We reported that the lipoapoptosis of beta-cells observed in fat-laden islets of obese fa/fa Zucker Diabetic Fatty (ZDF) rats results from overproduction of ceramide, an initiator of the apoptotic cascade and is induced by long-chain fatty acids (FA). Whereas the ceramide of cytokine-induced apoptosis may be derived from sphingomyelin hydrolysis, FA-induced ceramide overproduction seems to be derived from FA. We therefore semi-quantified mRNA of serine palmitoyltransferase (SPT), which catalyzes the first step in ceramide synthesis. It was 2-3-fold higher in fa/fa islets than in +/+ controls. [3H]Ceramide formation from [3H]palmitate was not fully explained by the foregoing studies. Because the high triglyceride (TG) content of these islets provided an excellent substrate for de novo ceramide synthesis rather than by causing sphingomyelin hydrolysis to ceramide, we tested this possibility. We studied [3H]ceramide formation from [3H]palmitate and observed a marked increase in islets from fa/fa ZDF rats (4). This overproduction of ceramide was blocked with fumonisin-B1, an inhibitor of ceramide synthetase (8), which also blocked the apoptosis (4). Thus de novo synthesis of ceramide appeared to be important in fatty acid-induced apoptosis.

Recent studies from this laboratory have demonstrated that long-chain fatty acids induce apoptosis of the beta-cells of obese ZDF rats (4). Furthermore, the evidence pointed to de novo ceramide synthesis as a major factor in the lipoapoptosis (4). Obese ZDF rats are homozygous for the fa mutation (fa/fa), a Gln-269 → Pro substitution in the leptin receptor (OB-R) (5, 6). The lipid content of their islets is markedly elevated because of complete unresponsiveness of their fat-laden islets to the lipopinic action of leptin (7). We theorized that the overproduction of ceramide was somehow linked to the overproduction of fat.

Because each molecule of ceramide contains two molecules of long-chain fatty acids (FA), it seemed likely that fatty acids stimulated ceramide-mediated apoptosis by providing excess substrate for de novo ceramide synthesis rather than by causing sphingomyelin hydrolysis to ceramide. To test this possibility, we studied [3H]ceramide formation from [3H]palmitate and observed a marked increase in islets from fa/fa ZDF rats (4). This overproduction of ceramide was blocked with fumonisin-B1, an inhibitor of ceramide synthetase (8), which also blocked the apoptosis (4). Thus de novo synthesis of ceramide appeared to be important in fatty acid-induced apoptosis.

However, the mechanism of de novo ceramide overproduction was not fully explained by the foregoing studies. Because the high triglyceride (TG) content of these islets provided an expanded source of palmitoyl-CoA for de novo ceramide formation (4), we considered an increase within the metabolic pathway of ceramide synthesis. The first step in the pathway involves serine palmitoyltransferase (SPT), the enzyme that catalyzes the condensation of palmitoyl-CoA and serine to form dehydro sphinganine, a precursor of sphingosine (9, 10); sphingosine is then acylated to form ceramide (11, 12). The following study was designed to determine whether there is an increase in SPT activity and, if so, whether fatty acid-induced ceramide-mediated apoptosis of beta-cells in islets of ZDF rats could be blocked by inhibiting SPT activity and if this prevents the partial destruction of beta-cells that accompanies this form of obesity.

**MATERIALS AND METHODS**

**Animals**—Lean wild-type (+/+ ) male ZDF rats and obese homozygous (fa/fa) male ZDF rats were bred in our laboratory from ZDF(Drft-F10) rats originally purchased from Dr. R. Peterson (University of Indiana School of Medicine, Indianapolis, IN). In some rats (n = 6), 25 mg/kg/day of l-cycloserine (freshly dissolved in phosphate-buffered saline (PBS)) was injected intraperitoneally for 2 weeks. In controls (n = 6), only PBS was given.

**Islet Isolation and Culture**—Pancreatic islets were isolated by the method of Naber et al. (13) with modifications (14). Isolated islets were cultured as described previously (14). In some experiments, islets were cultured with or without 1 mM 3-mercaptopropanesulfonate (Sigma) in 2% bovine serum albumin in the absence or presence of 20 ng/ml recombinant mouse leptin (kindly provided by Ron Chance, Ph.D., Lilly), or 10 μM Triacsin-C (Biomol Research Laboratories, Plymouth Meeting, PA).

**De Novo Ceramide Synthesis From [3H]Serine**—Groups of 100–200
islets were cultured for 2 days with [3H]serine (Amersham Pharmacia Biotech) in the absence or presence of 10 μM Triacsin-C or 2 mM L-cycloserine (Sigma). Lipids were extracted as described (15) with modification (7). Lipid extracts dissolved in a volume of 80 μl of chloroform:methanol:water (65:25:4, v/v/v). The radioactive spot corresponding to [3H]ceramide was counted as described (4).

Semiquantitation of SPT mRNA by Reverse Transcription Polymerase Chain Reaction—SPT mRNA expression was analyzed in cultured islets using the reverse transcription polymerase chain reaction protocol described previously in detail (14). Briefly, total RNA was extracted using TRIzol isolation kit (Life Technologies) and treated with RNase-free DNase. First-strand cDNA was obtained using a first strand cDNA synthesis kit (CLONTECH). Primers used to amplify SPT and β-actin cDNA were: 5′-GGTGTGGGCTGCTGATGA-3′ (1501–1520) and 5′-AACCTCTTCCCAAAACTGA-3′ (2031–2050) for SPT (GenBank™ accession number X95641, 550-base pair fragment) and 5′-TGTGAAACCTGGACGATATGG-3′ (1552–1575) and 5′-GATC TTGATCTTCATGGTGCTAGG-3′ (2991–2844) for β-actin (GenBank™ accession number J00691, 764-base pair fragment). The products were electrophoresed on a 1.2% agarose gel. After transferring to a Hybond-N nylon membrane (Amersham Pharmacia Biotech), DNA samples were electrophoresed on a 1.2% agarose gel. After transferring to a Hybond-N nylon membrane (Amersham Pharmacia Biotech), DNA samples were electrophoresed on a 1.2% agarose gel. After transferring to a Hybond-N nylon membrane (Amersham Pharmacia Biotech), DNA samples were electrophoresed on a 1.2% agarose gel. After transferring to a Hybond-N nylon membrane (Amersham Pharmacia Biotech), DNA samples were electrophoresed on a 1.2% agarose gel. After transferring to a Hybond-N nylon membrane (Amersham Pharmacia Biotech), DNA samples were electrophoresed on a 1.2% agarose gel.

DNA Fragmentation Assay—DNA fragmentation was assayed by a modification (4) of the method of Duke and Sellins (17). Groups of ~ 200 islets were washed twice with cold PBS and suspended in 100 μl of PBS (10 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0). Islets were then homogenized every 5 min by gentle pipetting. Incubation was carried out in an ice bath for 20 min. After centrifugation for 20 min at 4 °C (14000 × g), the supernatant containing fragmented (soluble) DNA was transferred to another tube. Lysis buffer (100 μl) was added to the pellet containing insoluble DNA. Both samples were treated with RNase A (0.5 mg/ml) for 1 h at 37 °C and then with Proteinase K (Sigma, 0.4 mg/ml) for 1 h at 37 °C. After adding 20 μl of 5 M NaCl and 120 μl of isopropyl alcohol, the samples were incubated overnight at -20 °C, and the DNA concentrations were measured by the method of Hopcroft et al. (18). Fragmented DNA was calculated as 100% × soluble DNA/soluble + insoluble DNA). The soluble fraction of DNA was determined by electrophoresis on 1.5% agarose gel and has a ladder-like appearance.

Statistical Analysis—All values shown are expressed as mean ± S.E. Statistical analysis was performed by unpaired Student’s t test or by one-way analysis of variance.

RESULTS

Comparison of Serine Palmitoyltransferase mRNA—To determine whether the increase in de novo ceramide synthesis in fa/fa islets (4) was the consequence of overexpression of SPT, its mRNA was semiquantitated by reverse transcription-polymerase chain reaction. At 7 and 14 weeks of age, the SPT/β-actin mRNA ratio was, respectively, 2 and 3 times greater than that of +/+ controls (Fig. 1).

Comparison of Ceramide Synthesis from Serine—To determine whether the increase in SPT mRNA was associated with an increase in de novo ceramide formation from serine, we compared the rate of [3H]ceramide formation from [3H]serine in +/+ and fa/fa ZDF islets (Fig. 2). The rate of [3H]ceramide formation in fa/fa islets was 2.2 times that of +/+ controls islets at 7 weeks of age and 3.5 times greater at 14 weeks of age. Thus, there was enhancement of ceramide synthesis from serine.

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in +/+ and fa/fa islets, evidence that the increase in synthetic activity was limited to the serine → ceramide pathway.

Effects of Metabolic Blockers on Ceramide Synthesis—For additional evidence that the increased ceramide formation in fa/fa islets reflected de novo ceramide formation, we prevented fatty acyl-CoA formation in fa/fa ZDF islets by culturing them in 10 μM Triacsin-C, which blocks fatty acyl-CoA synthetase (19, 20), or with 2 mM L-cycloserine, which blocks SPT activity (21). As shown in Fig. 3, both completely blocked the formation of [3H]ceramide from [3H]serine. This indicated that L-cycloserine treatment of 7-week-old prediabetic fa/fa rats for 2 weeks on [3H]ceramide formation from [3H]serine in islets. Results in +/+ islets are shown for reference purposes. Bars represent the mean ± S.E. of arbitrary densitometric units (n = 3). *, p < 0.01 versus 0 mM FA.

**FIG. 6.** Effects of L-cycloserine treatment of 7-week-old prediabetic fa/fa rats for 2 weeks on [3H]ceramide formation from [3H]serine in islets. Results in +/+ islets are shown for reference. Bars represent the mean ± S.E. of arbitrary densitometric units (n = 3). *, p < 0.01 versus +/+; †, p < 0.05 versus fa/fa islets without cycloserine. B, Effects of in vivo L-cycloserine treatment of prediabetic fa/fa rats on DNA fragmentation in islets. +/+ islets are shown for a reference. Bars represent the mean ± S.E. of arbitrary densitometric units (n = 3). *, p < 0.01 versus +/+; †, p < 0.05 versus fa/fa islets without cycloserine.

**TABLE II**

**Concentration relationship of FFA to DNA fragmentation (% total DNA) in islets of prediabetic ZDF rats (n = 3)**

| FFA (mM) | % DNA laddering |
|----------|-----------------|
| 0        | 7.2 ± 0.24      |
| 0.1      | 7.0 ± 0.93      |
| 0.5      | 13.7 ± 3.23     |
| 1        | 19.2 ± 1.18     |

**FIG. 7.** A possible mechanism for increased ceramide synthesis in islets of fa/fa ZDF rats. SPT, serine palmitoyltransferase. Arrow width indicates putative enzyme activity. Only SPT was actually quantified; the width of arrows is inferred from the metabolic studies of Fig. 2.

**FIG. 5.** Effects on SPT mRNA expression of 1 mM FA with or without 20 ng/ml recombinant leptin in islets of obese diabetic fa/fa ZDF rats that overexpress either OB-Rb (AdCMV-OB-Rb) or β-galactosidase (AdCMV-β-gal). Representative blots are displayed. Bars represent the mean ± S.E. of arbitrary densitometric units (n = 3). *, p < 0.01 versus 0 mM FA; †, p < 0.05 versus 1 mM FA.

**TABLE I**

**Effect of recombinant leptin on islet triglyceride content (ng/islet)**

Data are the mean ± S.E. of three-four samples. *, p < 0.01 versus 0 leptin.

| Fatty acids (mM) | FA (ng/ml) | 0 | 0.1 | 0.5 | 1 |
|-----------------|------------|---|-----|-----|---|
|                   | Leptin (ng/ml) | 0 | 20 | 20 | 20 |
| AdCMV-β-gal      | 48 ± 0.1    | 46 ± 0.1 | 155 ± 9 | 146 ± 8 |
| AdCMV-OB-Rb      | 50 ± 0.2    | 27 ± 0.2* | 161 ± 12 | 52 ± 7* |

**FIG. 4.** Effects of 1 mM FA on SPT mRNA expression in islets from 7-week-old lean +/+ and obese fa/fa ZDF rats. Representative blots are displayed. Bars represent the mean ± S.E. mRNA ratio of SPT/β-actin (n = 3). *, p < 0.01 versus 0 mM FA.

**Effects of Long-chain Fatty Acids on SPT mRNA—**Next, we attempted to identify the cause of the overexpression of SPT. Based on the mutation in the leptin receptor, the most plausible explanation for ceramide overproduction seemed to be either lack of a direct inhibitory action of leptin on ceramide synthesis or an indirect effect resulting from the excess lipid content of these islets. Because leptin swiftly depletes lipid content in leptin-responsive islets (7), it would be next to impossible to distinguish between direct lowering of SPT expression by leptin and indirect lowering by leptin-induced reduction in lipids. Therefore, we studied the effect of 1 mM FA on SPT expression in +/+ and fa/fa islets. In both groups of islets, FA caused a significant increase in SPT/β-actin mRNA ratio (Fig. 4). However, the base-line SPT expression (without added FA) in the fa/fa islets was 90% higher, and the fatty acid-induced increment in SPT expression (above the base line) was 60% higher in fa/fa islets than in control +/+ islets. Both the base-line TG content and the fatty acid-induced increase in TG were proportionately higher in fa/fa islets (Table I), raising the possibility that SPT expression was influenced by the ambient islet lipid content.

**Effects of Leptin on SPT Overexpression in fa/fa Islets Over-expressing Wild-type OB-Rb—**To obtain further insight into the mechanism of the exaggerated SPT expression, adenoviral gene transfer technology was employed to overexpress the wild-
type leptin receptor in the fa/fa islets (16). We perfused recombinant adenovirus containing either the OB-Rb cDNA (Ad-CMV-OB-Rb) or, as a control, the β-galactosidase cDNA (Ad-CMV-β-gal) into fa/fa pancreata and immediately thereafter cultured the islets for 2 days. On the third day, we added either 1 mM FA alone or 1 mM FA plus 20 ng/ml recombinant leptin for an additional 24 h. In the absence of FA, SPT mRNA was virtually identical in OB-Rb-overexpressing and β-galactosidase-overexpressing islets. When 1 mM FA was added, SPT mRNA increased to the same degree in both groups of islets. However, the presence of leptin completely blocked the FA-induced increase in SPT mRNA in OB-Rb-overexpressing islets and actually lowered it by 20% below the baseline (p < 0.05) (Fig. 5). Leptin had no effect on SPT in the β-galactosidase-overexpressing control islets.

In Vivo Effects of L-cycloserine on de Novo Ceramide Formation and DNA Fragmentation in Islets—To determine whether in vivo SPT blockade would reduce de novo ceramide synthesis and spare the beta-cells of fa/fa rats from apoptosis, we treated prediabetic ZDF rats with 25 mg/kg/day L-cycloserine for 2 weeks. Control rats were given injections of saline. After 2 weeks, all rats were sacrificed and the ceramide content of their islets measured, together with percentage of DNA fragmentation. As shown in Fig. 6, ceramide content of L-cycloserine-treated rats was decreased by 50%, and DNA fragmentation was reduced by 43% (from 14 to 8%) (Fig. 6, A and B). The concentration relationship of FFA to lipoapoptosis in islets of prediabetic rats is shown in Table II.

**DISCUSSION**

The results of this study provide several lines of new evidence that the high ceramide content observed in pancreatic islets of obese fa/fa ZDF rats, and implicated in beta-cell lipoapoptosis and adipogenic diabetes (4), represents newly synthesized ceramide rather than a product of sphingomyelin hydrolysis. First, expression of the enzyme SPT, the enzyme that catalyzes the condensation of serine and palmitoyl-CoA to form the ceramide precursor, dehydrosphinganine (8–12), was strikingly increased in the fa/fa islets with high ceramide content. Second, [3H]ceramide formation from [3H]serine, evidence of SPT enzyme activity, was increased in those islets, confirming our previous report of increased [3H]ceramide from [3H]palmitate (4). Third, the increased ceramide synthesis was inhibited by Triacsin-C blockade of fatty acyl-CoA synthetase, indicating that the excess ceramide formation is dependent on activation of long-chain fatty acids. Triacsin-C also blocked fatty acid-induced apoptosis (4), providing further evidence of their link.

Finally, we tested the possibility that SPT blockade might protect the fat-laden islets of prediabetic ZDF rats from ceramide-mediated lipoapoptosis of beta-cells (4), which may be a factor in their diabetes. Prediabetic ZDF rats were treated with L-cycloserine for 2 weeks, and ceramide content and DNA fragmentation were measured. The results suggest that agent was partially effective, reducing [3H]ceramide formation from [3H]serine and DNA fragmentation by ~50%.

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