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Induction of Apoptosis by All-*trans*-Retinoic Acid and C2-Ceramide Treatment in Rat Stromal–Vascular Cultures

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Apoptosis of preadipocytes and adipocytes contributes to the balance of adipose tissue mass by reducing adipocyte number. To address this phenomenon, we treated cultured rat S-V cells with all-*trans*-retinoic acid (RA) (10 μM) or C2-ceramide (50 μM) during adipogenesis. Gel electrophoresis of DNA from treated cells cultured in serum-free medium showed that 10 μM RA or 50 μM ceramide induced a distinct laddering pattern of DNA fragments. Cellular caspase 3 activity, another marker of apoptosis, was increased by RA (10 μM) ($P < 0.05$), but not by 50 μm C2-ceramide. RT-PCR results showed that RA (10 μM) decreased the expression of Bcl-2 mRNA. These results suggest that fat cell loss by apoptosis can be regulated, in part, by RA (10 μM) which increases caspase 3 activity and decreases Bcl-2 expression in rat S-V cells. C2-ceramide apparently works through a different cellular mechanism to induce apoptosis. © 2000 Academic Press

Key Words: **apoptosis; rat stromal–vascular (S-V) cells; all-*trans* retinoic acid; C2-ceramide.**

Adipose tissue mass is regulated by both the average volume and the number of adipocytes. Reduction of adipocyte number can result from preadipocyte and adipocyte apoptosis, as well as adipocyte dedifferentiation (1). Several lines of evidence suggest that apoptosis is inducible in adipocytes. *In vitro,* apoptosis in adipocytes can be mediated by growth factor deprivation, mild heat injury or TNF-α treatment (2, 3). In addition, in humans certain types of malignancies can result in adipocyte apoptosis (4). In ob/ob mice that have been depleted of insulin by streptozotocin treatment, apoptosis of adipocytes has been demonstrated (5). Therefore, under these conditions, apoptosis may be an important factor regulating adipose tissue mass.

All-*trans*-retinoic acid (RA), a vitamin A metabolite, is a ligand for the retinoic acid receptor (RAR), which is a member of nuclear hormone receptor superfamily (6). Vitamin A supplementation in rats has been shown to reduce adiposity (7), but the mechanism by which this occurs is unknown. RA has previously been reported to inhibit adipocyte differentiation (8–10), and other studies have shown that RA treatment induced apoptosis during chick limb bud development and in HL-60 leukemic cells (11, 12). RA has also been found to induce apoptosis in 3T3-L1 cells when cells are treated with delipidated serum (13). Therefore, it is possible that RA plays a role in the regulation of adipose tissue mass via inhibition of adipocyte differentiation and induction of apoptosis.

Ceramide, a product of sphingomyelin breakdown by sphingomyelinases, can also act as a second messenger that mediates apoptotic processes (14, 15). Studies have shown that ceramide can induce apoptosis in pancreatic β cells of Zucker diabetic fatty (ZDF) rats and fa/fa rats (16, 17). However, the effects of ceramide on adipocytes is unknown.

Caspase 3 is a member of the caspase family of cysteine proteases, which is composed of caspase 1-10 (18). It cleaves substrates at aspartate residues and activation of this proteolytic activity appears to be an early event in apoptosis (19, 20). Bcl-2, one of the antiapoptotic members, is expressed when programmed cell death is inhibited (21). In this study, we tested the hypothesis that all-*trans* RA and C2-ceramide reduce adipose cell number by induction of apoptosis in rat S-V cells. To explore the possible mechanisms by which all-*trans* RA and C2-ceramide induce apoptosis in rat S-V cells we measured the caspase 3 activity and the expression of Bcl-2 mRNA following these treatments.

MATERIALS AND METHODS

Cell cultures. Rat S-V cells from the inguinal adipose tissue of Sprague–Dawley (SD) rats (77-95 g) were collected by collagenase digestion as previously described (22). S-V cells were cultured for 1 day in medium containing DMEM/F12 Ham's and 10% fetal bovine serum. Then, cells were treated in serum-free medium (ITTS) con-

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taining 850 nM insulin, 64 nM transferrin, 29 nM sodium selenite, and T3 (2 nM triiodothyronine) for 3 days. Cells were treated with all-*trans* RA (10 μM) or C2 ceramide (50 μM) in ITTS medium containing 8.5 nM insulin for 1 day prior to analysis. For treatments, all-*trans* RA (Sigma Chemicals, St. Louis, MO) and C2-ceramide (Sigma Chemicals, St. Louis, MO) were dissolved in ethanol and added to ITTS medium at 1% and 0.1% on a volume basis for RA and C2-ceramide, respectively.

DNA fragmentation analysis (gel electrophoresis apoptotic assay). DNA fragmentation analysis was performed as a modified procedure described elsewhere (23). Following treatment in cell culture, the cells were scraped from the petri dishes and resuspended in ice-cold PBS. Cells were then centrifuged for 5 min at 500 $\times$ g. The genomic DNA was collected from the cells following an overnight incubation in 600 μl of lysis buffer [10 mM Tris-Cl, pH 8.0; 25 mM EDTA; 100 mM NaCl, 0.5% SDS and 0.1 μg/μl proteinase K (Sigma Chemicals, St. Louis, MO)] at 55°C. Then, DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was ethanol precipitated with ammonium acetate and the resulting DNA pellet was washed with 70% ethanol and dissolved in TE. Subsequently, the DNA was treated with RNase (1 u/μg DNA, for 2 h at 37°C) and 4–6 μg of genomic DNA was loaded onto a 1.8% agarose gel containing ethdium bromide (EtBr).

RT-PCR for the expression of Bcl-2 mRNA. Total RNA was isolated from treated cells using the TRIzol reagent and subsequently treated with RQ1 DNase (1 u/μg RNA) for 1 h at 37°C. RNA was incubated at 65°C for 10 min to inactivate the DNase. The PCR primers used to detect the expressions of Bcl-2 (GenBank accession no. L14680, 474-bp fragments) and β-actin (Accession No. J00691, 764-bp fragment) were 5′-gcaaccgaacgcccgctgtg-3′ (398-417, sense) and 5′-gtgatgcaggcccccaccag-3′ (842-871, antisense); and 5′-ttgtaaccaactgggacgatatgg-3′ (1552-1575, sense) and 5′-gatcttgatcttcatggtgctagg-3′ (2991-2884, antisense), respectively (24). RT-PCR using 1 μg of total RNA was performed using a commercially available RT and PCR system (Promega, Madison, WI). The reverse transcription reaction was performed at 48°C for 45 min. To inactivate the AMV RT enzyme the mixture was heated at 94°C for 2 min, followed by 35 PCR cycles, at 94°C for 40 sec for denaturation, at 58°C for 1 min for annealing, and at 72°C for 1 min for extension. The final extension was performed at 72°C for 5 min. The PCR products were electrophoretically separated on a 1.2% agarose gel stained with EtBr. β-actin expression was used to normalize the relative level of Bcl-2 expression.

Caspase 3 assay. Caspase 3 activity was measured using a modified procedure described elsewhere (25). Cells ($\sim 1 \times 10^7$ cells) were lysed in a buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (Hepes) at pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml aprotinin and 0.1% (w/v) 3-[3 chloramidopropyl dimethylammonio]-1-propanesulfonate (Chaps) (Sigma Chemicals, St. Louis, MO). The cell lysate was centrifuged at 15,000*g* for 15 min at 4°C and the supernatant fraction was used to quantitate caspase 3 activity. The protein concentration in cell lysate was determined by the bicinchoninic acid assay procedure (BCA) [Pierce Chemical Co. (Rockford, IL)] (26). 10 μg of cell lysate protein was incubated for 3 hours at 37°C in assay buffer containing 100 mM Hepes at pH 7.5, 5 mM dithiothreitol, 0.1% Chaps, 10% sucrose and 16 μM caspase 3 substrate (Ac-Asp-Glu-Val-Asp-*p*-nitroanaline) (Calbiochem-Novabiochem Corp., San Diego, CA). The amount of p-nitroaniline released by caspase 3 activity was quantitated by measuring the optical density at 400 nM. Caspase 3 activity was expressed as μM *p*-nitroaniline released per hour per μg cellular protein.

Statistical analysis. All experiments were replicated at least twice using different rats and there were three replicates per treatment. The data represent the mean among triplicate values from a representative experiment $\pm$ one standard deviation. Data analysis was performed using an analysis of variance (ANOVA), SAS (Cary, NC). Statistically significant differences were defined at the 95% confidence level using Duncan's Multiple Range test.

RESULTS

All-trans retinoic acid (RA) treatment of rat stromal–vascular (S-V) cells. To test if all-*trans* RA induces apoptosis in rat S-V cells, DNA fragmentation analysis was performed. S-V cells incubated in defined media and treated with 10 μM of all-*trans* RA demonstrated a clear laddering pattern of DNA fragments when analyzed by agarose gel electrophoresis (Fig. 1A). To further investigate the mechanism of all-*trans* RA-triggered apoptosis in rat S-V cells, caspase 3 activity was measured (Fig. 1B). Figure 1B shows that all-*trans* RA (10 μM) significantly increased caspase 3 activity compared to groups that were not treated with all-*trans* RA ($P < 0.05$). RT-PCR analysis of Bcl-2 mRNA expression was performed to further investigate the mechanism of all-*trans* RA-induced apoptosis in rat S-V cells (Fig. 1C). The expression of Bcl-2 was decreased by all-*trans* RA treatment (Fig. 1C), compared to control cells.

The effect of insulin or T3 on rat S-V cells. To determine if insulin (8.5 nM) and/or T3 (2 nM) affect the survival of S-V cells in rat S-V cultures, cells were cultured in defined media in the presence or absence of insulin and/or T3 and DNA fragmentation analysis and measurement of caspase 3 activity were performed (Figs. 2A and 2B). Camptothecin, an inhibitor of topoisomerase I, was used as a positive control for apoptosis induction since it induces apoptosis in several cell types (27, 28). Camptothecin treatment resulted in a very distinctive DNA laddering pattern compared to the other treatments (Fig. 2A).

Insulin (8.5 nM) or T3 (2 nM) had little affect on DNA laddering pattern (Fig. 2A). 10% FBS treatment showed minimal DNA laddering pattern (Fig. 2A). Cells grown in ITTS had much higher levels of caspase 3 activity than cells grown in 10% FBS (Fig. 2B); however, the presence or absence of insulin or T3 had no significant effect on caspase 3 activity (Fig. 2B).

C2-ceramide treatment of rat S-V cells. To determine if C2-ceramide induces apoptosis in rat S-V cultures, cells were treated with 50 μM C2-ceramide and DNA fragmentation analysis and measurement of caspase 3 activity were performed. Figure 3A shows that this treatment induced a distinctive laddering pattern of DNA fragments compared to groups treated with 10% FBS or ITTS without C2-ceramide. However, the C2-ceramide treatment did not increase caspase 3 activity when compared to ITTS treatment (Fig. 3B).

DISCUSSION

This study demonstrated that all-*trans* retinoic acid (RA) (10 μM) or C2-ceramide (50 μM) induces apopto-

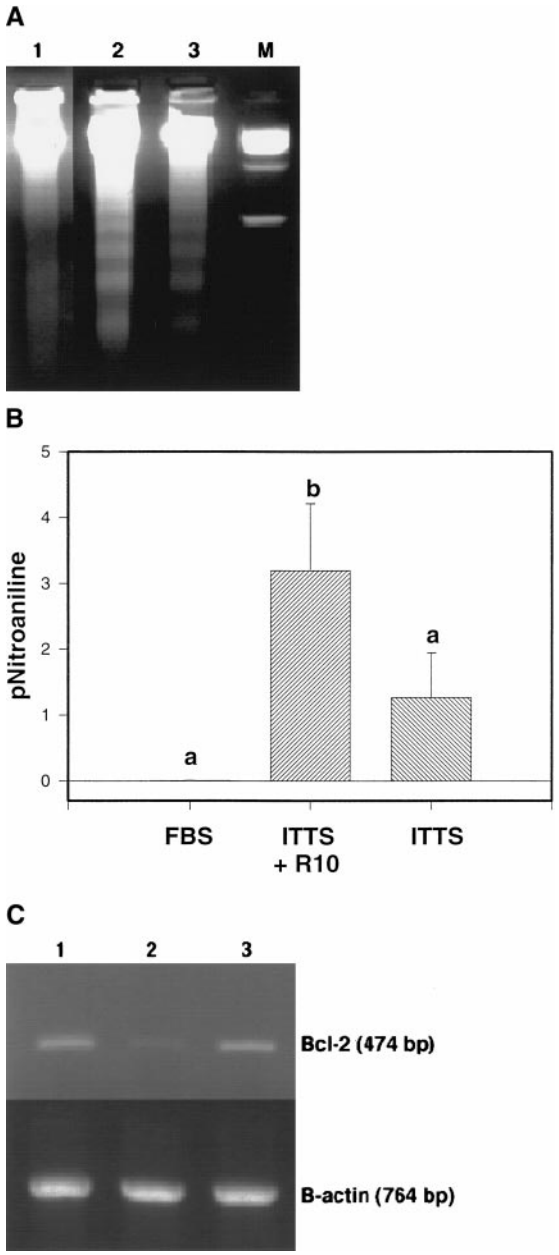

FIG. 1. Analysis of apoptosis induced by 10 μM of retinoic acid (RA) in rat stromal–vascular (S-V) cultures. Rat S-V cells were cultured in ITTS medium containing 850 nM insulin for 3 days following a 1-day culture in 10% FBS. Cells were then exposed to the treatments for 1 day and collected for analysis of apoptosis. The results in this figure are representative of three replicates per experiment and the experiment was replicated twice. (A) DNA fragmentation analysis of RA (10 μM) treatment. Lane 1, 10% FBS treatment. Lane 2, RA (10 μM) treatment with ITTS medium containing 8.5 nM insulin. Lane 3, ITTS medium containing 8.5 nM insulin without treatment. M, 1 kb DNA ladder markers. (B) Analysis of caspase 3 activity in rat S-V cells treated with RA (10 μM). The relative caspase 3 activity was measured spectrophotometrically and is expressed as μM *p*-nitroanaline released/hour/μg cellular protein. The bar graph represents the mean value (mean + SD, $n = 6$). Means with different letters indicate significant ($P < 0.05$) differences. (C) RT-PCR demonstrates RA decreases the level of Bcl-2 mRNA. Lane 1, 10% FBS. Lane 2, RA (10 μM) treatment with ITTS medium containing 8.5 nM insulin. Lane 3, ITTS medium containing 8.5 nM insulin without treatment.

sis in rat S-V cultures. Thus, it appears that during adipogenesis, adipocyte number can be regulated by all-*trans* RA or C2-ceramide mediated apoptosis.

RA is known to affect growth, differentiation and development (29, 30). All-*trans* RA has been reported to induce cell death by apoptosis in several cell types including cancer cells (31), and all-*trans* RA (10 μM) treatment of 3T3-L1 cells in the presence of delipidated

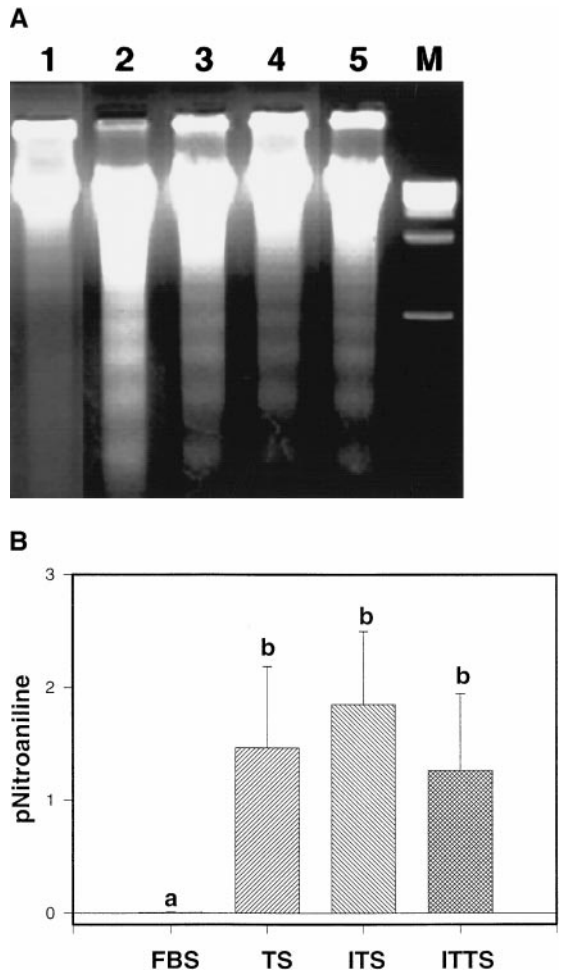

FIG. 2. Analysis of apoptosis to determine if insulin or T3 affects apoptosis in rat S-V cultures. After 4 days of cell culture, cells were treated for 1 day with or without insulin (8.5 nM) and/or T3 (2 nM). The results are representative of three replicates per experiment and each experiment was replicated twice. (A) DNA fragmentation analysis. Lane 1, 10% FBS. Lane 2, Camptothecin (inhibitor of topoisomerase I, apoptosis inducer) with ITTS medium containing 8.5 nM insulin and T3. Lane 3, TS (64 nM transferrin and 29 nM sodium selenite) medium without insulin and T3. Lane 4, ITS (8.5 nM insulin, 64 nM transferrin, and 29 nM sodium selenite) without T3. Lane 5, ITTS medium (8.5 nM insulin, 2 nM T3, 64 nM transferrin, and 29 nM sodium selenite). M, 1 kb DNA ladder markers. (B) Analysis of caspase 3 activity in rat S-V cells in the presence or absence of insulin and/or T3. Caspase 3 activity was measured in detergent lysates of rat S-V cells by the method described under Materials and Methods. The bar graph represents the mean value (mean + SD, $n = 6$). Means with different letters indicate significant ($P < 0.05$) differences.

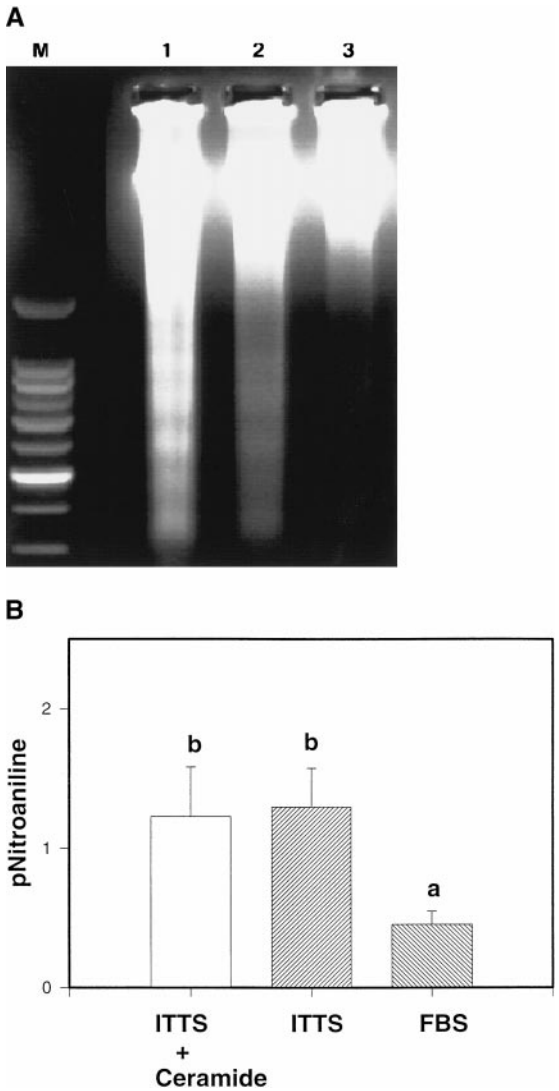

FIG. 3. Analysis of apoptosis induced by 50 μM of C2-ceramide in rat stromal–vascular (S-V) cultures. After 4 days of cell culture, cells were exposed to 0 or 50 μM of C2-ceramide in ITTS medium containing 8.5 nM insulin for 1 day. The results are representative of three replicate per experiment and each experiment was replicated twice. (A) DNA fragmentation analysis. M, 100 bp DNA ladder markers. Lane 1, C2-ceramide (50 μM) treatment in ITTS medium. Lane 2, ITTS medium without C2-ceramide. Lane 3, 10% FBS. (B) Analysis of caspase 3 activity in lysates of rat S-V cells treated with or without C2-ceramide (50 μM) in ITTS medium containing 8.5 nM insulin. The group treated with 10% FBS was used as a control. The bar graph shows the mean value (mean + SD, $n = 6$). Means with different letters represent significant differences ($P < 0.05$).

serum induces preadipocyte apoptosis (13). A previous study (7) suggests that dietary vitamin A supplementation in rats may contribute to energy homeostasis but the authors did not discuss the possibility that RA-triggered apoptosis might be involved in decreasing adiposity. In the present study, 10 μM of all-*trans* RA induced apoptosis in rat S-V cells treated with serum-free medium, (ITTS medium). Therefore, it is possible that all-*trans* RA (10 μM) regulates adiposity by reducing the number of rat S-V cells via apoptosis.

The Bcl-2 gene product is considered to be a prominent regulator of apoptosis (32). The RT-PCR results presented in this study showed that all-*trans* RA treatment decreased the expression of Bcl-2 mRNA. Thus, all-*trans* RA induced apoptosis of S-V cells may be mediated by a decrease in Bcl-2 expression.

Ceramide is generally produced by two different ways: one pathway is *de novo* synthesis of ceramide from free fatty acids, and the other pathway is via cytokine-induced sphingomyelinases such as TNF-α (16, 33, 34). Studies have shown that *de novo* ceramide synthesis from fatty acid induced lipoapoptosis in beta-cells of obese fa/fa Zucker diabetic fatty (ZDF) rats (16). Data in this study showed that C2-ceramide (50 μM) induced a distinctive laddering pattern of DNA fragments suggesting that apoptosis in rat S-V cells can be induced by C2-ceramide during adipogenesis. Furthermore, cytokine or free fatty acid may affect the adipocyte number by increasing C2-ceramide.

Caspase 3 activation is related to the proteolytic cleavage of several key proteins that regulate DNA-repair (19, 20). The results of this study showed that caspase 3 activity correlated with RA-induced DNA fragmentation (Fig. 1A). However, caspase 3 activity does not always reflect apoptosis (35–37) since the function of caspase 3 can be carried out by other proteases in the caspase family (18). In our study, caspase 3 activity in cells treated with C2-ceramide, did not parallel the DNA fragmentation analysis that we found with RA. Therefore, the mechanisms of apoptosis in rat S-V cells induced by RA and C2-ceramide appear to be different.

Previous work suggests that insulin treatment protect adipocytes from apoptosis (5). The relative concentration of insulin in rat S-V cultures appears to modulate the apoptotic pathway (38). For example, 85 nM insulin protected S-V cells from undergoing TNF-α-induced apoptosis whereas 8.5 nM insulin was ineffective. In the current study, the presence of 8.5 nM of insulin in ITTS medium of rat S-V cultures did not prevent apoptosis induced by either all-*trans* RA or C2-ceramide. Therefore, this concentration of insulin does not appear to alter all-*trans* RA- or C2-ceramide-triggered apoptosis. Thyroxine treatment has also been shown to affect apoptosis of several cell types (39–41). However, in this study the presence or absence of T3 (2 nM) or insulin (8.5 nM) did not have an effect on DNA fragmentation or caspase 3 activity. Thus, the levels of T3 (2 nM) or insulin (8.5 nM) tested in our experiments may not have been sufficient to influence apoptosis in rat S-V cultures. Other work has demonstrated that serum deprivation of human adipocytes can induce apoptosis (2). Our data suggest that factors present in FBS actually protect S-V cells from undergoing apoptosis. In addition, all-*trans* RA and C2-ceramide treat-

ment of rat S-V cells cultured with FBS did not induce apoptosis (data not shown). This suggests that factors in serum may protect S-V cells from apoptosis induced by RA and C2-ceramide *in vivo.*

In summary, apoptosis of rat S-V cells induced by all-*trans* RA (10 μM) appears to be mediated by elevated caspase 3 activity and decreased Bcl-2 expression. Insulin (8.5 nM) or T3 (2 nM) treatment of S-V cells did not appear to significantly affect caspase 3 activity. However, inclusion of FBS in cell culture medium appears to decrease caspase 3 activity and protect rat S-V cells from undergoing apoptosis. C2-ceramide (50 μM) induced apoptosis in rat S-V cells but it did not increase caspase 3 activity. Collectively, these results suggest that all-*trans* RA and C2-ceramide may contribute to adipocyte loss via apoptosis by reducing the number of S-V cells during adipogenesis in rat S-V cultures.

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