

Role of Nitric Oxide in Obesity-induced β Cell Disease

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Abstract

Here we report that free fatty acid–induced suppression of insulin output in prediabetic Zucker diabetic fatty (ZDF) rats is mediated by NO. When normal islets were cultured in 2 mM FFA, NO production and basal insulin secretion increased slightly. In cultured prediabetic ZDF islets, FFA induced a fourfold greater rise in NO, upregulated mRNA of inducible nitric oxide synthase (iNOS), and reduced insulin output; both nicotinamide and aminoguanidine, which lower NO, prevented the FFA-mediated increase in iNOS mRNA, reduced NO, and minimized the loss of insulin secretion. In vivo nicotinamide or aminoguanidine treatment of prediabetic ZDF rats prevented the iNOS expression in islets and decreased β cell dysfunction while blocking β cell destruction and hyperglycemia. We conclude that NO-lowering agents prevent adipogenic diabetes in obese rats. (*J. Clin. Invest.* 1997. 100:290–295.) Key words: nitric oxide • obesity • diabetes • inducible nitric oxide synthase • Zucker diabetic fatty rat

Introduction

Obesity-linked non–insulin-dependent diabetes mellitus (NIDDM)¹ is preceded by years of insulin resistance, during which normal blood glucose levels are maintained through effective compensation by pancreatic β cells (1). In \sim 20% of obese individuals, the compensation wanes, hyperglycemia appears, and overt NIDDM is diagnosed. The mechanisms by which obesity initially enhances and subsequently depresses β cell function are unknown. One possibility is that the excess of free fatty acids released from adipocytes in obesity (2) may initially stimulate, but ultimately impair, the function of β cells, and thus limit their compensatory capability. Chronic elevations in FFA have been shown to interfere with glycolysis and glucose oxidation (3–6), and could thereby contribute to both

insulin resistance (5, 6) and the alterations in β cell function (7–10) that characterize obesity.

We have questioned the concept that the severe progressive functional and morphologic alterations of β cells (lipotoxicity) at the onset of NIDDM in Zucker diabetic fatty (ZDF) rats (11, 12) are entirely the consequence of interference by FFA in the glucose metabolism of β cells. We have examined the possibility that some of the FFA-induced β cell changes, particularly the loss of β cells that occurs late in the disease, are the result of excessive nitric oxide. NO has previously been shown to mediate IL-1 β -induced impairment of β cell function (13–16), and ultimately cause β cell death (17). This study provides evidence that this same mechanism may be operative in the deterioration of β cells that occurs in obese ZDF rats. In addition, it provides in vitro and in vivo evidence that therapeutic strategies to reduce NO production in islets may prevent adipogenic NIDDM.

Methods

Animals. Male Wistar rats were obtained from Sasco (Omaha, NE). Obese homozygous (*fa/fa*) ZDF-drt rats and lean heterozygous (*fa/+*) ZDF littermates were bred in our laboratory from (ZDF/drt-*fa* [F10]) rats purchased from Dr. R. Peterson (University of Indiana School of Medicine, Indianapolis, IN). Male rats from our colony exhibiting the previously described phenotype (18) were used.

Islet isolation and culture. Pancreatic islets were isolated and maintained in suspension culture in 60-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO₂/95% air as described previously (11). The culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (200 U/ml), streptomycin (0.2 mg/ml), and 2% BSA (fraction V; Miles Inc., Kankakee, IL), either with or without 2 mM long-chain fatty acids (oleate/palmitate, 2:1, sodium salt; Sigma Chemical Co., St. Louis, MO). The final glucose concentration was 8.0 mM, which is required for long-term survival of islets (> 80%).

Nitrite determination. 250 μ l of culture medium were collected at different time points and incubated with an equal volume of the Griess reagent (1% sulfanilamide in 0.1 mol/liter HCl and 0.1% naphthyl ethylenediamine dihydrochloride) for 10 min at room temperature as described (19). The nitrite concentration was determined from the absorbance at 550 nm using sodium nitrite as standard. The background nitrite levels in the culture media were subtracted from each sample. The background level in islet-free medium was unaffected by an addition of the FFA mixture for 72 h.

Reverse transcriptase-PCR. From \sim 300 to 500 cultured islets, total RNA was extracted using the TRIzol total RNA isolation kit (Life Technologies, Inc., Gaithersburg, MD). Total RNA from each sample were treated with RNase-free DNase (Promega Corp., Madison, WI) for 30 min at 37°C to remove any contaminating DNA. First strand cDNA was carried out by oligo(dT) 12–18 primed reverse transcription of 2 μ g of each total RNA using first strand cDNA synthesis kit (Clontech, Palo Alto, CA). Primers used for the cDNA synthesis were designed to span introns in respective genes, allowing a size discrimination of amplified cDNA from genomic DNA. Primers used to amplify inducible nitric oxide synthase (iNOS) cDNA were 5'-CGT GTG CCT GCT GCC TIC CTG CTG T-3' and 5'-GTA ATC CTC AAC CTG CTC CTC ACT C-3' (nucleotides 2679–2703 and 3326–3350, a 672-bp fragment) (20). As an internal standard, β -actin cDNA was amplified using primers 5'-TTG TAA CCA ACT GGG ACG

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1. Abbreviations used in this paper: AG, aminoguanidine; iNOS, inducible nitric oxide synthase; NAME, N^G-arginine methylester; NIC, nicotinamide; NIDDM, non–insulin-dependent diabetes mellitus; ZDF rats, Zucker diabetic fatty rats.

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ATA TGG-3' and 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3' (nucleotides 1552–1575 and 2991–2844, a 764-bp fragment). 2 μ l of first strand cDNA mixture was used for PCR amplification in 50 μ l of 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTP, 0.4 μ M of each primer, and 2.0 U of Taq DNA polymerase (Promega Corp.). PCR amplification consisted of a 2-min hot start at 94°C, followed by 35 cycles (iNOS) and 30 cycles (β -actin) 92°C for 45 s, 55°C for 45 s, and 72°C for 1 min. The amplification products were analyzed on a 1.2% agarose gel and transferred to Hybond™-N Nylon membrane (Amersham Corp., Arlington Heights, IL) by the capillary transfer method. DNA was cross-linked to the membrane by an ultraviolet irradiation using GS Gene Linker™ UV Chamber (Bio-Rad Laboratories, Hercules, CA). Oligonucleotide probes for internal 30 nucleotides (5'-TGC GAC ATG ATT AAT GGC ACA GAT GCA GCC-3' for iNOS (20) and 5'-GGT CAG GAT CTT CAT GAG GTA GTC TGT CAG-3' for β -actin) were endlabeled with 6,000 Ci/mmol γ 32P-ATP (Amersham Corp.) using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN). After pre-hybridization at 42°C for 30 min in a Rapid-Hyb buffer (Amersham Corp.), hybridization with the radiolabeled probes (10 ng of radiolabeled oligonucleotide probe per milliliter hybridization buffer) was carried out for 1 h at 42°C. The filters were washed once for 15 min at room temperature in 5 \times SSC with 0.1% SDS, and twice for 15 min at 42°C in 1 \times SSC with 0.1% SDS. The relative amounts of PCR products were quantified using the Molecular Imager System (GS-363; Bio-Rad Laboratories) and Molecular Analyst software (Bio-Rad Laboratories).

Perfusion of cultured islets. For perfusion, 50–100 islets were collected under a stereoscopic microscope, washed twice with Krebs-Ringer bicarbonate-Hepes buffer (pH 7.4, 3 mM glucose), and loaded into a 13-mm chamber containing an 8- μ m nylon membrane filter (Millipore Corp., Bedford, MA). Islets were perfused with buffer containing 3 or 23 mM glucose at a flow rate of 0.8 ml/min for 15 min each (21, 22). Effluent fractions were collected at 2-min intervals and stored at -20°C until insulin assay. Immunoreactive insulin was determined by radioimmunoassay using charcoal separation as described (21).

Plasma measurements. Blood samples were obtained between 0900 and 1100 hours from tail veins with capillary tubes coated with

EDTA. Plasma glucose was measured by the glucose oxidase method using the glucose analyzer II (Beckman Instruments, Inc., Brea, CA). Plasma FFAs were determined with the kit (Boehringer Mannheim Biochemicals) using the enzymatic colorimetric assay.

Pancreatic perfusion. Immunoreactive insulin was determined in perfusate collected at 1-min intervals during perfusion at glucose concentrations of 5.6 mM (basal) and 20 mM (glucose stimulated) for 10 min each using the previously described perfusion technique as modified (23).

Immunohistochemistry. Bouin-fixed paraffin-embedded serial sections of perfused pancreata (5- μ m thickness) were stained for insulin and GLUT-2 by indirect immunofluorescence (24). In brief, serial sections were layered in either a guinea pig anti-pork insulin antibody (1:100) or a rabbit anti-rat GLUT-2 antibody (1:1,000) for 16 h at 4°C. After washing, the sections were incubated with fluorescein isothiocyanate-conjugated IgG (1:20) for 2 h at room temperature and examined under a fluorescein microscope. The percentage of GLUT-2-positive β cells was determined from the ratio of the area of GLUT-2- to insulin-positive cells using the method of Weibel (25).

Statistical analysis. Values shown are expressed as the mean \pm SEM. Statistical analysis was performed by two-tailed unpaired Student's *t* test or by one-way analysis of variance.

Results

Effects of FFA on NO production by islets. To test the possibility that NO mediates FFA-induced β cell impairment, we cultured normal and prediabetic islets in 0 or 2 mM FFA in 2% BSA for 3 d (Fig. 1 A). We used the nitrite method of Green (19) to quantify NO in islets from lean Wistar, lean *fa/+* ZDF, or obese prediabetic *fa/fa* ZDF rats. In the absence of FFA, NO in islets of Wistar rats reached a plateau of 40 pmol/islet at 72 h; in the presence of 2 mM FFA, NO rose to 200 pmol/islet. In the presence of FFA, NO rose to 400 pmol/islet in islets of lean *fa/+* rats, and 900 pmol/islet in islets from prediabetic obese *fa/fa* ZDF rats. The presence in the culture medium of 25 mM nicotinamide (NIC), which prevents induction of iNOS

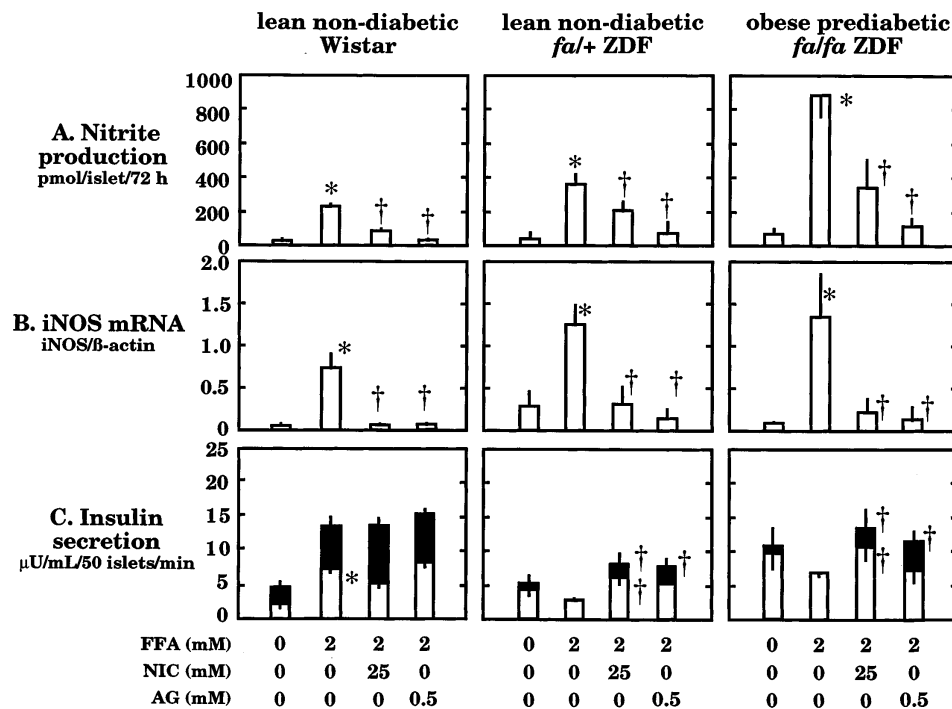


Figure 1. Effects of long-chain FFA with and without nicotinamide (NIC) or aminoguanidine (AG) on islets isolated from 6–7-wk-old Wistar rats, prediabetic obese ZDF rats (*fa/fa*), and lean heterozygous littermates (*fa/+*). Islets were cultured for 72 h in medium containing either 0 or 2 mM FFA plus either 0 or 25 mM NIC, or 0 or 0.5 mM AG. (A) Effects on NO formation determined spectrophotometrically as nitrite. (B) Effects on iNOS/ β -actin mRNA ratio semiquantified by reverse transcriptase-PCR. (C) Effects on insulin secretion. Islets were perfused for 10 min with 3 (\square) and 23 (\blacksquare) mM glucose for 15 min. Bars represent the mean \pm SEM of the sum of all measurements in each experiment ($n = 3$ –5). Significant differences are indicated as follows: * $P < 0.05$ vs. 0 mM FFA group, and † $P < 0.05$ vs. 2 mM FFA group.

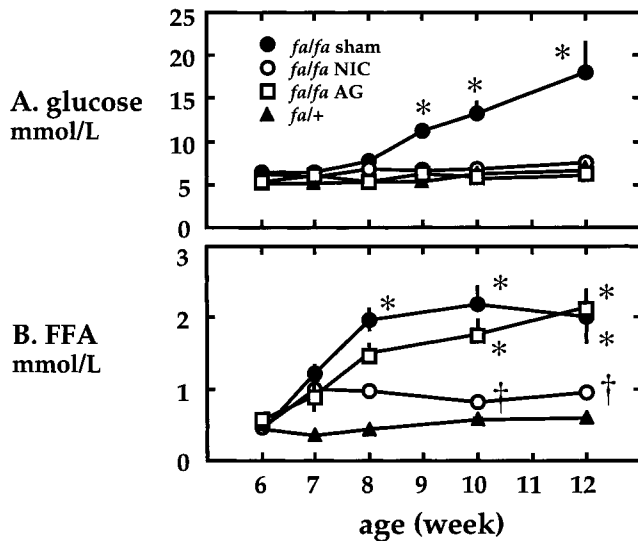


Figure 2. Effects of a 6-wk course of NIC and AG treatment on blood glucose (A) and FFA levels (B) of obese prediabetic *fa/fa* ZDF rats (levels in untreated lean *fa/+* controls are also shown). Values represent the mean \pm SEM of three to six animals. Significant differences are marked as follows: * $P < 0.05$ vs. untreated lean *fa/+* ZDF group, and $\dagger P < 0.05$ vs. sham-treated obese *fa/fa* ZDF group.

by IL-1 β in islets (26), reduced the FFA-induced increase in NO in islets from all groups (Fig. 1A). Similarly, 0.5 mM aminoguanidine (AG), which is also a competitive inhibitor for iNOS (27) but lowers iNOS expression as well (28), reduced islet NO release (Fig. 1A).

Effects of FFA on inducible NO synthase expression. To determine the relationship between FFA-induced NO observed in these groups and iNOS expression, the mRNA for the enzyme was semiquantified by reverse transcriptase-PCR (Fig. 1B). FFA dramatically increased iNOS mRNA in islets from all groups; the induction was somewhat greater in the islets of heterozygous and homozygous ZDF rats than in islets of Wistar rats ($P < 0.05$). The addition of 25 mM NIC to the culture medium lowered iNOS mRNA in all groups, indicating that this may contribute to the lowering of FFA-induced NO levels by NIC in vitro. AG also lowered iNOS.

NO and β cell function. To evaluate the relationship of the foregoing changes in NO and iNOS to β cell function, the effects of 2 mM FFA on insulin secretion were compared by perfusing islets from various rat groups with 3 or 23 mM glucose

Table I. Insulin Secretion by Pancreata Isolated from *fa/+* and *fa/fa* ZDF Rats Treated with NIC, or AG (Microunit per milliliter per Minute, Mean \pm SEM)

	Lean <i>fa/+</i> ZDF sham-rx <i>n</i> = 3	Obese prediabetic <i>fa/fa</i> ZCF		
		sham-rx <i>n</i> = 6	NIC-rx <i>n</i> = 3	AG-rx <i>n</i> = 3
5.6 mM glucose	10.2 \pm 1.4	43.3 \pm 7.7	36.3 \pm 10.9	45.2 \pm 8.6
20 mM glucose*	83.6 \pm 6.6	2.8 \pm 2.0 [‡]	26.9 \pm 12.2 [§]	22.2 \pm 11.1 [†]

*Insulin values represent increments above the levels at 5.6 mM glucose; [‡] $P < 0.05$ vs. lean *fa/+*; [§] $P < 0.05$ vs. sham-treated obese *fa/fa*.

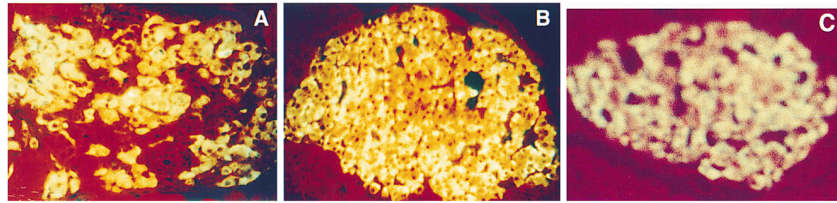
(Fig. 1C). In confirmation of our earlier reports (21, 22), in islets from Wistar rats basal and glucose-stimulated insulin secretion were both enhanced by 2 mM FFA; by contrast, in islets from lean *fa/+* and obese *fa/fa* ZDF rats, the presence of 2 mM FFA paradoxically reduced basal insulin secretion and glucose-stimulated insulin responses to below control levels (22). In normal islets, the addition of 25 mM NIC to the culture medium attenuated the stimulatory effect of FFA on basal insulin secretion but did not alter glucose-stimulated secretion; 0.5 mM AG did not cause a statistically significant change in either.

By contrast, the effects of NIC and AG on islets of ZDF rats were dramatic. 25 mM NIC or 0.5 mM AG in the culture medium reduced the inhibitory effects of FFA on insulin production by islets of the heterozygous lean and homozygous obese ZDF rats (Fig. 1, A and C). These results suggest that NO plays a role in FFA-induced suppression of insulin secretion observed in islets of rats with a mutant *fa* allele. For this reason, we studied the effect of 1 mM *N*^G-arginine methylester (NAME), a competitive inhibitor of iNOS, on insulin secretion. NAME, like NIC and AG, caused a marked improvement in glucose-stimulated insulin secretion in the presence of 2 mM FFA (6.3 \pm 0.16 vs. 11.2 \pm 0.7 U/ml per 50 islets/min).

Effect of anti-NO therapy on development of NIDDM in prediabetic ZDF rats. NIC has been shown to prevent the deleterious effects of IL-1 β -induced NO on β cells (26), and is currently being evaluated in autoimmune insulin-dependent diabetes (29). To determine if NIC and AG prevent obesity-related NIDDM in vivo, we treated prediabetic obese ZDF rats (*fa/fa*) for 6 wk with daily intraperitoneal injections of 0.5 g NIC or 0.4 g AG/kg body wt beginning at the age of 6 wk. None of the animals treated with NIC or AG became diabetic; their blood glucose levels averaged 7.5 \pm 0.1 and 6.7 \pm 0.2 mmol/liter, respectively, at the end of the treatment period, compared with 18.0 \pm 0.4 mmol/liter in untreated controls (Fig. 2A). Plasma FFA levels of NIC-treated rats were lower than in the sham-treated controls, although they exceeded the values in lean rats (Fig. 2B). In AG-treated rats, FFA levels were no different than in untreated controls. Glucose tolerance was normal in NIC- and AG-treated prediabetic ZDF rats, with blood glucose returning to the fasting level at 2 h after an intraperitoneal injection of glucose (2 g/kg) (data not shown). The profound reduction in the number of β cells was prevented by both NIC and AG therapy (Fig. 3A), as was the β cell GLUT-2 loss (Fig. 3B). GLUT-2 loss is a morphological marker of NIDDM (24), and is now recognized to be secondary to metabolic changes (30). Perfusion of pancreata from NIC-treated rats showed marked improvement in β cell function; instead of the negative insulin response to glucose observed in untreated diabetic controls, there was a positive response in the low normal range (Table I) that was nine times that of sham-treated animals. AG caused a sevenfold improvement over sham-treated controls. Thus, two agents that lower NO production by different mechanisms prevented the development of the NIDDM phenotype in β cells. By contrast, 6 wk of daily treatment with 0.05 g/kg 3-aminobenzamide, which, like NIC, inhibits poly-ADP-ribose synthetase (31) but has no other known action in common with NIC, failed to prevent diabetes (data not shown).

In vivo iNOS expression in diabetic ZDF rats. To determine if iNOS expression is increased in β cells of diabetic ZDF rats, we semiquantified iNOS mRNA in islets freshly isolated from

A. Islet of 12-week old obese *fa/fa* ZDF rats stained for insulin



sham

NIC+

AG+

B. β -cell volume fraction

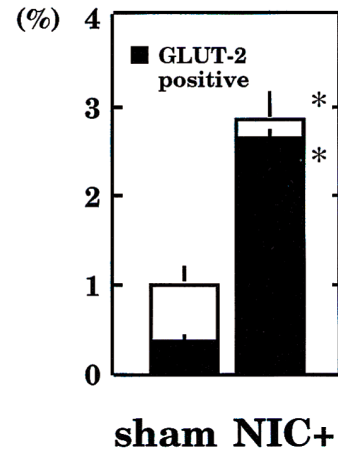


Figure 3. (A) Representative photomicrographs comparing insulin-positive cells by immunofluorescent staining in the pancreas of sham-, NIC-, and AG-treated obese *fa/fa* ZDF rats. (B) Effects of a 6-wk course of NIC treatment on morphometrically determined β cell volume fraction and GLUT-2 positivity in obese *fa/fa* ZDF rats. From 6 to 12 wk of age, obese prediabetic *fa/fa* ZDF rats received daily intraperitoneal injections of 0.5 g/kg⁻¹ NIC (NIC+), 0.4 g/kg⁻¹ AG (AG+), or 0.9% saline (*sham*). Values shown are the mean \pm SEM of three experiments. Significant differences are marked as follows: * $P < 0.05$ vs. untreated obese *fa/fa* ZDF group.

6- and 12-wk-old obese *fa/fa* ZDF and lean *fa/+* ZDF rats (Fig. 4). We also examined pancreatic sections immunocytochemically for the presence of iNOS using an iNOS antibody (anti-iNOS; Transduction Laboratories, Lexington, KY). In addition, islets from the NIC- and AG-treated prediabetic rats were examined for iNOS mRNA and immunostainable iNOS. iNOS mRNA could not be detected in islets in any of the 6-wk-old rats; iNOS mRNA was measurable in both homozygous and heterozygous 12-wk-old groups, but was 20 times higher in the homozygous rats, all of which were diabetic. In 12-wk-old “prediabetic” ZDF rats in which the diabetes had been prevented by 6 wk with NIC and AG treatment, iNOS mRNA was reduced almost to normal (Fig. 4). Immunostaining for iNOS was positive only in the diabetic ZDF rats (data not shown).

Discussion

This report provides the first evidence that long-chain fatty acids influence pancreatic β cells via the NO system. In other tissues, NO is thought to have a dual role, serving as a regulator under physiologic conditions (32) and as a cytotoxin under pathophysiologic circumstances (31–33). As a physiologic regulator, NO mediates diverse functions in many organs (32), including the cardiovascular, neuromuscular, neurological, genitourinary, gastrointestinal, and renal systems; in pancreatic islets, NO regulates islet blood flow (34). The constitutive forms of nitric oxide synthase, NOS I and III, have been identified in rat islets and in β cell lines (32), and iNOS (NOS II) has been induced in islets by IL-1 β (13–17, 26). The induction of iNOS by IL-1 β results in cytotoxicity (13, 14, 16, 17). NO donors have been shown to cause both β cell dysfunction and damage (15).

The present studies suggest that the cytotoxic role of NO can be induced by FFA in islets of rats predisposed to NIDDM. FFA caused a reduction in both basal and glucose-stimulated insulin secretion in islets from lean *fa/+* and obese prediabetic

fa/fa ZDF rats in association with a > 20-fold FFA-induced increase in NO. It is possible that the higher NO levels in islets of ZDF rats result in greater production of toxic hydroxyl ions from peroxynitrite (35). The addition of an inhibitor of iNOS expression, NIC, to the culture medium prevented the induction of iNOS by FFA, reduced NO production in all groups, and prevented the FFA-induced decrease in insulin secretion in islets from *fa/+* and *fa/fa* rats. AG, both a competitive inhibitor of iNOS and a suppressor of its expression (27, 28), also

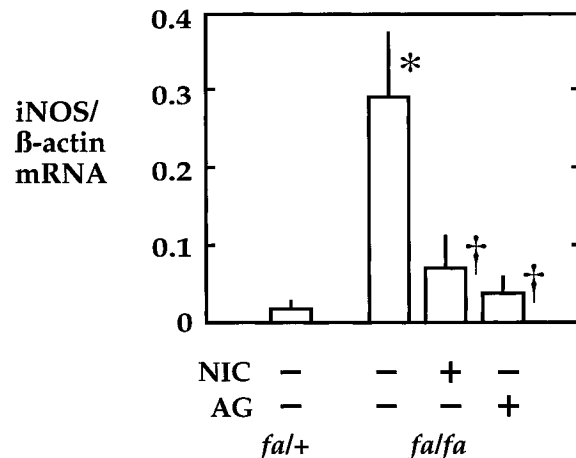


Figure 4. Effects of a 6-wk course of NIC and AG treatment on iNOS/ β -actin in RNA ratio in freshly isolated islets of 12-wk-old obese ZDF (*fa/fa*) rats that were sham-treated (diabetic), or treated for 6 wk with either NIC (0.5 g/kg⁻¹ body wt) or AG (0.4 g/kg⁻¹ body wt) administered intraperitoneally. The mean ratio in islets from lean heterozygous ZDF control (*fa/+*) rats is also shown. All values represent the mean \pm SEM of three experiments. Differences are marked as follows: * $P < 0.05$ vs. untreated lean *fa/+* ZDF group and † $P < 0.05$ vs. obese *fa/fa* ZDF group.

prevented the decrease in insulin secretion in islets from *fa/+* and *fa/fa* rats, as did NAME, a pure competitive inhibitor. This provides support for the hypothesis that NO causes, or is required for, FFA-induced attenuation of insulin secretion in prediabetic rat islets, at least in vitro. The relevance of the in vitro findings to clinical diabetes in vivo is suggested by the fact that iNOS mRNA was 20 times higher in diabetic rats than in lean nondiabetic controls and that immunostainable iNOS was detectable only in islets of diabetic obese ZDF rats. The cellular source of iNOS and NO may well be the β cells rather than macrophages. NO production by purified β cells has been reported previously (13) and iNOS expression in β cells has been documented (14). Moreover, we found no evidence of macrophages in pancreatic sections from ZDF rats, using two immunohistochemical stains specific for macrophages (data not shown).

In these rats, plasma FFA were elevated and triacylglycerol content of islets was increased, evidence that intracellular FFA was high (11, 12). ZDF rats are leptin-resistant because of a Glu 269→Pro mutation in the leptin receptor (36), and their islets have an increased capacity to esterify and a decreased capacity to oxidize FFA (37). We speculate that this defect, which must be related to the leptin resistance, somehow caused the greater induction of iNOS expression by FFA, perhaps by increasing intracellular levels of FFA. Although the mechanism by which FFA or high triacylglycerol increases iNOS expression and NO production in pancreatic islets is unknown, increased levels of diacylglycerol and/or ceramide are among the possibilities.

NIC decreased plasma FFA levels and inhibited iNOS expression in obese prediabetic ZDF rats, while AG suppressed FFA-induced iNOS mRNA expression without lowering the high plasma FFA. This raises the possibility that clinical adipogenic NIDDM and its associated β cell abnormalities (loss of glucose-stimulated insulin secretion, loss of GLUT-2, and a reduction in β cell mass) might be prevented by treatment with agents that reduce the FFA levels and/or decrease FFA-mediated NO generation. These rational approaches preventing the β cell changes and the NIDDM of obesity are already available for clinical trial.

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