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Saturated FFAs, Palmitic Acid and Stearic Acid, Induce Apoptosis in Human Granulosa Cells

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Obesity is associated with insulin resistance and some reproductive abnormalities. Circulating FFAs are often elevated in obese subjects and are also closely linked to insulin resistance. In this study, we demonstrated that saturated FFAs, such as palmitic acid and stearic acid, markedly suppressed the granulosa cell survival in a time- and dose-dependent manner. Polyunsaturated FFA, arachidonic acid, had no effect on the cell survival, even at supraphysiological concentrations. The suppressive effect of saturated FFAs on cell survival was caused by apoptosis, as evidenced by DNA ladder formation and annexin V-EGFP/propidium iodide staining of the cells. The apoptotic effects of palmitic acid and stearic acid were unrelated to the increase of ceramide generation or nitric oxide production and were also completely blocked by Triacsin C, an inhibitor of acylcoenzyme A synthetase. In addition, acylcoenzyme A, pamitoylcoenzyme A, and stearylcoenzyme A markedly suppressed granulosa cell survival, whereas arachidonoylcoenzyme A had no such effect, and this finding was consistent with the effect of the respective FFA form. Surprisingly, arachidonic acid instead showed a protective effect on palmitic acid- and stearic acid-induced cell apoptosis. A Western blot analysis showed the apoptosis of the granulosa cells induced by palmitic acid to be accompanied by the down-regulation of an apoptosis inhibitor, Bcl-2, and the up-regulation of an apoptosis effector, Bax. These results indicate that saturated FFAs induce apoptosis in human granulosa cells caused by the metabolism of the respective acylcoenzyme A form, and the actual composition of circulating FFAs may thus play a critical role in the apoptotic events of human granulosa cells. These effects of FFAs on granulosa cell survival may be a possible mechanism for reproductive abnormalities, such as amenorrhea, which is frequently observed in obese women. (*Endocrinology* 142: 3590–3597, 2001)

LONG CHAIN FFAs play an important role in the cellular biological functions. They serve as a source of metabolic energy, as the substrates for cell membrane biogenesis (glyco- and phospholipid), and as precursors of many intracellular signaling molecules such as PGs, leukotrienes, thromboxanes, platelet-activating factor, and others (1–3). Under normal conditions, the plasma total FFA concentrations range from 200–600 μM in women. This wide variation is induced by an individual's diet and changes in the serum insulin level (4–6). In pathological states such as diabetes, obesity, and cancer, the plasma total FFA concentrations increase to levels ranging from 400-2000 μM (7–9). The elevation of the plasma FFA is one important link between obesity and insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM), and also increases the risk of cardiovascular diseases (10–12). The FFA pattern was reported to differ between the normal control and obese patients, in which obese patients had a low unsaturated-to-saturated ratio (13). More recent studies indicate that an increase in FFAs inhibits T lymphocyte signaling and induces pancreatic β-cell apoptosis (14, 15).

Obese women are frequently associated with reproductive abnormalities, including amenorrhea (16, 17). This abnormality in obese women is generally thought to be attributable to the dysregulation of the hypothalamic-pituitary axis, regarding the ovarian function (16, 18). Hyperandrogenism that is secondary to hyperinsulinemia may also be another cause for such reproductive abnormalities in obese women. A few molecules such as FFA, leptin, and TNFα have been reported to be elevated in obese subjects and have also been intensively discussed as candidates that may contribute to insulin resistance (19–23). Especially, a close relationship between the elevated plasma FFA level and insulin resistance is commonly reported in obese subjects (24–26). In the present study, to test the hypothesis that these factors may also cause ovarian dysfunction, we examined the effect of these molecules on the cell proliferation of human ovarian granulosa cells obtained from *in vitro* fertilization. We found that saturated FFAs affect the cell survival of human granulosa cells by inducing apoptosis, whereas a polyunsatu-

Abbreviations: AA, Arachidonic acid; AG, aminoguanidine; CoA, coenzyme A; EGFP, enhanced green fluorescent protein; iNOS, aminoguanidine; LA, linoleic acid; NIDDM, non-insulin-dependent diabetes mellitus; NO, nitric oxide; OA, oleic acid; PA, palmitic acid; PI, propidium iodide; PPAR, peroxisome proliferator-activated receptor α; SA, stearic acid; SNAP, S-nitroso-*N*-acetylpenicillamine.

rated FFA, arachidonic acid (AA), instead antagonizes saturated FFA-induced apoptosis. Because the mechanism of FFA-induced apoptosis has not been well elucidated, we also investigated this mechanism in our system and discussed the clinical implications of this phenomenon.

Materials and Methods

Materials

FFAs [including palmitic acid (PA) and stearic acid (SA)], oleic acid (OA), linoleic acid (LA) and AA, palmitoylcoenzyme A (palmitoyl-CoA) stearoyl-CoA and arachidonoyl-CoA, fumonisin B1, C2-ceramide, bovine insulin, and S-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Sigma (St. Louis, MO). PA and SA were dissolved in 0.1 N NaOH, and the final concentration of NaOH in the cell growth medium was 0.1 mN. Triacsin C was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). DME/F12, FCS, and proteinase K were purchased from Life Technologies, Inc. (Grand Island, NY). All antibodies, including antihuman Bcl-2, Bax, and β-actin used for the Western blot analysis, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Annexin V-EGFP/PI Apoptosis Detection Kit was obtained from the MBL Co., Ltd. (Nagoya, Japan). TNFα and leptin were purchased from PeproTech LTD (London, UK).

Cells and cell survival assay

Human ovarian granulosa cells were obtained from patients who underwent *in vitro* fertilization. Written informed consent was obtained from each subject before starting the study. The cells were maintained in DME/F12 supplemented with 10% FCS in an atmosphere of 5% CO_2 at 37 C. The purity of the human granulosa cells was estimated to be almost 90% of all cells, based on the expression of cytochrome P450arom examined by immunohistochemical staining of the cultured cells with the antibody against human cytochrome P450 (27).

For a cell survival assay, the cells were plated onto a 24-well plate, at 1×10^4 cells/well, in DME/F12 supplemented with 10% FCS, 1 d before treatment. The cells (30–40% confluence) were treated with various FFAs or other reagents, whereas control cells were incubated in medium with carrier (0.1 mN NaOH), and the medium with or without FFAs was changed every 2 d. After washing, the cells were trypsinized and then counted using a hemocytometer. Cell viability was assessed by the trypan blue dye exclusion method.

DNA fragmentation assay

DNA fragmentation was examined as previously described (28). Briefly, the cells treated with or without FFAs were trypsinized and washed with PBS and then were spinned down and resuspended in 100 μl lysing buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA-2Na, 0.5% SDS). Heat-treated ribonuclease A (QIAGEN, GmbH, Germany) was then added to a concentration of 0.5 mg/ml and incubated at 37 C for 60 min. Next, the protein was degraded using 0.5 mg/ml proteinase K at 37 C for 60 min. DNA was extracted using two extractions, first with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). DNA was then precipitated from the upper aqueous phase, using 0.1 vol of 3 M sodium acetate with 2.5 vol of ice-cold ethanol, and left at −20 C for 60 min before centrifugation. DNA pellets were resuspended in 50 μl sterile water. DNA fragments (10–12 μg) were separated by electrophoresis in 1.2% agarose gel and then visualized by staining with ethidium bromide.

Detection of apoptosis by fluorescence microscopy

Early and late apoptotic changes in human granulosa cells were also determined using an Annexin V-EGFP/PI Apoptosis Detection Kit. One day before treatment, the cells were divided into 35-mm glass-bottom dishes (MatTek Corporation, Ashland, MA) and then were treated with 300 μM PA or SA for 24 h and 48 h, respectively. After the treatment, the cells were washed once with PBS and then were incubated with 200 μl 1× binding buffer containing 1 μl annexin V-EGFP and 1 μl propidium iodide (PI) at room temperature, for 5 min, in the dark. The cells were then scanned using confocal laser scanning microscopy (TCS-SP system, Leica Corp. Microsystems, Heidelberg, Germany) using a dual-filter set for fluorescein isothiocyanate and TRITC. The cell membrane was imaged for green fluorescence (stained by annexin V-EGFP) by excitation with the 488-nm line from an argon laser, and the emission was viewed through a 460- to 505-nm band pass filter. The cell nucleus was imaged for red fluorescence (stained by PI) by excitation with the 560-nm line from an argon laser, and then the emission was viewed through a 520- to 580-nm band pass filter.

Western blot analysis

The expression of apoptosis-related proteins, Bax and Bcl-2, and β-actin were examined by Western blot analysis, as described previously (28), using specific antibodies. Human granulosa cells were cultured with 100 μM or 300 μM PA for 48 h. The cells were then washed with PBS and lysed with SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.2 mM/liter phenylmethylsulfonlyfluoride supplemented with 0.2 trypsin inhibitory units/ml aprotinin, 0.7 μg/ml pepstatin, and 1 μg/ml leupeptin]. The samples were sonicated for a few seconds to shear the DNA and reduce the viscosity. Twenty micrograms of protein were mixed with an equal volume of 2× electrophoresis buffer, then boiled for 3 min. The samples were electrophoresed on a 12% SDS-polyacrylamide gel, and proteins were transferred to a Hybond-P, polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were rinsed in Tris-buffered saline-0.1% Tween-20 and blocked with 4% BSA overnight at 4 C. The blots were incubated with rabbit antihuman bax (1:2000), human bcl-2 (1:2000), and goat antihuman β-actin (1:200) antibodies, respectively, for 1 h at room temperature. After washing with Tris-buffered saline-0.1% Tween-20 for three times, blots were probed with horseradish peroxidase-labeled antirabbit IgG (1:2000) or antigoat IgG (1:500), respectively, in blocking buffer. Proteins were detected using a chemiluminescence luminol reagent (Santa Cruz Biotechnology, Inc.), and the bands were visualized by autoradiography.

Statistical analysis

For the cell survival assay, experiments were performed in triplicate, and the results were expressed as the mean ± SD from three or four independent experiments. A statistical analysis was performed using the Statview software. All data were evaluated for unpaired variables to compare two or more groups. $P < 0.05$ was considered to be statistically significant.

Results

Saturated FFAs induce a dose-dependent reduction in cell survival

To determine whether FFAs induce a reduction in granulosa cell survival, we investigated the cell viability, by trypan blue exclusion, using a hemocytometer. The cells were treated with or without various concentrations of FFAs for 3 d, and then the cell viability was determined. As shown in Fig. 1, a dose-dependent reduction in the cell survival was observed at doses of PA (C16:0) and SA (C18:0) ranging from 50–300 μM. The cell survival was less than 20% after exposure to 300 μM PA or SA for 3 d. PA and SA seemed to be similar in the potency to reduce the cell survival. The unsaturated FFAs present different effects on cell survival. Namely, OA (C18:1) and LA (C18:2) induced a 39% and 31% reduction, respectively, in survival at a 300-μM concentration, which is about 2–3 times the normal plasma level (13). AA (C20:4) had no effect on the granulosa cell survival at concentrations ranging from 1–10 μM.

Neither physiological nor supraphysiological concentration of TNFα (1–20 ng/ml), leptin (10–200 ng/ml), and insulin (100–2000 ng/ml) had an effect on cell survival (data

not shown). These results are consistent with previous studies (29, 30).

The effect of saturated FFAs was also time-dependent, because the cell survival declined dramatically as the time of treatment increased from 1 d to 3 d (Fig. 2). After 3 d of treatment, the cell viabilities were 9% and 12% in the cells exposed to 300 μM PA and SA, respectively, whereas the cell viability was more than 60% in cells treated with 300 μM of unsaturated FFAs like OA and LA. Another polyunsaturated FFA, AA, had no effect on the cell survival within the 6-d treatment period.

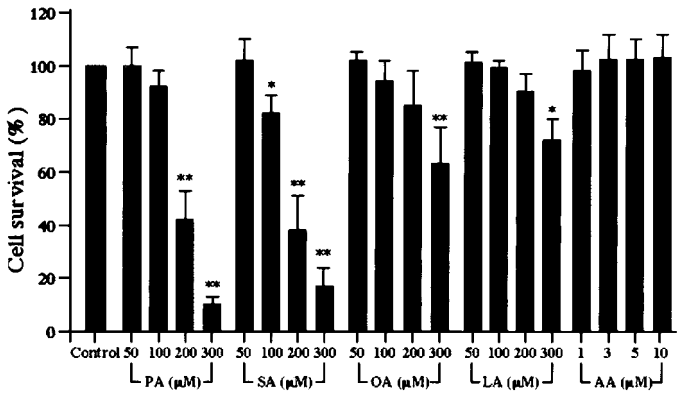

FIG. 1. The effects of FFAs on granulosa cell viability. Human granulosa cells were treated with various concentrations of saturated FFAs (including PA and SA) or unsaturated FFAs (including OA, LA, and AA). After treatment for 3 d, the cell viability was determined as described in *Materials and Methods*. The data represent the mean ± SD of four independent experiments with triplicate wells and are presented as the percentage of the control cell number. *, $P < 0.05$ *vs.* control cells; **, $P < 0.01$ *vs.* control cells.

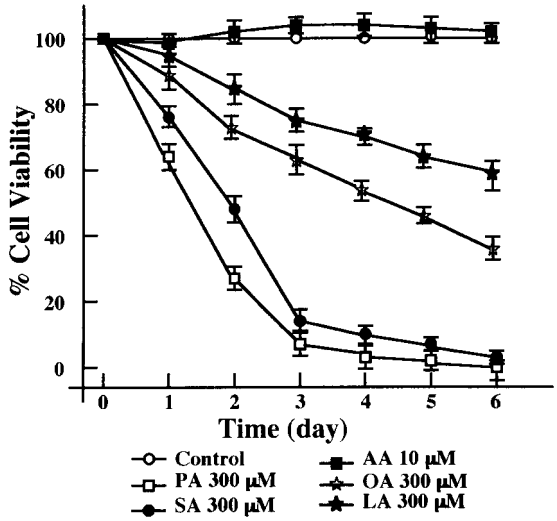

FIG. 2. The effects of various FFAs on the cell survival. Human granulosa cells were incubated in media containing various FFAs indicated, whereas the control cells were incubated in the medium with the carrier (0.1 mM NaOH). The cells were harvested at 24-h intervals and counted, and viability was assessed by trypan blue exclusion. The data represent the mean ± SD of three separate experiments with triplicate wells and are presented as the percentage of the control cell number.

Evidence that apoptosis mediates the saturated FFAs-induced reduction in granulosa cell survival

To determine whether apoptosis was the cause of the loss of cell survival after saturated FFAs treatment, we measured the effects of PA and SA on DNA fragmentation using DNA electrophoresis and fluorescent staining of the cells. After treating the cells with 300 μM PA or SA, respectively, for 72 h, the genomic DNA extracted from cells was subjected to 1.2% agarose gel electrophoresis. In each case, DNA ladders, which are typical of apoptosis, were visible on agarose gel after staining with ethidium bromide in granulosa cells (Fig. 3). In contrast, in the control cells treated with carrier (0.1 mN NaOH), no DNA fragmentation was observed. Saturated FFA-induced apoptosis was further confirmed using Annexin V-EGFP/PI staining. In healthy cells, the phospholipids of the plasma membrane are distributed asymmetrically over the two leaflets of the bilayer. Phosphatidylserine (PS) is located exclusively in the inner membrane leaflet because of an inhibition of aminophospholipid translocase and a subsequent activation of scramblase (31, 32). Because annexin V binds with a high affinity to negatively charged PS, it has been used in combination with PI to detect early and late apoptotic or necrosis cells (33). In unexposed control cells, most cells stained for neither annexin V-EGFP (green) nor PI (red). After being treated with 300 μM PA or SA, early apoptotic cells with a cell membrane stained by green color (annexin V positive/PI negative) were detected at 24 h; and late apoptotic cells, with a cell membrane demonstrating green staining and red staining for nuclear staining (annexin V positive/PI positive) and chromatin fragmentation were seen (Fig. 4).

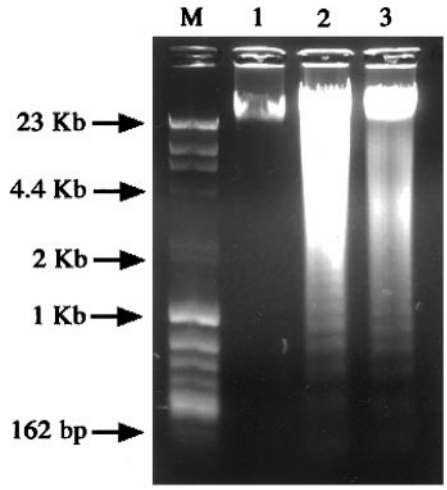

FIG. 3. The induction of DNA fragmentation by saturated FFAs in granulosa cells. The cells were cultured for 3 d with saturated FFAs (300 μM each) or carrier (0.1 mM NaOH) alone as control. DNA was extracted as described in *Materials and Methods* and separated by electrophoresis in 1.2% agarous gel. Band formation from a DNA molecular weight marker is shown in lane M, and the sizes of the migration fragments are indicated. Both PA (lane 2) and SA (lane 3) induced DNA fragmentation in granulosa cells, whereas the carrier did not induce DNA fragmentation (lane 1).

FFA-induced apoptosis is not mediated by an increase of ceramide generation or nitric oxide (NO) production

Because FFA has been demonstrated to induce pancreatic β-cell apoptosis by increasing both ceramide generation and NO production (15), we investigated whether DNA fragmentation in granulosa cells was caused by exogenous ceramide and NO donor SNAP. As shown in Fig. 5, 100 μM C2-ceramide induced DNA fragmentation in CEM leukemic cells (lane 3) but did not induce apoptosis in the granulosa cells (lane 4); and 50 μM fumonisin B1, the ceramide synthase inhibitor, did not prevent the PA (300 μM)-induced apoptosis (lane 5). These results indicate that PA-induced apoptosis in granulosa cells is not by an increase of ceramide generation.

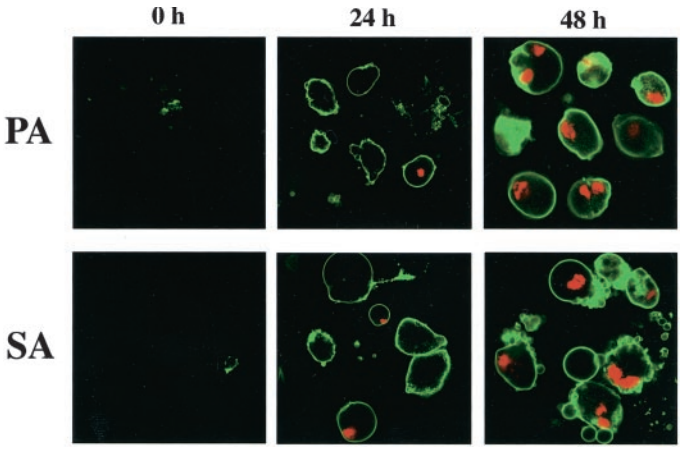

FIG. 4. Morphological evidence for apoptosis induced by saturated FFAs. After incubating the granulosa cells with 300 μM PA or SA for 24 h and 48 h, as indicated, the cells were stained by annexin V-EGFP and PI, as described in *Materials and Methods*. The cells were then scanned using a confocal laser scanning microscopy. The early apoptotic cells, with the cell membrane stained by annexin V-EGFP (*green*), and the late apoptotic cells, with both the cell membrane stained by annexin V-EGFP and nucleus stained by PI (*red*), were detected (0 h, 24 h, and 48 h indicate before treatment, 24 h after treatment, and 48 h after treatment, respectively.

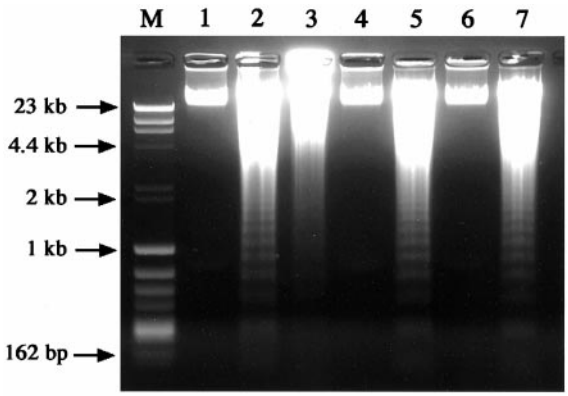

FIG. 5. The effects of ceramide and NO donor on DNA fragmentation induced by PA. The cells were incubated with the reagents described below, for 3 d, and the DNA from the cells was extracted and electrophoresed in 1.2% agarose gel. Lane M, DNA molecular weight marker; lane 1, control; lane 2, 300 μM PA; lane 3, treatment of CEM leukemic cells with 100 μM C2-ceramide; lane 4, treatment of granulosa cells with 100 μM C2-ceramide; lane 5, 300 μM PA plus 100 μM fumonisin B1; lane 6, 1 mM SNAP; lane 7, 300 μM PA plus 1 mM AG.

We further determined that the increase of NO production was not the cause of PA- or SA-induced apoptosis in granulosa cells, because: 1) a high concentration (1 mM) of NO donor SNAP did not induce DNA fragmentation (lane 6); 2) aminoguanidine (AG), an inducible NO synthase (iNOS) inhibitor (34), which has been reported to profoundly reduce NO production *in vitro* and effectively prevent β-cell loss (15), did not block the apoptotic effect of PA in granulosa cells (lane 7); and 3) even PA and SA increased NO production and iNOS protein level determined by Western blot analysis in granulosa cells (data not shown), but the NO levels in culture medium were much lower than those in the cells treated with 1 mM SNAP (about 1:200) (data not shown). The doses of C2-ceramide, fumonisin B1, and AG used in this experiment were optimal, above which levels these reagents themselves were toxic to the cells. From the above results, we concluded that saturated FFA-induced apoptosis in granulosa cells is not mediated by either the increase of ceramide generation or NO production.

Effects of fatty acyl-CoA blockade on DNA fragmentation

Next, to examine whether FFA-induced apoptosis is caused by FFA itself or by its metabolites, we investigated the effects of fatty acyl-CoA blockade on granulosa cell survival. Exogenous FFAs traverse the cellular membrane by means of nonmediated passive diffusion (35, 36). Upon entry into the cell, FFAs are esterified into their active form, acyl-CoA, and then are further metabolized. Triacsin C has been shown to be an effective inhibitor of acyl-CoA synthetase (37–39). As shown in Fig. 6, though Triacsin C itself had no effect on cell survival, 5 μM of this compound completely prevented the apoptotic effects of various concentrations (ranging from 100–500 μM) of PA and SA. To further determine whether acyl-CoA can directly induce granulosa cell death, we treated the cells with various concentrations of palmitoyl-CoA,

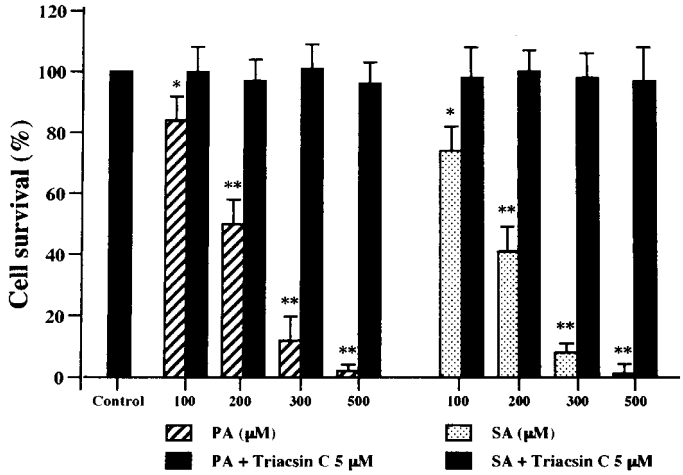

FIG. 6. The inhibitory effect of Triacsin C on saturated FFA-induced apoptosis in granulosa cells. The cells were treated with various concentrations of PA or SA alone or with PA or SA plus 5 μM Triacsin C for 3 d. The cell viability was determined as described in *Materials and Methods*. The data represent the mean ± SD of three independent experiments with triplicate wells and are presented as the percentage of control cell number. *, $P < 0.05$ *vs.* control cells; **, $P < 0.01$ *vs.* control cells.

stearoyl-CoA, and arachidonoyl-CoA for 3 d As shown in Fig. 6, both palmitoyl-CoA and stearoyl-CoA induced a reduction in cell survival in a dose-dependent manner; 200 μM of either palmitoyl-CoA or stearoyl-CoA nearly killed all of the cells. In line with the observed effect of AA, arachidonoyl-CoA itself had no effect on granulosa cell survival (Fig. 7). On the other hand, Triacsin C had no effect on palmitoyl-CoA- and stearoyl-CoA-induced apoptosis (data not shown).

AA suppresses PA- and SA-induced cell apoptosis

AA has been determined to promote tumor growth by enhancing DNA synthesis and cell proliferation (40). To test the hypothesis that AA may also function as a survival factor for granulosa cells, we cultured the cells with various concentrations of PA or SA alone, and PA or SA plus 15 μM AA for 3 d. As shown in Fig. 8A, AA provided a significantly protective effect on PA- and SA-induced cell death. The AA effect was dose-dependent and was first observed at 3 μM, and later plateaued at approx. 10–15 μM and could be observed up to 20 μM, above which level AA itself was toxic to granulosa cells (Fig. 8B). These results indicate that AA may be important in the prevention of apoptosis induced by saturated FFAs in granulosa cells.

PA increases Bax and decreases Bcl-2 expression in granulosa cells

An apoptosis suppressor, Bcl-2, has been reported to be suppressed in FFA-induced pancreatic β-cell apoptosis (41). To determine whether saturated FFA-induced apoptosis in granulosa cells is related to the suppression of an apoptosis suppressor, Bcl-2, or the induction of an apoptosis effector, Bax, we measured the expression levels of Bcl-2 and Bax in granulosa cells, either treated or not treated with PA, by a Western blot analysis. As shown in Fig. 9, PA treatment

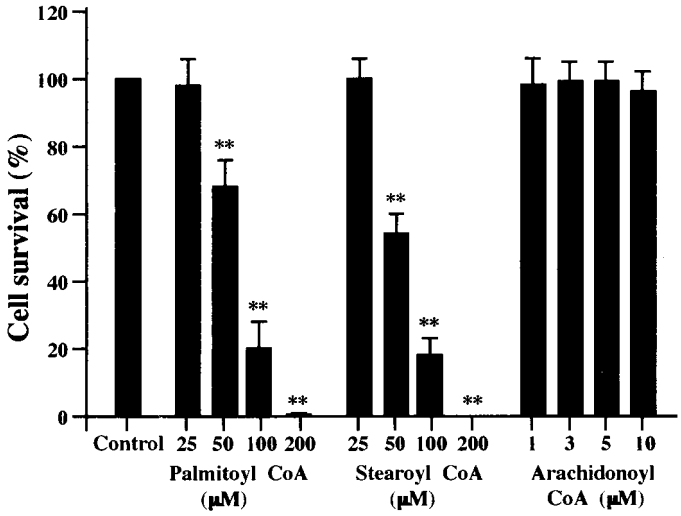

FIG. 7. The effects of palmitoyl-CoA, stearoyl-CoA, and arachidonoyl-CoA on granulosa cell viability. Cells were incubated in media with various concentrations of acyl-CoAs, as indicated, for 3 d. The cell viability was determined as described in *Materials and Methods*. The data represent the mean ± SD of four independent experiments with triplicate wells and are presented as the percentage of the control cell number. **, $P < 0.01$ *vs.* control cells.

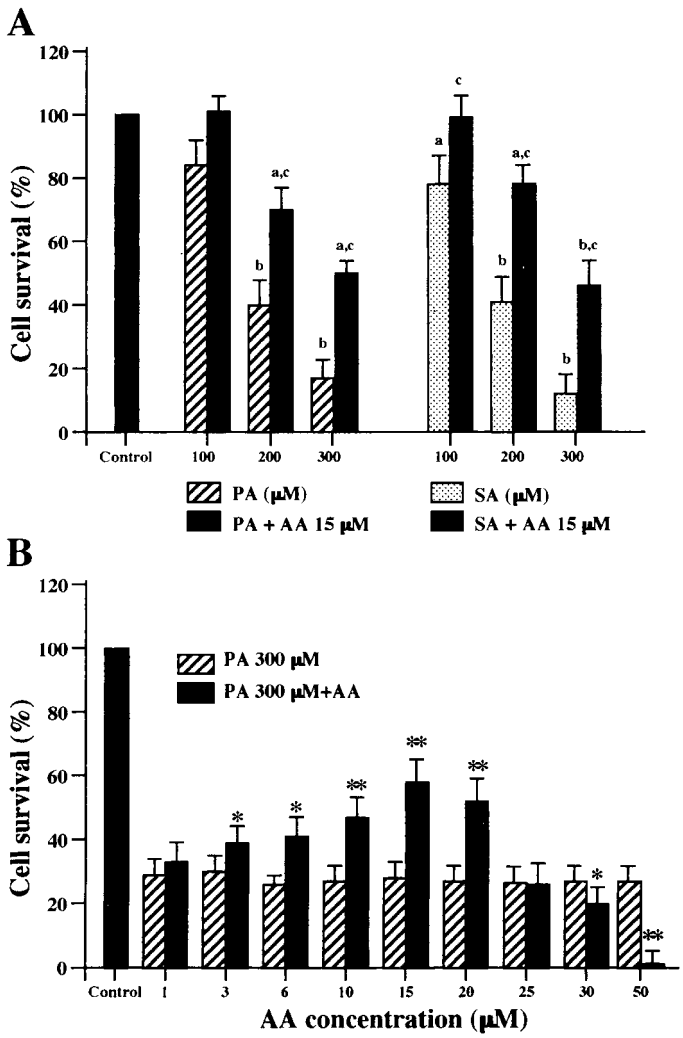

FIG. 8. The protective effect of AA on saturated FFA-induced apoptosis in granulosa cells. A, The cells were incubated in media containing 100–300 μM PA or SA, with or without 15 μM AA, for 3 d. B, The cells were incubated in media containing 300 μM PA alone or 300 μM PA plus various concentrations of AA, as indicated, for 3 d. The cell viability was determined as described in *Materials and Methods*. The data represent the mean ± SD of three independent experiments with triplicate wells and are presented as the percentage of the control cell number. a, $P < 0.05$ *vs.* control cells; b, $P < 0.01$ *vs.* control cells; c, $P < 0.05$ *vs.* cells treated with saturated FFA alone; *, $P < 0.05$ *vs.* cells treated with 300 μM PA; **, $P < 0.01$ *vs.* cells treated with 300 μM PA.

resulted in a dramatic down-regulation of Bcl-2 and an up-regulation of Bax.

Discussion

Obesity is the most common cause of insulin resistance and is frequently associated with reproductive abnormalities, especially in women. Obese women show ovarian dysfunction, leading to various degrees of menstrual abnormalities, including amenorrhea. At least three candidate molecules, which are increased in the circulation of obese subjects, have been implicated in the development of insulin resistance (19–24). One is FFA, which inhibits the glucose uptake and is used in muscle (19, 24). Second, TNFα has been

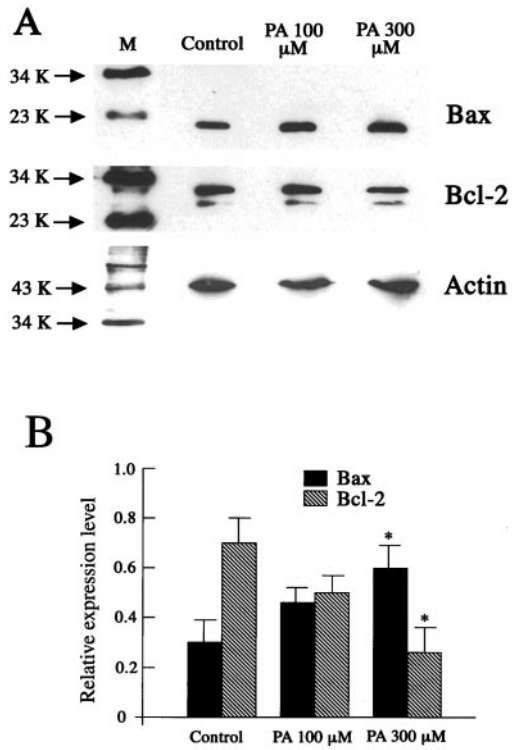

FIG. 9. Expression of Bcl-2 and Bax during apoptosis induced by PA in the granulosa cells. A, The cells were treated with 100 or 300 μM PA for 2 d. The cell lysates were then prepared, and a Western blot analysis was performed, with the use of specific antibodies to Bcl-2, Bax, and β-actin. B, The relative expression levels of Bcl-2 and Bax were determined by measuring the intensity using ImageQuant. The data represent the mean ± SD of three independent experiments and are presented as the fold of β-actin. *, $P < 0.05$ *vs.* cells treated with 300 μM PA.

reported to be at least partially responsible for the insulin resistance caused by obesity (22, 23). Third, leptin may cause insulin resistance, especially in the liver and adipocytes (20, 21); however, this issue remains highly controversial. We tested the possibility that these candidate molecules for insulin resistance may also be a possible cause for the ovarian dysfunction by affecting ovarian granulosa cell survival. Leptin and TNFα caused no effect on the cell survival of human granulosa cells, which also correlated with the results observed by others (41, 42). In the present study, we demonstrated that mainly saturated FFAs, such as PA (C16:0) and SA (C18:0), induce cell death via apoptosis in human ovarian granulosa cells, whereas unsaturated FFAs do not essentially have such an effect. The same phenomenon was also observed in a human granulosa cell line, KGN, which was most recently established by our group (27) (data not shown). These results indicate that the human granulosa cell is truly a target for apoptosis by FFAs.

The nonfasting serum FFA levels in patients with hyperlipidemia are reported to be 140.9 ± 77.7 μM, 109.79 ± 70.0 μM, 89.5 ± 74.5 μM, 45.9 ± 44.0 μM, and 0.8 ± 1.3 μM for PA, SA, OA, LA, and AA, respectively (13). As a result, the dose-dependent reduction in cellular viability observed at doses of PA and SA ranging from 100–300 μM is considered to mimic the hyperlipidemic condition. The unsaturated FFAs had only a slight effect on cell survival. Namely, OA (C18:1) and LA (C18:2) caused a 39% and 31% reduction, respectively, in survival at a 300-μM concentration, which is a level 2–3 times the normal plasma level (13). On the other hand, AA had no effect on granulosa cell survival at concentrations ranging from 1–20 μM. The predominant effect of saturated FFAs over unsaturated FFAs, regarding the suppression of human granulosa cell proliferation, is very similar to the situation observed in insulin resistance; namely, a higher proportion of saturated FFAs, relative to unsaturated FFAs, is reported to be associated with insulin resistance (42). As a result, we may safely say that obese women with insulin resistance may be susceptible to ovarian dysfunction via elevated saturated FFA concentration in the circulation. Interestingly, although AA itself has no effect on the cell survival of human granulosa cells, it had a highly protective effect against PA- or SA-induced apoptosis of human granulosa cells. This protective effect is dose-dependent at concentrations from 1–15 μM. The suppressive effect of AA on apoptosis induced by lipoxygenase inhibitor, nordihydroguaiaretic acid, is also reported in the W256 carcinosarcoma cell (40). These results may clinically suggest that not only the circulating levels of FFAs, but also the composition of FFAs, may play an important role in the apoptotic event. Interestingly, it is also reported that FFA destabilizes LH receptor and inhibits LH responsiveness in porcine ovarian granulosa cells (43). Such an effect of FFA may also be one additional mechanism for the ovarian dysfunction in insulin resistance. However, to elucidate the exact role of FFAs on human granulosa cells, the examinations of actual concentrations of FFAs in human follicular fluid may be needed.

FFA-induced apoptosis has been reported in the pancreatic β-cells (15), hepatocytes (44), and brain tumors (45). However, the mechanism of FFA-induced apoptosis is still not well understood. It is not clear even whether FFA-induced apoptosis is a direct effect of FFA or an indirect effect through its metabolite. The complete block of FFA-induced apoptosis in human granulosa cells by fatty acyl-CoA synthetase inhibitor, Triacsin C, and the direct apoptotic effects of acyl-CoA form in the cells clearly indicate that FFA itself does not have an apoptotic effect; instead, it shows that the metabolite acyl-CoA form plays an important role in the induction of apoptosis. This is the first demonstration of the important fact that FFA induces apoptosis through its metabolite to acyl-CoA forms.

Ceramide, which is one of the known sphingolipids, has recently attracted much attention attributable, in part, to its role as a second messenger of cell death. Ceramide can be generated by the hydrolysis of the membrane phospholipid and sphingomelin, which is enriched in the outer leaflet of the plasma membrane (46, 47), or derived from *de novo* synthesis in response to inducers of apoptosis (48). Fatty acid-induced apoptosis has been reported in the pancreatic β-cells (15) of Zucker diabetic fatty rats. Under these conditions, the increase of ceramide generation or NO production was implicated as a mechanism of FFA-induced β-cell apoptosis (15). However, in human granulosa cells, neither mechanism seems to be involved, because neither exogenous ceramide nor NO donor SNAP-induced apoptosis and because the ceramide synthase inhibitor, fumonisin B1, which has been

reported to effectively block the apoptotic effect of ceramide (15, 49), or AG, an iNOS inhibitor, did not prevent FFA-induced apoptosis. The difference in the mechanism between human granulosa cells and β-cells may be attributable to a difference in the cell types or to a difference in the FFA concentration used in the experiment, because a relatively higher concentration of FFAs, namely 1mM long-chain FFAs, was used in a previous study of β-cell apoptosis, than that (1–300 μM) in our study. Several recent papers have suggested rather that NO production by iNOS suppresses apoptosis in granulosa cells (50) and may act as a cytostatic factor in ovarian follicles (51).

FFAs are known to be endogenous ligands for peroxisome proliferator-activated receptor α (PPARα) (52). However, another PPARα ligand, fenofibrate (10 μM), did not cause apoptosis of human granulosa cells, suggesting that PPARα is not involved in the mechanism.

Bcl-2 is known to extend cell survival by suppressing apoptosis, thus suggesting that a reduction in Bcl-2 might play a role in promoting the apoptotic process. Conversely, the overexpression of one of the apoptosis effector genes, Bax, can antagonize the function of Bcl-2 and increase the susceptibility of the cells to apoptosis. Both Bcl-2 and Bax are expressed in ovarian granulosa cells. The gonadotropin-induced follicular growth and survival were associated with a relatively constitutive level of Bcl-2 and Bcl-XL expression but markedly reduced levels of Bax mRNA (53, 54). In our study, the induction of apoptosis by the treatment with PA in human granulosa cells was accompanied by a dramatic reduction in the expression of Bcl-2 and by the induction of the heterodimer partner Bax proteins. As a result, the PA-induced apoptotic mechanism in human granulosa cells seems to be clearly explained by the observed changes in Bcl-2 and Bax. Recently, both tumor suppressor genes, such as p53 (55, 56), and death genes, such as Fas antigen/Fas ligand (57, 58), were found to play an important role in controlling granulosa cell death during follicular atresia. In the present study, FFA-induced apoptosis was not accompanied by any change in the expression levels of p53 and Fas antigen, based on the findings of either a Western blot or a flow cytometry analysis of human granulosa cells (data not shown).

In summary, we demonstrated, for the first time, that FFAs, especially saturated FFAs, induce apoptosis in human granulosa cells, not directly but through the metabolite of acyl-CoA forms. These effects of FFAs on granulosa cell proliferation may therefore be a possible mechanism for the reproductive abnormalities, including amenorrhea, which are frequently observed in obese women.

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