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Regulation of Apoptosis Through Arachidonate Cascade in Mammalian Cells

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Abstract

The arachidonate cascade includes the cyclooxygenase (COX) pathway to form prostanoids and the lipoxygenase (LOX) pathway to generate several oxygenated fatty acids, collectively called eicosanoids. Eicosanoids are suggested to play a dual role in regulating cell survival and apoptosis in various types of cells through an unknown mechanism. We found apoptosis in cultured Madin-Darby canine kidney (MDCK) cells treated with 12-*O*-tetradecanoylphorbol β-acetate (TPA), a potent tumor promoter, and nordihydroguaiaretic acid (NDGA), a LOX inhibitor. The effect of TPA was synergistically stimulated along with NDGA. Aspirin, a COX inhibitor, was not effective. The target of NDGA might be different from the mechanism involving a LOX activity in some kinds of carcinoma cells because the increased expression of 12-LOX was not detected in MDCK cells treated with TPA. Caspase and poly(ADP-ribose) metabolites were found to be involved in the signal transduction pathway of the TPA- and NDGA-induced apoptosis in MDCK cells. Alternatively, hydrogen peroxide–induced apoptosis was not affected by NDGA. Thus, the TPA-induced response involved the mechanism independent of the oxidative stress. Obesity is a risk factor for severe diseases including noninsulin–dependent diabetes and atherosclerosis characterized by the changes of cell properties of adipocytes. We found that conjugated linolenic acid from bitter gourd was able to induce apoptosis in mouse preadipogenic 3T3-L1 cells. The findings provide the potential use of conjugated fatty acids to regulate obesity.

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Index Entries: Adipocyte; apoptosis; arachidonate cascade; conjugated linolenic acid; cyclooxygenase; lipoxygenase; Madin-Darby canine kidney; nordihydroguaiaretic acid; obesity; 12-*O*-tetradecanoylphorbol β-acetate.

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 potent bioactive molecules by two different pathways such as cyclooxygenase (COX) and lipoxygenase (LOX) pathways, collectively called arachidonate cascade *(1,2)*. The arachidonate metabolites are involved in various biologic events such as cell proliferation, differentiation, immune system, inflammation, and apoptosis *(3–7)*. In addition, both COX and LOX metabolites are suggested to play a dual role in regulating cell survival and apoptosis in various types of cells *(8–10)*. For example, cyclopentenone prostaglandins such as PGA_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2, a known prostanoid inducer of adipocyte differentiation *(11,12)*, have been shown to cause tumor cell apoptosis *(13)*. By contrast, a variety of COX inhibitors were reported to promote apoptosis of mammalian cultured cells, indicating the suppressive effect of the COX pathway *(14)*. Thus, the roles of the COX metabolites in cell survival and death remain controversial.

As for the LOX pathways, their products have also been shown to play contrasting roles in the control of cell survival and apoptosis *(15,16)*. 15-HPETE induced the apoptosis of human immunodeficiency virus–infected human T-cells owing to the action of lipid hydroperoxides *(17)*. In addition, tumor necrosis factor α-induced apoptosis of multiple cells was presumably mediated by LOX metabolites *(18)*, whereas nonspecific inhibitors of the LOX activity and 5- or 12-LOX-selective inhibitors have been shown to be effective in inducing cell death *(19,20)*, indicating the function of LOX metabolites in tumorigenic cell lines as a critical survival factor.

We have been employing cultured Madin-Darby canine kidney (MDCK) cells derived from tubular epithelium as a useful model system. The MDCK cells showed the increased production of prostanoids in response to 12-*O*-tetradecanoylphorbol β-acetate (TPA), a tumor-promoting phorbol diester, and calcium ionophere A23187. The effect of TPA required the induced *de novo* synthesis of COX *(21,22)*. The dietary regulation of the arachidonate cascade was also studied by modifying the cell membranes with dietary fatty acids *(23)*. Recently, TPA was reported to induce apoptosis in cultured MDCK cells *(24)*.

Obesity is a risk factor for the severe diseases including noninsulin-dependent diabetes, atherosclerosis, and certain cancers *(25,26)*. 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 *(27)*. We have begun to use cultured mouse preadipogenic 3T3-L1 cells as a model system to study the dietary regulation of obesity by food-derived factors for their ability to induce or repress apoptosis. The 3T3-L1 cells have

been useful because of the ability of adipocyte differentiation in response to a mixture of insulin, dexamethasone, and 1-methyl-3-isobutyl xanthine *(28)*. Recently, we found that some kinds of food-derived conjugated linolenic acid (CLN) from bitter gourd as well as conjugated linoleic acid (CLA) induced apoptosis in mouse preadipogenic 3T3-L1 cells. The effect of CLA has also been reported just recently *(29)*.

In the present study, we attempted to determine the involvement of the arachidonate cascade in the two types of apoptosis in mammalian cells such as the TPA-induced apoptosis in MDCK cells and the CLN-induced apoptosis in mouse preadipogenic 3T3-L1 cells.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium with 25 m*M* HEPES (DMEM-HEPES), TPA, nordihydroguaiaretic acid (NDGA), 3-aminobenzamide (3-AB), glutaraldehyde, and Hoechst 33342 were obtained from Sigma (St. Louis, MO). Aspirin, *n*-propyl gallate (*n*-PrG), tannic acid, *N*-acetyl cysteine (NAC), 2-mercaptoethanol (2-ME), Pronase (protease from *Streptomyces griseus*), and ascorbic acid were purchased from Wako (Osaka, Japan). Fetal calf serum (FCS) and newborn bovine serum (NBS) were supplied by Biological Industries (Kibbutz Beth Haemek, Israel) and Biowhittacker (Walkersville), respectively. z-VAD-fmk was obtained from Funakoshi (Tokyo, Japan). RAV-2 reverse transcriptase and ribonuclease inhibitor were from Takara Shuzo (Kyoto, Japan). KOD-Plus- DNA polymerase was purchased from Toyobo (Osaka, Japan). Authentic free fatty acids and prostaglandins were purchased from Cayman (Ann Arbor, MI). CLN (18:3 [9,11t,13t]) was extracted and purified from seeds of bitter gourd *(30)*. NDGA, aspirin, n-PrG, tannic acid, free fatty acids, and prostaglandins were dissolved in ethanol; TPA, 3-AB, NAC, and z-VAD-fmk in dimethyl sulfoxide; 2-ME and ascorbic acid in water. All other chemicals were of reagent grade or tissue culture grade.

Cell Culture

The MDCK (JCRB9029) and 3T3-L1 (JCRB9014) cell lines were obtained from Health Science Research Resources Bank (Osaka, Japan). MDCK cells and 3T3-L1 cells were grown in DMEM-HEPES medium supplemented with 5 or 10% FCS at 37°C under 7% CO_2, respectively. 3T3-L1 cells required 200 μ*M* ascorbic acid for the medium. When MDCK cells and 3T3-L1 cells were grown to near confluence (80%), the culture medium was replaced with fresh DMEM-HEPES medium supplemented with 2% NBS or charcoal-treated 2% FCS for stripping off endogenous fatty acids, respectively, to which the reagents to be tested were added. After MDCK cells and 3T3-L1 cells were treated with various reagents for the indicated period, the apoptotic response was analyzed under a Nikon fluorescence microscope,

model Optiphot-2. To protect CLN and CLA from peroxidation, 10 μ*M* α-tocopherol was added to the medium when the effects of CLN and CLA were examined on apoptosis in 3T3-L1 cells for 24 h *(29)*.

DNA Fragmentation Analysis

After MDCK cells were treated with apoptotic inducers for the indicated period, the cells were harvested and suspended in 20 μL of the lysis buffer consisting of 50 m*M* Tris-HCl buffer (pH 7.8), 10 m*M* EDTA-Na, and 0.5% (w/v) sodium *N*-lauroyl sarkosinate. Then, the cell suspension was incubated with 10 μg of RNase A at 50°C for 30 min, followed by the reaction of 10 μg of Pronase for 60 min. The reaction mixture was applied to 2% agarose gel electrophoresis.

Nuclear Condensation Analysis

MDCK cells treated with apoptotic inducers were trypsinized at 37°C for 10 min, harvested, and rinsed with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS(–)). The cells were suspended in 100 μL of 2% glutaraldehyde solution and fixed for more than 2 h. An aliquot (10–20 μL) of the cells was stained with 2 μL of 1 m*M* Hoechst 33342 in PBS (–). The cells were spotted onto a glass slide and observed with a fluorescence microscope. In the 3T3-L1 cells, the adherent cells were fixed with 1% glutaraldehyde, stained with 1 m*M* Hoechst 33342, and observed with a fluorescence microscope as previously reported *(31)*.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted by an acid guanidinium thiocyanate–phenol–chloroform mixture *(32)*, and used for reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA (0.5 μg) was added to a reaction mixture (5 μL) of 8.2 m*M* Tris-HCl buffer (pH 8.3), 0.82 m*M* EDTA, 204.5 m*M* KCl, 10 μ*M* oligo$(dT)_{12–18}$, and 10 μ*M* random hexamer. This mixture was treated for 3 min at 80°C followed by 1 h of incubation at 31°C. To the resulting annealing mixture, 8.7 U of ribonuclease inhibitor and 2.7 U of RAV-2 RT were added by dissolving in 5 μL of cDNA buffer containing of 24 m*M* Tris-HCl (pH 8.3), 16 m*M* $MgCl_2$, 8 m*M* dithiothreitol, and 100 μ*M* dNTPs. After a 1-h incubation at 43°C, the reaction mixture (10 μL) was heated for 5 min at 98°C. The resulting 1-μL aliquot was used for the PCR amplification in a total volume of 20 μL containing of 1X PCR buffer for KOD-Plus- DNA polymerase (Toyobo), 200 μ*M* dNTPs, 0.3 μ*M* sense primer, 0.3 μ*M* antisense primer, 0.4 U of KOD-Plus- DNA polymerase, supplemented with either 1 m*M* $MgSO_4$ for both COX-II and GAPDH or 2.5 m*M* $MgSO_4$ for both COX-I and 12-LOX. After heating at 94°C for 2 min, the amplification was performed for 35 cycles involving the denaturation at 94°C for 15 s, primer annealing at 53°C for 30 s, and extension at 68°C for

Table 1
Oligonucleotides of Sense and Antisense Primers Used for RT-PCR

Primer	Size (mer)	Sequence
5'-Primers		
COX-I-S	17	5'-ATTCTGCCCTC(CT)GT(AG)CC-3'
COX-II-S	20	5'-GAGATGATCTACCCTCCTCA-3'
12-LOX-S	19	5'-(GT)G(AG)(AC)CTGGCT(CG)(CT)T(AG)GC(CT)AA-3'
GAPDH-S	20	5'-GTCTTCACCACCATGGAGAA-3'
3'-Primers		
COX-I-AS	21	5'-(AT)GC(AT)GCCAT(CT)TC(CT)TT(CT)TC(AT)CC-3'
COX-II-AS	21	5'-(CG)CC(CT)TTCACATT(AG)TTGCAGAT-3'
12-LOX-AS	19	5'-(AG)TGC(CT)(GT)I(CG)C(ACG)G(AT)GCA(CG)GTG-3'
GAPDH-AS	20	5'-TCCAC(AC)AC(AC)C(GT)GTTGCTGTA-3'

90 s. A 1-µL aliquot of the reaction mixture was subjected to the separation by 2% agarose gel electrophoresis. The sense and antisense primers used are listed in Table 1.

Results and Discussion

Synergistic Stimulation of Apoptosis with TPA and NDGA in Cultured MDCK Cells

TPA was reported to induce apoptosis after exposure to 40 ng/mL of TPA for 12 h *(24)*. TPA also has the ability to induce the synthesis of prostaglandins in cultured MDCK cells in the presence of A23187 *(21)*. TPA at 100 ng/mL was found to stimulate apoptosis of MDCK cells in the presence of 2% NBS to allow the quiescent condition. To determine whether the activation of the arachidonate cascade was involved in the TPA-induced apoptosis, we examined the effects of aspirin, a COX inhibitor, as well as NDGA, a LOX inhibitor, on the TPA-induced apoptosis in MDCK cells. As shown in Fig. 1, NDGA at 50 µ*M* synergistically stimulated the TPA-induced apoptosis, but aspirin at 50 µ*M* was not effective in this type of apoptosis. Another LOX inhibitor, *n*-PrG, at 50 µ*M* was also effective in stimulating the TPA-induced apoptosis (data not shown). Therefore, the TPA-induced apoptosis in MDCK cells might possibly be regulated by lipoxygenase products.

Expression of COX and LOX mRNA Levels in MDCK Cells Treated with TPA

The expression of the COX isoforms I and II, and 12-LOX was determined in MDCK cells treated with TPA for 24 h. As shown in Fig. 2, mRNA of COX-I and COX-II was detected, but the level of COX-I was much lower. On the other hand, 12-LOX was not detectable although 12-HETE was

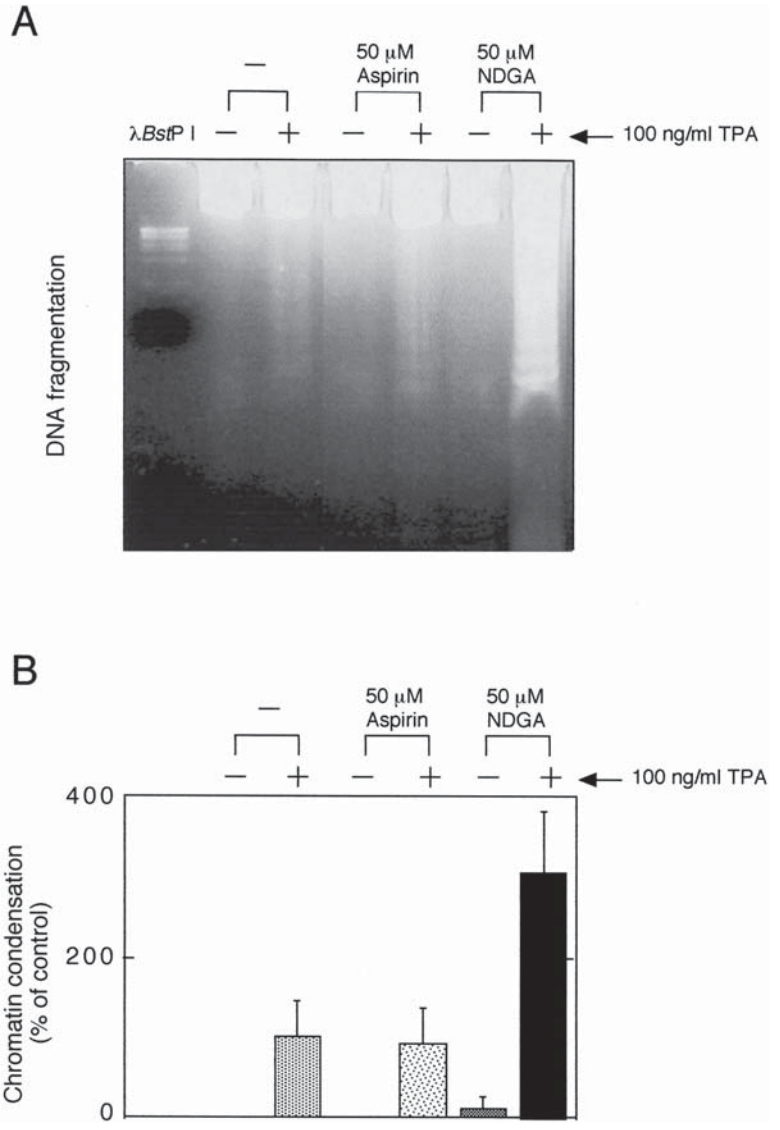

Fig. 1. Effects of COX and LOX inhibitors on TPA-induced apoptosis in MDCK cells. MDCK cells were grown to 80% confluence in the growth medium with 5% FCS, and then the culture medium was replaced by DMEM-HEPES medium containing 2% NBS. The cells were then pretreated for 1 h with 50 μ*M* aspirin or 50 μ*M* NDGA and stimulated with 100 ng/mL of TPA for 24 h. **(A)** DNA fragmentation analysis; **(B)** nuclear condensation analysis. Values are the results from at least three independent experiments. Data represent the mean ± SD.

reported to be detected in rat kidney tubular tissue *(33)*. These observations suggested that the NDGA-stimulated apoptosis did not reflect the inhibition of 12-LOX activity, which has been reported as a survival factor in some types of cancer cells *(20)*.

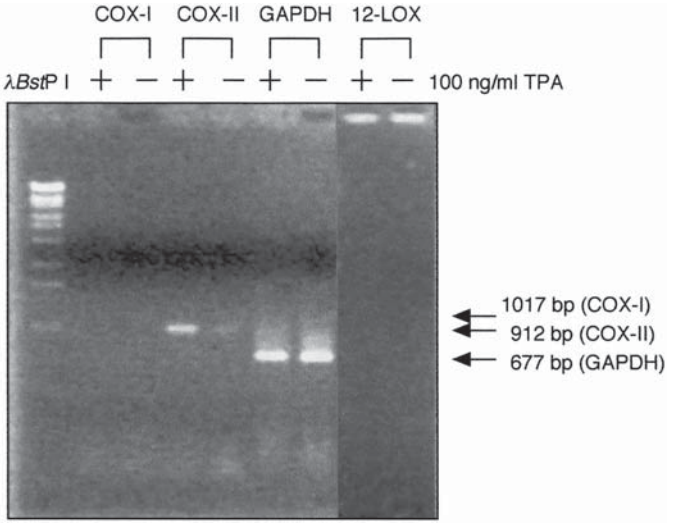

Fig. 2. RT-PCR analysis of expression of mRNAs levels of COX-I, COX-II, and 12-LOX in MDCK cells treated with TPA. Total RNA was extracted from MDCK cells treated with 100 ng/mL of TPA for 24 h. Expression of GAPDH was examined as control. Gene-specific primers in RT-PCR analysis were listed in Table 1.

Effects of Various Antioxidants on Apoptosis in MDCK Cells Induced by TPA and NDGA

A previous report has described NDGA-induced apoptosis in carcinoma expressing 12-LOX *(20)*. The induction was reported to be inhibited by the treatment of antioxidant such as NAC and 2-ME. It was suggested that this type of apoptosis was presumably the result of a decrease in the level of intracellular glutathione. Hence, we examined the effect of various antioxidants such as NAC, ascorbic acid, and 2-ME on the NDGA-stimulated apoptosis in our MDCK cells. Although only 50 μ*M* 2-ME had a significant inhibitory effect on this type of apoptosis (Fig. 3), other antioxidants were without significant effects, suggesting that the TPA- and NDGA-induced apoptosis in MDCK cells was not explained by the decrease in the level of glutathione, as reported elsewhere *(20)*.

Apoptotic Signal Transduction Pathway in MDCK Cells Induced by TPA and NDGA

Nucleosomal DNA ladder formation and chromatin condensation are hallmarks of apoptosis *(34,35)*. We tested the effect of caspase inhibitor z-VAD-fmk on those processes. The apoptosis was almost completely inhibited by 40 μ*M* z-VAD-fmk, indicating that the activation of caspases would be involved in apoptosis induced by TPA and NDGA (Fig. 4).

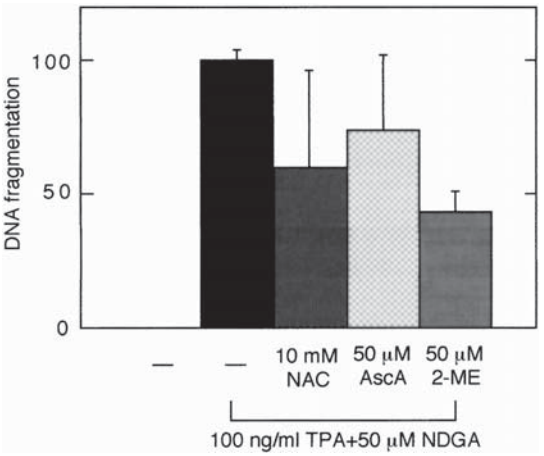

Fig. 3. Effects of various antioxidants on apoptosis induced by TPA and NDGA. MDCK cells were pretreated for 1 h with 50 μ*M* ascorbic acid (AscA), 1 m*M* NAC, or 50 μ*M* 2-ME before being stimulated with a mixture of 100 ng/mL of TPA and 50 μ*M* NDGA for 24 h. Nuclear condensation was analyzed. Values are the results from at least three independent experiments. Data represent the mean ± SD.

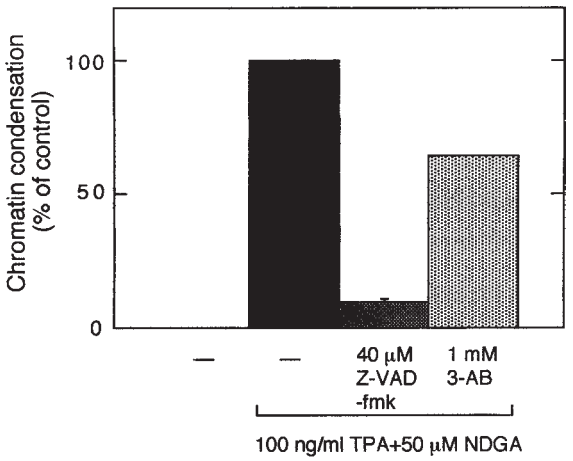

Fig. 4. Effects of z-VAD-fmk and 3-AB on apoptosis induced by TPA and NDGA. MDCK cells were pretreated for 24 h with 40 μ*M* z-VAD-fmk and 1 m*M* 3-AB. Nuclear condensation was analyzed by a fluorescence microscope. Values are the results from at least three independent experiments. Data represent the mean ± SD.

Poly(ADP-ribose) polymerase (PARP) was one of the targets of caspase-3. Thus, the limited cleavage of PARP by caspase-3 has been used as an index for apoptosis *(36)*. As previously reported, 3-AB, a PARP inhibitor, was reported to inhibit morphologic changes during apoptosis *(37)*. The effect of 3-AB was examined on the induced apoptosis by TPA and NDGA in MDCK cells. 3-AB had an inhibitory effect on the chromatin condensation (Fig. 4) and nucleosomal DNA ladder formation (data not

Fig. 5. Effect of COX or LOX inhibitors on hydrogen peroxide–induced apoptosis in MDCK cells. MDCK cells were treated for 24 h with or without 50 μ*M* aspirin or 50 μ*M* NDGA in the presence of 1 m*M* hydrogen peroxide, and then DNA fragmentation was analyzed.

shown) in this type of apoptosis, indicating that poly(ADP-ribose) metabolites could be involved in induced apoptosis in the presence of TPA and NDGA.

Effects of COX or LOX Inhibitors on Hydrogen Peroxide-Induced Apoptosis in MDCK Cells

When we examined the action of COX or LOX inhibitors on other types of apoptosis in MDCK cells, we found hydrogen peroxide-induced apoptosis in MDCK cells as reported in pig kidney (LLC-PK1) cells *(38)*. This apoptosis was stimulated with 1 m*M* hydrogen peroxide after treating for 24 h (Fig. 5). Both aspirin and NDGA had little effects on this type of apoptosis, indicating that the regulatory mechanism of the TPA-induced apoptosis might be independent of that by hydrogen peroxide.

CLN- and CLA-Induced Apoptosis in Mouse Preadipocyte 3T3-L1 Cells

Mouse preadipogenic 3T3-L1 cells have been reported to produce various types of prostaglandins during adipogenesis *(39)*, and such prostaglandins have been thought to be involved in the regulation of adipogenesis. However, the roles of prostaglandins still seem controversial. Recently, CLA has been reported to induce apoptosis mainly in cancer cells *(40)*.

Fig. 6. Effect of CLN on mouse preadipogenic 3T3-L1 cells. Mouse preadipogenic 3T3-L1 cells were grown to 80% confluence, and the culture medium was replaced by fresh medium containing charcoal-treated 2% FCS. The cells were then treated for 24 h with 200 μ*M* CLN or 200 μ*M* CLA. The cells were stained with Hoechst 33342 and visualized under a fluorescence microscope. **(A)** Control; **(B)** 200 μ*M* CLN; **(C)** 200 μ*M* CLA. Arrows indicate typical apoptotic cells. Magnification of images is ×100.

Although CLA was reported to activate peroxisome-proliferator activated receptors (PPARs), which required endogenous ligands for the activation *(41)*, the effect of CLA on the arachidonate cascade in adipocytes remains unclear. More recently, one of the PGJ_2 derivatives, 15-deoxy-$\Delta^{12,14}$-PGJ_2, was shown to bind directly to PPARγ *(12)*. In addition, a peroxisome-proliferator response element was found to regulate the expression of COX mRNA *(42)*. Recently, Miyashita and his group found that the distinct level of CLN was present in the flesh of the bitter gourd *(30)*. In terms of the potential use of regulating obesity by food therapy, we tested the effect of CLN on preadipogenic 3T3-L1 cells.

CLN at 200 μ*M* induced apoptosis in preadipogenic 3T3-L1 cells. The effect of CLN seemed to be more potent than 200 μ*M* CLA (Fig. 6). We are in the process of studying the involvement of eicosanoids in the CLN-induced apoptosis in 3T3-L1 cells. The apoptotic responses of the adipocytes by CLA were also investigated *(29)*.

In conclusion, we found that a LOX inhibitor, NDGA synergistically stimulated the TPA-induced apoptosis in MDCK cells. The effect of NDGA appeared not to involve the inhibition of LOX activities because the increased level of 12-LOX mRNA was not detectable in the MDCK cells treated with TPA by RT-PCR analysis. Aspirin at a higher level promoted apoptosis in the presence of TPA and NDGA (data not shown) suggesting that TPA-induced synthesis of prostaglandins might be involved in the prevention of apoptosis in MDCK cells. Further studies need to be conducted. In preadipogenic 3T3-L1 cells, CLN was found to be a novel apoptotic inducer. The involvement of COX or LOX in apoptosis induced by CLN in 3T3-L1 cells is under investigation.

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