Leptin Normalizes the Impaired Response of Proinsulin mRNA to Long Chain Fatty Acids in Heterozygous Zucker Diabetic Fatty Rats

Yan-Ting Zhou, Michio Shimabukuro, Young Lee, Kazunori Koyama, Falguni Trieu, and Roger H. Unger‡

From the Gifford Laboratories, Center for Diabetes Research, Departments of Internal Medicine and Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235 and the Department of Veterans Affairs Medical Center, Dallas, Texas 75216

To determine if underleptinization of islets of Zucker diabetic fatty (ZDF) rats is the proximal cause of their inability to compensate for obesity, we compared the proinsulin/β-actin mRNA ratio in heterozygous (fa/+) ZDF rats with that of wild-type (+/+ ) and homozygous (fa/fa) ZDF rats. In +/+ islets cultured with 2 mM free fatty acids (FFA) the proinsulin mRNA ratio rose 2.4-fold at 12 h. In fa/fa islets, the ratio rose only 65% above normal. There was no change in fa/fa islets. The presence of leptin (20 ng/ml) in the culture medium increased the FFA-induced response of proinsulin mRNA of fa/+ islets to that of +/+ islets while reducing FFA incorporation into triglycerides. The leptin-induced improvement in the proinsulin mRNA response was independent of any changes in glucose usage. These findings support a causal relationship between diminished leptin action on islets and the impaired β-cell response to FFA in ZDF rats.

Resistance to leptin is a common feature in both human and rodent obesity (1–3). In db/db mice and fa/fa rats, it is the consequence of mutations in the leptin receptor (OB-R) (4–6), whereas in obese leptin-resistant humans an unidentified postreceptor defect may be the cause (7). Recent studies in our lab suggest that β-cell compensation for the insulin resistance of adiposity is, at least in part, driven by the accompanying elevations of long-chain free fatty acids (FFA) in tissues and plasma (8, 9). Since leptin regulates the metabolic fate of FFA in tissues by blocking intracellular esterification and by enhancing intracellular oxidation (10), leptin action may influence the ability of β-cells to augment insulin production in response to FFA. Indeed, in leptin-resistant obese Zucker diabetic fatty (ZDF) rats with defective leptin receptors (5, 6), there is a marked increase in the triglyceride (TG) content in islets during the development of the β-cell defects of adipogenic diabetes, so-called “lipotoxicity” (11). In other words, “underleptinization” might be the proximal cause of the β-cell dysfunction that results in the noninsulin-dependent diabetes mellitus of ZDF rats.

To test this hypothesis, we compared the effect of leptin upon FFA-induced up-regulation of proinsulin mRNA in islets of lean wild-type ZDF rats (+/+), of obese prediabetic ZDF rats, which are homozygous for the mutation in the leptin receptor (fa/fa), and of lean heterozygous ZDF rats (fa/+). The islets of +/+ ZDF rats are entirely normal and fully responsive to the lipogenic action of leptin (10), whereas the fat-laden islets of fa/fa ZDF rats are completely unresponsive to leptin (10) and cannot be used to study leptin action. The islets of lean heterozygous ZDF rats with a single normal OB-R allele, therefore, became the focus of this study. Their fat content is slightly higher than islets of wild-type ZDF rats (12), and, as in homozygous rats, they do not mount a normal compensatory increase in insulin production in response to FFA (9). Nevertheless, since high levels of leptin will reduce their fat content (10), they can be employed to test the premise that leptin action plays a role in the FFA-induced increase in proinsulin gene expression. The following studies were designed to test this hypothesis.

EXPERIMENTAL PROCEDURES

Animals—All groups of rats were studied at 7 weeks of age. Homozygous male obese prediabetic ZDF-drt rats (fa/fa), which become diabetic at 8–10 weeks of age, heterozygous lean ZDF littermates (+/+), and wild-type lean ZDF rats (-/-) were bred in our laboratory from ZDF/drt-fa (F10) stock purchased from Dr. Richard Peterson (University of Indiana School of Medicine, Indianapolis, IN). All rats received standard rat chow (Teklad F6 8664, Teklad, Madison, WI) ad libitum and had free access to water. The institutional guidelines for animal care and use were followed.

Genotyping of ZDF Animals—DNA was extracted from rat tails (~8 mm) by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation (13). Primers 5'-GTTTGCGTATGCAAGTCA-3' and 5'-ACCAGCAGAGTCCGAG-3' were used to amplify products from 5 ng of genomic DNA in a 50-µl reaction mixture. A polymerase chain reaction (PCR) protocol of 30 cycles of 94 °C for 1 min, 55 °C for 90 s, and 68 °C for 5 min was employed. PCR products were digested with the restriction enzymeMspI for 2 h at 37 °C and analyzed on a 2% agarose gel (5).

Islet Culture—Pancreatic islets were isolated from 7-week-old rats by the method of Naber et al. (14) as modified by Lee et al. (15). They were maintained for 3 days in suspension culture in 60-mm glass Petri dishes at 37 °C in a humidified atmosphere of 5% CO2 and 95% air as described previously (8). The culture consisted of RPMI 1640 medium supplemented with 8.0 mM glucose, 10% fetal bovine serum, 200 units/ml penicillin, 0.2 mg/ml streptomycin, and 2% bovine serum albumin, fraction V (Bayer Corp., Kankakee, IL). In some experiments, 20 ng/ml recombinant leptin (kindly provided by Todd Kirchgessner, Bristol-Myers Squibb) and/or 2 × 105 of a 2:1 oleate:palmitate mixture in 2% albumin were added to the culture medium (Sigma).

* This work was supported by National Institutes of Health Grant DK02700–37, a National Institutes of Health/Juvenile Diabetes Foundation Diabetes Interdisciplinary Research Program, and the Department of Veterans Affairs Institutional Research Support Grant SMI 821–109. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Center for Diabetes Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235–8854. Tel.: 214-648-6742; Fax: 214-648-9191.

1 The abbreviations used are: FFA, free fatty acid; ZDF, Zucker diabetic fatty; TG, triglyceride; PCR, polymerase chain reaction; OB-R, leptin receptor; NO, nitric oxide.
Leptin and Proinsulin mRNA

25649

FIG. 1. Time course of changes in the proinsulin/β-actin mRNA ratio in normal islets from wild-type (+/+) ZDF rats cultured in 2 mM FFA or 16 mM glucose.

Measurements of Glucose Usage—Glucose usage in cultured islets was measured by the method of Zawalich and Matchinsky (16) and Zawalich et al. (17), as described previously (8).

RNA Extraction and Reverse Transcription PCR—Total RNA was extracted from islets by the TRIzol isolation method (Life Technologies, Gaithersburg, MD) from about 200 isolated islets. 2-μg aliquots of total RNA were treated with RNase-free DNase (Promega), and the first-strand cDNA synthesis was carried out using the oligo(dT) primer in 1st-strand cDNA synthesis kit (CLONTECH). The primers used were: 5′-GGTCCCGGGCTTTTGTTCAACAG-3′ for insulin sense primer and 5′-CCCTAGTGCAAGGCTGAAAT-3′ for insulin antisense primer, corresponding to 4244–4263 and 4411–4430 base pairs of the proinsulin sequence (GeneBankTM Data Bank accession number J00691); and 5′-GGTCATACACTGGGAGATATGGG-3′ for β-actin sense primer and 5′-GATCTTGATCTTCATGGTCGTAG-3′ for β-actin antisense primer, corresponding to 1552–1575 and 2968–2991 base pairs of the β-actin sequence (GeneBankTM Data Bank accession number J00691). Linearity of the PCR reaction was tested by amplification of 100 ng first-strand cDNA per reaction from 10 to 45 cycles. The linear range was found to be between 10 and 35 cycles. In no case did the amount of first-strand cDNA used for PCR reaction exceed 100 ng per reaction.

The samples were amplified for 22 cycles using the following parameters: 92 °C for 1 min, 53 °C for 45 s, and 72 °C for 45 s. The PCR products were subjected to electrophoresis on an agarose gel, and Southern blotting was carried out on a nylon membrane. The membrane was hybridized with 32P-labeled internal oligo primers (5′-ACA-CACACGAGCTCACACGATTG-3′ for proinsulin and 5′-GGTCATACACTGGGAGATATGGG-3′ for β-actin). Insulin mRNA and β-actin mRNA levels were quantified using the phosphor-imager. β-actin was employed as an internal standard for normalizing proinsulin mRNA.

Measurements of Cell Viability—After the 3-day culture period in medium containing either 0 or 2 mM FFA, islets from all groups of rats were randomly selected, washed twice with PBS, and stained with fluorescein diacetate and ethidium bromide for 1 min. Approximately 500 cells from each of the six groups were counted at random under a fluorescence microscope (Nikon Optiphot UFX 1-A, Garden City, NY). Green cells were counted as viable and red cells as dead. The data were expressed as percent viability.

Statistical Analysis—All results are expressed as ± S.E. Statistical significance was evaluated using Student’s t test on two-way analysis of variance followed by Scheffe’s multiple comparison.

RESULTS

Effect of FFA on Proinsulin/β-Actin mRNA Ratio in Normal Islets—The effect of 2 mM FFA on the proinsulin/β-actin mRNA ratio is shown in Fig. 1. FFA up-regulated proinsulin mRNA, a peak 2.4-fold of the control level at 12 h after the start of culture. By contrast, up-regulation of the proinsulin mRNA ratio by 16 mM glucose reached a peak 3 times the control value at 4 h (Fig. 1).

Effect of FFA on Proinsulin/β-Actin mRNA Ratio in Islets with Mutated Leptin Receptors—Enhancement of insulin production by FFA does not occur in islets from rats that are either homozygous (fa/fa) or heterozygous (fa/+) for a mutant leptin receptor (9). We determined if the failure of islets of ZDF rats to increase insulin production in response to FFA was associated with impairment of the response of the proinsulin mRNA/β-actin mRNA ratio. In contrast to the 2.4-fold increase in proinsulin mRNA in islets from normal wild-type (+/+) ZDF rats, it rose by only 65% in islets from heterozygous (fa/+) and homozygous (fa/fa) ZDF rats, respectively. These small in-group differences in viability were regarded as insufficient to explain the large differences in FFA-induced insulin gene expression.

Comparison of FFA- and Glucose-induced Increase in Insulin Gene Expression in Islets with Mutated Leptin Receptors—To determine whether the failure of insulin gene expression to respond to FFA represented an FFA-restricted defect or a non-specific and general inability to respond to increased insulin demand, we compared the proinsulin/β-actin mRNA ratio in islets from the three groups of rats cultured for 3 days in the medium containing either 0 or 2 mM FFA.

FIG. 2. Comparison of the proinsulin/β-actin mRNA ratio in islets from wild-type (+/+) homozygous (fa/fa), and homozygous (fa/fa) ZDF rats after 3 days in culture medium containing 0 or 2 mM FFA.

FIG. 3. Comparison of proinsulin/β-actin mRNA ratio in islets from wild-type (+/+), heterozygous (fa/+), and homozygous (fa/fa) ZDF rats after 3 days in culture medium containing 8 or 16 mM glucose.
presence of 8 or 16 mM glucose. As shown in Fig. 3, the proinsulin mRNA ratio in islets from fa/fa rats was not increased by glucose, perhaps because the massive increase in fat content had seriously impaired the ability of β-cell to respond to any increase in insulin need. However, in islets from heterozygous ZDF rats, 16 mM glucose induced a greater than 2.5-fold increase in proinsulin mRNA, not significantly different from the proinsulin mRNA response to glucose of islets from wild-type ZDF rats, whereas the response to FFA was only 30% of that of wild-type rats. Thus, the defect in islets of heterozygous ZDF rats seemed to be FFA-restricted without impairment of the glucose-induced response of proinsulin mRNA.

Effects of Leptin on FFA-induced Proinsulin mRNA—Previous findings from this laboratory indicate that in normal islets leptin promotes the channeling of FFA into oxidative rather than lipogenic pathways (10). Since islets of fa/fa rats cannot respond to leptin (10), they have an increased rate of FFA esterification (12), islet TG content rises to extremely high levels (15), and FFA enhancement of β-cell function is somehow blocked. Islets of heterozygous rats, by contrast, have a much less marked increase in TG content (12), but FFA enhancement of β-cell function is, nevertheless, attenuated. However, their islets respond partially to 20 ng/ml leptin with a lowering of TG content, as shown in Table I (10). To determine if exposure to leptin would repair the partial defect in the response of proinsulin mRNA to FFA, we added 20 ng/ml recombinant hormone to the culture medium and reassessed the effect of FFA on proinsulin mRNA. The presence of leptin in the culture medium reduced the increase in TG otherwise observed in islets of heterozygous rats cultured in the presence of 1 mM FFA from 27 ng/islet to only 4.6 ng/islet (Table I). Concomitantly, the response of proinsulin mRNA to FFA was restored to normal (Fig. 4); by contrast, in islets of homozygous ZDF rats, neither the TG content nor FFA induction of proinsulin mRNA were affected by leptin.

Relationship of FFA-mediated Increase in Low $K_m$ Glucose Usage to the Increase in Proinsulin mRNA—We have previously reported that FFA cause an increase in low $K_m$ glucose usage by islets (8, 9). Since insulin production varies with glucose metabolism by islets (8, 9), it seemed possible that FFA effects on insulin gene expression were mediated by the increase in glucose usage. We, therefore, compared the chronology of the FFA-induced proinsulin mRNA response and the rise in low $K_m$ glucose usage. As shown in Table II, glucose usage was rising at 12 h, the time at which the FFA-induced increase was at its peak. This indicated that the improved effect of FFA on proinsulin mRNA might have been mediated via enhanced glucose metabolism.

Since the foregoing result did not prove a relationship between low $K_m$ glucose usage and proinsulin mRNA up-regulation, we exploited the fact that the normal up-regulatory effect of FFA on proinsulin mRNA is absent in cultured islets of heterozygous ZDF rats but is restored by exposure of the islets to leptin (Fig. 4). Low $K_m$ glucose usage should also be improved by leptin if the leptin-induced improvement in the proinsulin mRNA response to FFA were mediated by increased glucose metabolism. As shown in Table II, leptin did not increase low $K_m$ glucose usage, indicating that the leptin-mediated normalization of proinsulin mRNA does not depend upon an increase in low $K_m$ glucose usage.

### DISCUSSION

There is now substantial evidence to indicate that the increased insulin production required to compensate for obesity-induced insulin resistance is stimulated by fatty acids reaching the islets (8, 9). As a consequence of higher circulating FFA levels (15), coupled with a dramatically increased esterification capacity resulting from the lack of leptin action (12), the TG content of islets from obese prediabetic ZDF rats, in which the β-cells have successfully compensated for the concomitant insulin resistance, is ∼13–17-fold that of lean Wistar rats (12). The increased insulin production observed in vivo in these obese compensated prediabetic rats can be duplicated in vitro by culturing normal islets from lean rats in 1 or 2 mM FFA (8). We refer to these changes as “compensatory.”

Later in the course of prediabetic obesity, islet fat content rises to 50–100-fold that of lean Wistar rats, at which time basal and stimulated insulin production significantly decline (15). These in vivo changes can be duplicated in vitro by culturing islets from compensated prediabetic obese rats in 1 or 2 mM FFA (9). We have referred to this effect of FFA on β-cell function as “lipotoxic” since we attribute it to the lipid overload (11). More recent work suggests that lipotoxicity is mediated by excessive levels of nitric oxide (NO) in islets (20).

#### TABLE I

| FFA | Leptin | Lean +/+ ZDF | Lean fa/+ ZDF | Obese fa/fa ZDF |
|-----|--------|--------------|---------------|-----------------|
| mM  | ng/ml  | mM  | ng/ml  | mM  | ng/ml  | mM  | ng/ml  | mM  | ng/ml  | mM  | ng/ml  | mM  | ng/ml  |
| 0   | 0      | 0   | 0      | 0   | 0      | 0   | 0      | 0   | 0      | 0   | 0      |
| 0.1 | 20     | 2.6 | 1.2   | 7.7 | 1.2b   | 5.1 | 9.3   | 5.6 | 2.3b   | 5.1 | 9.3   |
| 1   | 20     | 34.7| 4.3   | 5.7 | 1.2e   | 10.4| 8.7   | 10.4| 8.7   |
| 10  | 20     | 6.0 | 2.8   | 15.7| 2.3e   | 13.2| 10.2  | 13.2| 10.2  |

| $p < 0.01$ versus 0 leptin. | $p < 0.01$ versus 0 FFA.

#### Fig. 4. Comparison of proinsulin/β-actin mRNA ratio in islets from wild-type (+/+), heterozygous (fa/+), and homozygous (fa/fa) cultured for 24 h with or without 2 mM FFA after 2 days of pretreatment with or without 20 ng/ml of leptin.

| FFA (mM) | Leptin-pre-treated | Leptin | /-cell |
|---------|--------------------|--------|--------|
| 0       | 2                  | 20     | 0      | 2      | 20     | 0      | 2      | 20     |
| +/+     |                     |        | -      |        |        | -      |        |        |
| fa/+    |                     |        | -      |        |        | -      |        |        |
| fa/fa   |                     |        | -      |        |        | -      |        |        |

#### TABLE II

| 2 mM FFA | 0 h     | 4 h     | 12 h    | 24 h    |
|----------|---------|---------|---------|---------|
| Lean +/+ ZDF | 24.9 ± 0.6 (5) | 30.5 (2) | 37.7 ± 1.3 (4) | 48.8 (2) |
| Lean fa/+ ZDF | 34.7 ± 1.2 (3) | 35.9 (2) | 40.9 ± 1.7 (4) | 42.2 (2) |
| Obese fa/fa ZDF | 182.7 (2) | 157.1 (2) | 170.3 ± 3.4 (3) | 175.8 (2) |
Since leptin reduces islet TG via the leptin receptor (10), it was logical to ascribe the fat overload of ZDF rats and the β-cell dysfunction in these animals to their abnormal leptin receptor. However, since islets of homozygous ZDF rats cannot respond to leptin, we could not test the validity of the premise that underleptinization causes their β-cell phenotype. We therefore employed islets of heterozygous fa/+ rats, which are responsive to high concentrations of leptin (10) but which do not mount a normal compensatory increase in insulin production response to FFA in vitro. Leptin reduced the rise in islet TG that otherwise occurs when islets from heterozygous ZDF islets are cultured in FFA, and this was associated with normalization of the attenuated FFA-induced response of the proinsulin/β-actin mRNA ratio. However, a causal relationship between the elevated islet TG and the impairment in FFA-induced compensatory changes of β-cells in fa/+ rats is difficult to substantiate inasmuch as the differences in fat content from +/+ controls are unimpressive. While it is possible that by shifting FFA from esterification to other pathways in fa/+ islets, leptin provided the FFA substrate required to up-regulate β-cell function; however, a clearcut relationship between the leptin-mediated improvement in FFA-induced proinsulin mRNA response to the observed changes in lipid metabolism could not be established.

Nor does it appear that the FFA-induced up-regulation of proinsulin mRNA is mediated by the previously demonstrated FFA-induced increase in low K_m glucose metabolism (8, 9). If the proinsulin mRNA response to FFA had been secondary to increased low K_m glucose usage, leptin treatment, which restored the impaired FFA-induced proinsulin mRNA response of fa/+ islets to normal, should also have restored to normal the FFA-mediated increase in low K_m glucose usage. Since leptin failed to improve FFA induction of low K_m glucose usage, we concluded that the amelioration in the proinsulin mRNA response attributed to leptin action was independent of changes in low K_m glucose metabolism.

Acknowledgments—We thank Dr. Chris Newgard for critically reviewing this paper, Kay McCorkle for technical support, and Sharryn Harris for secretarial assistance.

REFERENCES
1. Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R. L., Ranganathan, S., Kern, P. A., and Friedman, J. (1995) Nat. Med. 1, 1155–1161
2. Considine, R. V., Sinha, M. K., Heiman, M. L., Krianciunas, A., Stephens, T. W., Nye, M. R., Ohannesian, J. P., Marco, C. C., McKee, L. J., Bauer, T. L., and Caro, J. F. (1996) N. Engl. J. Med. 334, 292–295
3. Chua, B. C., Chung, W. K., Wu-Peng, X. S., Zhang, Y., Lui, S. M., Tartaglia, L., and Leibel, R. L. (1996) Science 271, 994–996
4. Chen, H., Charlat, O., Tartaglia, J. A., Woolf, E. A., Weng, X., Ellis, S. J., Lakey, N. D., Culpepper, J., Moore, K. J., Breitbart, R. E., Dayk, G. M., Tepper, R. L., and Morgenstern, J. F. (1996) Cell 84, 491–495
5. Phillips, M. S., Liu, Q. Y., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. T., and Hess, J. F. (1996) Nat. Genet. 13, 18–19
6. Iida, M., Murakami, T., Ishida, K., Mizuno, A., Kuwajima, M., and Shima, K. (1996) Biochem. Biophys. Res. Commun. 224, 597–604
7. Caro, J. F., Sinha, M. K., Kolaczynski, J. W., Zhang, P. L., and Considine, R. V. (1996) Diabetes 45, 1455–1462
8. Milburn, J. L., Hirose, H., Lee, Y. H., Nagasawa, Y., Ogawa, A., Ohneda, M., BeltrandRio, H., Newgard, C. B., Johnson, J. H., and Unger, R. H. (1995) J. Biol. Chem. 270, 1295–1299
9. Hirose, H., Lee, Y. H., Inman, L. R., Nagasawa, Y., Johnson, J. H., and Unger, R. H. (1996) J. Biol. Chem. 271, 5633–5637
10. Shimabukuro, M., Koyama, K., Chen, G., Wang, M. Y., Trieu, F., Lee, Y., Newgard, C. B., and Unger, R. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4637–4641
11. Unger, R. H. (1995) Diabetes 44, 863–870
12. Lee, Y., Hirose, H., Zhou, Y.-T., Eeser, V., McGarry, J. D., and Unger, R. H. (1997) Diabetes 46, 408–413
13. Bli, N., and Stafford, D. W. (1976) Nucleic Acids Res. 3, 2303–2308
14. Naber, S. P., McDonald, J. M., Jarett, L., McDaniel, M. L., Ludvigsen, C. W., and Lacy, P. E. (1980) Diabetes 29, 439–444
15. Lee, Y., Hirose, H., Ohneda, M., Johnson, J. H., McGarry, J. D., and Unger, R. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10878–10882
16. Zawalich, W. S., and Matchinsky, F. M. (1977) Endocrinology 100, 1–8
17. Zawalich, W. S., Pagliara, A. S., and Matchinsky, F. M. (1977) Endocrinology 100, 1276–1283
18. Matchinsky, F. M., Trus, M., Burch, P., Berner, D., Ghosh, A., Zawalich, W., and Weill, V. (1980) in Diabetes 1979 (Waldhaus, W. K., ed) pp. 154–159, Excerpta Medica, Amsterdam
19. Meglissan, M. D., and Matchinsky, F. M. (1986) Diabetes Metab. Rev. 2, 163–214
20. Shimabukuro, M., Ohneda, M., Lee, Y., and Unger, R. H. (1997) J. Clin. Invest. 100, 290–295