleic acid would serve as survival factors in 3T3-L1 preadipocytes.

Because CLN from pomegranate was also reported to induce cell death in human monocytic leukemia U937 cells through oxidative stress *(32)*, CLN-induced apoptosis might also be mediated by reactive oxygen species.

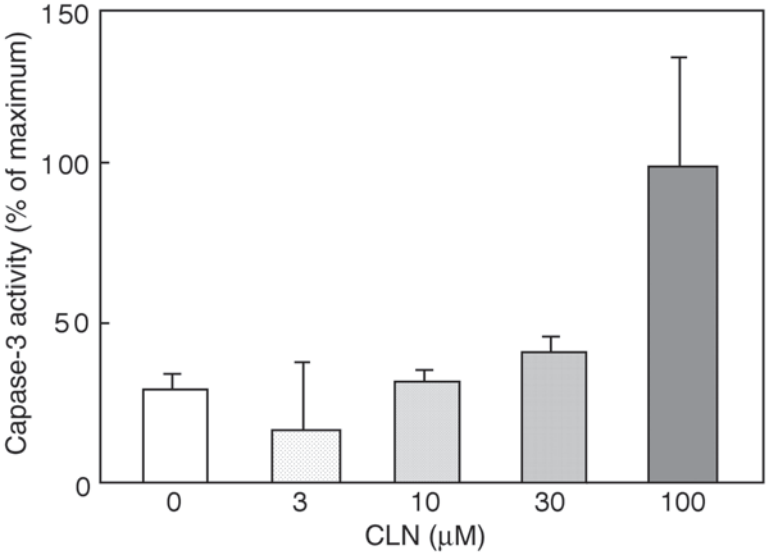

Fig. 5. Effect of CLN from bitter gourd on 3T3-L1 preadipocytes. The cells were grown to 70–80% confluence in growth medium, and then the culture medium was replaced with fresh growth medium. The cells were treated with an increasing concentration of CLN for 24 h. Then, the cells were harvested to determine caspase-3 activity. Data represent the mean ± SD.

Further investigation is needed to understand the molecular regulation of CLN-induced apoptosis.

Synergistic Stimulation of TNF-α-Induced Apoptosis by CLN in Mouse Preadipogenic 3T3-L1 Cells

As shown in Fig. 3, exposure of 3T3-L1 preadipocytes to 40 ng/mL of TNF-α effectively stimulated the induction of apoptosis. To determine whether dietary lipids modulate TNF-α-induced apoptosis in 3T3-L1 preadipocytes, we examined the effects of CLN on this type of apoptosis induced by TNF-α. As shown in Fig. 7A, treatment of cells with a lower concentration of 30 μ*M* CLN alone caused almost no apoptosis. However, caspase-3 activity induced by TNF-α was synergistically stimulated in the presence of 30 μ*M* CLN. Enhanced chromatin condensation was confirmed when cells were exposed to a mixture of TNF-α and CLN (Fig. 7B). Although CLA is known to inhibit proliferation of 3T3-L1 preadipocytes *(45)*, CLN had the additional effect of stimulating apoptosis. Because CLA was shown to bind PPARγ *(30)*, the activation of PPARγ by CLN might be considered for the stimulation of TNF-α-induced apoptosis by CLN in 3T3-L1 preadipocytes. However, the level of PPARγ mRNA at the proliferation phase is known to be substantially lower than at the maturation phase. Hence, the involvement of the action of PPARγ remains unclear. We are in the process of studying the involvement of eicosanoids in CLN-induced apoptosis in 3T3-L1 cells.

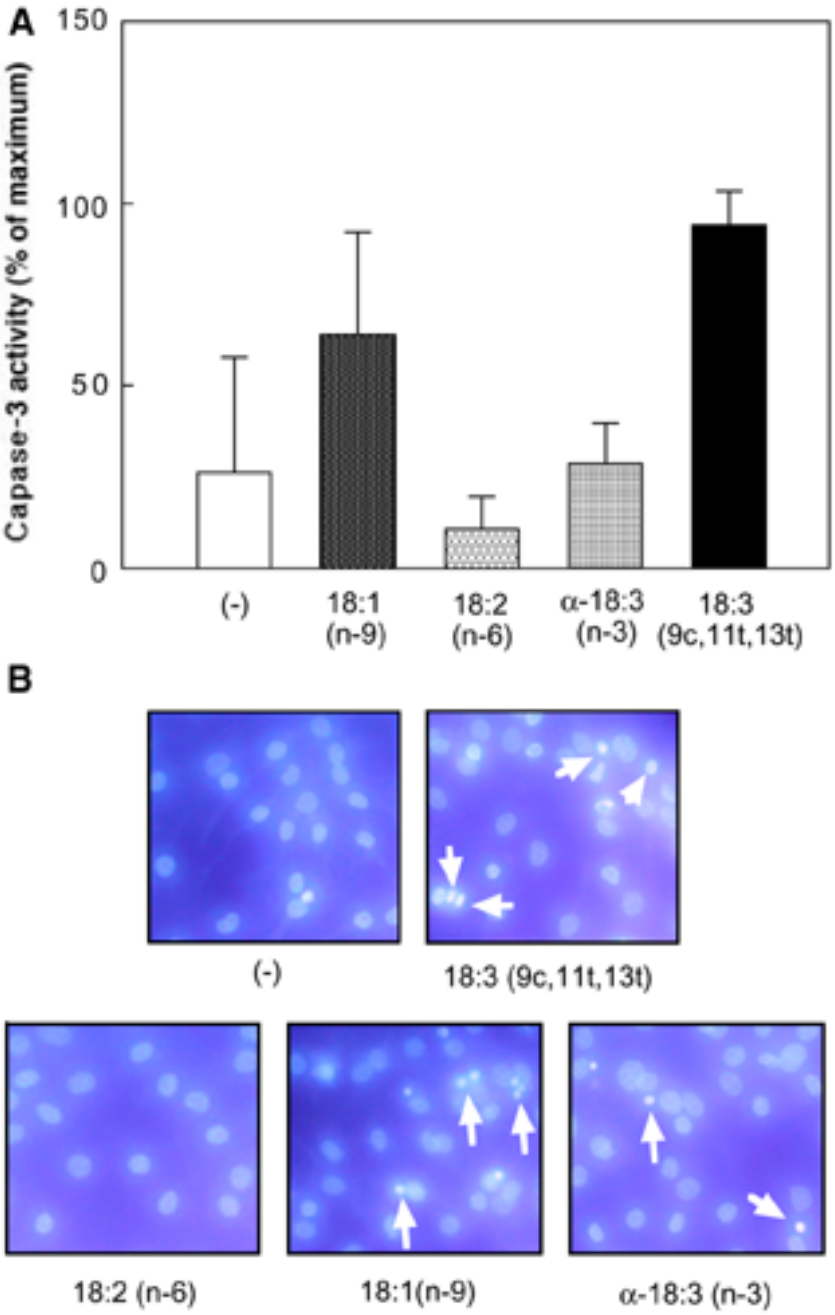

Fig. 6. Effect of various 18-carbon unsaturated fatty acids on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were grown to 70–80% confluence in growth medium, and then the culture medium was replaced with medium containing DMEM-HEPES supplemented with 2% FCS treated with charcoal, and with 200 μ*M* ascorbic acid and 10 μ*M* vitamin E. The cells were treated with various types of unsaturated octadecanoic acids at 100 μ*M* for 24 h. **(A)** Stimulation of caspase-3 activity by oleic acid and CLN in 3T3-L1 preadipocytes. The cells were harvested for determination of caspase activity. Data represent the mean ± SD. **(B)** Fluorescence microscopic images of nuclear condensation (×100 magnification). The cells were stained with Hoechst 33342. Arrows indicate typical apoptotic cells.

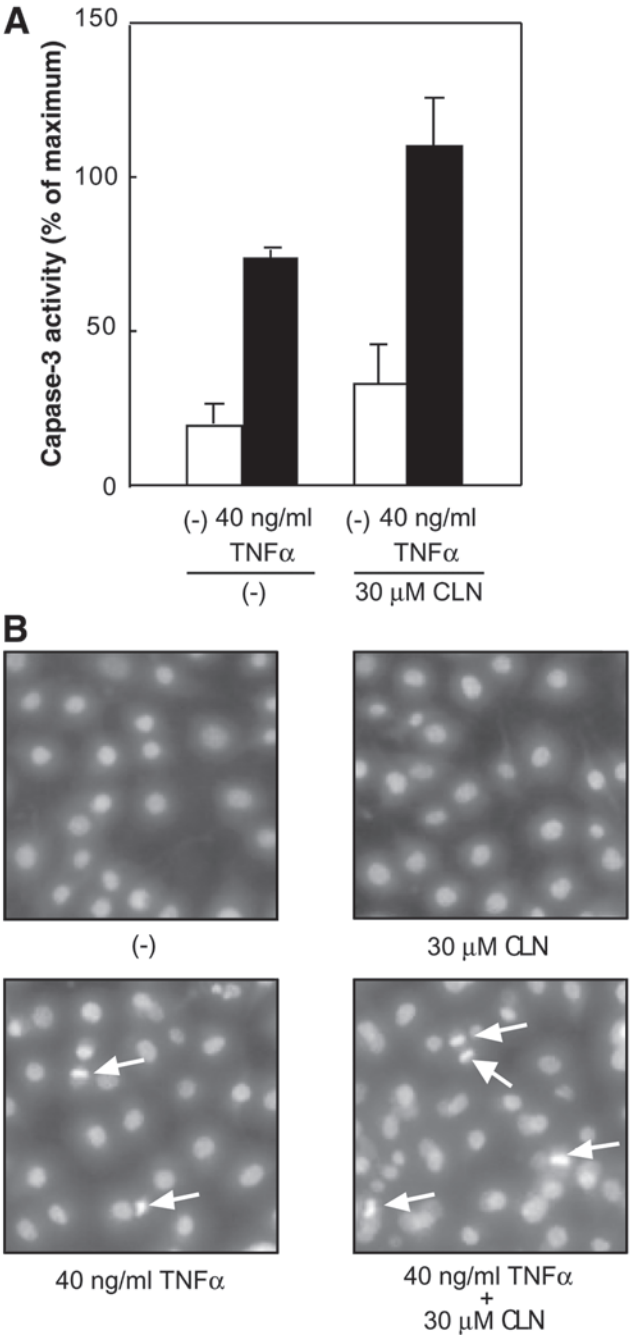

Fig. 7. Effect of CLN from bitter gourd on TNF-α-induced apoptosis in 3T3-L1 preadipocytes. 3T3-L1 cells were grown to 70–80% confluence in growth medium, and then the culture medium was replaced with growth medium including 10 μ*M* vitamin E. 3T3-L1 cells were pretreated for 1 h with 30 μ*M* CLN, followed by incubation for 24 h with 40 ng/mL of TNF-α. **(A)** Stimulation of TNF-α-induced caspase-3 activity by COX inhibitors in 3T3-L1 cells. The 3T3-L1 cells were harvested for determination of caspase-3 activity. Data represent the mean ± SD. **(B)** Fluorescence microscopic images of nuclear condensation (×100 magnification). The cells were stained with Hoechst 33342. Arrows indicate typical apoptotic cells.

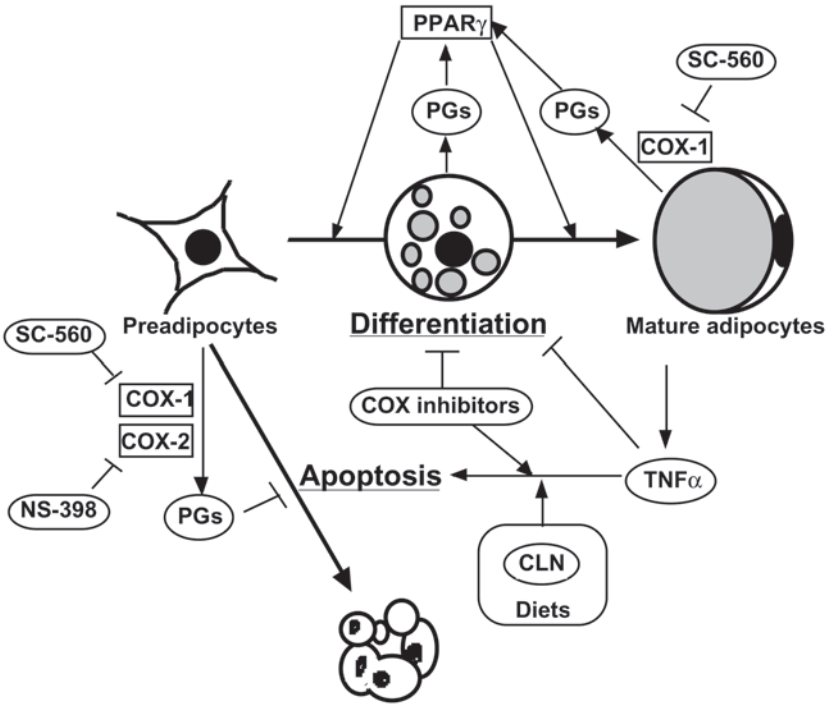

Fig. 8. Schematic representation of possible action of COX inhibitors and CLN on life cycle of adipogenic 3T3-L1 cells. Thick arrows indicate the processes of changes in the life cycle. The COX-1 inhibitor SC-560 blocked adipogenesis in mature 3T3-L1 adipocytes, as shown by a T-head line, indicating that prostanoids including PGD_2 and PGJ_2 derivatives secreted by mature adipocytes through the action of COX-1 might promote adipocyte differentiation through the activation of PPARγ. On the other hand, both COX-1 and COX-2 inhibitors stimulated apoptotic cell death induced by TNF-α, as shown by a line with arrowhead. Thus, prostanoids such as PGE_2 and $PGF_{2\alpha}$ produced by preadipocytes through the action of both COX-1 and COX-2 protected the cells from apoptosis induced by TNF-α. Alternatively, CLN synergistically stimulated TNF-α-induced apoptosis. In animal adipose tissues, CLN might stimulate the secretion of TNF-α in mature adipocytes, which causes the stimulation of apoptosis in preadipocytes. Thus, the roles of endogenous eicosanoids in the control of the life cycle of adipocytes should be multifunctional and different depending on the individual stages of the life cycle of adipocytes.

Postulated Action of Prostanoids and CLN in Life Cycle of 3T3-L1 Adipocytes

Figure 8 illustrates the postulated action of PGs and CLN in 3T3-L1 cells. Based on the described results, we propose the distinct roles of prostanoids in adipogenesis and apoptosis in 3T3-L1 cells.

With respect to adipocyte differentiation, the COX-1 inhibitor SC-560 attenuated adipogenesis in 3T3-L1 adipocytes from the differentiation phase to the maturation phase, suggesting the predominant action of COX-1 in the stimulation of adipogenesis (Fig. 2). Thus, the constitutive COX-1 is

involved in the basal synthesis of PGD_2 and PGJ_2 derivatives in mature adipocytes even though the action of COX-2 cannot be excluded completely. In support of this, we have just recently confirmed the stimulation of adipogenesis by PGD_2 and PGJ_2 derivatives (data not shown).

As for apoptotic cell death, both COX-1 and COX-2 inhibitors were effective in synergistically stimulating apoptosis induced by TNF-α in 3T3-L1 preadipocytes, as shown in Fig. 4, indicating the involvement of both COX-1 and COX-2 activities in inhibiting this type of apoptosis. Hence, PGE_2 and $PGF_{2\alpha}$ produced by 3T3-L1 preadipocytes can serve as the survival factors. In a more recent study, we confirmed the action of exogenous PGE_2 and $PGF_{2\alpha}$ to attenuate apoptotic cell death (data not shown). In addition, a recent report has shown that $PGF_{2\alpha}$ induced phosphorylation of PPARγ via mitogen-activated protein kinase *(46)*. Hence, phosphorylation of PPARγ might be required for the antiapoptotic effect of $PGF_{2\alpha}$. Similarly, we have recently reported that $PGF_{2\alpha}$ inhibited the promotion of apoptosis induced by a tumor promoter and nordihydroguaiatretic acid in Madin-Darby canine kidney cells *(37)*. However, it remains unclear whether the antiapoptotic effect of $PGF_{2\alpha}$ is ubiquitous in mammalian cells.

Apoptosis induced by TNF-α in 3T3-L1 preadipocytes was also synergistically stimulated with CLN (Fig. 7). Previously, CLA was reported to activate PPARγ *(30)*, and the agonist of PPARγ induced apoptosis in HT29 human colon cancer cells *(47)*. Therefore, it is possible that CLN caused the effect through the action of PPARγ. This seems unlikely, however, because of the lower expression of PPARγ in 3T3-L1 preadipocytes. Alternatively, CLN might promote the secretion of TNF-α from adipose tissues. However, the exact mechanism of the stimulatory effect of CLN remains unclear. Nonetheless, CLN has the potential for novel dietary control of the life cycle of adipocytes.

In conclusion, we found that the COX-1 inhibitor blocked adipocyte differentiation in mature adipocytes, whereas both COX-1 and COX-2 inhibitors stimulated TNF-α-induced apoptosis in preadipocytes. Therefore, endogenous PGD_2 and PGJ_2 derivatives formed by mature adipocytes stimulate adipogenesis. By contrast, PGE_2 and $PGF_{2\alpha}$ produced by preadipocytes contribute to the cell survival of preadipocytes themselves. Thus, the roles of PGs in preadipocytes and mature adipocytes depend differently on the phase of the life cycle of adipocytes. Since CLN, along with TNF-α, can decrease the cell number of preadipocytes, these might be promising for potential food therapy for the control of obesity. The role of COX and LOX pathways in apoptosis induced by these dietary factors or metabolic factors in 3T3-L1 cells is still under investigation.

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