Adaptation to intermittent stress promotes maintenance of β-cell compensation: comparison with food restriction

Holly E. Bates,1 Adam Sirek,1 Michael A. Kiraly,1 Jessica T. Y. Yue,1 Michael C. Riddell,4 Stephen G. Matthews,1,2,3 and Mladen Vranic1,3

Departments of 1Physiology, 2Obstetrics and Gynecology, and 3Medicine, University of Toronto; and 4School of Kinesiology and Health Science, York University, Toronto, Ontario, Canada

Submitted 18 April 2008; accepted in final form 13 August 2008

Bates HE, Sirek A, Kiraly MA, Yue JT, Riddell MC, Matthews SG, Vranic M. Adaptation to intermittent stress promotes maintenance of β-cell compensation: comparison with food restriction. Am J Physiol Endocrinol Metab 295: E947–E958, 2008. First published August 19, 2008; doi:10.1152/ajpendo.90378.2008.—Intermittent restraint stress delays hyperglycemia in ZDF rats better than pair feeding. We hypothesized that intermittent stress would preserve β-cell mass through distinct mechanisms from food restriction. We studied temporal effects of intermittent stress on β-cell compensation during pre-, early, and late diabetes. Six-week-old obese male ZDF rats were restraint-stressed 1 h/day, 5 days/wk for 0, 3, 6, or 13 wk and compared with age-matched obese ZDF rats that had been food restricted for 13 wk, and 19-wk-old lean ZDF rats. Thirteen weeks of stress and food restriction lowered cumulative food intake 10–15%. Obese islets were fibrotic and disorganized and not improved by stress or food restriction. Obese pancreata had islet hyperplasia and showed evidence of neogenesis, but by 19 wk old β-cell mass was not increased, and islets had fewer β-cells that were hypertrophic. Both stress and food restriction partially preserved β-cell mass at 19 wk old via islet hypertrophy, whereas stress additionally lowered α-cell mass. Concomitant with maintenance of insulin responses to glucose, stress delayed the sixfold decline in β-cell proliferation and reduced β-cell hypertrophy, translating into 30% more β-cells per islet after 13 wk. In contrast, food restriction did not improve insulin responses or β-cell hyperplasia, exacerbated β-cell hypertrophy, and resulted in fewer β-cells and greater α-cell mass than with stress. Thus, preservation of β-cell mass with adaptation to intermittent stress is related to β-cell hyperplasia, maintenance of insulin responses to glucose, and reductions in α-cell mass that do not occur with food restriction.

The global prevalence of people with diabetes is predicted to rise to 300 million in 2025 (68), most with type 2 diabetes (T2DM). T2DM is characterized by insufficient insulin secretion to compensate for the relative degree of insulin resistance (6). Reduction of hyperglycemia is important to prevent the long-term complications of diabetes (1), and therefore interventions that maintain appropriate insulin secretion and thus reduce glycemia are important for prevention of diabetes-associated comorbidities.

β-Cell compensation occurs in insulin-resistant states such as obesity, in which pancreatic islets increase insulin secretion (32, 55) and β-cell mass (10, 19, 23, 25, 46, 55) to maintain euglycemia. Peripheral signals suggested to promote β-cell compensation include free fatty acids (FFA) (29, 35, 39, 40, 55), glucose (7, 44, 54, 55), insulin/IGF-I (44), and glucagon-like peptide-1 (GLP-1) secretion (61, 63). In T2DM, this compensation is insufficient, leading to hyperglycemia. This β-cell dysfunction manifests as a reduction in insulin responses to glucose (3, 26, 45) and nonglucose secretagogues (64), altered insulin pulsatility (41, 48), and inefficient proinsulin processing (24). Pancreata from T2DM patients also have a 30–60% loss of islet β-cell volume (10, 50, 67), primarily through increased β-cell apoptosis, since β-cell proliferation is normal (10). Additionally, T2DM patients demonstrate increased islet α-cell volume or area (50, 67) and islet fibrosis (14). Patients with impaired fasting glycemia also have reduced β-cell volume (10), suggesting that reduced β-cell mass precedes diabetes.

Similar to humans with T2DM, development of hyperglycemia in male Zucker diabetic fatty (ZDF) rats is associated with a decline in relative β-cell mass (46), β-cell function (56, 57), and vascular integrity of the islet (31). An early increase in β-cell proliferation (19, 46) is eventually overridden by a larger increase in β-cell apoptosis (19, 46, 52, 53). ZDF rat islets also have reduced relative glucose-stimulated insulin secretion and altered insulin pulsatility (42, 56). Thus, ZDF rats are a suitable model for examination of the dynamic changes in β-cell compensation with development of T2DM.

It is widely assumed that “stress” worsens T2DM; consequently, patients are often advised to avoid stressful situations. However, this assumption does not consider the variability, complexity, or divergence between effects of chronic and intermittent stress and has resulted from the paucity of adequately controlled human studies examining these in the context of T2DM. We (4, 5) and others (27) have demonstrated that intermittent stress delays development of hyperglycemia in genetic models of T2DM. This contrast with common views that all stressors are deleterious for diabetes and illustrates that intermittent exposure to stressors and the ensuing adaptations may instead be important for normal physiological functioning by preparing the body to deal with threats to homeostasis.

In the male ZDF rat, 13 wk of intermittent restraint stress delays development of hyperglycemia (5) and increases the basal insulin-to-glucose ratio primarily in the morning (5), suggesting that adaptation to stress leads to maintenance of β-cell compensation for insulin resistance. However, it is unknown whether adaptation to intermittent stress alters the deterioration in β-cell compensation during development of diabetes.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
diabetes. Therefore, the current study examines the effect of intermittent stress on basal hyperinsulinemia, insulin responses to intraperitoneal glucose, and β-cell mass dynamics beginning at 6 wk of age (prediabetes) and during progressive stages of diabetes development: the prediabetic (9 wk old), early diabetic (12 wk old), and late diabetic (19 wk old) phases (after 0, 3, 6, or 13 wk of intermittent stress, respectively). Second, the amelioration of hyperglycemia by intermittent restraint stress is partially mediated by stress-induced reductions in food intake (4, 5). However, intermittent restraint stress further reduces glycemia compared with pair feeding, illustrating beneficial effects of intermittent stress independent of food intake. It is well known that food restriction ameliorates diabetes at least partially via improvements in β-cell function (22). Therefore, we hypothesized that adaptation to 13 wk of intermittent stress would maintain β-cell compensation through different mechanisms better than a comparable food restriction, which may therefore explain the further lowering of glycemia by intermittent stress per se. Thus, the effects of 13 wk of intermittent stress were compared with mild 15–20% food restriction, a reduction in hyperphagia comparable to that induced by intermittent stress (4, 5).

MATERIALS AND METHODS

Animals. Male obese ZDF/Crl-Lepr+/− rats and lean (ZDF/Crl-Lepr−/−) rats were obtained at 5 wk old from Charles Rivers Laboratories (Wilmington, MA) and individually housed in opaque microisolation cages in temperature (22–23°C)- and humidity-controlled rooms at the University of Toronto. Rats were kept on a 12:12-h light-dark cycle (lights on from 0700 to 1900) and were fed normal rat chow (Purina 5001) in wire cage lids. Food intake was monitored daily. All experiments were performed according to protocols approved by the University of Toronto Animal Care Committee and followed guidelines from the Canadian Council for Animal Care. At 5 wk old and before experimental manipulation, all rats were acclimatized by daily handling for 1 wk. At the end of the study, all animals were euthanized by decapitation within 1 min of disturbing the animal from its home cage.

Treatment. Obese rats were intermittently restrained stressed starting at 6 wk old for 1 h/day, 5 days/wk for 0 (Basal), 3 (Ob-Stress 3), 6 (Ob-Stress 6), or 13 wk (Ob-Stress 13) to allow analysis of the progressive changes in β-cell compensation with age (Fig. 1). Restraint stress, a potent psychological stressor (43), involved placing a nonanesthetized rat in a Broome rodent restrainer (Harvard Apparatus, Saint Laurent, QC, Canada). Once the head and forelimbs were inside the restraint tube, rats would enter the restraint tube under their own volition. Exit from the tube was prevented by a rear stop that could be adjusted according to the size of the rat. Three sizes of tubes were utilized in the study to account for the growth of the rats and to ensure that they were well restrained at all sizes. Basal and stress-induced corticosterone levels in the rats intermittently restraint stressed for 13 wk have been presented previously (4, 5). The corticosterone responses to restraint stress were elevated for the first 4 wk, after which responses habituated. Thus, intermittent exposure to restraint stress for an extended time causes habituation of corticosterone responses as is commonly seen with repeated exposure to the same type of stress (2, 15, 33, 34, 65). However, we (5) have previously shown that this is accompanied by sustained reductions in food intake and inhibition of growth hormone, illustrating persistent effects of chronic stress independent of corticosterone responses. RestRAINT-stressed rats were compared with age-matched, obese ZDF control rats (Ob-Control 3; Ob-Control 6; Ob-Control 13). In addition, for comparison with the effects of food intake, obese ZDF rats that had their food intake restricted in a gradual fashion for 13 wk from −5% up to −20% of a pattern mimicking the reduction in food intake induced by intermittent restraint stress (Ob-Food Rest 13) (4, 5). This group was originally intended to act as a pair-fed control group. However, hoarding behavior led to a further reduction in food intake compared with intermittently stressed rats, and therefore this group is referred to as a food-restricted group. Finally, lean ZDF rats were monitored for 13 wk (Ln-Control 13) to act as a nonobese control group. Treatment in control and food-restricted groups included 1 h of food removal to ensure that all groups had the same timing of food availability as those exposed to daily restraint stress.

Basal insulin. We previously showed that basal insulin levels were increased after 13 wk of intermittent stress (5). Thus, basal plasma glucose and insulin were measured from trunk blood obtained at euthanasia after 0, 3, 6, and 13 wk of intermittent restraint stress and compared with age-matched ZDF control rats. Samples were obtained from ad libitum-fed rats between 0900 and 1300. Relative insulinemia ([the ratio of basal insulin to glucose (ng insulin/mmol glucose)] was calculated to control for group differences in glycemia. Insulin levels after 13 wk of intermittent restraint stress are reproduced with permission (Metabolism) for the purpose of determining the time course of effects of intermittent stress on insulinemia. Fasting glucose levels from rats intermittently restrained stressed for 13 wk have been presented previously (4) and confirmed in a second study (5).

Glucose-stimulated insulin secretion. Following an overnight fast, the ratio of the insulin response/glucose response (ΔInsulin/ΔGlucose) 30 min after intraperitoneal injection of 50% dextrose (2 g/kg; Abbott Laboratories, Mississauga, ON, Canada) was measured after 0, 3, 6, 9, and 13 wk of intermittent stress and compared with age-matched obese ZDF control rats. Responses were also compared with those in food-restricted rats after 9 and 13 wk of treatment. Tests after 9 and 13 wk were done in the same rats. These tests were done 1 h prior to treatment such that it had been 24 h since the last restraint. Glucose tolerance curves from rats treated for 13 wk have been presented previously (4). Fasting insulinemia in Lean rats was below the detection limit of the assay after the dilution necessary for comparison with obese ZDF hyperinsulinemic samples. Since undiluted samples did not follow a linear dilution curve compared with diluted samples, insulin responses in Lean rats are not shown.

β-Cell mass dynamics. Pancreata were obtained after 0, 3, 6, and 13 wk of intermittent restraint stress and compared with pancreata from age-matched obese ZDF control rats, 19-wk-old lean ZDF rats, and 13-wk food-restricted obese ZDF rats. Six hours prior to pancreas removal, rats were injected intraperitoneally with 100 mg/kg of 2-bromodeoxyuridine (BrDU) (Sigma-Adrich Canada, Oakville, ON, Canada) for quantification of proliferating β- and α-cells within the 6 h prior to euthanasia, the estimated duration of the G2+S phase of the β-cell (58). Within 10 min of euthanasia, the pancreas was isolated and extraneous fat removed. The pancreas was weighed and placed in
Bock's fixative. After fixation, tissue samples were cut into ~20 equivalent-size pieces and randomly placed into tissue cassettes to ensure equal representation of head and tail segments. Cassettes were placed in 70% ethanol until paraffin embedding. Sections (4 μm) were double stained by immunohistochemistry for insulin and BrdU for measurement of β-cell mass, individual β-cell area, β-cell proliferation, ductal proliferation, insulin-stained ductal cells, and islet size distribution measurements. Sections were double stained for glucagon and BrdU for measurement of α-cell mass and proliferation.

**Insulin/glucagon and BrdU double immunohistochemistry.** Slide preparation and double-immunohistochemical staining for insulin and BrdU have been described previously (30). Glucagon and BrdU double-immunohistochemical staining was done similar to insulin/BrdU staining. Briefly, for insulin/glucagon, slides were incubated in rabbit anti-insulin IgG (1:100; Dako, Mississauga, ON, Canada) or rabbit anti-glucagon (1:300; Novacstra, Norwell, MA) primary antibodies, followed by biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories, Burlington, ON, Canada). Color development was done using 3,3'-diaminobenzidine (DAB; Dako). For BrdU, slides were incubated with mouse anti-BrdU (1:1,000; Invitrogen, Burlington, ON, Canada), followed by biotinylated horse anti-mouse IgG (1:500; Vector Laboratories). Color development for BrdU was done with Nickel DAB (Dako). Mayer's hematoxylin was used as a counterstain, and sections were cleared and mounted.

**β-Cell and α-cell masses.** Since each pancreas was cut into ~20 pieces prior to embedding and sectioning, β-cell and α-cell masses were quantified using one random slide per animal representing all regions of the pancreas (200–500 islets per animal or 2,200–4,600 islets per group) as described previously (30). The slides were chosen on the basis of quality of fixation, embedding, and sectioning. Slides were scanned at ×20 magnification using a slide scanner (Aperio Scanscope CS, Vista, CA). The total tissue area and insulin/glucagon stained areas were quantified using an image analysis system (Aperio Imagescope, v. 7.1.32.1024, Vista, CA) and the Aperio positive pixel count algorithm, which is preconfigured for quantification of brown and nonbrown pixels. α-Cell mass for Lean rat is not shown due to high nonglucagon background staining in acinar tissue of this group that prevented accurate quantification of positive pixels. β-Cell and α-cell masses were calculated by dividing the total cross-sectional area of insulin/glucagon by total tissue area and multiplying this by the wet weight of the pancreas.

**β-Cell and α-cell proliferation.** β-Cell and α-cell proliferation were examined by two blinded observers. Scanned images (×20) were digitally magnified to ×400, and the percentages of BrdU stained β- or α-cells were counted. Over 1,150 cells per animal were counted for quantification of β-cell proliferation after 0, 3, 6, and 13 wk of treatment. α-Cell proliferation was examined only after 13 wk of treatment, and more than 500 cells per animal were counted.

**Individual β-cell area.** β-Cell area was measured using digitally magnified ×400 images. For each islet, the total number of β-cells within that islet was counted and divided by the insulin-stained area (mm²). At least 1,000 β-cells per animal were counted.

**Islet areas.** Islet areas were quantified in insulin-stained ×20 scanned slides. One slide per animal was analyzed, representing all regions of the pancreas (200–500 islets per animal, 2,200–4,600 islets per group). All islets within the slide were circled, and the insulin-stained area (mm²) for each islet was quantified (Aperio Imagescope, v. 7.1.32.1024). Islet areas were then divided by the average β-cell area to quantify β-cell hyperplasia. This analysis allowed calculation of the number of islets per square millimeter, average islet area (mm²), the number of β-cells per average islet, the number of β-cells per large islet (>10 000 μm²), the islet size distribution, and the number of islets less than five β-cells in size, a measurement used to estimate β-cell neogenesis (23).

**Ductal proliferation and insulin-stained ductal cells.** Ductal cell proliferation and transdifferentiation of ductal cells into endocrine cells have been suggested to be linked to neogenesis of β-cells during β-cell compensation (23, 51). Although proliferation of β-cells is the most important mechanism for normal β-cell compensation (16, 21), neogenesis may play a role under more severe circumstances (9, 51, 62).

Ductal cell proliferation and insulin-stained ductal cells were quantified from ×400 digitally magnified images of slides used for β-cell proliferation and are expressed as a percentage. At least 1,000 ductal cells per animal were counted by a blinded observer (7,900–11,400 cells per group).

**Assays.** Blood for glucose (<2 μl) was taken from trunk blood or by tail prick with a 27-gauge needle and measured with a Glucometer Elite XL (Bayer, Toronto, ON, Canada). Blood for insulin (<20 μl) was obtained by tail nick with a scalpel blade or from trunk blood at euthanasia and collected into heparinized microvetic tubes (Sarstedt, Montreal, QC, Canada). Plasma was stored at −20°C until analysis. Plasma insulin was assayed using a rat insulin ELISA kit (Crystal Chem, Downers Grove, IL) after dilutions of 10× for glucose-stimulated/fed insulin in obese ZDF rats, 2.5× for fed insulin in lean rats, and 5× for fasting insulin in obese ZDF rats. Fasting insulin after any dilution from Lean rats was below the detection limit of the assay. Undiluted plasma did not follow a linear dilution curve with diluted plasma; therefore, fasting insulin in lean rats could not be directly compared with that of obese ZDF rats and is not shown. Data was analyzed using I-SMART (v. 2.0, Packard Instruments).

**Statistics.** Data are presented as means ± SE. Statistical analysis was performed with Statistica 6.0 (Statsoft, Tulsa, OK), and P < 0.05 was considered statistically significant. Factorial ANOVA or repeated-measures ANOVA (RM-ANOVA) were used for analysis of the effects of treatment over time and main effects, with Duncan's post hoc test for multiple comparisons performed if the ANOVA revealed a P of <0.05. Comparisons between ages and treatments were done by unpaired Student's t-test because of age and strain differences (Lean vs. obese ZDF rat, 6 wk old vs. 19 wk old). Individual correlations were done using linear regression with hormones and metabolites obtained from trunk blood at euthanasia after 0, 3, and 6 wk of intermittent stress (Supplementary Table S1) and after 13 wk of intermittent stress (published previously (46)).

**RESULTS**

**Cumulative food intake.** Food intake after 3 wk of intermittent restraint stress was not affected but was modestly reduced after 6 wk (P = 0.02 vs. Control; Table 1). After 13 wk of intermittent stress, cumulative food intake tended to be reduced (P = 0.07 vs. Control). As expected, food restriction reduced cumulative food intake by ~15% (P = 0.0001 vs. Control), resulting in ~10% lower food intake than that induced by 13 wk of intermittent stress (P < 0.001). Neither intermittent stress nor food restriction lowered final body weights after 3, 6, or 13 wk of treatment (data not shown).

**Basal insulin over 13 wk of intermittent stress.** We (4, 5) previously demonstrated that basal glycemia is reduced and

Table 1. Cumulative food intake

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>3 wk</th>
<th>6 wk</th>
<th>13 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>583 ± 9</td>
<td>1,260 ± 22</td>
<td>2,511 ± 798</td>
</tr>
<tr>
<td>Stress</td>
<td>573 ± 14</td>
<td>1,174 ± 255</td>
<td>2,333 ± 498</td>
</tr>
<tr>
<td>Food restricted</td>
<td>ND</td>
<td>ND</td>
<td>2,071 ± 318</td>
</tr>
<tr>
<td>Lean</td>
<td>ND</td>
<td>ND</td>
<td>1,606 ± 29</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE in grams; n = 8–9 per group. *P < 0.0001 vs. Lean, †P < 0.05 vs. age-matched Control, ‡P < 0.001 vs. Stress.
Insulinemia is increased after 13 wk of intermittent stress. Therefore, we examined the time course of changes in fed insulinemia with intermittent stress. Glycemia was affected by intermittent restraint stress over time (treatment × time interaction, RM-ANOVA P < 0.005), such that glycemia was not affected after 3 wk of stress (prediabetic phase) but was >50% lower (P < 0.005) after 6 wk of stress (early diabetic phase) and ~40% lower (P < 0.001) after 13 wk of stress (late diabetic phase) (Fig. 2A). Corresponding insulinemia was also affected by intermittent stress (treatment × time, P = 0.001) such that it was reduced by 40% after 3 wk of stress (P < 0.05) but tended to be maintained 30% higher after 6 wk of stress (P = 0.06) and 40% higher after 13 wk of stress (P < 0.05). When normalized to glucose levels, the effect of intermittent stress on relative insulinemia became more apparent (treatment × time, P < 0.0005). Relative insulinemia dropped by 65% in obese ZDF control rats between the prediabetic and early diabetic phases (3 and 6 wk, P = 0.002), which was prevented by intermittent stress. Thus, levels were maintained 2.5-fold higher after 6 wk (P = 0.001) and 3.5-fold higher after 13 wk (P < 0.005) of intermittent stress.

**Insulin response to intraperitoneal glucose.** After a 16- to 18-h fast, we examined the insulin response to intraperitoneal (i.p.) glucose 30 min postinjection (Δinsulin/Δglucose) after 0, 3, 6, 9, and 13 wk of intermittent stress. Intermittent stress affected the insulin response to glucose over time (treatment × time, P = 0.05) such that it was similar to that of control rats after 3, 6, and 9 wk of intermittent stress (P > 0.49) but higher during the late diabetic phase (19 wk old) after 13 wk of intermittent stress (P = 0.022) (Fig. 2B). Although the insulin response to glucose challenge after 9 and 13 wk of mild food restriction was similar to that in intermittently stressed rats (P > 0.1), it did not show improvements compared with control rats. Diluted fasting insulin levels in lean rats were below assay detection as described in MATERIALS AND METHODS; therefore, insulin responses could not be calculated.

**β-Cell mass.** Islets in all ZDF animals were significantly enlarged, with disarray of islet architecture and irregular islet boundaries that became worse with age (Fig. 3). Neither intermittent stress nor mild food restriction appeared to improve this islet architecture.

β-Cell mass changed significantly over time (P < 0.0001) such that in control rats β-cell mass grew rapidly and peaked at 9 wk old (prediabetic phase after 3 wk of treatment) and declined thereafter to levels similar to those in lean rats at 19 wk old (13 wk of treatment, late diabetic phase; Fig. 4A). Although intermittent stress did not affect β-cell mass over time, the peak in β-cell mass was delayed until 12 wk old, such that, even though β-cell mass declined thereafter, β-cell mass at 19 wk old (after 13 wk of intermittent stress) was still higher than in control rats (P < 0.02). Thirteen weeks of food restriction led to comparably higher β-cell mass than in controls (P < 0.05).

To examine whether the greater β-cell mass after 13 wk of intermittent stress was caused by islet hypertrophy or hyperplasia, we quantified the number and size distribution of islets (300–500 islets per animal) in each slide at 19 wk old. All 19-wk-old obese groups compensated with a more than 80% increase in the number of islets compared with lean rats (P < 0.00001) and an over 30% increase compared with 6-wk-old prediabetic basal rats (P < 0.0005; Fig. 4B). Both intermittently stressed and diet-restricted rats further compensated by increasing the mean islet area compared with lean (P < 0.02), and control (P < 0.05) rats (Fig. 4C), likely because of an increase in the percentage of large-size islets (>10,000 μm², P ≤ 0.056 vs. Control; Fig. 4D).

β-Cell hypertrophy vs. hyperplasia. We examined individual β-cell size at 19 wk old after 13 wk of treatment, by taking the total area of an islet, and dividing that by the number of β-cell nuclei within the islet (≥1,000 nuclei counted). Using this methodology, β-cell area was likely slightly overestimated but consistently so between groups.

Individual β-cell area was larger in obese than in lean rats (P < 0.03), even at 6 wk old (P < 0.05; Fig. 5A). β-Cell area was further increased in 19-wk-old Control rats compared with 6-wk-old basal levels (P < 0.005). Intermittent stress prevented this compensatory β-cell hypertrophy (P = 0.02 vs. Control), whereas food restriction exacerbated the hypertrophy (P < 0.005 vs. Control and Basal). Thus, β-cells in diet-restricted rats were ~20% larger, whereas they were ~15%
smaller, in stressed rats compared with control rats. An average-size control islet therefore contained ~30% fewer β-cells than the average-size lean or 6-wk-old prediabetic basal islet (P < 0.01), a reduction prevented by intermittent stress (P < 0.02) but not food restriction (Fig. 5B). Since both intermittent stress and food restriction increased the proportion of large-size islets (>10 000 μm²), these large-size islets contain 30% more β-cells after 13 wk of intermittent stress (P < 0.001 vs. Control) but not food restriction (Fig. 5C). Thus, intermittent stress increases the proportion of large-size islets through β-cell hyperplasia, whereas β-cell hypertrophy plays a greater role with food restriction.

β-Cell proliferation. To examine dynamic changes in β-cell proliferation, the percentage of BrdU-stained β-cells was quantified in pancreata after 0, 3, 6, and 13 wk of intermittent restraint stress. β-Cell proliferation in all obese groups, regardless of age, was two- to eightfold higher than in 19-wk-old lean rats (P < 0.05; Fig. 5D), demonstrating the importance of β-cell proliferation for β-cell mass compensation. Intermittent stress delayed the decline in β-cell proliferation (treatment × time, P = 0.0006). This was reflected by the more than fourfold drop in β-cell proliferation during the early diabetic phase (12 wk old, 6 wk of treatment) in Control rats, which was prevented by intermittent stress. However, proliferation was no longer maintained at higher rates after 13 wk of intermittent stress and was similar to that in diet-restricted rats.

Since β-cell proliferation was affected primarily during the first 6 wk of treatment, we used data from these animals to...
examine the relationship between β-cell proliferation and hormones/metabolites obtained at euthanasia that could be altered by intermittent stress and signal to increase β-cell proliferation. 

β-Cell proliferation showed a strong negative correlation with fed glycemia \( r = -0.645, P = 0.0002 \) and positive correlation with basal insulinemia \( r = 0.573, P = 0.0001 \), but not with basal corticosterone, FFA, triglycerides, glucagon, growth hormone, or adiponectin (for data see Supplemental Table S1).

**Ductal proliferation, insulin-stained ductal cells, and small islet clusters.** To examine dynamic changes in putative markers of neogenesis, we quantified the percentage of proliferating ductal cells, insulin-stained ductal cells, and number of small islet (<5 cells) clusters (Fig. 6). Ductal proliferation decreased...
intermittent stress over time (treatment × time P = 0.013) such that it was reduced ∼50% during the prediabetic phase after 3 wk of intermittent stress (P = 0.03) and elevated approximately twofold during the early diabetic phase after 6 wk of intermittent stress (P = 0.013). Ductal proliferation after 13 wk of intermittent stress was not different from Control or diet-restricted rats.

The percentage of insulin-stained ductal cells increased over time (P < 0.0001), such that levels in 19-wk-old diabetic control rats were threefold higher than in 6-wk-old prediabetic basal rats (P ≤ 0.05), and levels were at least threefold higher in all obese compared with lean rats (P < 0.05). The percentage of insulin-stained ductal cells was, however, not affected by intermittent stress or food restriction.

When we examined the number of small β-cell clusters (<5 cells) in pancreata from 19-wk-old rats treated for 13 wk, the number of small β-cell clusters increased at least twofold in all 19-wk-old obese groups compared with 6-wk-old prediabetic basal rats (P < 0.001) and to 19-wk-old lean rats (P < 0.001). The number of β-cell clusters was not affected by intermittent stress or food restriction.

**Correlations.** We used linear regression to examine which characteristics of β-cell dynamics are most related to increases in β-cell mass (Table 2) in obese 19-wk-old rats treated for 13 wk. Mean islet area positively correlated with the number of β-cells per islet (r = 0.8052, P = 0.00001) but not β-cell size (r = 0.311, P = 0.17). Similarly, β-cell mass was positively related to an increase in the mean number of cells per islet (r = 0.508, P = 0.02) and the mean islet area (r = 0.467, P = 0.03) but not an increase in β-cell size (r = -0.077, P = 0.73). However, when separated into groups, β-cell mass in diet-restricted rats showed a surprisingly strong negative correlation with β-cell size (r = -0.85, P = 0.014), whereas it showed no relationship in intermittently stressed or control rats.

We next examined the relationship between peripheral metabolic signals [values published previously (4)] that may play a role in β-cell mass compensation (Table 3). Absolute β-cell mass was positively related to reduced glycemia (r = -0.5971, P = 0.002) and increased leptin (r = 0.399, P = 0.049) and adiponectin (r = 0.6151, P = 0.001). Remarkably, β-cell mass did not correlate with plasma insulin levels (r = 0.1573, P = 0.463). In contrast, β-cell hyperplasia (increase in the number of β-cells per average islet) positively correlated with reduced glycemia (r = -0.7851, P < 0.0001) and increased insulinemia (r = 0.5131, P = 0.017). Basal corticosterone tended to be lower with β-cell hyperplasia (r = -0.3833, P = 0.086), whereas leptin (r = 0.4345, P = 0.049) and adiponectin (r = 0.629, P = 0.0023) were higher.

**α-Cell mass and proliferation.** Since α-cell mass is increased in human T2DM (50, 67), we examined α-cell mass after 0, 3, 6, and 13 wk of intermittent stress and compared these values to age-matched diabetic control rats, and rats diet restricted for 13 wk (Fig. 7, A and B).

α-Cell mass in Control rats remained relatively constant with age, although α-cell proliferation tended to decline with age (P = 0.06 19-wk-old Control vs. 6-wk-old prediabetic Basal rats). Interestingly, α-cell mass was slightly reduced by intermittent stress (treatment, P = 0.046) and after 13 wk was also 25% lower than in diet restricted rats (P = 0.04). However, this was not due to reduced rates of α-cell proliferation after 13 wk of intermittent stress. This reduction in α-cell mass was also not associated with reduced fed glucagonemia from single measurements made at euthanasia [values published
previously (4), although this may not be representative of local glucagon concentrations within the islet or portal glucagon levels.

By use of data from 19-wk-old obese rats treated for 13 wk, α-cell mass did not correlate with glucose, insulin, FFA, triglycerides, or even glucagon but showed a strong positive correlation with basal corticosterone levels \( r = 0.58, P = 0.0018 \). This relationship with corticosterone became even stronger when the corresponding β-cell mass was taken into account, such that the ratio of β-cell to α-cell mass decreased \( r = -0.695, P = 0.0002 \) with increases in basal corticosterone. The ratio of β-cell to α-cell mass further correlated with both increased glycemia \( r = -0.64, P = 0.0009 \) and decreased basal insulinemia \( r = 0.51, P = 0.015 \).

**DISCUSSION**

We (4, 5) previously demonstrated that intermittent restraint stress delays the development of fed and fasting hyperglycemia in the ZDF rat, which is partially mediated by stress-induced reductions in food intake. The present study examined whether the beneficial glycemic effects of intermittent restraint stress were mediated by improved β-cell compensation and whether similar effects were induced by food restriction, which would be expected to have a lesser effect on islet mass preservation and glycemia/insulinemia. However, intermittent restraint stress was more beneficial. Thus, preservation of β-cell mass with adaptation to intermittent stress is related to β-cell hyperplasia, maintenance of insulin responses to glucose, and reductions in α-cell mass that do not occur with food restriction and thus may explain how intermittent stress per se lowers glycemia.

Although the deterioration in basal hyperinsulinemia and the insulin response to glucose were prevented by intermittent stress, we previously showed that glucose tolerance was not improved, whereas absolute glucose levels were (4). This suggests that the maintenance of basal hyperinsulinemia by intermittent stress is more important for the attenuation of hyperglycemia than maintenance of β-cell responsiveness to glucose. Similarly, high-fat-fed C57BL/6 mice compensate for insulin resistance through increasing basal insulinemia but not the insulin response to glucose (18), and dogs fed a cafeteria diet maintain euglycemia through basal hyperinsulinemia despite reduced glucose tolerance (37). Zucker fatty rats, which in contrast with ZDF rats compensate for their insulin resistance, have enhanced insulin responses to substimulatory glucose concentrations and β-cell hyperplasia (36) but are still glucose intolerant (46). Thus, we hypothesize that the maintenance of basal hyperinsulinemia by intermittent stress plays an important role in the delay of hyperglycemia.

We postulate that this basal hyperinsulinemia is related to the islet hypertrophy. Zucker fatty rats compensate by increasing the size of islets (23), which are hypersensitive to glucose (11). Furthermore, we postulate that the improved insulinemia and insulin responses to glucose with intermittent stress are related to their β-cell hyperplasia as opposed to the β-cell hypertrophy in food-restricted rats. Dexamethasone-induced β-cell mass expansion occurs through β-cell hypertrophy and is associated with reduced glucose disposal, whereas exercise-induced β-cell mass expansion occurs through β-cell proliferation and improves glucose disposal (13). Likewise, hypertrophy, but not hyperplasia, of β-cells is important for normal increases in β-cell mass in older rats, which may reflect a limitation in the β-cell replicative capacity (38). Hypertrophic or growth-arrested cells have initially enhanced function but eventually may be more susceptible to apoptosis (8, 17, 66), and the lack of a correlation between hypertrophy and glycemia or insulinemia suggests that hypertrophy may not increase basal function. In fact, food-restricted rats had β-cell hypertrophy, but β-cell size correlated negatively with β-cell mass. In contrast, β-cell hyperplasia correlated with amelioration of hyperglycemia and an increase in insulinemia. Thus, it is tempting to speculate that the hypertrophy in food-restricted rats represents a final effort for β-cell compensation, as is suggested by the incline in glycemia at this age (4, 5) and that β-cell hyperplasia represents a more successful means of β-cell compensation.

We postulate that the peripheral signal responsible for β-cell hypertrophy vs. hyperplasia in our study is the stress hormone corticosterone. Hypercortisolemia occurs in T2DM patients and is related to poor metabolic control (12). In ZDF rats, basal corticosterone levels are increased by food restriction, an effect that is ameliorated by intermittent stress (4, 5). The glucocorticoid dexamethasone can induce β-cell mass expansion secondarily to reducing glucose disposal by β-cell hypertrophy.

### Table 2. Linear correlations between β-cell mass and β-cell mass characteristics in ZDF rats after 13 wk of intermittent stress, food restriction, or no treatment

<table>
<thead>
<tr>
<th>β-Cell mass, mg</th>
<th>Mean Islet Area, μm²</th>
<th>Individual β-Cell Area, μm²</th>
<th>No. of β-Cells per Mean Islet Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Cell mass, mg</td>
<td>Mean islet, μm²</td>
<td>( r = 0.467, P = 0.03 )</td>
<td>NS</td>
</tr>
<tr>
<td>Mean islet, μm²</td>
<td>NS</td>
<td>( r = 0.508, P = 0.02 )</td>
<td>ND</td>
</tr>
<tr>
<td>Individual β-cell area, μm²</td>
<td>NS</td>
<td>NS</td>
<td>ND</td>
</tr>
<tr>
<td>No. of β-cells per mean islet area</td>
<td>( r = 0.508, P = 0.02 )</td>
<td>( r = 0.81, P = 0.00001 )</td>
<td>ND</td>
</tr>
</tbody>
</table>

Not significant (NS), \( P > 0.05 \); ND, not done.

Downloaded from http://ajpendo.physiology.org/ by 10.220.33.1 on October 29, 2017.
Table 3. Linear correlations between β-cell mass characteristics and hormones and metabolites obtained in the basal state at euthanasia in ZDF rats after 13 wk of intermittent stress, food restriction, or no treatment.

<table>
<thead>
<tr>
<th>Glucose, mM</th>
<th>Insulin, ng/ml</th>
<th>Corticosterone, ng/ml</th>
<th>Triglycerides, mM</th>
<th>FFA, mEq/l</th>
<th>Somatostatin, pmol/ml</th>
<th>Leptin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>r</em> = 0.475, <em>P</em> = 0.047</td>
<td><em>r</em> = 0.449, <em>P</em> = 0.049</td>
<td><em>r</em> = 0.450, <em>P</em> = 0.021</td>
<td><em>r</em> = 0.364, <em>P</em> = 0.012</td>
<td><em>r</em> = 0.282, <em>P</em> = 0.099</td>
<td><em>r</em> = 0.369, <em>P</em> = 0.010</td>
<td><em>r</em> = 0.382, <em>P</em> = 0.008</td>
</tr>
</tbody>
</table>

- β-Cell Mass, mg
- Mean Islet Area, μm²
- %Islets

**Fig. 7.** Effect of intermittent restraint stress and food restriction on α-cell mass (A) and proliferation (B). α-Cell mass was reduced by intermittent stress (factorial ANOVA, main effect treatment *P* = 0.046), and after 13 wk of intermittent stress was 25% lower than in diet-restricted rats (4), which was not caused by reduced α-cell proliferation after 13 wk of stress (B). Data are presented as means ± SE; *n* = 5–9 per group.

(13) and also directly stimulates β-cell apoptosis (49). Reduced basal corticosterone was associated with a higher ratio of β-cell to α-cell mass, which was associated with improvements in glycemia and insulinemia. Although the correlations between basal corticosterone levels and hyperplasia or hypertrophy after 13 wk were not significant, these effects of corticosterone likely occurred at an earlier time point. We (4) previously demonstrated, by multiple regression analysis, that changes in basal corticosterone levels can predict 20% of the variance in glucose levels in ZDF rats intermittently stressed or food restricted for 13 wk. This may explain the differing mechanisms of β-cell mass compensation with intermittent stress and dietary restriction and thus their differences in insulinemia.

Maintenance of elevated β-cell proliferation rates up to 12 wk of age by intermittent stress resulted in β-cell hyperplasia. However, reduced β-cell apoptosis may have also contributed. We attempted quantification of β-cell apoptosis by immunohistochemical double-staining for insulin and TUNEL, cleaved caspase-3, DAPI, or propidium iodide without success. A number of studies that quantified apoptosis did not double-stain for insulin to identify β-cells (47, 59). In our study, the alterations in α-cell mass and disorganized islet structure makes it important for β-cells to be distinguished from other endocrine types.

In addition to delaying the deterioration in β-cell mass, intermittent stress reduced α-cell mass. Postprandial hyperglucagonemia (60) as well as α-cell hyperplasia (50, 67) occurs in patients with T2DM despite their hyperinsulinemia. We (4, 5) previously demonstrated that basal corticosterone levels are...
increased by food restriction and that this is prevented by intermittent restraint. We now show that there is a strong positive correlation between basal corticosterone and α-cell mass, suggesting that corticosterone levels modulate α-cell dynamics, which may represent an additional mechanism for the improved glycemia in intermittently stressed ZDF rats.

It is possible that β-cell compensation after 13 wk of intermittent stress is secondary to improvements in insulin sensitivity. In three weeks after the initiation of intermittent stress, insulinulinemia was reduced without an increase in glycemia, suggesting improved insulin sensitivity. β-Cell mass at 19 wk of age was positively correlated with plasma leptin levels, albeit with a relatively low r value. Since leptin is higher with increased fat mass (20), this likely reflects increased adiposity and insulin resistance and compensatory β-cell mass expansion. Adiponectin levels showed a stronger positive correlation with β-cell mass at 19 wk of age. Transgenic overexpression of adiponectin in ob/ob mice increases adipose mass but improves islet morphology and glycemia, lipidemia, and inflammation, all of which can promote β-cell apoptosis (28). Thus, it is not surprising that adiponectin is associated with a higher β-cell mass. However, since neither leptin nor adiponectin levels were affected by intermittent stress or food restriction (4), they are likely not responsible for the divergent effects on β-cell compensation. Increased β-cell mass at 19 wk of age did not correlate with plasma insulin, lipid, corticosterone, growth hormone, or glucagon levels, but our measurements are based only on single time points rather than 24-h circadian levels. Furthermore, although intermittent stress maintained the insulin response to intraperitoneal glucose, under in vivo conditions this may have been a reflection of differences in peripheral handling of glucose.

Obese diabetic humans could be considered to be in a state of chronic metabolic stress and psychological stress associated with adherence to diet, exercise, and medications. However, these forms of stress are different from the intermittent stress that lasted only 1 h per day in the current study. Although one cannot fully equate the restraint stress of a rat to any stressor in humans, it is primarily psychological, which extends the possibility that restraint stress is similar to mental stressors in humans. For example, driving through rush hour traffic is mentally stressful. However, as it is repeated on a daily basis, it becomes more predictable and less taxing, akin to habituation of ZDF rats to repeated restraint stress. Due to the difficulties with obtaining human pancreata, there are no human studies that examine the relationship between chronic or intermittent stress and β-cell morphology in humans. However, if one considers human T2DM itself a chronic, metabolic stressor, then diabetes is associated with a reduction in β-cell mass and increased β-cell apoptosis (10, 67), effects that were not observed in the present study in ZDF rats in response to intermittent, psychological stress. However, this is a difficult interpretation, since in the case of human T2DM the loss of β-cell mass is to some degree causal of diabetes, since β-cell mass is already reduced in patients with impaired fasting glycemia (10).

In sum, intermittent restraint stress delays development of hyperglycemia in the ZDF rat, which is mediated, at least in part, by improvements in β-cell compensation. Intermittent stress prevents the deterioration in basal hyperinsulinemia and the insulin response to glucose and delays the deterioration in β-cell mass, resulting in amelioration of hyperglycemia. Although these effects may be partially mediated by a modest reduction in food intake, the effects of intermittent stress tend to be more pronounced (4, 5), with intermittent stress, but not food restriction, maintaining the insulin response to glucose. Intermittent stress increases the number and proportion of large islets through delaying the decline in β-cell proliferation and increasing β-cell hyperplasia. In contrast, β-cell hypertrophy plays a more dominant role in increasing the proportion of large islets with food restriction. Since β-cell hyperplasia correlates with amelioration of hyperglycemia and increased insulinemia whereas β-cell hypertrophy does not, hyperplasia may be a more beneficial mechanism for β-cell mass compensation. Furthermore, only intermittent stress lowers α-cell mass. Thus, in contrast with common views that all stressors are deleterious for diabetes, intermittent exposure to mild stressors and the ensuing adaptations, including those with respect to the β-cell, may prepare the body to deal with threats to glycemic homeostasis such as T2DM.

ACKNOWLEDGMENTS

We thank Elena Burdett for technical support.

GRANTS

This work was supported by a research grant to M. Vranic, M. C. Riddell, and S. G. Matthews from the Canadian Institutes of Health Research (CIHR) (MOP-2197). H. E. Bates was supported by a CIHR Canada Graduate Scholarship Doctoral Award (CGD-76341). M. A. Kiraly is a recipient of a Natural Sciences and Engineering Research Council of Canada (NSERC) Doctoral Award and Banting and Best Diabetes Centre (BBDC) Novo Nordisk Scholarship, and J. T. Y. Yue is a recipient of a CIHR Doctoral Award and a BBDC Novo Nordisk Scholarship.

REFERENCES


35. Sharma A, Zangen DH, Reitz P, Taneja M, Lissauer ME, Miller CP, Weir GC, Habener JF, Bonner-Weir S. The homeodomain protein...


