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Mitochondrial Dysfunction Is Involved in Apoptosis Induced by Serum Withdrawal and Fatty Acids in the β-Cell Line Ins-1

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The potential toxic effects of high extracellular concentrations of fatty acids were tested in β(INS-1)-cells cultured in the absence of serum, a condition known to alter cell survival in various systems. This may in part mimic the situation in type 1 or 2 diabetes where β-cells are already insulted by various stressful conditions, such as cytokines and oxidative stress. Serum removal caused, over a 36-h period, oxidative stress and an early impairment of mitochondrial function, as revealed by increased superoxide production and markedly reduced mitochondrial membrane potential, but a lack of cytochrome *c* and apoptosis-inducing factor release in the cytosol. The fatty acids palmitate and oleate considerably accelerated the apoptosis process in serum-starved cells, as revealed by fluorescence-activated cell sorting analysis, morphological changes, chromatin condensation, DNA laddering, poly(ADP-ribose) polymerase cleavage, cytochrome *c* and apoptosis-inducing factor release, and increased levels of Bax and cytosolic caspase-2. The fatty acids also increased nitric oxide production, apparently independently of inducible nitric oxide synthase induction. Under the same experimental conditions, elevated glucose alone had only a marginal effect on β-cell apoptosis. Together the results indicate that elevated concentrations of fatty acids are particularly efficient in accelerating the rate of apoptosis of already stressed β(INS-1)-cells displaying altered mitochondrial function, and that the mitochondrial arm of the apoptosis process is involved in β-cell lipotoxicity. (*Endocrinology* 144: 335–345, 2003)

REDUCTION OF pancreatic β-cell mass has been documented in autoimmune diabetes through the action of several cytokines produced by circulating inflammatory cells. These include IL-1β, TNFα, and interferon-γ (IFNγ) which impair cell function, produce DNA damage, and favor cell death in rodent and human β-cells (1). The mechanism seems to involve nitric oxide (NO) production through the increased expression of the inducible NO synthase (iNOS) gene and disruption of mitochondrial function, leading to the release of apoptogenic factors, such as cytochrome *c*, which recruits and activates procaspase 9 and additional executioner caspases, and apoptosis-inducing factor (AIF), which induces nuclear chromatin condensation, progressing to apoptosis (2, 3).

Several lines of evidence also support a possible role for apoptosis in noninsulin-dependent diabetes mellitus. In this context, prolonged exposure to an excess of circulating nutrients (glucose or fatty acids) seems to be a key event in the initiation of this death process. The Zucker diabetic fatty (ZDF) rat model has been extensively studied to decipher the molecular mechanisms implicated in the apoptosis process induced by lipids (4–7). Results obtained by different laboratories suggest that lipid accumulation in islet tissue is harmful for β-cell function, leading to the development of the so-called toxic effect of lipids, or lipotoxicity. This is correlated with impaired insulin secretion and changes in the expression of genes involved in the lipogenic and fat oxidation pathways (8, 9).

In the ZDF rat model, *de novo* ceramide formation and increased NO production have been proposed to be involved in final β-cell decompensation (4, 5). Thus, β-cell apoptosis is prevented by pharmacological blockade of serine palmitoyltransferase, which catalyzes the first step in ceramide synthesis, as well as inhibition of acyl-coenzyme A (acyl-CoA) synthetase, which catalyzes the formation of palmitoyl-CoA, the carbon precursor of ceramides. This suggests that the rise in ceramides in islets treated with a palmitate/oleate mixture (1:2) might be due to their accelerated *de novo* synthesis and not from sphingomyelin hydrolysis (7). However, the actions of individual fatty acids (palmitate and oleate alone) were not reported in this study (7).

Accelerated lipolysis, which should avoid intracellular deposition of fat and ceramide synthesis, reduces the apoptotic effect of fatty acids in the β-cell. Thus, leptin acts by depleting triglyceride deposits in adipose and nonadipose tissues bearing the corresponding receptors. Leptin increases the expression of enzymes implicated in fatty acid metabo-

Abbreviations: acyl-CoA, Acyl-coenzyme A; AIF, apoptosis-inducing factor; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; G5, 5 mM glucose; GDH, glutamate dehydrogenase; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; KRB, Krebs-Ringer bicarbonate; NO, nitric oxide; O_2^-, superoxide radical; PARP, poly(ADP-ribose) polymerase; PMSF, phenylmethylsulfonylfluoride; PVDF, polyvinylidene difluoride; ZDF, Zucker diabetic fatty.

lism and uncoupling protein-2 in rat islets, whereas the hormone reduces the expression of those activities implicated in lipid storage processes (10–12). Furthermore, leptin blocked the suppressor effect of fatty acids on the expression level of the antiapoptotic protein Bcl-2 (6). All of these data suggest that the β-cell adapts to an elevated circulating concentration of fatty acids through the induction of fatty acid metabolism genes, allowing intracellular lipid detoxification (13).

To gain insight into the mechanisms by which elevated concentrations of fatty acids cause β-cell death, we studied the actions of palmitate and oleate on the β(INS-1)-cell apoptosis process. In this paper we present evidence that serum deprivation (a widespread working model for apoptosis in several tissues) (14) produces apoptosis in β-cells, and that fatty acids considerably accelerate this process. We propose the participation of the mitochondrial arm of the apoptotic process in the toxic action of fatty acids, as evidenced from disruption of mitochondria function and release of cytochrome *c* and AIF.

Materials and Methods

Cell culture

INS-1 cells were grown in regular RPMI medium (11.2 mM glucose) supplemented with 10% heat-treated fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 10 mM HEPES (pH 7.4). When cells were 80% confluent, they were washed twice with Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) containing 5 mM glucose and 0.07% BSA and incubated for an additional 2 d in RPMI medium containing 5 mM glucose and 10% FCS. The day of the experiment, cells were washed with KRB buffer and incubated for different times at 37 C in RPMI medium containing 5 mM glucose in the presence of 10% FCS or in the absence of serum with 0.5% defatted BSA with or without fatty acids and staurosporine. The stock solutions of fatty acids bound to BSA were prepared as previously described (15).

Fluorescence-activated cell sorting (FACS) analysis

Cells (10^6) incubated under different experimental conditions were washed in cold PBS and fixed with cold 75% ethanol for 1 h at −20 C. Attached cells were harvested by centrifugation, resuspended in 0.5 ml PBS containing 0.5% Triton X-100 and 0.05% ribonuclease A, and incubated for 1 h at room temperature. DNA was estimated by measuring red fluorescence after staining with 50 μg/ml propidium iodide (Molecular Probes, Inc., Leiden, The Netherlands) for 15–30 min at room temperature. Before flow cytometry, samples were passed through a 16-gauge needle to retain aggregates. Fluorescence was measured in an EPICS flow cytometer (Coulter, Hialeah, FL), and results were analyzed in real time using the NEURODNA program and displayed as two-parameter histograms: cell number *vs.* DNA content.

Detection of chromatin condensation

Cells were grown on poly-L-lysine-treated glass slides. After incubation with the different test substances, cells were washed twice in PBS and stained with 10 μg/ml Hoechst 33342 dye (Molecular Probes, Inc.) for 1 min. After washing twice in PBS, samples were analyzed using a fluorescence microscope (Carl Zeiss, New York, NY).

DNA ladder analysis

After cell centrifugation at 300 × *g*, cell pellets were quickly resuspended in 50 mM Tris (pH 8.0), 50 mM NaCl, 10 mM EGTA, 0.5% sodium dodecyl sulfate, and 200 μg/ml proteinase K, and DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated in 2.5 vol ethanol (16). DNA was resolved in 1.5% agarose gel containing ethidium bromide and visualized in a transilluminator.

Superoxide anion ($O_2^{\cdot -}$) production and mitochondrial membrane potential determinations

For superoxide determinations, cells were gently resuspended in a KRB buffer, collected by centrifugation at 300 × *g*, resuspended in KRB, and incubated at room temperature with 1 μg/ml dihydroethidine (Molecular Probes, Inc.) for 2 min. After two washes in PBS, the change in fluorescence from blue to red (oxidized dye) was determined in an EPICS flow cytometer. For mitochondrial potential measurements, cells were harvested by centrifugation in KRB, resuspended, and incubated for 5 min at room temperature with 10 μg/ml rhodamine 123 (Molecular Probes, Inc.). After two washes in PBS, fluorescence was determined in an EPICS cytometer.

Subcellular fractionation

After incubation, cells were scraped from the dishes, collected by centrifugation at 700 × *g* for 4 min at 4 C, washed twice with ice-cold PBS, and centrifuged at 700 × *g* for 4 min. Cell pellets were resuspended in 50 μl extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM HEPES (pH 7.5), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml phenylmethylsulfonylfluoride (PMSF), 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. After incubation on ice for 30 min, cells were homogenized by 50 strokes of a polypropylene homogenizer (motor-driven micropistil) and spun at 15,000 × *g* for 15 min. Supernatants (cytosolic fraction) were then recovered and assayed for glutamate dehydrogenase (GDH) enzymatic activity (17). Less than 5% of the total cellular GDH activity (mitochondrial marker) was detected in supernatants after sample homogenization. The yield of GDH in both fractions was not affected by fatty acids or staurosporine.

Western blot analysis of cytochrome c, AIF, and caspase-2

Aliquots of 15 μg protein of cytosolic fractions and 10 μg protein of particulate fractions were boiled for 30 min for cytochrome *c* and for 3 min for AIF and caspase-2 measurements, resolved on 12.5% sodium dodecyl sulfate-polyacrylamide gels, and then blotted to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were incubated overnight at 4 C with an anticytochrome *c* monoclonal antibody (1:2,000; BD Pharmingen, San Diego, CA), an anti-AIF polyclonal antibody (1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or an anticaspase-2_L polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc.). After removal of excess primary antibody, membranes were incubated for 2 h with conjugated peroxidase antimouse IgG (Sigma, St. Louis, MO; 1:10,000) for cytochrome *c*, with antigoat IgG (Sigma; 1:5,000) for AIF, or with antirabbit IgG (Sigma; 1:500) for caspase-2. Bound antibodies were detected by enhanced chemiluminescence using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Poly(ADP-ribose) polymerase (PARP) cleavage detection

Total proteins from cells incubated for 36 h under the different experimental conditions were extracted with a buffer containing 62.5 mM Tris (pH 6.8), 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml antipain, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 6 M urea, and 0.00125% bromophenol blue and incubated for 8 min at 65 C. Aliquots of 15 μg protein were immediately resolved on 10% sodium dodecyl sulfate-polyacrylamide gels. The time between cell lysis and the start of electrophoresis was less than 30 min so as to minimize PARP cleavage during manipulations. Gels were then blotted to PVDF membranes that were incubated overnight at 4 C with an anti-PARP monoclonal antibody (1:1000; Oncogene Research Products, Boston, MA). After removal of the primary antibody, membranes were incubated for 2 h with conjugated peroxidase antimouse IgG (Sigma; 1:5000). Bound antibodies were detected by chemiluminescence.

Bcl-2, Bax, and iNOS detection

Induction of iNOS was performed by incubating INS-1 cells for 24 h in the presence of 50 IU/ml rat IL-1β, 100 IU/ml rat IFNγ, and 50 ng/ml rat TNF-α (R&D Systems, Inc., Weisbaden, Germany). Total proteins

from cells incubated for 36 h for Bcl-2 and Bax and for 24 h for iNOS under the different experimental conditions were extracted with a buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml antipain, 10 mM β-mercaptoethanol, and 1% Triton X-100. Aliquots of 25 μg protein were resolved on 12.5% sodium dodecyl sulfate-polyacrylamide gels for Bcl-2 and Bax and 7,5% SDS gels for iNOS, and then blotted to PVDF membranes that were then incubated overnight at 4 C with an anti-Bcl-2 polyclonal antibody (1:2000; Santa Cruz Biotechnology, Inc.), an anti-Bax polyclonal antibody (1:500; Santa Cruz Biotechnology, Inc.), or an anti-macrophage iNOS polyclonal antibody (1:2000; Transduction Laboratories, Inc., Lexington, KY). After removal of the primary antibody, membranes were incubated for 2 h with conjugated peroxidase antirabbit IgG (Sigma; 1:5000). Bound antibodies were detected using an enhanced chemiluminescence kit.

NO production

NO was determined as nitrite by the method of Green *et al.* (18) with modifications. Briefly, 100 μl of a 1:1 mix of 1% *N*-1-naphtyl-ethylenediamine dihydrochloride and 10% sulfonilamide (Sigma) were added to 50 μl culture supernatant. After 20-min incubation at 37 C in the absence of light, absorbance was measured at 540 nm and compared with standard curves of different concentrations of $NaNO_2$ in the presence of the different test substances added to the cells to correct for interferences.

Reagents

RPMI 1640 medium, FCS, and culture supplements were purchased from Life Technologies, Inc. (Gaithersburg, MD). Staurosporine, fatty acids, and protease inhibitors were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Statistical analysis

Data were expressed as the mean ± SEM and were analyzed by *t* test.

Results

To better understand the mechanism by which high concentrations of fatty acids may be toxic to β-cells, we decided to use INS-1(β) cells as a model because the amount of available cellular material allows more biochemical determinations to be performed than with isolated islets. In addition, cell death measurements in isolated islets are rendered difficult due to the high background of central necrosis. In initial experiments we tested the actions of fatty acids in the presence of 10% FCS, but noticed that fatty acids were barely toxic to INS cells under our culture conditions and time span (36 h). We therefore tested their actions in the absence of serum (14), a situation known to alter cell survival in various systems, and noticed that fatty acids were toxic to the β-cell under this experimental condition where cells are already "fragilized." This may in part mimic the situation in type 1 or 2 diabetes where β-cells have encountered the assault of various stressful conditions, such as cytokines and oxidative stress (1, 19–21).

FACS analysis of β(INS-1)-cells incubated in the presence of different nutrients and test substances

One way to study cell survival and death is by FACS analysis, which also provides information about cell cycle progression. INS cells incubated with or without serum and various concentrations of glucose and fatty acids were then stained with propidium iodide and sorted according to DNA content. Staurosporine was used as a positive control, because this drug efficiently induces apoptosis in several cell types (22, 23).

As indicated in Fig. 1, A and B, cells incubated with 5 mM glucose in the presence of the growth factors supplied by the serum are mainly quiescent in the G_0 phase, with very low levels of subdiploid (cell death) events or DNA synthesis (S phase). The subdiploid population increased when serum was absent from culture medium. No major changes where observed when the glucose concentration was increased to 25 mM, except for a significant ($P < 0.05$) rise in the cell population corresponding to the S phase of the cell cycle, thus confirming the mitogenic action of the sugar. The addition of oleate, palmitate, and staurosporine to the serum-deprived culture medium dramatically increased the number of subdiploid events in association with a decrease in the G_0 population. Oleate, but not palmitate, significantly increased the number of cells in the S phase with a poor progression to the M phase. The increased level of S phase events noticed in oleate-treated cells is comparable to that caused by 25 mM glucose. This observation is in accordance with tritiated thymidine experiments in INS cells incubated under similar experimental conditions (15). Together the results indicate that serum deprivation over 36 h induces a modest β(INS-1)-cell death that is markedly enhanced by fatty acids, but not by elevated glucose. In addition, palmitate and oleate cause differential aberrations in cell cycle phase distribution.

The percentage of live cells (determined from the total and subdiploid dead cell populations in FACS analysis) was evaluated under different conditions over a 72-h period (Fig. 2A). Dead cells reached a value of approximately 10% at either 5 or 25 mM glucose after 36 h. However, death events strongly increased under the same period in cells incubated in the presence of palmitate or oleate or with the positive control staurosporine. The percentage of dead cells in all conditions, except for incubation in the presence of staurosporine, remained relatively stable during the 36- to 60-h period. Incubation times longer than 60 h without serum caused cell detachment and increased cell death in all conditions. The data indicate that serum-deprived β(INS-1)-cells enter a death process that is markedly accelerated by fatty acids.

We also incubated INS-1 cells for 36 h in the absence of serum, but exposed them to elevated concentrations of fatty acids for different periods of time, followed by a further incubation in the absence of fatty acids. Interestingly, a 2-h transient incubation of cells in the presence of fatty acids was sufficient to trigger the death process after 24–36 h (Fig. 2B).

The study of the dose dependence of the effect of fatty acids on β(INS-1) cell death indicated that a fixed concentration of BSA (0.5%) and 0.1 mM palmitate or oleate caused a very modest cell death, and 0.3 mM of either fatty acid induced approximately half the death process observed at 0.5 mM.

Characterization of the apoptotic population

Next we wished to determine whether the death process caused by fatty acids corresponds to apoptosis or necrosis mechanisms. The formation of a DNA ladder is indicative of apoptotic events (24, 25). Figure 3 shows that a DNA ladder occurred mainly in cells incubated in the presence of palmi-

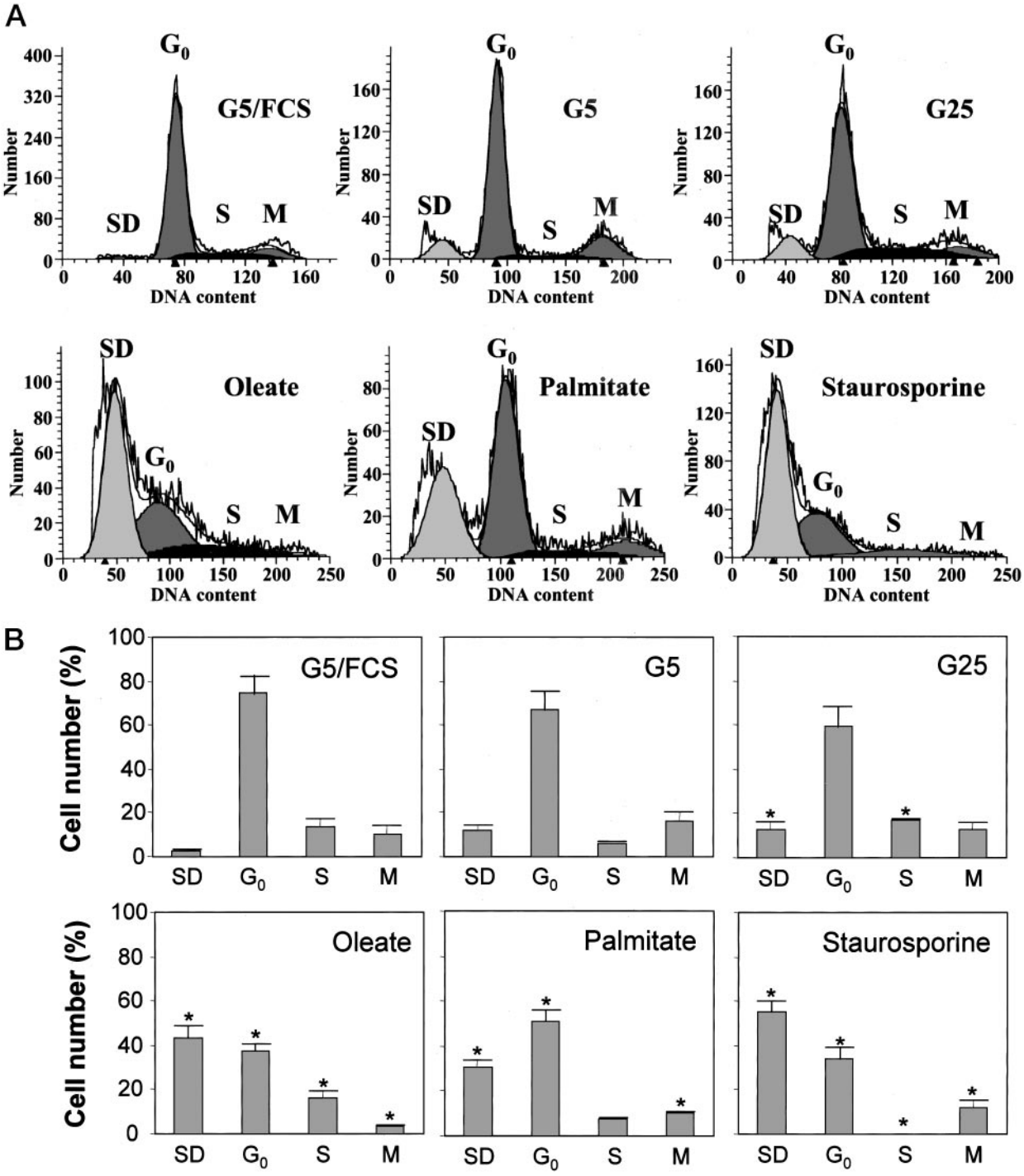

FIG. 1. A, FACS analysis of INS-1 cells incubated in the presence of various test substances. Cells were cultured for 2 d with 5 mM glucose in the presence of 10% FCS. Cells were subsequently incubated for 36 h in culture medium containing 5 mM glucose plus 10% FCS (G5/FCS) or medium containing 0.5% defatted BSA with 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose plus 0.5 mM oleate, 0.5 mM palmitate, and 0.2 μM staurosporine. At the end of the incubation period, cells were harvested, and FACS analysis was performed. The figure shows histograms from a representative experiment. SD, Subdiploid population; G_0, diploid population in the G_0 phase of the cell cycle; S, cell population in the S phase; M, tetraploid population in the M phase. B, Quantitation of the FACS analysis of INS-1 cells cultured in the presence of various test substances. Values shown are the mean ± SEM of three independent experiments. *, $P < 0.05$, *vs.* control (G5).

tate and oleate as well as with staurosporine. DNA laddering was much less apparent in cells incubated in the absence of serum at 5 or 25 mM glucose only. No ladder was observed in cells incubated with the basal glucose level in the presence of FCS.

Cells tended to detach from the culture dishes after 36 h

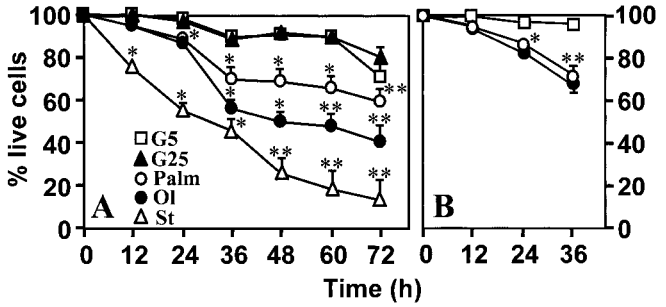

FIG. 2. Effect of a constant or transient exposure of INS-1 cells to elevated fatty acids on β-cell death. A, Time course of β-cell death in INS-1 cells cultured with various test substances. Cells were incubated for different times in medium containing 0.5% defatted BSA in the presence of 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose plus 0.5 mM oleate (Ol); 0.5 mM palmitate (Palm); and 0.2 μM staurosporine (St). Subsequently, apoptosis and/or necrosis (subdiploid population) were determined by FACS analysis as described in Fig. 1. Each *point* represents the mean ± SEM of three independent experiments. *, $P < 0.01$; **, $P < 0.005$ [*vs.* control (G5)]. B, A 2-h transient incubation of INS cells in the presence of an elevated fatty acid concentration is sufficient to promote β-cell death. INS cells were cultured for 2 h in medium containing 0.5% defatted BSA in the presence of 5 mM glucose (G5), 5 mM glucose plus 0.5 mM oleate (Ol), or 0.5 mM palmitate (Palm). Subsequently, media were removed, and incubation was continued for 34 h in fresh medium containing 5 mM glucose plus 0.5% defatted BSA. At the end of the incubation period, cells were harvested, and β-cell death was determined as described in A. Values shown are the mean ± SEM of three independent experiments. *, $P < 0.01$; **, $P < 0.005$ [*vs.* control (G5)].

when incubated in the absence of serum, and detachment markedly increased when either fatty acids or staurosporine were present. Cells tended also to shrink and round up in the presence of fatty acids, displaying a high nucleus/cytoplasm ratio and a typical morphology (Fig. 4A) of apoptotic cells (24, 25). The mean percentages ± SEM of cells displaying an apoptotic and/or necrotic phenotype were 2.5 ± 0.1 [5 mM glucose (G5)/FCS], 10.4 ± 0.5 (G5), 12.0 ± 0.9 (G25), 45.4 ± 5.4 (oleate; $P < 0.001$ *vs.* G5), 27.1 ± 2.8 (palmitate; $P < 0.001$ *vs.* G5), and 65.2 ± 6.2 (staurosporine; $P < 0.001$ *vs.* G5; n = 6). The number of cells presenting these phenotypic changes fits with the percentage of subdiploid cells determined by FACS analysis. In addition, palmitate and oleate caused chromatin condensation, as revealed by the positive staining with Hoechst dye (Fig. 4B). These DNA and chromatin alterations were accompanied by an increase in PARP cleavage (data not shown) in cells treated with oleate, palmitate, and staurosporine. Therefore, these phenotypic changes observed in the presence of fatty acids correspond to the induction of an apoptotic process.

Mechanisms of the apoptotic process induced by fatty acids

Previous studies in several cellular models documented that altered mitochondria function plays a prominent role in the induction of apoptosis under serum-starved conditions (14, 26–29). Mitochondria dysfunction can be assessed by several parameters, including superoxide production, loss of mitochondria membrane potential, cytochrome *c*, and AIF release.

Disruption of the membrane-associated electron transport chain leads to incomplete reduction of O_2 and production of

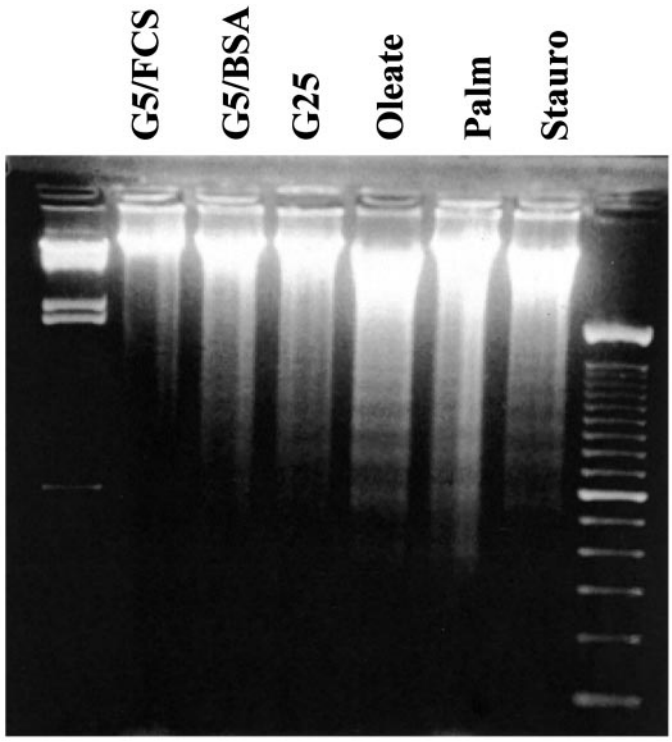

FIG. 3. Oleate and palmitate increase DNA fragmentation in serum-starved INS-1 cells. DNA ladder analysis was carried out in extracts of INS-1 cells cultured for 36 h in the presence of 5 mM glucose plus 10% FCS (G5/FCS) or in medium containing 0.5% defatted BSA with in 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose plus 0.5 mM oleate; 0.5 mM palmitate (Palm); and 0.2 μM staurosporine (Stauro). DNA was extracted, and 10 μg were resolved in a 1.5% agarose gel. DNA markers are a λ/*Hin*dIII digest (*left*) and a 100-bp DNA ladder (*right*). The gel shown is representative of three independent experiments.

reactive oxygen species, such as the superoxide radical. Serum removal considerably increased superoxide production, and oleate and palmitate caused a further rise in O_2^- production by β(INS)-cells (Fig. 5A; $P < 0.05$ *vs.* cells incubated with 5 mM glucose in the presence of BSA).

Consistent with the O_2^- measurements, serum removal caused a pronounced reduction of the mitochondrial membrane potential, as evaluated using rhodamine 123 (Fig. 5B). Both fatty acids had only a minor additive effect on the top of the serum removal condition ($P < 0.05$ *vs.* the G5/BSA condition).

The release of different proteins from mitochondria is implicated in the late stages of the apoptotic process (24, 27). Therefore, we determined whether cytochrome *c*, AIF, and caspase-2 relocate from the mitochondrial compartment to the cytosol in INS-1 cells exposed to fatty acids. Figure 6 shows that no measurable release of cytochrome *c* and AIF was observed when cells were incubated with 5 mM glucose in the absence or presence of FCS. High glucose caused a modest release of cytochrome *c*. However, both palmitate and mainly oleate induced a prominent release of cytochrome *c* and AIF to the cytosol similar to that observed with staurosporine. The release of cytochrome *c* in the presence of oleate (Fig. 7) and palmitate (not shown) was gradual over time, reaching a maximum after 30–36 h of incubation. On

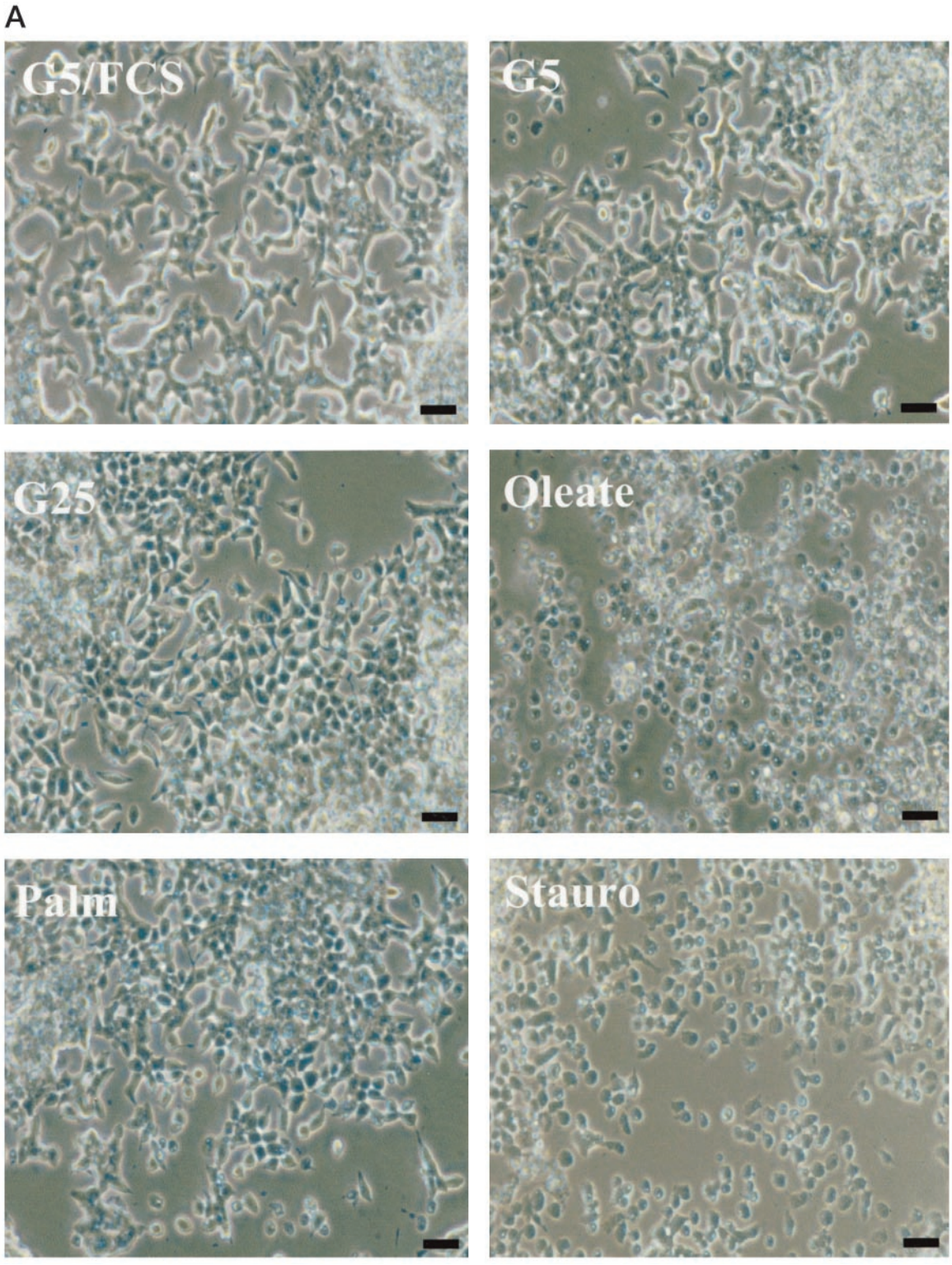

FIG. 4. A, Morphological analysis of INS cells cultured in the presence of various test substances. INS cells were cultured for 36 h with 5 mM glucose plus 10% FCS (G5/FCS) or with medium containing 0.5% defatted BSA in the presence of 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose plus 0.5 mM oleate, 0.5 mM palmitate (Palm), and 0.2 μM staurosporine (Stauro). Images of representative fields are captured at a magnification of ×40. *Bars*, 12 μm. B, Chromatin condensation analysis of INS cells determined by Hoechst 33342 staining. Cells were grown on poly-L-lysine-treated glass slides and incubated under the same conditions as described in A. Figures are representative of three independent experiments. Images are captured at ×40. *Bars*, 12 μm. *, Representative staining of mitosis; *arrow*, representative staining of condensed chromatin.

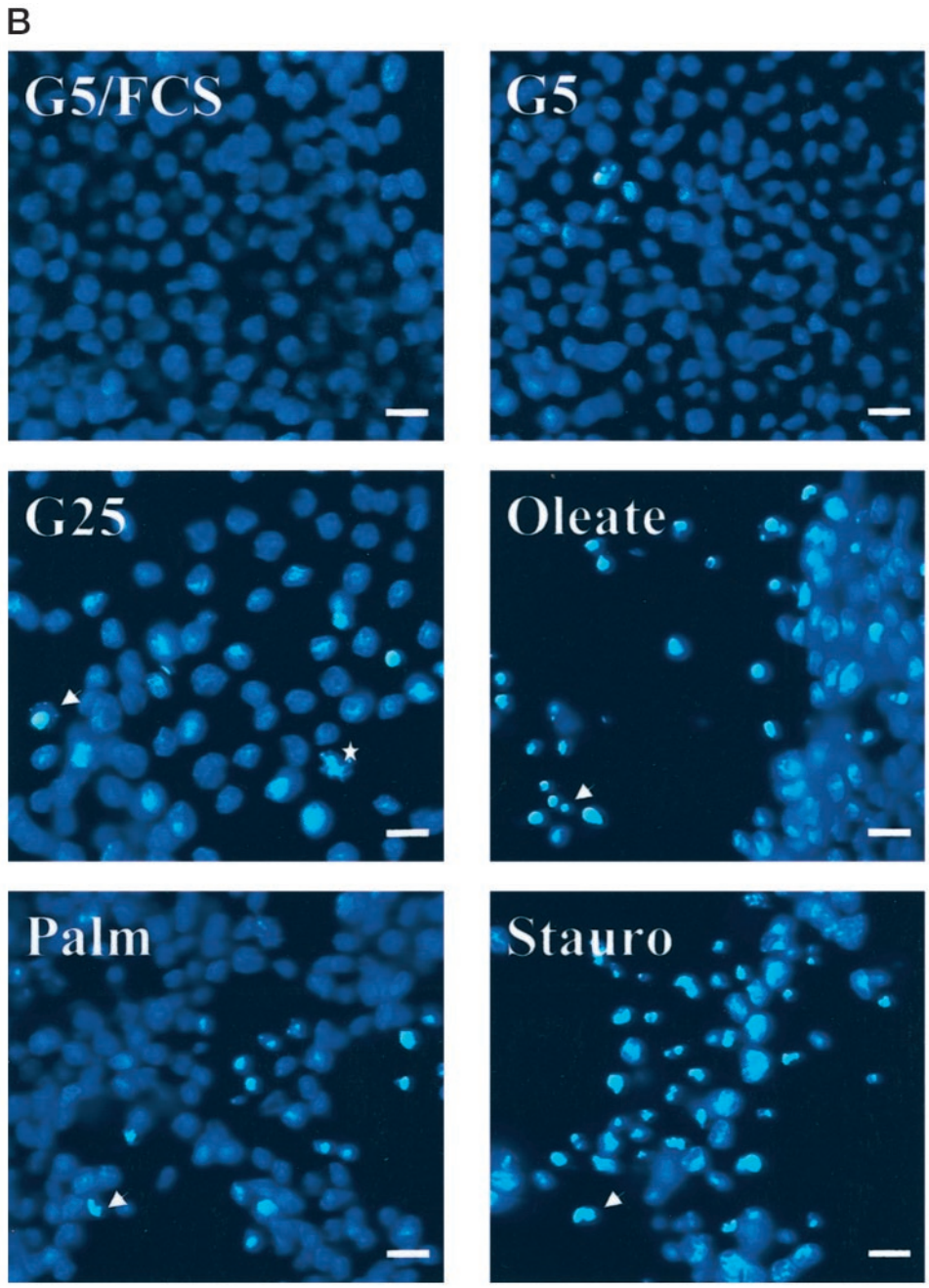

FIG. 4. Continued.

the other hand, AIF translocation from mitochondria in the presence of palmitate (not shown) and oleate (Fig. 7) was delayed with respect to that of cytochrome *c*, and the protein started to be detected in the cytosolic fraction only after 30–36 h of incubation.

Caspase-2 is an important zymogen distributed in cytosol and mitochondria. After specific stresses, mitochondrial caspase-2 as well as cytochrome *c* and AIF can be released from mitochondria to the cytosol, thus triggering apoptosis (30). Caspase-2 activation has been implicated in the induction of apoptosis by GTP depletion in HIT-T15 cells (31). In contrast to cytochrome *c* and AIF, caspase-2 was not apparently redistributed from mitochondria to cytosol when cells were incubated in the presence of fatty acids (Fig. 7). However, the amount of caspase-2 increased in the cytosolic fraction of cells incubated in the presence of oleate after a 30- to 36-h period (Fig. 7). Similar results were observed with palmitate, although the effect was somewhat smaller.

It has been described in human (32) and rat islets (6) incubated in the presence of fatty acids that the progression to apoptosis is associated with reduced mRNA levels of the antiapoptotic protein Bcl-2, although the expression level of the Bcl-2 protein was not measured in one of these studies (32). Immunoblot analysis revealed that the amount of the Bcl-2 protein remained relatively stable over a 36-h period in the presence of elevated glucose or fatty acids, decreasing only in staurosporine-treated cells (Fig. 8). Similar observations were made at 60 h (not shown). However, the proapoptotic protein

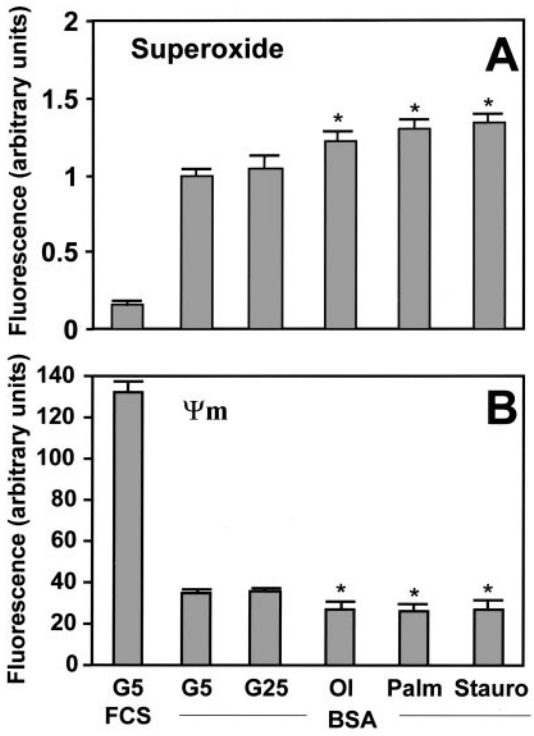

FIG. 5. Effect of serum starvation and elevated glucose and fatty acids on superoxide production and the mitochondrial membrane potential (ψm) of INS-1 cells. A, INS cells were cultured for 36 h in the presence of 5 mM glucose plus 10% FCS (G5/FCS) and 0.5% defatted BSA at 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose plus 0.5 mM oleate (Ol), 0.5 mM palmitate (Palm), and 0.2 μM staurosporine (Stauro). Cells were then detached from the flasks and incubated in suspension at room temperature with 1 μg/ml dihydroethidine for 2 min. Superoxide production was determined by the change in fluorescence from blue to red in an EPICS flow cytometer. B, For ψm determinations cells were harvested after 36-h culture and incubated in suspension at room temperature with 10 μg/ml rhodamine 123 for 5 min. ψm was assessed through rhodamine fluorescence measurements, determined in an EPICS cytometer. Values shown are the mean ± SEM of four independent experiments. *, $P < 0.05$ *vs.* G5/BSA.

Bax increased in cells incubated in the presence of oleate and to a lesser degree with palmitate (Fig. 8).

Several studies have provided evidence that NO production is involved in the apoptotic death of pancreatic β-cells induced by cytokines (1, 2) and possibly fatty acids as well, via iNOS induction (4). Oleate increased NO production ($P < 0.05$ *vs.* cells incubated in the presence of 5 mM glucose/BSA), further indicating that it causes oxidative stress (Fig. 9A). Palmitate had a modest, but significant, effect on NO production ($P < 0.05$ *vs.* G5/BSA condition). We also measured the expression level of iNOS. A mixture of three cytokines (IL-1β, IFNγ, and TNFα), known to be efficient in inducing iNOS gene in this cell type (33), was used as a positive control. The results shown in Fig. 9B indicate that the expression level of iNOS under basal conditions was below the detection limit of the Western blot and that fatty acids did not appear to induce iNOS.

Discussion

Elevated circulating fatty acids, such as occurs during fasting or the early stages of obesity, are not very toxic to the β-cell because the β-cell is expected to adapt via the induction of genes of the fat oxidation pathways, resulting in fat oxidation and lipid detoxification (13). However, it can be hypothesized that under conditions where the β-cell is stressed, such as high glucose, islet inflammation, and cytokine production by lymphocytes or oxidative stress, elevated fatty acids may become very toxic to the β-cell because they cannot be efficiently detoxified. With this background in mind we used serum removal as a model of stressed β-cell and studied the action of fatty acids under this condition. Interestingly, we found that serum removal causes oxidative stress and impairs β(INS-1)-cell mitochondrial function. In this respect it is established that the β-cell is particularly sensitive to oxidative stress, possibly because it contains low levels of oxygen radicals scavengers, such as superoxide dismutase, catalase, and glutathione peroxidase (34).

The mitochondrial-dependent apoptotic process can be divided into three phases: initiation, progression, and execution (25, 26). During the first phase, particular proapoptotic signals rise in the cell and act on mitochondria to alter the permeability of their membranes. Mitochondrial dysfunction can be monitored by several parameters, including overproduction of superoxide radical, as a result of respiratory chain failure, and loss of mitochondrial membrane potential (28). During the progression phase, which in this study spans from approximately 18–30 h, mitochondrial membrane permeabilization is achieved, allowing the gradual relocation of cytochrome *c* in the cytosolic compartment. The final execution phase, coincident with the 30- to 36-h period of this study, culminates with the maximum release of cytochrome *c* and AIF from mitochondria, the induction of mitochondrial-destabilizing proapoptotic factors (*i.e.* Bax), and the activation of catabolic proteases (such as cytosolic caspase-2) and nucleases (such as PARP) that are responsible for the DNA ladder and chromatin pattern observed in the programmed cell death process (31, 35–38). Taking into account this general knowledge of apoptosis, we conclude that serum deprivation predisposes INS-1 cells to enter into a preapoptotic stage. This is supported by the fact that under these conditions mitochondria are severely affected in terms of free radical production and membrane potential decay, both considered early markers in the apoptotic process (26–28). The low levels of subdiploid population in serum-free conditions as well as the undetectable amounts of cytochrome *c* and AIF release, condensed chromatin, and DNA laddering, are indicative that the progression to a further apoptotic stage was not accomplished, at least during the first 36 h of serum removal in the absence of fatty acids. However, the apoptotic programs appear to be executed when fatty acids are present under the same culture conditions. This is supported by the fact that late apoptotic events, such as increases in subdiploid cell population, morphological changes, cytochrome *c* and AIF detection in the cytosolic fraction, increased Bax and caspase-2 expression, PARP activation, chromatin condensation, and DNA breaks, are clearly monitored when fatty acids are present in the culture medium. Time dependence studies revealed that fatty acids cause an early release of cytochrome *c*, followed by AIF release and subsequently increased expression of caspase-2 in the cytosol. Increased caspase-2 levels in the cytosol do not

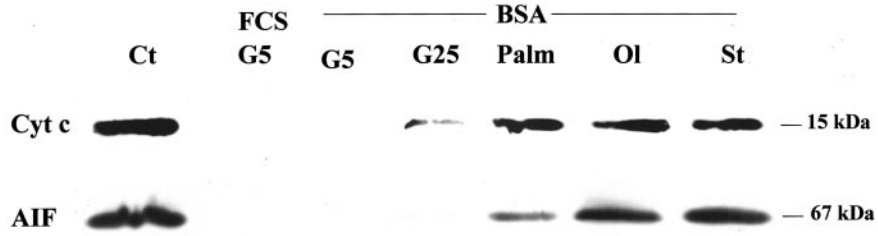

FIG. 6. Elevated fatty acids promote cytochrome *c* and AIF release in INS-1 cells. Cytosolic fractions were obtained from treated cells after 36-h incubation as described in *Materials and Methods*. Aliquots of 20 μg protein were resolved on a 12.5% SDS-polyacrylamide gel, blotted, and revealed by a monoclonal cytochrome *c* antibody or an AIF polyclonal antibody. Experimental conditions were 5 mM glucose plus 10% FCS (G5/FCS), and 0.5% defatted BSA at 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose with 0.5 mM palmitate (Palm), 0.5 mM oleate (Ol), and 0.2 μM staurosporine (St). Ct, Purified cytochrome *c* control from bovine heart and Jurkat whole cell lysate control for AIF. The immunoblots shown are representative of four independent experiments.

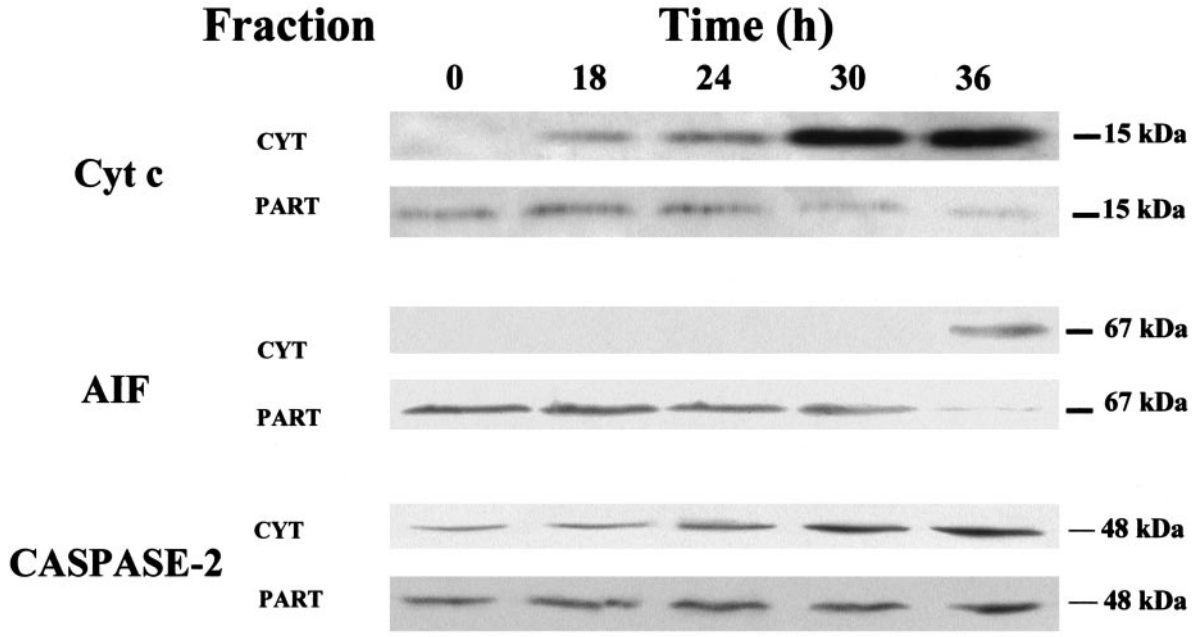

FIG. 7. Time course of cytochrome *c* and AIF release from mitochondria and cytosolic caspase-2 accumulation in INS-1 cells incubated in the presence of oleate. Cells were cultured for the indicated times in the presence of 0.5 mM oleate. Subcellular fractionation was performed as described in *Materials and Methods*. Aliquots of 15 μg protein for the cytosolic (CYT) fraction and 10 μg protein for the particulate (PART) fraction were resolved on 12.5% SDS-polyacrylamide gels, blotted, and revealed using cytochrome *c*, AIF, and caspase-2 antibodies. The immunoblots shown are representative of three independent experiments.

appear to result from its release from mitochondria. The mechanisms underlying this phenomenon remain to be defined.

How do fatty acids considerably accelerate the apoptotic process initiated by serum deprivation? This is a complex question in view of the wide variety of effects that these nutrients have on β-cell function. Nevertheless, there are several possibilities. The first is through NO production. Previous reports from Unger's group (4) using isolated islets from the ZDF rat model have suggested that elevated concentrations of fatty acids [mixture oleate/palmitate (2:1) in the presence of 2% BSA, 10% FCS, and 8 mM glucose] promote β-cell apoptosis via iNOS induction and NO production. Consistent with this possibility we found that NO release is increased by both palmitate and oleate. However, iNOS was not detected under basal conditions nor apparently induced by fatty acids in INS cells despite a robust

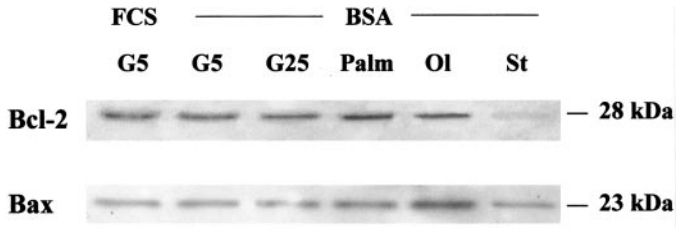

FIG. 8. Elevated fatty acids do not affect Bcl-2 levels, but induce Bax accumulation in INS-1 cells. Cellular extracts were obtained at 36 h from treated cells as described in *Materials and Methods*. Aliquots of 20 μg protein were resolved on 12.5% SDS-polyacrylamide gel, blotted, and revealed by Bcl-2 or Bax polyclonal antibodies. Experimental conditions were 5 mM glucose plus 10% FCS (G5/FCS), and 0.5% defatted BSA at 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose with 0.5 mM palmitate (Palm), 0.5 mM oleate (Ol), and 0.2 μM staurosporine (St). The immunoblots shown are representative of three independent experiments.

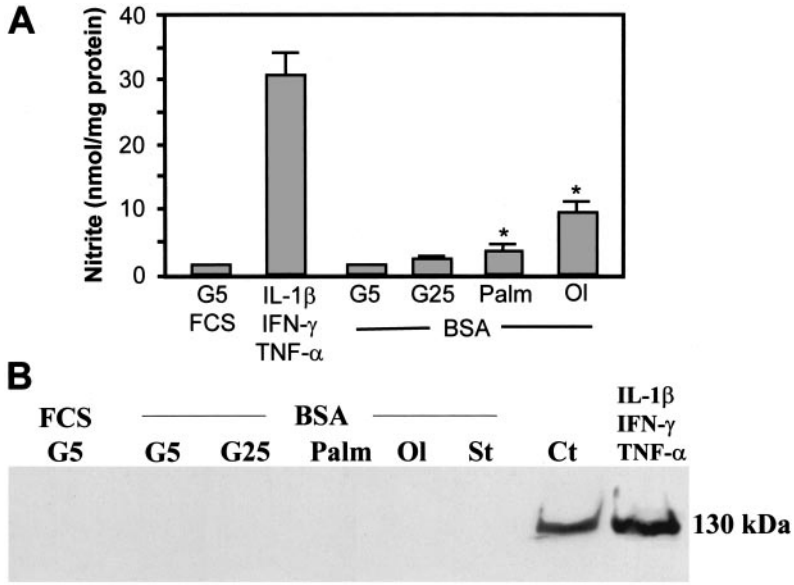

FIG. 9. Effect of various experimental conditions on NO production and iNOS protein expression level in INS cells. A, Cells were cultured for 36 h in the presence of 5 mM glucose plus 10% FCS (G5/FCS) in the presence or absence of cytokines (IL-1β, TNFα, and IFNγ), and 0.5% defatted BSA at 5 mM glucose (G5), 25 mM glucose (G25), or 5 mM glucose plus 0.5 mM palmitate (Palm) and 0.5 mM oleate (Ol). NO was determined as nitrite as indicated in *Materials and Methods*. Values shown are the mean ± SEM of three independent experiments. *, $P < 0.05$ *vs.* G5/BSA. B, Cells were incubated for 24 h in the presence of different test substances. Aliquots of 25 μg protein were resolved on 7.5% SDS-polyacrylamide gels, blotted, and revealed using an iNOS polyclonal antibody. The immunoblot shown is representative of three independent experiments. Conditions were 5 mM glucose plus 10% FCS (G5/FCS), 0.5% defatted BSA with 5 mM glucose (G5), 25 mM glucose (G25), 0.5 mM palmitate (Palm), 0.5 mM oleate (Ol), 0.2 μM staurosporine (St), and 5 mM glucose, 10% FCS, and cytokines (IL-1β + IFNγ + TNF-α). Ct, Activated macrophage cell extract.

induction by cytokines. These results are in agreement with recent reports in rat and human β-cells (32, 39). Thus, we do not favor the view that iNOS induction is implicated in accelerated production of NO caused by fatty acids in INS-1 cells, but do not discount activation of the enzyme by fatty acids or an action of fatty acids on the constitutive isoforms of the enzyme (40).

The second possibility is ceramide synthesis, in view of the work by Shimabukuro *et al.* (7) in ZDF islets. This possibility is not favored because both palmitate and oleate are pro-apoptotic in serum-starved INS cells, yet only palmitate serves as a substrate for the *de novo* synthesis of ceramides (41, 42).

Third, the proapoptotic action of free fatty acids might in part be related to the induction of the immediate-early gene *nur-77*. Thus, a recent study has described translocation of the nuclear orphan receptor Nur-77 to mitochondria, triggering mitochondrial membrane permeabilization and apoptotic cell death (43). Interestingly, we showed that both palmitate and oleate are very efficient in inducing *nur-77* gene expression in isolated islets and INS-1 cells (15).

A fourth possibility that we favor is a general toxicity of fatty acids, simply due to the fact that they cannot be oxidized in serum-starved cells because of the mitochondrial membrane potential collapse and mitochondrial function impairment. Thus, the fate of the excess free fatty acids in this condition is membrane binding as well as lipid esterification processes, leading to the accumulation of reactive long chain acyl-CoAs, acylcarnitines, lysophosphatidic acid, phosphatidic acid, and diacylglycerol (44–48).

In conclusion, serum deprivation is harmful for β-cell mitochondrial function, and fatty acids appear to be particularly efficient in accelerating the rate of apoptosis of β(INS-1)-cells with already altered mitochondrial function. The precise mechanisms involved in this toxic action of free fatty acids remain to be defined, but are probably multifactorial.

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