Effects of Conjugated Linoleic Acid and Stearic Acid on Apoptosis of the INS-1 β-cells and Pancreatic Islets Isolated from Zucker Obese (*fa/fa*) Rats

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ABSTRACT : To determine whether dietary fatty acids affect pancreatic β-cell function, the INS-1 β-cells and the pancreatic islets isolated from Zucker obese (*fa/fa*) rats were cultured with stearic acid and conjugated linoleic acid (CLA). As a result, DNA fragmentation laddering was substantially decreased in the INS-1 β-cells and the isolated pancreatic islets cultured with 2 mM CLA compared to those cultured with stearic acid. To investigate the mechanism by which CLA alleviates cell apoptosis under DNA fragmentation assay, we examined mRNA expressions of apoptosis-related proteins including Bax and Bcl-2 associated with cell death agonist and antagonist, respectively, in both INS-1 cells and islets cultured with 2 mM fatty acids. Bax mRNA expression was not altered by either stearic acid or CLA, whereas Bcl-2 mRNA expression was enhanced by CLA when compared to the stearic acid cultures. However, there were no changes in cell apoptosis and apoptotic-regulating gene products in either INS-1 cells or isolated islets treated with or without 2 mM CLA. It is concluded that CLA maintains β-cell viability via increased Bcl-2 expression compared to the stearic acid cultures, which may help to alleviate, at least somewhat, the onset of NIDDM in the physiological status. More detailed study is still needed to elucidate the effect of CLA on the prevention of fatty acid-induced β-cell apoptosis. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 7 : 1060-1065)***

Key Words : INS-1 β-cells, Pancreatic Islets, CLA, Apoptosis

INTRODUCTION

Conjugated linoleic acid (CLA), a group of positional and geometric isomers derived from linoleic acid, has attracted considerable attention over last decade due to its protective actions against cancer, atherosclerosis and obesity (Whigham et al., 2000). CLA naturally exists in meat and dairy products of ruminants and can also be synthesized from safflower oil or corn oil rich in linoleic acid (Chin et al., 1992; Wang et al., 2002). Recent evidence has indicated that CLA is clearly associated with fat reduction in pigs, rats and mice (Dugan et al., 1997; DeLany et al., 1999; Rahman et al., 2001; Dunshea et al., 2002). Moreover, improvement of glucose tolerance in ZDF rats has indicated that CLA might play an important role in the prevention and treatment of non insulin-dependent diabetes (NIDDM; Houseknecht et al., 1998). It has thus been suggested that a beneficial effect of CLA-mediated hypodystrophy is responsible for an improvement in and a prevention of insulin resistance (Tseboyama-Kasaoka et al., 2000). However, there is still a lack of evidence of the underlying mechanisms by which dietary CLA modulates diabetes.

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Received October 9, 2002; Accepted April 23, 2003

It is evident that pancreatic β-cell dysfunction is causally implicated in the development of NIDDM (Milburn et al., 1995). Recent studies have demonstrated that accumulated fatty acids altered pancreatic β-cell function by reducing a substantial number of β-cells from fat-laden islets through apoptosis (Lee et al., 1994; Shimabukuro et al., 1998b). This evidence shows that dietary fatty acids played a critical role in pancreatic islet dysfunction and subsequent NIDDM development. The direct application of CLA or stearic acid to the pancreatic islets would be able to indicate either a preventive or toxic effect on β-cell function.

Therefore, the present study was undertaken to evaluate the efficacy of dietary CLA on the maintenance of β-cell function using both the INS-1 cells and the pancreatic islets isolated from Zucker obese (*fa/fa*) rats, one of diabetic model animals associated with adipogenic NIDDM. Furthermore, our goal was to understand the mechanisms associated with the onset of pancreatic β-cell dysfunction from Zucker obese (*fa/fa*) rats. In an effort to demonstrate pancreatic β-cell function in response to dietary fatty acids, we examined DNA fragmentation laddering and mRNA expressions of Bcl-2 and Bax in both the INS-1 cell line and intact isolated islets.

MATERIALS AND METHODS

Experimental animals

Male, Zucker obese (*fa/fa*) rats were bred from our

colony originated from Charles River (USA) and maintained under a barrier system regulated temperature (23±2°C), humidity (50±10%) and light: dark cycle (light on 07:00-19:00 h). All rats were allowed free access to commercial lab chow and water. All animal experiments were approved by our institutional laboratory animal care and use committee and performed in compliance with committee guidelines.

Pancreatic islet isolation:

Briefly, we sacrificed 6-wk old Zucker obese (*fa/fa*) rats, prediabetic stage, to isolate the pancreatic islets immediately after anesthetizing with sodium pentobarbital (100 mg/kg) followed by deprived food for 6-8 h. The pancreatic duct in the opened abdominal cavity was clamped with a hemostat and expanded by injection of 10 ml HBSS (Gibco-BRL, NY). The pancreas was harvested, washed with HBSS and minced finely (1 mm) with scissors. The obtained tissues were treated with freshly prepared 2 ml HBSS containing collagenase P (BM, Germany; 1.6 mg/HBSS) at 37°C for 6 min and 30 sec and the suspended tissues were then poured into a sieve (400 μm) to eliminate undigested debris. After that, the digested islets were placed in an HBSS solution and washed several times by centrifugation. The resultant pellet was resuspended in a tube containing 35% Ficoll (Sigma, USA). The resuspended pellet was then applied to the gradient Ficoll solution consisting 23%, 20.5% and 11% Ficoll and centrifuged at 2,000 rpm for 20 min. Residual fat was removed from harvested islets by centrifugation-twice at 4,500×g for 10 min. To evaluate isolated islet purity, 500 μl of harvested islets were stained with the same volume of dye solution containing 1:2 ratio of diphenylthiocarbonate to HBSS and observed under a stereoscopic microscope (S1-Trw/acc, Olympus).

The INS-1 cell and pancreatic islet incubation

The INS-1 cells were cultured in RPMI-1640 medium containing 10% FBS, 50 μg/ml penicillin and 50 μg/ml streptomycin (Gibco-BRL, NY) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After preincubation, the cultured INS-1 cells reaching 80% confluency were incubated in the RPMI-1640 medium containing stearic acid (Sigma, USA) bound to 2% BSA and CLA to determine time- (0, 1, 3, 6 and 12 h) and dose- (0, 1, 2, 4 and 8 mM) dependent changes in DNA fragmentation. CLA containing isomers 40.88% of cis9/trans11 and 53.53% of *trans*10/*cis*12 was obtained from LiveMax (Korea). Approximately 300 islets per dish were cultured for 12 h in an M199 medium containing 10% FBS at 37°C in a 5% CO_2 humidified atmosphere. After 12 h preincubation, the isolated islets were incubated to determine the effects of 2 mM stearic acid bound to BSA and 2 mM CLA on time- (0, 3, 6 and 12 h) dependent changes in cell apoptotic index. To examine mRNA expressions of Bax and Bcl-2, the intact islets were cultured for 2 h with 2 mM stearic acid and CLA after 12 h preincubation.

DNA laddering assay

The INS-1 β-cells and the isolated pancreatic islets cultured with fatty acids were harvested by centrifugation at 1,000 rpm and PBS washing. The INS-1 cells (2×10^5 cells) and harvested islets (350-400) were suspended in 100 μl of lysis buffer containing 0.6% SDS and 10 mM EDTA (pH 7.5) and added NaCl to be 1 M of final concentration. After incubating overnight for 4°C, the resultant supernatant containing fragmented DNA was separated by centrifugation at 15,000 rpm. The resultant supernatant was treated with 10 μg/ml RNAase at 37°C for 40 min and added phenol/chloroform solution to remove protein. The pellets were then incubated with isopropanol and washed with 70% alcohol. After drying, the fragmented DNA fractions loaded in a buffer containing 30% glycerol, 0.25% BPB and 0.25% XC were determined by electrophoresis on 1.2% agarose gel and visualized laddering like appearance with EtBr.

Semiquantification of Bcl-2 and Bax mRNA

Total RNA was isolated from the INS-1 β-cells and pancreatic islets by the method of RNAsol B (Tel-Test, TX). The chilled lysate was transferred to a tube and added to 1/10 volumes of chloroform to remove protein extract. The aqueous phase was separated by centrifugation for 15 min at 15,000 rpm. Total RNA was precipitated with the same volume of isopropanol and centrifuged for 15 min at 15,000 rpm. The precipitated total RNA with 75% ethyl alcohol was dried and diluted with DEPC treated water. The concentration of total mRNA was determined by spectrophotometer and confirmed 18S and 28S on a 1.0% agarose gel stained with EtBr. Semiquantification of mRNA using RT-PCR was performed to quantify mRNA of Bcl-2 (349 bp) and Bax (306 bp). For synthesis of first strand cDNA, 5 μg of total RNA was incubated at 62°C for 10 min with 0.5 μg of oligo dT. The resulting solution was incubated at 42°C for 50 min in a reaction mixture containing 2.5 mM dNTP and 200 units reverse transcriptase (Gibco-BRL, NY). After that, 3.2 unit RNAase H was treated to remove RNA produced by cDNA for 30 min at 37°C. The amplification was performed for 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec and extension at 72°C for 7 min with PCR system in a reaction mixture containing 10 pmol primer, 0.5 μg cDNA, 2 mM dNTP and 1 unit Taq polymerase (BM, Germany). The products were confirmed on a 1.0% agarose gel stained

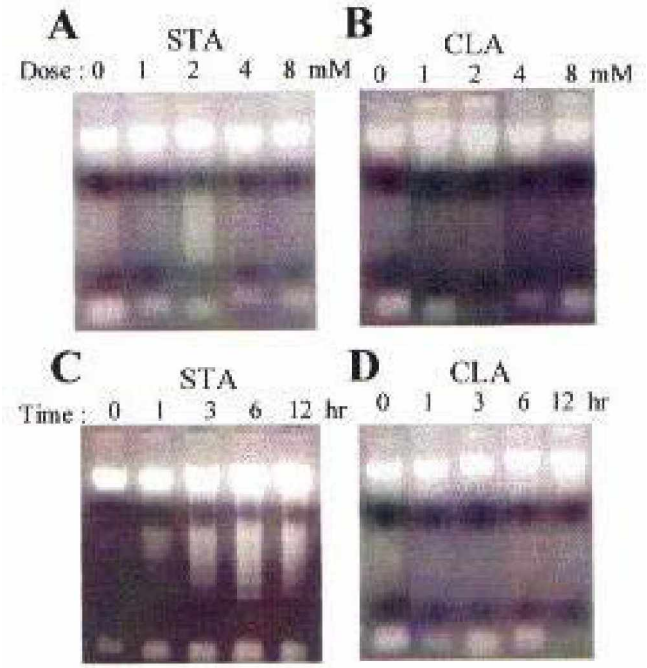

Figure 1. Effects of stearic acid (STA) and CLA on the INS-1 cell apoptosis: The INS-1 cells were cultured for 6 h with 0, 1, 2, 4 and 8 mM stearic acid (A) and CLA (B) to determine dose- dependent DNA fragmentation laddering. The INS-1 cells were cultured for 0, 1, 3, 6 and 12 h with 2 mM stearic acid (C) and CLA (D) to determine time-dependent DNA fragmentation laddering.

with EtBr. The cDNA primers used to amplify each gene were as follows: 5'-TGGCGATGAACTGGACAACAAC-3' and 5'-CCCGAAGTAGGAAAGGAGGGC-3' for Bax (GeneBank U52966) and 5'-CACCCCTGGCATCTTCTC CT-3' and 5'-GTTGACGCTCCCCACACACA-3' for Bcl-2 (GeneBank Y12882). The internal standard primers were 5'-GTGGGGCGCCCCAGGCACCAGGGC-3' and 5'-CT CCTTAATGTCACGCACGATTTC-3' for β-actin. We determined the number of cycles and kept the products within the exponential phase. The density of each product in Agarose gel electrophoresis (1%) containing EtBr was quantified using a densitometer (Kodak EDAS 120, CT). Levels of all mRNAs were expressed as the ratio of signal intensity for genes relative to that for β-actin.

Statistical analysis

One-way analysis of variance, followed by Tukey's multiple range tests, was applied to analyze the results obtained from treatment of fatty acids. The level of probability for statistical difference was established at $p<0.05$.

RESULTS

Effects of dose- and time- dependences of fatty acids on apoptosis in the INS-1 cells

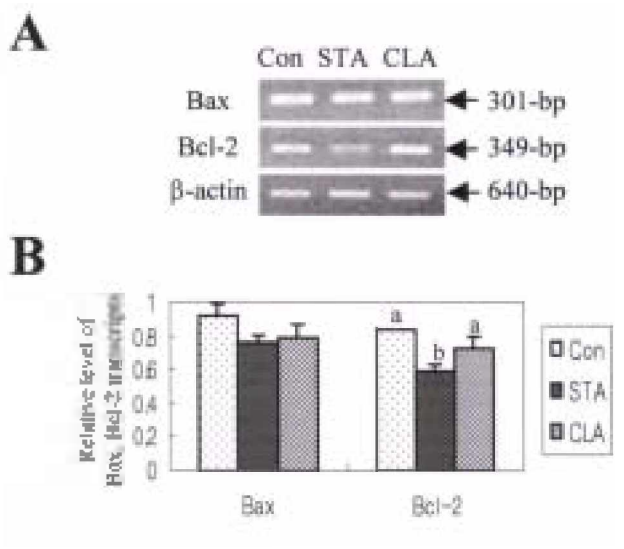

Figure 2. Semiquantification of mRNA expressions of Bax and Bcl-2 in the INS-1 cells cultured for 6 h without (Con) or with 2 mM of stearic acid (STA) and CLA. Each bar presents the mean±SD (n=3) of Bax and Bcl-2/β-actin mRNA ratio. ^{a,b} Values with different superscript differ significantly ($p<0.05$) among treatments.

To determine whether levels of stearic acid and CLA affect viability of pancreatic β-cells, the INS-1 cells were cultured with 0, 1, 2, 4 and 8 mM of these fatty acids for 6 h (Figure 1). The analysis of the effect of dose- dependent stearic acid on DNA fragmentation laddering in the INS-1 cells indicated that the most evident cell apoptotic effect was observed at 2 mM concentration of stearic acid (Figure 1, A). However, there was no change in DNA fragmentation laddering in the INS-1 cells treated with CLA with changing levels of concentrations (Figure 1, B). Thereafter, to verify the effect of time-dependence on β-cell viability, the INS-1 cells treated with 2 mM of steraic acid and CLA were cultured at various time intervals ranging from 0, 1, 3, 6 and 12 h after preincubation. The INS-1 cells treated with 2 mM stearic acid provided evidence for a time-dependent increase in DNA fragmentation laddering until 6 h after incubation (Figure 1, C). However, in response to CLA, we did not observe a time-dependent alteration in DNA fragmentation laddering (Figure 1, D). To investigate the mechanism by which dietary fatty acids induce cell death as indicated by DNA fragmentation assay, we examined expressions of apoptosis-related proteins including Bax and Bcl-2 in the INS-1 cells cultured with 2 mM fatty acids for 6 h (Figure 2). Bax mRNA expression was not markedly altered by either stearic acid or CLA, whereas Bcl-2 mRNA expression was noticeably increased by CLA but not by stearic acid. Apoptotic gene expression of the INS-1 cells cultured between the absence and presence of CLA was not changed.

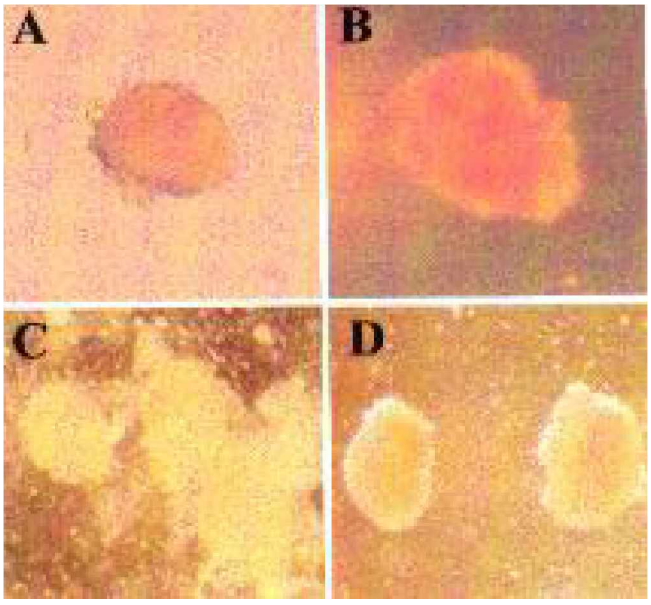

Figure 3. Photomicrographs (10×) representing freshly isolated pancreatic islets (A) and stained with diphenylthiocarbonate (B). Features of the isolated pancreatic islets cultured with 2 mM of stearic acid (C) and CLA (D) for 12 h.

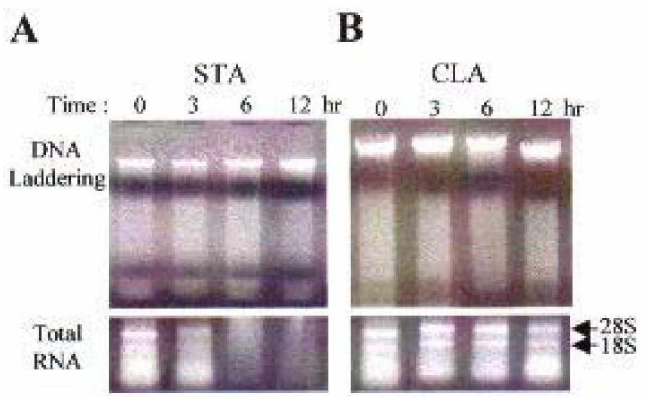

Figure 4. Effects of stearic acid (STA) and CLA on apoptosis of the pancreatic islets isolated from Zucker obese (*fa/fa*) rats. The intact pancreatic islets were cultured for 0, 3, 6 and 12 h with 2 mM stearic acid (A) and CLA (B) after 12 preincubation to determine time-dependent DNA fragmentation laddering and recovery of total RNA.

Effects of fatty acids on apoptosis in isolated pancreatic islets

To compare the morphological appearance in the pancreatic islets isolated from Zucker obese (*fa/fa*) rats in response to dietary fatty acids, we cultured the intact islets in the presence of 2 mM stearic acid and CLA for 6 h (Figure 3). The intact pancreatic islets cultured with 2 mM stearic acid had a larger small cluster around the cultured islets with apparently more loss of islet feature integrity compared to those cultured in CLA (Figure 3. C and D).

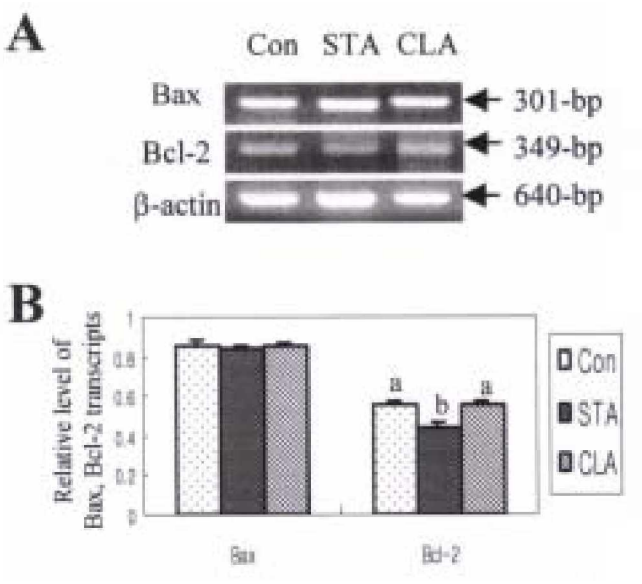

Figure 5. Semiquantification of mRNA expressions of Bax and Bcl-2 in the pancreatic islets cultured without (Con) or with 2 mM of stearic acid (STA) and CLA. Pooled pancreatic islets isolated from fifteen Zucker obese (*fa/fa*) rats were evenly divided into separate dished and about 350-400 islets per dish were incubated for 2 h with 2 mM stearic acid (STA) and CLA after 12 h preincubation. Replicates (n=3) mean the number of separated dishes culturing with 2 mM corresponding fatty acids. Each bar presents the mean±SD (n=3) of Bax and Bcl-2/β-actin mRNA ratio. $^{a, b}$ Values with different superscript differ significantly ($p<0.05$) among treatments.

This abnormality in the isolated islets in response to stearic acid could be associated with the incidence of pancreatic β-cell death. To assess the effect of time course dependence of 2 mM stearic acid and CLA on the pancreatic islets, DNA fragmentation assay was performed at various time intervals ranging from 0, 3, 6 and 12 h incubation with fatty acids after 12 h preincubation (Figure 4). Our observation indicated that DNA fragmentation of the pancreatic islets cultured with 2 mM stearic acid appeared to increase in a time-dependent manner until 6 h after incubation. Furthermore, recovery of total mRNA turned out to be undetectable as early as 3 h after treatment of stearic acid in the intact pancreatic islets (Figure 4. A). In contrast, the pancreatic islets cultured with 2 mM CLA indicated that DNA fragmentation laddering and recovery of total RNA were not affected by increased incubation time (Figure 4. B). Finally, we cultured the isolated islets for 2 h in the presence of 2 mM stearic acid and CLA, respectively, in order to examine mRNA expressions of Bcl-2 and Bax protein associated with cell death agonist and antagonist, respectively (Figure 5). A significant increase in Bcl-2 mRNA expression was observed in the islets cultured with CLA over those cultured with stearic acid. However, Bax

mRNA expression was unaffected by both stearic acid and CLA.

DISCUSSION

The dietary fatty acid, CLA, has been reported as a beneficial component associated with normalizing glucose tolerance, lowering plasma free fatty acids (FFA) and preventing insulin resistance in humans and rodents (Houseknecht et al., 1998; McCarthy, 2000; Whigham et al., 2000). Recent sophisticated work has demonstrated that pancreatic β-cell abnormality and cell death were observed just before and after the onset of NIDDM from a model animal of obesity and diabetes due to deposited pancreatic FFA (Lee et al., 1994; Roche et al., 2000). Therefore, to investigate the efficacy of dietary fatty acids on diabetes, we directly applied stearic acid and CLA to both the INS-1 cells and pancreatic islets isolated from Zucker obese (*fa/fa*) rats.

First, using the pancreatic β-cell line INS-1, DNA fragmentation analysis, commonly used for apoptosis detection, showed that the most evident cell apoptotic effect was observed among the β-cells cultured with 2 mM stearic acid for 6 h. The dose of stearic acid exceeding 2 mM (4 and 8 mM) did not show a dose-dependent increase in DNA fragmentation laddering, indicating that a measurable level of apoptosis from the INS-1 cells was correlated with substrate levels. It seemed that the cultured INS-1 cells exceeding 2 mM stearic acid were arrested by extrinsic factors, not intrinsic factors, although a conclusive evidence was needed in order to show whether a cell death process of the INS-1 cells exceeding 2 mM stearic acid occurred by apoptotic mechanism or not. Cattan et al. (2001) showed that DNA fragmentation from isolated islets was no longer detectable after 48 h culture, although DNA laddering was apparent from the islets cultured for 24 h. They also illustrated that the islets cultured for 48 h lost cell integrity. Plasma FFA levels ranging from 1.5 to 2 mM caused the onset of NIDDM in Zucker rats (Lee et al., 1994). The pancreatic islets cultured with a medium containing 2 mM FFA (oleate/palmitate, 2:1) exhibited significantly lower levels of glucose-stimulated insulin secretion (Lee et al., 1994), suggesting that this level of FFA was optimal for testing lipotoxicity of the isolated islets. Thereafter, combining the information from the above study with our result, we applied 2 mM stearic acid and CLA to the isolated islets. The intact pancreatic islets cultured with 2 mM stearic acid showed significant cell apoptosis and total RNA loss as early as 3 h after treatment with fatty acid. Similar to our study using the pancreatic islets, palmitic acid gave rise to a remarkable increase in DNA fragmentation compared to palmitoleic acid (Maedler et al., 2001). In contrast, the cultured INS-1 cells and the islets with 2 mM CLA for various time intervals exhibited no apparent time- or dose-dependent DNA fragmentation changes compared to those cultured with 2 mM stearic acid. It was clear from our observations that CLA did not induce β-cell apoptosis for a prolonged incubation time in contrast to stearic acid.

To elucidate a marked cell death difference in response to fatty acids, we investigated cell apoptotic-regulating gene products involved in a cell death program in both the INS-1 cells and the pancreatic islets. The Bcl-2 protein, mainly located in the integral membranes of mitochondria and endoplasmic reticulemn, is recognized as one of the most important antiapoptotic genes preventing cell death (Campani et al., 2001). The Bax protein, another transmembrane molecular, has been involved in causing cell death (Campani et al., 2001). In our data, Bcl-2 protein mRNA was highly expressed in both the INS-1 cells and the intact islets cultured with CLA as opposed to those cultured with stearic acid. This result provides evidence that lower DNA fragmentation laddering in the pancreatic islets by CLA was partially due to the activated antiapototic protein, Bcl-2. Bcl-2 protein expression was significantly reduced in the islets of ZDF (*fa/fa*) rats when compared to that of ZDF (+/+) rats at the onset of NIDDM, indicating that Bcl-2 protein is an important pancreatic β-cell death antagonist (Shimabukuro et al., 1998a). FFA was responsible for the induction of pancreatic β-cell death via reduced Bcl-2 protein in the cultured normal islets (Shimabukuro et al., 1998a). Pancreatic β-cell lipotoxicity occurring by FFA was directly associated with de novo ceramide formation and nitric oxide (NO), consequently leading to diabetic pathogenesis in rats (Shimabukuro et al., 1997; Shimabukuro et al., 1998a). Contradicting this study, FFA-induced apoptosis of the pancreatic islets could not be correlated to inducible NO synthase and NO, although enhanced β-cell apoptosis by palmitic acid was mainly due to ceramide formation in the islets (Cnop et al., 2001). In addition, the preventive effect of CLA on NIDDM was mediated through enhanced adipogenic factors including peroxisome proliferator-activated receptorγ (PPARγ) and, as a consequence, decreased circulatory FFA (Zhou et al., 1998). From their observations, it may be postulated that the preventive effect of CLA on pancreatic β-cells apoptosis is, at least somewhat, associated with increased removal of FFA via up regulation of adipogenic genes. A remarkably increased Bcl-2 mRNA expression by CLA over that of stearic acid suggests that CLA alleviates apoptosis in normal islets. Several studies have demonstrated that CLA noticeably reduces body fat deposition in mice, rats and pigs (Houseknecht et al., 1998; Park et al., 1999). It was also reported that dietary CLA had statistically lower plasma FFA and glucose (Rahman et al., 2001), suggesting that CLA can play a potential antidiatetic role because of

the presence of higher FFA, apparently promoting β-cell deaths.

In conclusion, the present study using INS-1 cells and intact islets from Zucker obese (*fa/fa*) rats shows evidence that CLA is, at least somewhat, associated with the maintenance of β-cell function via increased Bcl-2 protein expression when compared to that of stearic acid. Further detailed study is still needed to determine whether CLA has a direct therapeutic effect on impaired pancreatic β-cell function.

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