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# Susceptibility to Fatty Acid-Induced β-Cell Dysfunction Is Enhanced in Prediabetic Diabetes-Prone BioBreeding Rats: A Potential Link Between β-Cell Lipotoxicity and Islet Inflammation

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β-Cell lipotoxicity is thought to play an important role in the development of type 2 diabetes. However, no study has examined its role in type 1 diabetes, which could be clinically relevant for slow-onset type 1 diabetes. Reports of enhanced cytokine toxicity in fat-laden islets are consistent with the hypothesis that lipid and cytokine toxicity may be synergistic. Thus, β-cell lipotoxicity could be enhanced in models of autoimmune diabetes. To determine this, we examined the effects of prolonged free fatty acids elevation on β-cell secretory function in the prediabetic diabetes-prone BioBreeding (dp-BB) rat, its diabetes-resistant BioBreeding (dr-BB) control, and normal Wistar-Furth (WF) rats. Rats received a 48-h iv infusion of saline or Intralipid plus heparin (IH) (to elevate free fatty acid levels ~2-fold) followed by hyperglycemic clamp or islet secretion studies *ex vivo*. IH significantly decreased β-cell function, assessed both by the disposition index (insulin secretion corrected for IH-induced insulin resistance) and in isolated islets, in dp-BB, but not in dr-BB or WF, rats, and the effect of IH was inhibited by the antioxidant N-acetylcysteine. Furthermore, IH significantly increased islet cytokine mRNA and plasma cytokine levels (monocyte chemoattractant protein-1 and IL-10) in dp-BB, but not in dr-BB or WF, rats. All dp-BB rats had mononuclear infiltration of islets, which was absent in dr-BB and WF rats. In conclusion, the presence of insulitis was permissive for IH-induced β-cell dysfunction in the BB rat, which suggests a link between β-cell lipotoxicity and islet inflammation. **(*Endocrinology* 154: 89–101, 2013)**



The diabetes-prone BioBreeding (dp-BB) rat is an established animal model of type 1 diabetes. Diabetes in these rats is spontaneous and severe and is preceded by insulitis, similar to human type 1 diabetes (1). In contrast to human diabetes, diabetes in the dp-BB rat is associated with T-cell lymphopenia and can be prevented by regulatory RT6+ T cells. The diabetes-resistant BioBreeding (dr-BB) rats do harbor autoreactive cells but are resistant to

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Abbreviations: CD, Cluster of differentiation; COX-2, cyclooxygenase-2; DI, disposition index; dp-BB, diabetes-prone BioBreeding; dr-BB, diabetes-resistant BioBreeding; FFA, free fatty acid; Ginf, glucose infusion rate; GSIS, glucose-stimulated insulin secretion; IFN, interferon; IH, Intralipid plus heparin; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; M/I, glucose metabolism divided by plasma insulin; NAC, N-acetylcysteine; NF-κB, nuclear factor κB; SAL, saline; WF, Wistar-Furth.

autoimmune diabetes, unless depleted of regulatory RT6+ T cells (2).

β-Cell dysfunction and destruction in type 1 diabetes and BB rats are due to autoimmune inflammation of islets, where cytokines result in production of reactive oxygen species (3) and reactive nitrogen species (4) via up-regulation of inducible nitric oxide synthase (iNOS) (5). Both antioxidants (6) and inhibitors of iNOS (7) reduce the incidence of diabetes in dp-BB rats.

Studies in the past decade have suggested that islet inflammation is a shared etiology for both type 1 and type 2 diabetes (8). In type 2 diabetes, islet inflammation is not caused by autoimmunity but by excess of energy substrate (glucose and fat). Indeed, similar to cytokines, free fatty acids (FFAs) can induce alterations in both β-cell function and mass (*i.e.* β-cell lipotoxicity) (9) via mechanisms that include oxidative stress (10) and up-regulation of iNOS (11). FFAs are generally elevated in conditions that predispose to type 2 diabetes, such as obesity and insulin resistance, because of increased lipolysis from the expanded adipose tissue and resistance to the antilipolytic action of insulin. Therefore, in type 2 diabetes, lipotoxicity is thought to play an important role in the pathogenesis of β-cell failure. Although FFAs are sensitive markers of insulin deficiency (12) and increased FFAs have been described early in the pathogenesis of some animal models of type 1 diabetes (13, 14), lipotoxicity has not been traditionally associated with type 1 diabetes. However, reports of cytokine up-regulation (15, 16) and enhanced cytokine toxicity in fat exposed β-cells *in vitro* (17, 18) are consistent with the hypothesis that lipid and cytokine toxicity may be synergistic. Lipotoxicity could play a contributing role to β-cell failure if the inflamed islets of prediabetic type 1 individuals have increased susceptibility to the impairing effect of FFA. A chronic increase in FFA may be induced by incipient insulin deficiency or by concomitant obesity. Indeed, there is evidence that overweight, which is associated with elevated FFA, is a risk factor for latent autoimmune diabetes (19), which is a slow-onset form of type 1 diabetes, as well as for type 1 diabetes in general ("accelerator hypothesis," reviewed in Ref. 20).

Data in animals suggest that FFA may be one link between obesity and acceleration of autoimmune diabetes. For example, fat restriction (21) or depletion of n-6 fatty acids (22) reduced the incidence of diabetes in the nonobese diabetic mouse. To investigate whether the presence of autoimmune inflammation could predispose β-cells to the effects of fat, we evaluated β-cell secretory function during a two-step hyperglycemic clamp, and *ex vivo* in isolated islets, after prolonged (*i.e.* 48 h) iv fat infusion [standard Intralipid plus heparin (IH) method to elevate plasma FFA] in dp-BB, dr-BB, and normal Wistar-Furth (WF, the strain of origin of BB rats) rats.

## Materials and Methods

### Animals

Animal experiments were carried out according to protocols approved by the University of Toronto Animal Care Committee. Nine-week-old female dp-BB and dr-BB rats were obtained from Health Canada (Ottawa, Canada). The incidence of diabetes in dp-BB rats from this colony is 65.3 ± 14.9% (mean ± SD) (23). These animals carry a mutation of a member of the immunity-associated nucleotide binding protein family, which is important for T lymphocyte survival (24). The dr-BB rats are derived from a subline of the original BB rat colony that does not spontaneously develop diabetes. Age-matched female WF rats, which are major histocompatibility complex-compatible with BB rats (25), were also used as controls. These rats were obtained from Harlan (Indianapolis, IN), because Health Canada does not breed them. The rats were housed in the Department of Comparative Medicine. They were exposed to a 12-h light, 12-h dark cycle and were fed standard rat chow. Random plasma glucose levels were tested at least twice per week using a glucometer (Bayer, Toronto, Canada). Only 11-wk-old rats with glucose values less than 11 mmol/liter were used. We therefore excluded the dp-BB rats that developed diabetes (approximately one out of three dp-BB rats obtained at 9 wk). Animals were cannulated as in Ref. 26. The jugular catheter served for infusion and the carotid catheter for blood sampling. Rats were allowed at least 3 d after surgery to recover before infusions.

### Forty-eight-hour infusions

Rats were randomized to one of the following protocols: saline (SAL) or IH. Average body weights (in grams) before infusions were: dp-BB SAL, 227 ± 11, n = 9; dp-BB IH, 215 ± 8, n = 10; dr-BB SAL, 210 ± 5, n = 11; dr-BB IH, 212 ± 5, n = 11; WF SAL, 166 ± 3, n = 8; and WF IH, 174 ± 5, n = 9) ($P < 0.001$, WF *vs.* dp-BB or dr-BB). A subset of dp-BB rats was also infused with IH or SAL plus the antioxidant N-acetylcysteine (NAC) (2.76 μmol/kg·min), which protected against fat-induced β-cell dysfunction in normal Wistar rats (27). Intralipid (Baxter Corp., Toronto, Canada) is a commercially prepared triglyceride emulsion, which contains mostly n-6 polyunsaturated fatty acids (26). Heparin stimulates the breakdown of Intralipid to fatty acids by lipoprotein lipase. 20% Intralipid was infused at 2.5 μl/min in the dp-BB, 3 μl/min in the dr-BB rats, and 5.5 μl/min in the WF rats to approximately double plasma FFA. Heparin had been added to the Intralipid to reach a concentration of 20 U heparin/ml. Different doses of IH were necessary to obtain a similar FFA elevation in the different groups, presumably because of the previously reported elevation of apolipoprotein-CII (activator of lipoprotein lipase) in BB *vs.* Wistar rats (28). The infusion procedure has been described in Ref. 27. After 48 h of infusion and overnight fasting, we carried out one of the following protocols: 1) hyperglycemic clamp, 2) hyperinsulinemic clamp, or 3) islet isolation.

### Two-step hyperglycemic clamp

Insulin secretion was determined by measuring plasma insulin and C-peptide during a two-step (~13 and 22 mmol/liter) hyperglycemic clamp that was performed in conscious rats. The clamp details are described in Refs. 26, 27. At the end of the clamp, rats were anesthetized with a ketamine:xylazine:acepromazine cocktail

(27), and the whole pancreas was removed, fixed overnight in Bock's solution, and then stored in 70% ethanol. The samples were embedded in paraffin within 5 d of collection.

### Hyperinsulinemic-euglycemic clamp

A 120-min hyperinsulinemic-euglycemic clamp, as described in Ref. 29, was conducted in conscious rats fasted overnight to determine insulin sensitivity.

### Islet isolation and *ex vivo* evaluation of glucose-stimulated insulin secretion (GSIS)

Pancreatic islets were isolated using the Ficoll/Histopaque method as described in Ref. 30. GSIS was evaluated as in Ref. 30.

### Real-time PCR

Total rat islet RNA was extracted as previously described (16). Quantitative PCR was done using commercial TaqMan gene expression assays and the real-time PCR system 7500 of Applied Biosystems (Foster City, CA). TaqMan assays are available upon request. Changes in mRNA expression were calculated using difference of cycle threshold values compared with a housekeeping gene (18S), expressed relative to controls.

### Immunohistochemistry

Pancreatic slides were double-stained for insulin and glucagon using antibodies from Abcam, Inc. (Cambridge, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) coupled with an alkaline phosphatase and a peroxidase detection method, respectively. Cluster of differentiation (CD) 3 and 68 staining were performed using antibodies from Dako Corp. (Carpinteria, CA) and a peroxidase detection method. Adjacent sections were stained for insulin using an antiinsulin antibody from Biomeda (Foster City, CA) coupled with peroxidase detection.

The insulitis score for each islet was as follows. Score 0, no infiltration. Score 1, infiltration of lymphocytes and/or leukocytes, macrophages, dendritic cells in islet periphery. Score 2, infiltration into the islet core. Score 3, infiltration into the islet core associated with $\beta$-cell destruction (distorted islet). Score 4, complete loss of $\beta$-cells with variable infiltration in remnant islet.

Relative $\beta$-cell area was determined from the ratio between areas of insulin-positive cells and total pancreatic area. Three sections of the entire pancreas separated by 100 $\mu$m were used as in our previous studies (30).

### Plasma assays

Glucose was measured with a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA), FFA with a colorimetric kit (Wako Industries, Neuss, Germany), and insulin and C-peptide with RIA kits (Linco Research, Inc., St. Charles, MO) (intra- and interassay coefficients of variation $< 10\%$). Cytokines were run on Lincoplex.

### Calculations

#### *Insulin sensitivity*

The index of sensitivity M/I [glucose metabolism (M), which is represented by Ginf divided by plasma insulin (I)] (31) was calculated during both the hyperglycemic clamp and the hyperinsulinemic clamp by dividing the steady-state glucose infusion rate (Ginf) by the plasma insulin level.

#### *β-Cell function*

Plasma C-peptide was taken as an index of absolute insulin secretion, as insulin secretion rate cannot be calculated in rats, because C-peptide kinetics are unknown (the species-specific rat C-peptide is not available for injection). Notably, IH has not been found to affect C-peptide kinetics in humans (32). Insulin secretion *in vivo* has to be evaluated in the context of insulin sensitivity, because the normal $\beta$-cell compensates for insulin resistance by increasing secretion, independent of plasma glucose (33). The relationship between insulin sensitivity and insulin secretion in normal humans is hyperbolic, *i.e.* the product of insulin sensitivity and insulin secretion is a constant defined as disposition index (DI) and considered as a measure of $\beta$-cell function (33, 34). In our SAL-treated control rats, the relationship between C-peptide and M/I index (from the hyperglycemic clamp) was also hyperbolic (C-peptide $\times$ M/I = DI, a constant), that is there was a significant inverse relationship between the two variables after logarithmic transformation ($r^2 = 0.41$, $P < 0.001$ in WF rats; $r^2 = 0.60$, $P < 0.001$ in dr-BB rats) with slopes not significantly different from $-1$ ($-0.85 \pm 0.15$ and $-0.96 \pm 0.17$, respectively). Therefore, we calculated DI as the product of C-peptide and the M/I index (from the hyperglycemic clamp) during the last 40 min of each step of the hyperglycemic clamp.

We also calculated DI using the M/I index from the hyperinsulinemic-euglycemic clamp (gold standard method for assessing insulin sensitivity), multiplied by C-peptide from the hyperglycemic clamp. This was done with the group averages, as the hyperinsulinemic-euglycemic and hyperglycemic clamps were not performed in the same rat because of the invasiveness of a combined clamp protocol (see *Results*).

In addition, we corrected insulin secretion for insulin sensitivity with statistical methods. C-peptide was adjusted for M/I during the hyperglycemic clamp via covariance analysis as previously used in epidemiological studies (35). Both variables were logarithmically transformed and ln(M/I) was inserted as a covariate in a linear model between ln(C-peptide) as dependent variable, and group (dp-BB, dr-BB, and WF), treatment (SAL and IH) and their interaction as independent variables. The method is conceptually similar to the calculation of DI, as can be seen by the logarithmic transformation of the hyperbolic relationship C-peptide $\times$ M/I = DI, which yields ln(C-peptide) + ln(M/I) = ln(DI) and after rearrangement ln(C-peptide) = ln(DI) (*i.e.* a constant) $- 1 \times$ ln(M/I) (note the slope of $-1$). The hypothesis to be tested by the model was that the effect of treatment on the adjusted ln(C-peptide) levels would have significantly differed between groups (different interaction effect). The statistical method is a more conservative method of adjusting insulin secretion for insulin sensitivity than calculating a DI, because the error of the inverse relationship is taken into account.

### Statistical analysis

Data are presented as mean $\pm$ SE. A nonparametric one-way ANOVA for repeated measurements was used to examine the difference between treatments within each group. Data were also analyzed within treatments to examine the difference between groups using nonparametric ANOVA for repeated measurements followed by Tukey's *post hoc*. The calculations were performed using SAS (SAS Institute, Cary, NC). Significance was accepted at $P < 0.05$.

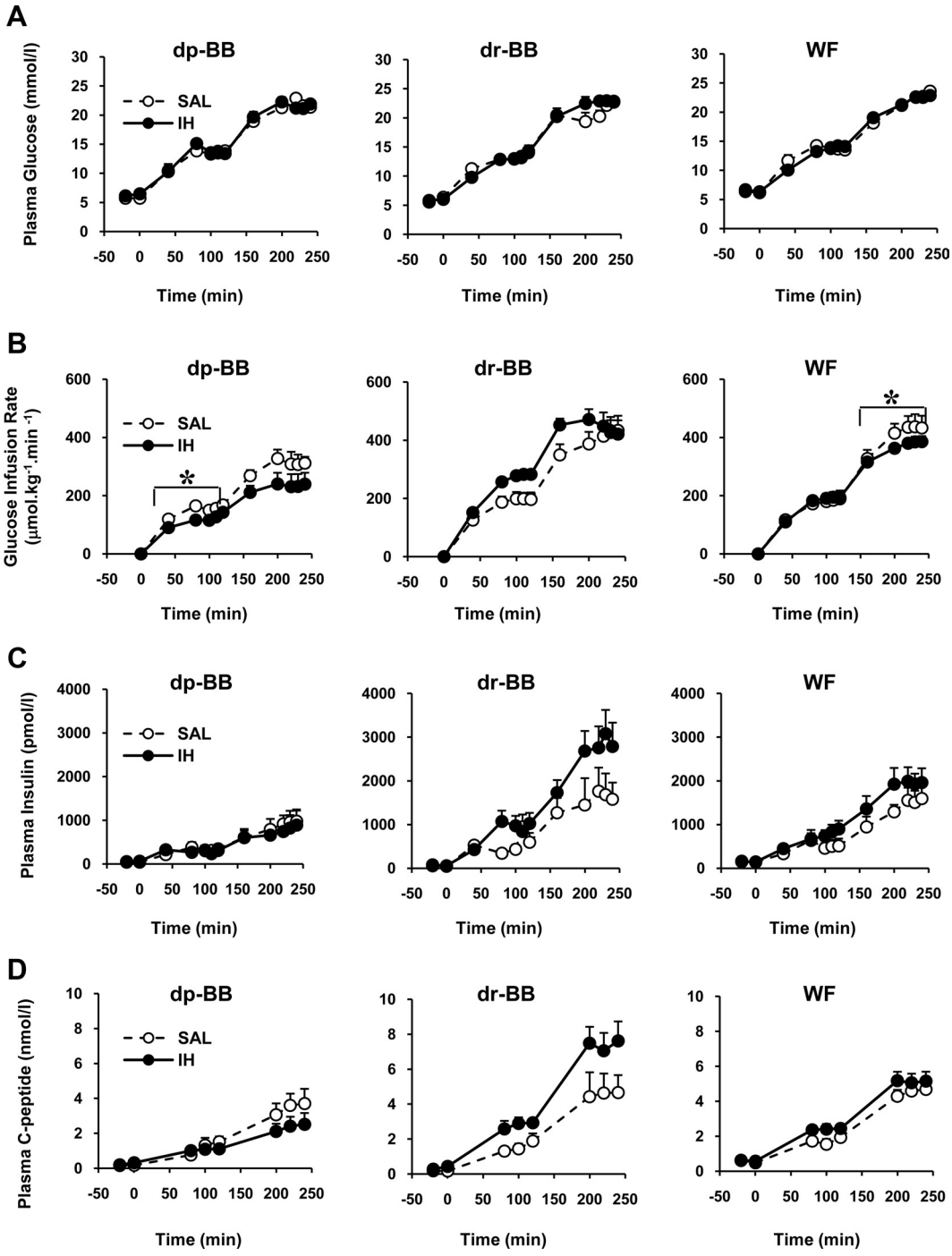


**FIG. 1.** Plasma glucose levels (A), Ginf (B), plasma insulin (C), and plasma C-peptide (D) during a two-step hyperglycemic clamp in dp-BB, dr-BB, and WF rats infused for 48 h with IH or equivolume SAL. Data are mean ± SE. Number of animals studied (n), 8-11 per group. During both steps of the hyperglycemic clamp, the glucose levels were superimposable in all groups (A). Ginf was reduced in IH-infused dp-BB and WF, but not dr-BB, rats (B). No differences in basal insulin and C-peptide were observed between IH- and SAL-infused rats. Clamp insulin and C-peptide tended to be higher with IH than SAL in dr-BB rats (C and D). *, $P < 0.05$ *vs.* SAL.

## Results

### Preclamp (48-h infusion) period

In the rats used for the hyperglycemic clamps, FFA levels were elevated to approximately 2-fold basal with prolonged IH infusion in all groups. In dr-BB rats, the FFA elevation appeared to be less than that in dp-BB rats; however, the difference was not significant. Furthermore, no significant differences in both basal and elevated FFA levels were found between groups (Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Total FFA elevation over the 48-h period was also similar among IH-

**TABLE 1.** Sensitivity index calculated during the two-step (13 and 22 mmol/liter) hyperglycemic clamp after 48 h of SAL or IH infusion in dp-BB, dr-BB, and WF rats

| | | | dp-BB | dr-BB | WF |
|---|---|---|---|---|---|
| Sensitivity index[a] | 13mmol/liter | SAL | 0.72 ± 0.13 | 0.67 ± 0.13 | 0.58 ± 0.13 |
| | | IH | 0.47 ± 0.05 | 0.42 ± 0.05 | 0.39 ± 0.05 |
| | 22mmol/liter | SAL | 0.57 ± 0.12 | 0.43 ± 0.08 | 0.36 ± 0.06 |
| | | IH | 0.49 ± 0.09 | 0.18 ± 0.03 | 0.24 ± 0.03 |

Number of animals studied (n), 8-11 per group.

[a] Units of sensitivity index, $\mu mol \cdot kg^{-1} \cdot min^{-1}$ glucose infusion per pmol/liter insulin (last 40 min).

infused rats used for islet secretion studies (dp-BB, 10,203 μEq/liter; dr-BB, 11,157 μEq/liter).

Baseline fed plasma glucose and plasma glucose levels during the 48-h IH/SAL infusion were not significantly different between groups or treatments (Supplemental Fig. 1B).

Baseline fed plasma insulin levels and insulin levels during the 48-h IH/SAL infusion were also similar between groups or treatments (Supplemental Fig. 1C).

Neither glucose nor insulin levels were different between groups or treatments in the rats used for the islet secretion studies (data not shown).

## Two-step hyperglycemic clamp

During the basal period (−20 to 0 min), plasma FFA levels were elevated by approximately 2-fold. Neither the FFA elevation nor the elevated FFA levels were significantly different between groups. FFA declined throughout the two-step hyperglycemic clamp as a result of hyperglycemia and hyperinsulinemia. However, FFA remained higher in all IH-treated groups (Supplemental Table 1).

There were no differences in basal glucose among groups or treatments. During the first step of the two-step hyperglycemic clamp, plasma glucose levels rose to 13 mmol/liter, which was maintained until 120 min. During the second step of the clamp, plasma glucose levels gradually rose to 22 mmol/liter, which was maintained until 240 min. During both steps of the clamp, the glucose levels were superimposable in all groups or treatments (Fig. 1A).

The Ginf necessary to achieve and maintain the hyperglycemic targets was lowered by IH infusion in dp-BB ($P < 0.05$ *vs.* SAL at 13 mmol/liter glucose) and WF ($P < 0.05$ *vs.* SAL at 22 mmol/liter glucose), but not dr-BB, rats (Fig. 1B).

As expected, plasma insulin and C-peptide concentrations rose in response to glucose during the clamp. IH did not significantly change insulin or C-peptide response in any group, although it tended to increase it in dr-BB rats (Fig. 1, C and D).

The ratio between C-peptide and insulin (data not shown), which is an approximate index of insulin clearance, was not significantly decreased by IH in any groups.

IH infusion tended to decrease insulin sensitivity, assessed by the sensitivity index (M/I index), in all groups; however, this was not significant (Table 1).

Because we could find a hyperbolic relationship between M/I and C-peptide levels in both our control groups (SAL-treated WF and dr-BB rats) as described in *Materials and Methods*, we calculated the DI, which is an index of

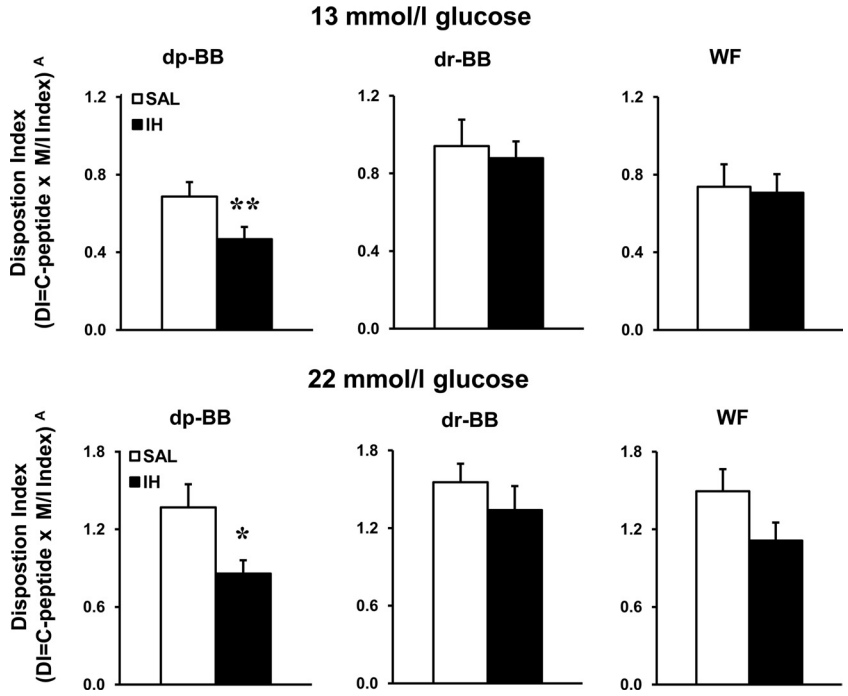


**FIG. 2.** DI (calculated as plasma C-peptide level multiplied by the sensitivity index M/I reported in Table 1) during the last 40 min of each step of the two-step hyperglycemic clamp (13 and 22 mmol/liter) in dp-BB, dr-BB, and WF rats infused for 48 h with IH or SAL. Data are mean ± SE. Number of animals studied (n), 8-11 per group. DI was reduced to the greatest extent by IH in dp-BB rats. IH tended to decrease DI at 22 mmol/liter glucose in WF rats. IH did not decrease DI in dr-BB rats both at 13 and 22 mmol/liter. A, Units of DI = C-peptide multiplied by $\mu mol \cdot kg^{-1} \cdot min^{-1}$ glucose infusion per pmol/liter insulin. *, $P < 0.05$ *vs.* SAL; **, $P < 0.01$ *vs.* SAL.

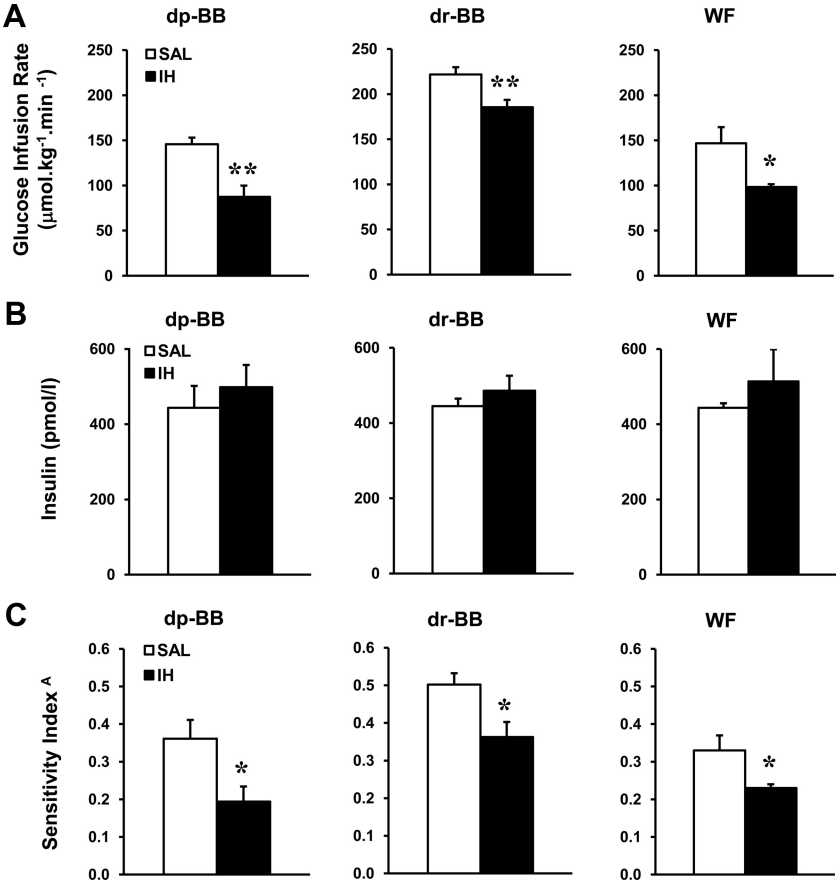


**FIG. 3.** Ginf (A), plasma insulin (B), and sensitivity index (C) during a hyperinsulinemic-euglycemic clamp in dp-BB, dr-BB, and WF rats infused for 48 h with IH or SAL. Data are mean ± SE. Number of animals studied (n), four to seven per group. Ginf (last 30 min) was reduced by IH in dp-BB, dr-BB, and WF rats (A). Similar levels of insulin (last 30 min) were achieved during the hyperinsulinemic-euglycemic clamp (B). Sensitivity index, calculated by dividing Ginf by insulin, was reduced to the greatest extent by IH in dp-BB rats (~52%) compared with dr-BB and WF rats (~30%) (C). A, Units of sensitivity index, $\mu mol \cdot kg^{-1} \cdot min^{-1}$ glucose infusion per pmol/liter insulin. *, $P < 0.05$ *vs.* SAL; **, $P < 0.01$ *vs.* SAL.

insulin secretion corrected for insulin sensitivity. IH decreased DI in dp-BB rats ($P < 0.01$ at 13 mmol/liter glucose; $P < 0.05$ at 22 mmol/liter glucose). IH tended to decrease DI in WF during the second step of the clamp and had no effect on DI in dr-BB rats (Fig. 2).

Supplemental Table 2 shows the whole clamp C-peptide levels (in logarithmic form) after adjustment for insulin sensitivity [ln(M/I)]. IH treatment significantly decreased adjusted ln(C-peptide) only in dp-BB rats. Thus, also using this conservative method of adjustment of insulin secretion for insulin sensitivity, the results were similar to those obtained using the DI method.

## Hyperinsulinemic-euglycemic clamp

Ginf during the last 30 min of the 2-h hyperinsulinemic-euglycemic clamp is an indicator of whole body insulin sensitivity. IH infusion significantly decreased Ginf in dp-BB, dr-BB, and WF rats compared with SAL infusion (Fig. 3A). Plasma insulin levels during the last 30 min of the clamp did not differ between SAL and IH in all groups (Fig. 3B). M/I index, calculated by dividing Ginf by insulin, was significantly reduced by IH infusion in all groups. dp-BB rats infused with IH had the greatest decrease in M/I (52%) compared with 30% in WF and 28% in dr-BB rats (Fig. 3C).

Because the M/I index derived from the hyperinsulinemic-euglycemic clamp is a better indicator of insulin sensitivity than that from the hyperglycemic clamp, we also calculated DI from the M/I index of the hyperinsulinemic-euglycemic clamp. We used group averages, and we could not calculate any SE, because the hyperinsulinemic and the hyperglycemic clamps were performed on separate animals. This had to be done because, although we commonly perform both clamps in the same subject in humans (36), it is too invasive to perform prolonged infusions followed by both clamps in the same small animal (*i.e.* there is a high experimental drop-out and the results the clamp performed last are heavily affected by stress). DI, calculated as the product of M/I index (group averages for hyperinsulinemic clamp) and C-peptide (group averages for hyperglycemic clamp), was lowered by IH infusion in dp-BB at both 13 and 22 mmol/liter glucose. IH infusion in WF lowered DI at 22 mmol/liter glucose but to a much lesser extent than that observed in IH-infused dp-BB rats. IH infusion in dr-BB rats did not decrease DI (Supplemental Fig. 2).

## Insulin secretion in isolated islets

Insulin secretion at 2.8 and 6.5 mmol/liter glucose did not differ between SAL- and IH-infused rats in all groups. In dp-BB rats, insulin secretion was significantly impaired by IH infusion at 13 mmol/liter ($P < 0.05$) and 22 mmol/liter ($P < 0.001$) glucose. In contrast, no impairment in insulin secretion at 13 and 22 mmol/liter glucose was observed in dr-BB and WF rats infused with IH compared with SAL infusion (Fig. 4). IH infusion did not significantly affect islet insulin content in any group. However, WF had low insulin content (in nanograms per islet) compared with dr-BB and dp-BB rats (dp-BB SAL, 138.9 ± 30.1, n = 5; dp-BB IH, 123.0 ± 4.1, n = 6; dr-BB SAL,

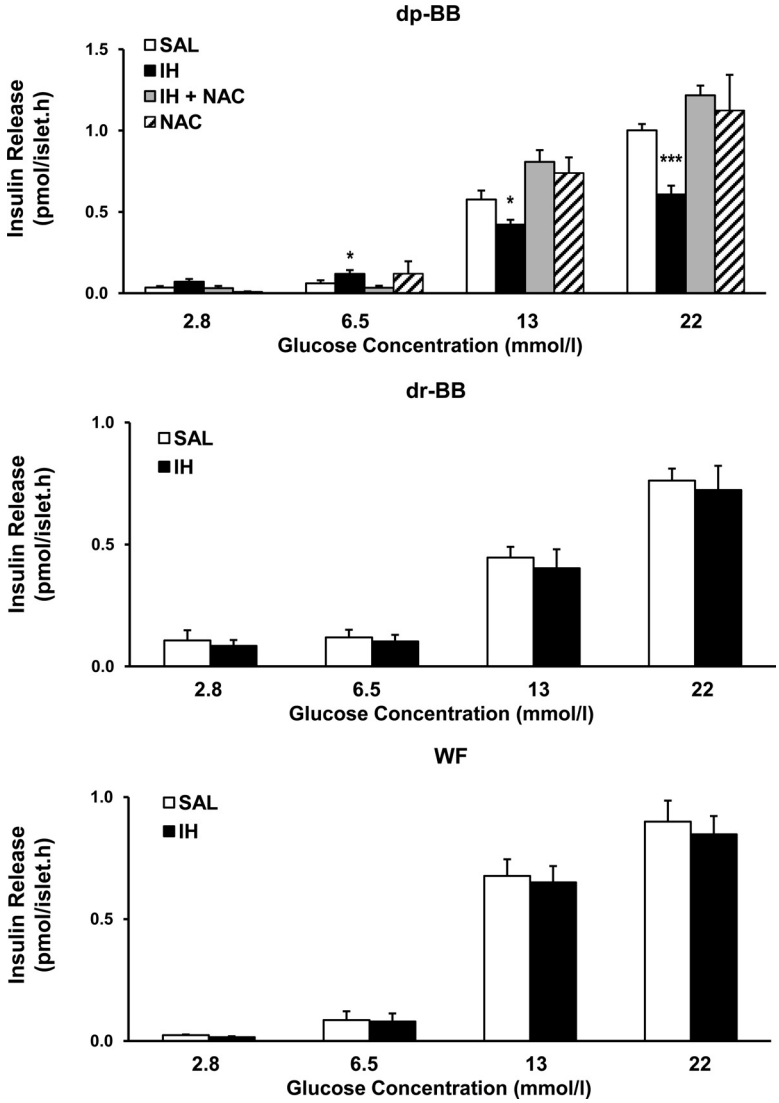


**FIG. 4.** GSIS in isolated islets of dp-BB, dr-BB, and WF rats infused for 48 h with IH or SAL. Data are mean ± SE, n = 5-12 per group; n represents the number of rats (each studied at all glucose concentrations in triplicate). IH had no effects on insulin release at 2.8 and 6.5 mmol/liter glucose, except in dp-BB at 6.5 mmol/liter. IH significantly impaired GSIS at 13 and 22 mmol/liter glucose in dp-BB rats. Coinfusion of the antioxidant NAC with IH completely restored GSIS (A). In contrast, IH did not impair GSIS in dr-BB or WF rats (B and C). *, $P < 0.05$ *vs.* SAL; ***, $P < 0.001$ *vs.* SAL.

148.5 ± 14.4, n = 4; dr-BB IH, 188.8 ± 16.5, n = 4; WF SAL, 39.2 ± 16.2, n = 6; and WF IH, 22.7 ± 5.4, n = 5).

## Effect of NAC

To determine whether the β-cell dysfunction induced by IH infusion in dp-BB rats is oxidative stress dependent, we coinfused the antioxidant NAC with IH. NAC completely prevented the impairment in GSIS induced by IH in islets of dp-BB rats ($P < 0.001$ at both 13 and 22 mmol/liter glucose). NAC alone without IH infusion did not have any significant effect (Fig. 4). NAC also prevented IH-induced β-cell dysfunction in dp-BB rats *in vivo* during the hyperglycemic clamp (Supplemental Fig. 3).

## Islet cytokine mRNA levels

IH infusion increased mRNA expression of proinflammatory (IL-1β and TNF-α), and type 1 [interferon (IFN)-γ] cytokines in islets of dp-BB, but not those of dr-BB or WF, rats. Likewise, the mRNA of monocyte chemoattractant protein-1 (MCP-1) was elevated by IH in dp-BB, but not dr-BB or WF, rats. This was similarly observed for the mRNA of type 2 cytokines (IL-4, IL-6, and IL-10), IL-1β receptor antagonist, a natural inhibitor of IL-1β, and TGF-β, a type 3 cytokine, which can suppress secretion of type 1 cytokines. The mRNA expression of cytokine-inducible isoform of NOS (iNOS) and cytokine-inducible isoform of cyclooxygenase-2 (COX-2), were also increased by IH in dp-BB rats (for iNOS, one in six detectable for dp-SAL; three in six detectable for IH) but not dr-BB or WF rats. Coinfusion of the antioxidant NAC with IH in dp-BB rats did not appear to reduce the mRNA of proinflammatory cytokines, with the exception of IFN-γ, where mRNA was detectable in only one of six samples. NAC did not significantly affect MCP-1 and COX-2 mRNA. NAC did appear to reduce iNOS mRNA, because this was only detectable in one of six samples (Fig. 5).

## Plasma cytokine levels

IH infusion elevated plasma levels of MCP-1 and IL-10 compared with SAL infusion in dp-BB rats. No other changes in plasma cytokine levels were noted between SAL and IH infusion in dp-BB, dr-BB, or WF rats. Interestingly, dp-BB rats had lower plasma IFN-γ levels compared with dr-BB or WF rats (Table 2). This is in accordance with the previously described findings that dp-BB rats have lower IFN-γ production in the gut lymphoid tissue (37). dp-BB rats also had lower levels of the type 2 (antiinflammatory) cytokines IL-4 and IL-10 (the SAL group), consistent with their susceptibility to autoimmune diabetes (38).

## Pancreas histology

Figure 6A shows a typical islet from control WF rats. Islets from dr-BB rats (Fig. 6B) were similar, *i.e.* there were no signs of insulitis in any of the rats. However, all dp-BB

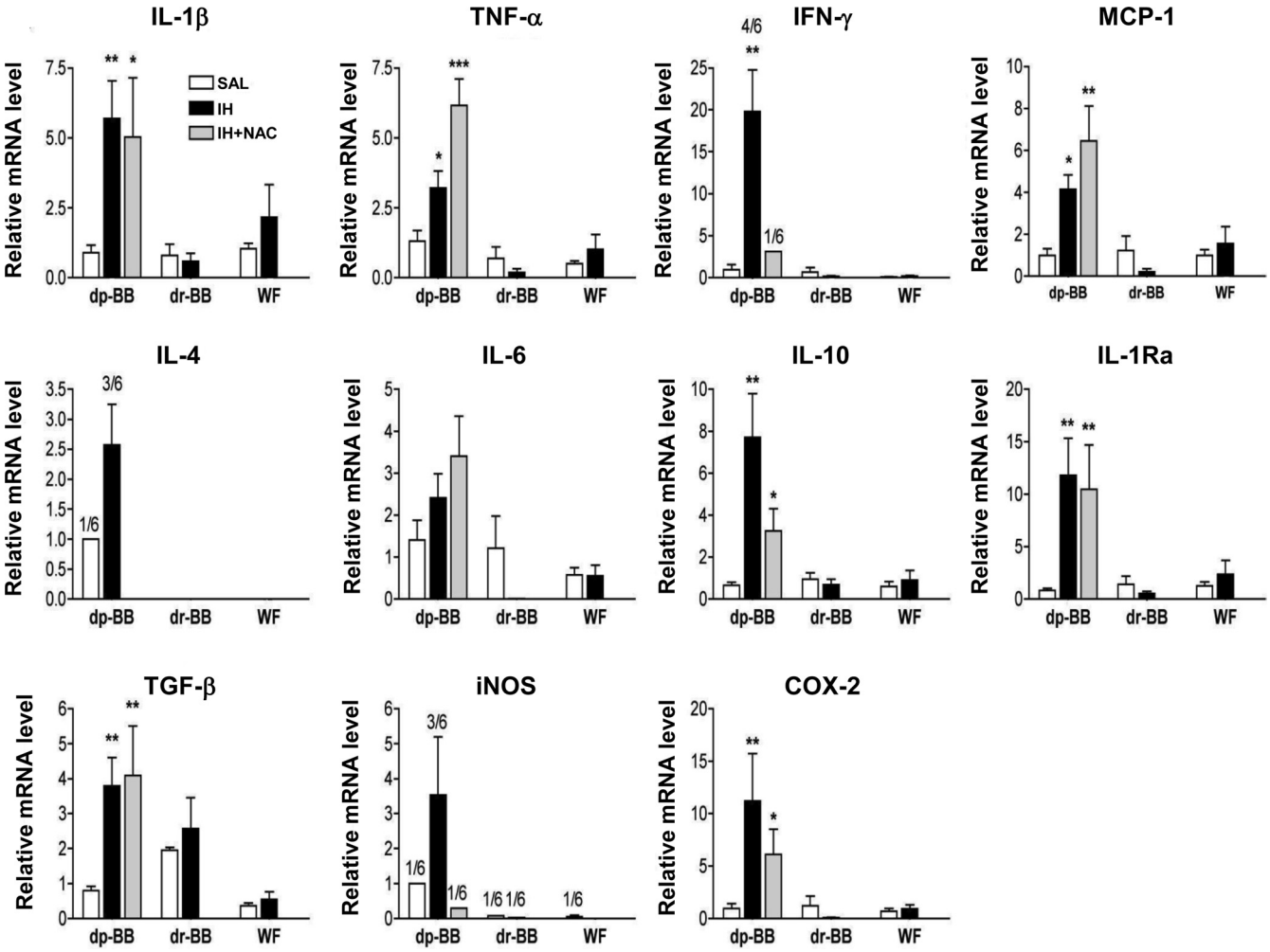


**FIG. 5.** mRNA levels of cytokines and inflammatory markers in isolated islets of dp-BB, dr-BB, and WF rats infused for 48 h with IH or SAL. Data are mean ± SE. The number of rats (n) studied, six per group. Changes in mRNA expression were calculated using difference of cycle threshold values compared with a housekeeping gene (18S) and expressed relative to SAL controls. IH infusion increased mRNA expression of proinflammatory (IL-1β and TNF-α) and type 1 (IFN-γ) cytokines in islets of dp-BB, but not dr-BB or WF, rats. Likewise, the mRNA of MCP-1 was elevated by IH in dp-BB, but not dr-BB or WF, rats. This was similarly observed for the mRNA of type 2 cytokines (IL-4, IL-6, and IL-10), IL-1β receptor antagonist (IL-1Ra), a natural inhibitor of IL-1β, and TGF-β, a type 3 cytokine, which can suppress secretion of type 1 cytokines. The mRNA expression of cytokine-inducible isoform of NOS (iNOS) and cytokine-inducible isoform of cyclooxygenase COX-2 was also increased by IH in dp-BB rats (for iNOS, one in six detectable for dp-SAL; three in six detectable for IH) but not dr-BB or WF rats. Coinfusion of the antioxidant NAC with IH in dp-BB rats did not appear to reduce the mRNA of proinflammatory cytokines, with the exception of IFN-γ, where mRNA was detectable in only one of six samples. NAC did not significantly affect MCP-1 and COX-2 mRNA. NAC did appear to reduce iNOS mRNA, because this was only detectable in one of six samples. *, $P < 0.05$ *vs.* SAL; **, $P < 0.01$ *vs.* SAL; ***, $P < 0.001$ *vs.* SAL.

rats had mononuclear infiltration of some islets (*i.e.* insulitis) (Fig. 6D), whereas other islets of the same rats appeared to be intact (Fig. 6C). Figure 6E shows that the mononuclear cells present in the islets of dp-BB rats were positive for CD3 (lymphocytic marker). Figure 6F shows the insulin staining of an adjacent section, thus confirming that the accumulation of CD3-positive cells is in islets. There were no CD3-positive cells in islets of dr-BB or WF rats (data not shown).

Insulitis tended to be reduced by IH in dp-BB rats, although this did not reach statistical significance (Fig. 6G). The ratio of the number of islets that have insulitis (categorized by insulitis scores 1-4) to the total number of islets present in the pancreas also tended to be reduced by IH (Fig. 6H). IH infusion significantly decreased the T lymphocyte marker CD3 (dp-BB SAL, 2.96 ± 0.38, n = 6; dp-BB IH, 0.32 ± 0.27, n = 5, expressed as average number of CD3-positive cells per islet; $P < 0.001$) and tended to decrease the macrophage marker CD68 (dp-BB SAL, 1.35 ± 0.41, n = 6; dp-BB IH, 0.62 ± 0.36, n = 5, expressed as average number of CD68-positive cells per islet) staining in the islets.

β-Cell area (expressed as percentage of pancreas area) was significantly increased by IH in dr-BB rats (dr-BB SAL, 0.563 ± 0.024%, n = 6, *vs.* dr-IH, 0.762 ± 0.022%, n = 6; $P < 0.05$) but not dp-BB rats (dp-BB SAL, 0.495 ±

**TABLE 2.** Plasma cytokine levels (mean ± SE) after 48 h of SAL or IH infusion in dp-BB, dr-BB, and WF rats

| | MCP-1 (pg/ml) | IL-4 (pg/ml) | IL-1β (pg/ml) | IL-2 (pg/ml) | IL-10 (pg/ml) | IFN-γ (pg/ml) |
|---|---|---|---|---|---|---|
| dp-BB SAL | 118 ± 21.4 *n*=6 | 28.6 ± 10.7 *n*=4 | 25.7 ± 13.4 *n*=5 | 417.2 ± 168.7 *n*=5 | 118.9 ± 22.4 *n*=4 | 73.2 ± 24.9 *n*=4 |
| dp-BB IH | 236.9 ± 26.1[b] *n*=6 | 37.4 ± 10.3 *n*=4 | 40.5 ± 8.1 *n*=6 | 309.1 ± 70.7 *n*=5 | 882.6 ± 200.5[a] *n*=6 | 26.7 ± 15.3 *n*=4 |
| dr-BB SAL | 72.9 ± 16.0 *n*=5 | 244.8 ± 144.3 *n*=6 | 14.3 ± 2.9 *n*=6 | 72.5 ± 31.6 *n*=6 | 316.0 ± 116.2 *n*=5 | 510.7 ± 169.7 *n*=5 |
| dr-BB IH | 140.0 ± 38.2 *n*=6 | 236.4 ± 77.8 *n*=5 | 22.1 ± 4.5 *n*=5 | 195.3 ± 111.2 *n*=4 | 546.2 ± 127.5 *n*=6 | 603.0 ± 211.9 *n*=6 |
| WF SAL | 189.8 ± 87.1 *n*=3 | 281.6 ± 77.2 *n*=6 | 12.3 ± 3.5 *n*=5 | 446.1 ± 102.2 *n*=5 | 485.3 ± 87.4 *n*=6 | 830.7 ± 108.0 *n*=4 |
| WF IH | 172.7 ± 20.1 *n*=5 | 201.6 ± 72.4 *n*=6 | 53.2 ± 40.4 *n*=4 | 198.0 ± 108.23 *n*=4 | 752.2 ± 170.4 *n*=6 | 1325.9 ± 442.7 *n*=6 |

TNF-α was measured but was undetectable in all groups.

[a] $P < 0.05$ *vs.* SAL.

[b] $P < 0.01$ *vs.* SAL.

0.075%, n = 9 *vs.* dp-BB IH, 0.552 ± 0.060%, n = 9) or WF rats (WF SAL, 1.000 ± 0.068%, n = 6 *vs.* WF IH, 0.863 ± 0.081%, n = 5). Interestingly, WF rats that had the lowest islet insulin content (see under *Insulin secretion in isolated islets*) had the highest β-cell area, suggesting that their pancreatic insulin stores were likely similar to those in the BB rats.

## Discussion

We here wished to determine whether the presence of autoimmune insulitis, such as that found in the islets of prediabetic dp-BB rats, sensitizes animals to the lipotoxic effect of fat on β-cell function. Pancreas histology showed insulitis in dp-BB rats, which was absent in dr-BB and WF rats. β-Cell function evaluated *in vivo* by the DI, and *ex vivo* in isolated islets, was not significantly affected by 48-h fat infusion in either dr-BB or WF rats but was reduced in dp-BB rats. IH infusion elevated plasma and islet cytokine and chemokine expression only in dp-BB, but not in dr-BB or WF, rats.

WF rats required a greater IH infusion rate to achieve a similar FFA elevation as dp-BB and dr-BB rats. This is presumably because of the previously described higher levels of lipoprotein lipase activator apolipoprotein-CII in BB rats (28). WF rats were also significantly smaller than BB rats. Despite these differences, we chose to use the WF rats as an additional control group, because dr-BB rats are not completely normal, because they develop autoimmune diabetes after depletion of regulatory RT6-positive T cells (2).

Plasma glucose and insulin levels did not differ during the 48-h infusion between IH- or SAL-infused rats in all groups. This suggests that the impairment in β-cell function in dp-BB rats infused with IH is likely due to FFA elevation and not to glucotoxicity and/or an increase in insulin demand. This is however not to say that the impairment in β-cell function in dp-BB rats is specific for fat. It is likely that a similar effect would be seen with sustained hyperglycemia, in which inflammation has also been implicated (39).

The relationship between insulin sensitivity and insulin secretion is hyperbolic, so that the DI, which is a product of insulin sensitivity and insulin secretion, remains constant in subjects with normal glucose tolerance (33, 34). Prolonged elevation of plasma FFA levels decreased insulin sensitivity, as assessed by the gold standard hyperinsulinemic-euglycemic clamp, in all three groups. dr-BB rats, and to a lesser extent WF rats, appropriately responded to this decrease in sensitivity by attempting to elevate their insulin secretion (as assessed by the C-peptide levels during the hyperglycemic clamp), so that DI did not change. dp-BB, in contrast, failed to increase insulin secretion despite the induction of insulin resistance by IH, thus DI of IH-infused dp-BB was reduced compared with SAL.

DI was calculated using insulin sensitivity from the hyperglycemic clamp as well as insulin sensitivity from hyperinsulinemic-euglycemic clamps. We also used a statistical method to adjust insulin secretion for insulin sensitivity. Irrespective of the method used to evaluate β-cell function *in vivo*, we obtained similar findings, *i.e.* that IH infusion decreases β-cell function only in dp-BB rats. That IH impaired β-cell function only in dp-BB rats is also suggested by our *ex vivo* islet secretion studies. It should be noted that islets of dp-BB rats tended to differ in the degree of insulitis (*i.e.* ~40% of islets of SAL-infused rats and 20% of IH-infused rats had insulitis), and thus, if islet isolation resulted in the selection of noninfiltrated islets (which we cannot totally exclude although is not

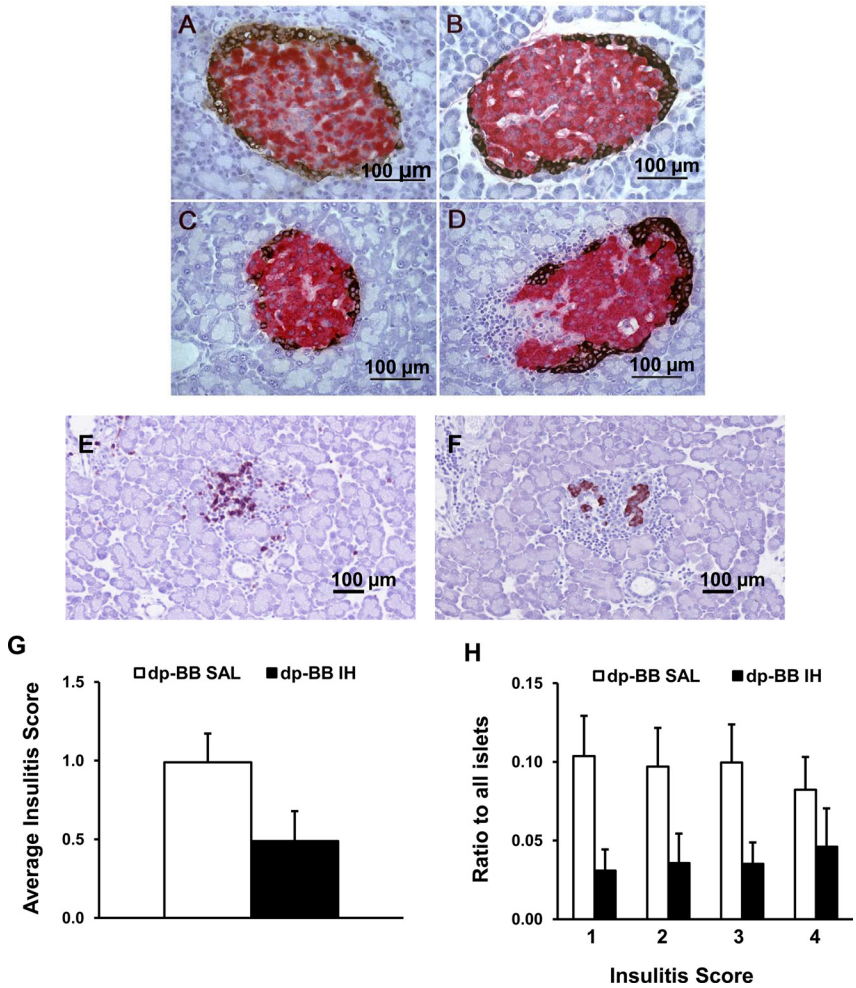


**FIG. 6.** Representative photographs of islets of WF (A), dr-BB (B), and dp-BB rats (C and D) (magnification, ×400). The islet of the dp-BB rat shown in D is infiltrated with mononuclear cells; however, the islet from the same rat in C appears to be intact, as are all islets of dr-BB rats (B) or WF rats (A). E and F, Infiltrated islets of dp-BB rats stained for CD3 (E) and insulin (F) in adjacent sections (magnification, ×200). CD3-positive cells were found in infiltrated islets of dp-BB rats. G, IH infusion tended to lower average insulitis score in dp-BB rats, although this was not significant. H, Ratio of the number of islets that have insulitis (categorized by insulitis scores 1-4) to the total number of islets present in the pancreas of dp-BB rats. G and H, Data are mean ± SE. Number of animals studied (n), five to nine per group.

supported by the cytokine mRNA results), the *ex vivo* GSIS may not fully represent the *in vivo* condition. Because of this possible limitation, the *ex vivo* data should be interpreted in conjunction with the *in vivo* hyperglycemic clamp study.

Our results suggest that insulitis predisposes dp-BB islets to fat-induced increase in cytokines and chemokines, presumably because of increased production by infiltrating inflammatory cells and/or up-regulation of inflammatory pathways in islet cells. The molecular mechanisms responsible for this up-regulation remain to be investigated, although activation of nuclear factor κB (NF-κB) and other transcription factors induced in inflammation, such as c-Jun, is likely. In INS-1 cells, both fatty acids and cytokines activated NF-κB in one study (40), although this was not confirmed in another study (41). Fatty acids interact with Toll-like receptors 2 and 4, which are expressed in islets (16) and activate NF-κB (42). In fact, Toll-like receptor 2-deficient mice are protected from β-cell dysfunction induced by high-fat diet (43). Fatty acids also induce oxidative stress (27), which is a known activator of NF-κB. However, with the exception of IFN-γ, coinfusion of NAC did not prevent this cytokine up-regulation. This suggests that this up-regulation is mainly upstream or independent of oxidative stress.

In contrast, the decrease in β-cell function appears to be mainly due to oxidative stress, because it is prevented by NAC. It is possible that fat increases cytokine production in islets to further increase oxidative stress in islets. Sources of oxidative stress contributed by up-regulation of inflammatory pathways may include increased activity of the enzymes iNOS and COX-2, whose mRNA expression levels are increased by IH infusion. Another possibility is that fat is a substrate for cytokine-induced free radical production (*e.g.* lipid peroxidation). It has been well established that β-cells are especially vulnerable to oxidative stress due to their low antioxidant capacity (44). Oxidative stress has been clearly shown to impair β-cell function (45) and to trigger β-cell death (46). Our results are in accordance with those of Shimabukuro *et al.* (17), who showed that the toxicity of cytokines is enhanced in fat rich islets, and those of Aarnes *et al.* (18), who showed that exposure to FFA enhances the toxicity of cytokines in INS-1 β-cell lines.

The observation of increased islet mRNA expression of cytokines and chemokines is consistent with our findings of plasma cytokine measurements, which showed that IH infusion increases MCP-1 and IL-10 levels (it is of note that although other cytokines were not increased by IH, this may be due to the difficulty of assaying cytokines, such as IL-1β, in plasma) (47). However, unexpectedly, we found that insulitis tended to be lower in IH-infused dp-BB rats. The mechanism for this is unclear. Previously, it has been reported that IH infusion decreases chemotactic and random migration of leukocytes (48, 49), possibly by in-

ducing alterations in the plasma membrane (50). Thus, it is possible that IH leads to decreased chemotactic migration of these cells to the islets, perhaps due to its n-3 fatty acids content (51), and thus exerts some antiinflammatory effects. Indeed, IH increased both type 1 and type 2 cytokines in plasma and islets, which is consistent with both inflammatory and antiinflammatory effects. Another possibility is that IH is increasing systemic inflammation, as suggested by elevated plasma MCP-1 levels with IH, which would partially remove the chemotactic gradient with respect to islets, and this would lead to a reduced attraction of immune cells to islets. How a decreased number of immune cells in the islets can coexist with increased cytokine and chemokine expression is unclear. One possibility is that IH increases the activation state of the immune cells (52, 53), despite decreasing their total number. One study has suggested that the chemokine MCP-1 mainly attracts activated macrophage (54). Also, studies performed by some of us in humans indicate that both glucose and fat increase ROS production and NF-κB activation in polymorphonuclear leukocytes and mononuclear cells (55-57). Another possibility may be that other islet cell types than immune cells, such as β-cells (58-61) and/or endothelial and ductal cells (62), are producing these cytokines and chemokines. Future studies are needed to investigate these possibilities.

Notably, in this study, IH did not alter β-cell function in dr-BB or WF rats. Previous studies from our group have shown that infusion of oleate or olive oil induced β-cell dysfunction in islets of normal Wistar rats, an effect prevented by antioxidants (27). IH was less effective than oleate (26), causing only a modest impairment in β-cell function in Wistar rats, a strain that is bigger than the WF and dr-BB rats used in the present study. We have previously shown that obesity predisposes β-cells to lipid-induced dysfunction (63, 64). The reason for the greater effect of oleate or olive oil than Intralipid is not clear but may be related to a greater inflammatory effect (or less antiinflammatory effect) of oleate and olive oil than Intralipid.

According to our findings, we would expect that diets high in fat should increase the incidence of type 1 diabetes in dp-BB rats. However, this has been shown not to be the case using diets with 10-15% fat in weight (65, 66). Further studies may be required with a greater dietary fat content. However, it should be pointed out that the BB rat is an extreme model of type 1 diabetes, which is suitable for demonstrating subtle impairments in β-cell function but where environmental factors do not easily increase diabetes incidence. Indeed, fat feeding (35% in kcal) has been shown to increase the incidence of diabetes in the nonobese diabetic mouse (21), which is well known to be a less extreme model of type 1 diabetes.

In summary, we have shown that autoimmune inflammation sensitizes the β-cell to metabolically induced dysfunction via increased cytokine production and that antioxidants prevent β-cell dysfunction. Our data suggest that β-cell lipotoxicity should be considered among the potential processes underlying overweight-induced acceleration of type 1 diabetes (accelerator hypothesis).

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