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Recurrent intermittent restraint delays fed and fasting hyperglycemia and improves glucose return to baseline levels during glucose tolerance tests in the Zucker diabetic fatty rat—role of food intake and corticosterone

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Abstract

Short-term elevations of stress hormones cause an increase in glycemia. However, the effect of intermittent stress on development of type 2 diabetes mellitus is unclear. We hypothesized that recurrent intermittent restraint stress would deteriorate glycemia. Male, prediabetic Zucker diabetic fatty (ZDF) rats were restrained 1 hour per day, 5 days per week for 13 weeks and compared with unstressed, age-matched diabetic controls and lean nondiabetic rats. To differentiate the effects of recurrent restraint stress per se vs restraint-induced inhibition of food intake, a pair-fed group of rats was included. Surprisingly, recurrent restraint and pair feeding delayed fed and fasting hyperglycemia, such that they were lowered 50% by restraint and 30% by pair feeding after 13 weeks. Rats that were previously restrained or pair fed had lower glucose levels during a glucose tolerance test, but restraint further improved the return of glucose to baseline compared to pair feeding ($P < .05$). This was despite pair-fed rats having slightly lowered food intake and body weights compared with restrained rats. Restraint and pair feeding did not alter insulin responses to an intraperitoneal glucose tolerance test (IPGTT) or fasting insulin, and did not lower plasma lipids. Interestingly, restraint normalized basal corticosterone to one third that in control and pair-fed rats, prevented increases in pretreatment corticosterone seen with pair feeding, and led to habituation of restraint-induced corticosterone responses. After 13 weeks of treatment, multiple regression analysis showed that elevations in basal corticosterone could explain approximately 20% of the variance in fed glucose levels. In summary, intermittent restraint and its adaptations delayed hyperglycemia and improved glucose control in Zucker diabetic fatty rats. These benefits can be partially explained by restraint-induced lowering of food intake, but additional improvements compared to pair feeding may involve lower overall corticosterone exposure with repeated restraint. Paradoxically, these novel investigations suggest some types of occasional stress may limit development of diabetes.

1. Introduction

Stressors constantly challenge daily life. Stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system has the following short-term effects: promotes alertness; suppresses feeding, reproduction and growth; mobilizes energy; and inhibits immune responses [1]. Chronic and repeated stress causes adaptation of these responses. However, as Hans Selye [2] suggested when coining "eustress" and "distress," not all stress is deleterious.

A number of studies suggest that stress hormones are deleterious to glycemic control in both human and animal models of diabetes [3-7]. However, in patients with type 1 diabetes mellitus, negative stressful events such as the death of a loved one were associated with decreased glycemic control, whereas positive life stress such as a job promotion or a wedding is associated with improved glycemic control [8,9]. Cortisol is increased in type 2 diabetes mellitus

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(T2DM), and patients with hypercortisolemia show deteriorations in glycemic control [10,11] and higher risk of diabetes complications [12]. HPA hyperactivity and altered glucocorticoid metabolism are associated with visceral adiposity, dyslipidemia, hyperglycemia, and insulin resistance [7,13-16]. Although glucocorticoids inhibit insulin secretion [17] and promote insulin resistance [17,18], intracerebroventricular injection of carbachol (a model of neurogenic stress) promotes insulin resistance in diabetic dogs [19,20], but lowers it in normal dogs [20]. Interestingly, although pharmacologic glucocorticoids induce diabetes, prolonged administration causes hypertrophy and hyperplasia of beta cells [21-23], although these effects may have been related to the prolonged moderate hyperglycemia. Furthermore, the diabetogenic effects of streptozotocin, alloxan, and partial pancreatectomy can be reduced by pretreatment with glucocorticoids and mild foot shock stress [24-27]. Thus, the effects of "stress" on diabetes are complex and may depend on the nature of the stressor (ie, positive vs negative stressors, heterotypic [different types] vs homotypic [same types] repeated stressors, psychological vs metabolic stressors), the stage of diabetes development, and may differ from the short-term effects of stress hormones.

Previous studies on the effect of stress on development of T2DM in humans included many confounding factors, making interpretation of the results complex and inconclusive [3,4,28-32]. These include dietary adherence, social support, individuality of stress responses, the inability to control duration, type, or severity of stress, and the assumption that these are similar in healthy, prediabetic, and diabetic patients. Animal studies have been much more effective at controlling these variables. Acute stress is well established to cause "stress hyperglycemia" in healthy and diabetic animals and has been shown to increase glycemia to diabetic levels in *ob*/*ob* mice [5]. However, long-term repeated homotypic stressors may have opposing effects. In Otsuka Long-Evans Tokushima fatty (OLETF) rats, repeated immobilization stress improved hemoglobin A_{1c} and glucose tolerance, and prevented diabetes in 80% of the rats, whereas short-term immobilization transiently increased blood glucose [33]. These effects were attributed to decreased body weight gain with chronic stress. Similarly, repeated restraint of high-fat–fed rats increases hepatic glucose uptake [34], effects again attributed to a temporary reduction in food intake, but permanent reduction in body weight with repeated restraint [35]. Regular exercise, which activates short-term stress responses and if performed regularly causes habituation of these responses [36-38], can prevent T2DM [39-41] by improving glucose uptake [42], insulin sensitivity [43], and preserving beta-cell mass [44,45] and proliferation [45] in animal models of T2DM. More importantly, the contribution of stress to exercise-induced benefits is unknown.

Thus, the effect of recurrent intermittent stress on development of T2DM without confounding effects on food intake and body weight remains unknown.

We hypothesized that intermittent restraint stress, a potent psychological stressor that activates the HPA axis and sympathetic nervous system [46,47] would deteriorate glycemia in male Zucker diabetic fatty (ZDF) rats. To test this hypothesis, we examined the effect of 13 weeks of intermittent restraint stress on development of glucose intolerance and hyperglycemia in male ZDF rats. More importantly, we examined whether intermittent restraint stress per se vs stress-induced reductions in food intake affect development of hyperglycemia.

2. Methods

2.1. Animals

Male ZDF/Crl-*Lepr*^{fa} rats were used as a model of human T2DM because their progression to hyperglycemia and diabetes resembles that of patients with T2DM. Male rats have initial hyperinsulinemia, insulin resistance, and impaired glucose intolerance, which progresses to hyperglycemia and relative hypoinsulinemia (Charles River Laboratories, Wilmington, MA). Obese ZDF (−/−) and lean ZDF (+/?) rats were obtained at 5 weeks of age, the earliest age they can be genotyped, from Charles River Laboratories and individually housed in opaque cages in temperature- and humidity-controlled rooms on a 0700 to 1900 hours light cycle. Animals were obtained in 9 batches and staggered to start in different weeks to control for variation between batches and any short-term environmental changes. Rats were fed normal chow (Purina 5001, St Louis, MO), which was supplied in wire lid food hoppers, making it easily accessible to the rats even when at low levels. All experiments were approved by the University of Toronto Animal Care Committee following guidelines from the Canadian Council for Animal Care. Animals were euthanized 24 hours after the last treatment by decapitation between 0830 and 1300 hours within 1 minute of agitating the animal. The adrenal glands and spleen were removed and stored at −80°C. Trunk blood was obtained and plasma was stored at −20°C for analysis of fed insulin, glucagon, free fatty acids (FFAs), triglycerides, leptin, adiponectin, basal corticosterone, and growth hormone levels.

2.2. Experimental protocol

This study examines the effect of a long duration (13 weeks) of recurrent intermittent restraint stress on the development of diabetes. To distinguish between the effects of stress per se vs the effects of stress-induced inhibition of food intake, a pair-fed group was included (Fig. 1). At 5 weeks of age, obese ZDF rats were randomly divided into 3 groups (n = 9 per group) and acclimatized by daily handling for 1 week. At 6 weeks old, rats were restrained 1 hour per day, 5 days per week between 0900 h and 1200 h (noon) in Broome rodent restrainers (Harvard Apparatus, Saint Laurent, QC, Canada) for 13 weeks (restraint); control rats had food and water removed 1 hour per day, 5 days per week for 13 weeks (control); and pair-fed rats were fed the

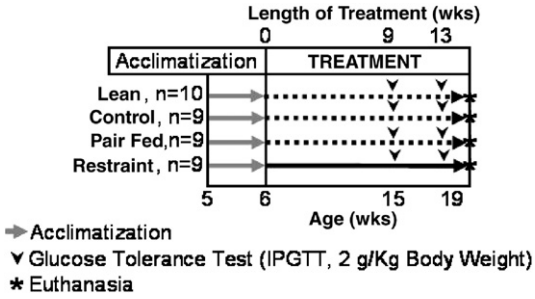

Fig. 1. Study design. All animals were obtained at 5 weeks old and acclimatized for 1 week. Rats were restrained (Restraint) (➞) for 1 hour/d, 5 days/wk for 13 weeks and compared with age-matched lean (ZLC), sedentary control (Control), and pair fed control (Pair Fed) rats that had food and water removed 1 hour/d, 5days/wk (---➞).

daily amount eaten by a restraint rat, and had food and water removed 1 hour per day, 5 days per week for 13 weeks (pair fed). All rats were transported together to the treatment room 30 to 60 minutes before treatment. In addition, lean ZDF (+/?) control rats (n = 10) were obtained at 5 weeks of age, and starting at 6 weeks of age were monitored daily (lean).

2.3. Animal monitoring

Body weight, food intake, and morning-fed glucose levels were monitored daily between 0900 and 1100 hours before treatment, and fasting glucose and insulin were monitored weekly before treatment after a 16- to 18-hour overnight fast. After 9 and 13 weeks of treatment, rats were given an intraperitoneal glucose tolerance test (IPGTT) (2 g/kg body weight of 50% dextrose) and glucose and insulin levels were measured every 30 minutes for 120 minutes. Pre- and posttreatment corticosterone samples were obtained weekly between 0900 h and 1200 h (noon).

2.4. Evaluation of food intake profile

After 4 weeks of treatment, food intake was measured every 2 weeks between 0700 and 0900, 0900 and 1030, 1030 and 1600, and 1600 and 0700 hours to determine whether recurrent intermittent restraint affected food intake between 0700 and 1030 when morning glucose was measured and whether stress affected feeding behavior.

2.5. Blood sampling and hormone assays

Blood for glucose (<2 μL) was taken by tail prick and measured by Glucometer Elite XL (Bayer, Toronto, ON, Canada). Blood for insulin and corticosterone (~20 μL) was obtained by tail nick within 30 seconds. Insulin was assayed using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem, Downers Grove, IL). Other hormones and adipokines were measured by using commercial radioimmunoassay kits: corticosterone (ICN Biomedicals, Costa Mesa, CA), glucagon, growth hormone, leptin, and adiponectin (Linco, St Charles, MO). Plasma FFAs (Wako Industrials, Richmond, VA) and triglycerides (Boehringer Mannheim, Indianapolis, IN) were measured using colorimetric kits.

2.6. Statistical analysis

Data presented are means ± SEM. Morning glucose, food intake, and body weights are reported as weekly means to control for daily variability. Statistical analysis was performed with Statistica 6.0 (Statsoft, Tulsa, OK), and $P < .05$ was considered statistically significant. Repeated-measures analyses of variance (RM ANOVAs) were used to analyze effects of treatment over time and main effects, with Duncan post hoc test performed if ANOVA revealed a $P < .05$. Weekly differences and differences at euthanasia were compared by unpaired Student t test because of strain differences. Area under the curve (AUC) was calculated using the trapezoidal rule from 0 or baseline (glucose tolerance test) using Origin 6.0 (Microcal Software, Northampton, MA). Multiple regression analysis using forward stepwise variable selection was used to investigate the influence of independent variables including insulin, corticosterone, FFA, triglycerides, leptin, adiponectin, glucagon, and growth hormone on the dependent variable fed glucose. Corticosterone, FFA, triglyceride, glucagon, and leptin levels were log transformed, and adiponectin was exponentially transformed to meet linear model assumptions.

3. Results

3.1. Food intake and body weight

Because repeated stress is well known to lower food intake and hence body weight gain [35], and this lower body weight gain has been suggested to be the cause of any improvements in glycemia in response to stress [33], we measured daily food intake and body weights.

3.1.1. Food intake

All groups ate more than lean rats (main effect of treatment, $P < .001$; post hoc, $P < .01$) (Fig. 2A). As expected, both restrained and pair-fed rats ate less than controls (main effect of treatment, $P < .001$; post hoc, $P < .01$). Surprisingly, pair-fed rats had lower food intake than restrained rats (main effect of treatment, $P < .001$; post hoc, $P < .01$), because approximately 3 g of food was uneaten on a daily basis.

3.1.2. Food intake profile

To examine whether recurrent restraint influenced feeding behavior, food intake of obese ZDF rats was measured between 0700 and 0900 and 0900 and 1030 (pretreatment), 1030 and 1600 (posttreatment), and 1600 and 0700 hours (nocturnal) after 4, 6, 8, 10, and 12 weeks of treatment (Fig. 2B and C).

Rats ate approximately 70% of their daily food intake at night between 1600 and 0700, and approximately 30% of their food during the day between 0700 and 1600 hours.

In the pretreatment period (0700-0900 or 0900-1030 hours), restraint did not affect the percent of total food intake compared with controls during any week

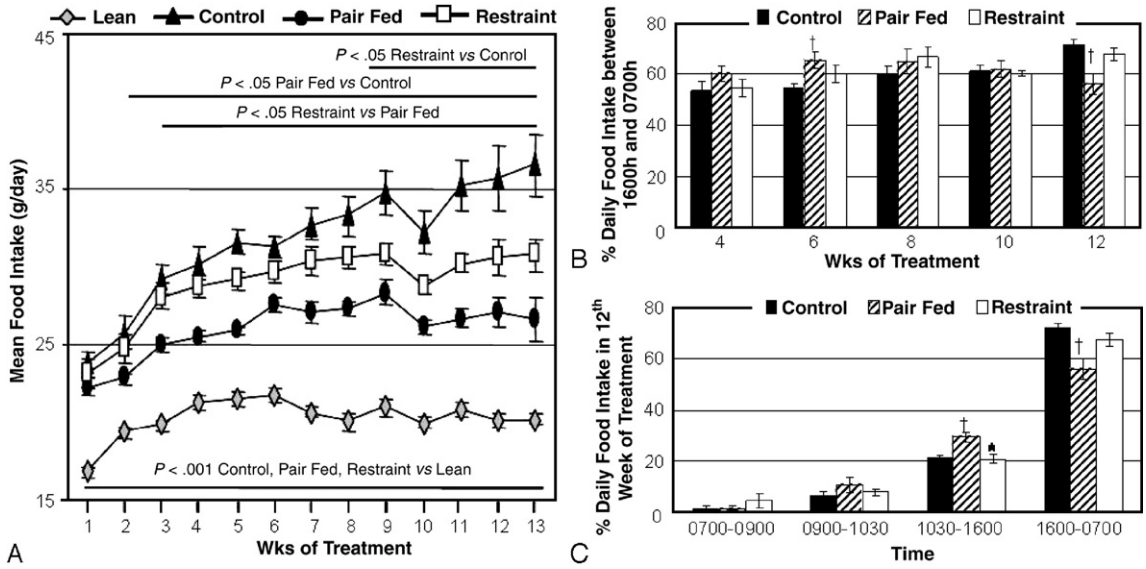

Fig. 2. Thirteen weeks of recurrent intermittent restraint lowers mean daily food intake ($P < .01$) (A) but does not affect feeding behavior (B, C). (A) Mean daily food intake. Food intake is lowered by recurrent restraint ($P < .01$) and pair feeding ($P < .01$), whereas pair feeding further lowers food intake compared with recurrent restraint ($P < .01$). (B) Percent of daily food intake eaten between 1600 and 0700 h after 4 to 12 weeks of treatment. (C) Percent of daily food intake after 12 weeks of treatment during different time intervals through the day. $^{\dagger}P < .05$ vs control, $^{*}P < .05$ vs pair fed.

(Fig. 2C, other data not shown), showing that morning food intake did not influence morning-fed glucose in restrained rats. Pair-fed rats ate less than control rats between 0900 and 1030 hours after 4 weeks of treatment (control, 13.4% ± 0.9%; pair fed, 7.1% ± 1.5%; restraint, 11.1% ± 1.2%; $P < .01$, pair fed vs control) and less than restrained rats between 0700 and 0900 hours after 10 weeks of treatment (control, 3.6% ± 2.5%; pair fed, 0.0% ± 0.0%; restraint, 7.7% ± 1.8%; $P < .0001$, restraint vs pair fed), although these differences were not apparent during any other week.

Restraint did not affect the percent of food intake during the posttreatment period (1030-1600 hours) compared with controls during any week measured (Fig. 2C, other data not shown). In contrast, pair feeding increased the percent of total food intake eaten during this posttreatment time compared with restrained rats after 10 weeks of treatment (control, 28.5% ± 2.1%; pair fed, 30.2% ± 1.1%; restraint, 21.7% ± 3.1%; $P < .05$, pair fed vs restraint) and with both restrained and control rats after 12 weeks of treatment (Fig. 2C).

During the dark phase of the light cycle, rats ate the largest proportion of their daily food intake. Restraint did not affect the percent of food intake between 1600 and 0700 hours (nocturnal food intake) during any week measured (Fig. 2B and C, other data not shown). In contrast, pair feeding increased the percentage of total food intake eaten nocturnally after 6 weeks of treatment (control, 54.5% ± 1.5%; pair fed, 65.4% ± 3.2%; restraint, 60.0% ± 3.5%; $P < .02$, pair fed vs control), but lowered it after 12 weeks of treatment (Fig. 2B).

In summary, recurrent restraint did not alter the food intake profile, whereas pair feeding altered the food intake profile during the posttreatment and nocturnal feeding phases such that by the end of the study (week 12) pair-fed rats ate a lower percentage of their food nocturnally and a greater percentage of their food during the posttreatment period. More importantly, this may have affected morning-fed glucose levels measured during these weeks, which may closer reflect fasting glucose as opposed to fed glucose levels in pair-fed rats.

3.1.3. Body weight

All groups had higher body weights than lean rats (main effect of treatment, $P < .001$; post hoc, $P < .001$) (Fig. 3A). RM ANOVAs comparing individual groups showed that restraint did not affect body weight gain compared with controls. However, pair-fed rats had lower body weights than controls (treatment-time interaction, pair fed vs control, $P < .01$) and restrained rats (treatment-time interaction, pair fed vs restraint; $P < .00001$). Control, pair-fed, and restrained animals had similar ratios of body weight gain to food intake (Fig. 3B), suggesting any differences in body weight were due to differences in food intake.

3.2. Evaluation of diabetes development

To determine whether recurrent intermittent restraint affected development of diabetes, morning-fed glucose (analogous to random blood glucose in humans), fasting glucose and insulin, and intraperitoneal glucose tolerance were measured.

3.2.1. Morning-fed glucose

Control and pair-fed rats had higher morning glucose than lean rats (main effect of treatment, $P < .001$; post hoc, $P < .05$), whereas it did not reach significance in restrained rats (post hoc, $P = .056$) (Fig. 4A). Thirteen weeks of restraint

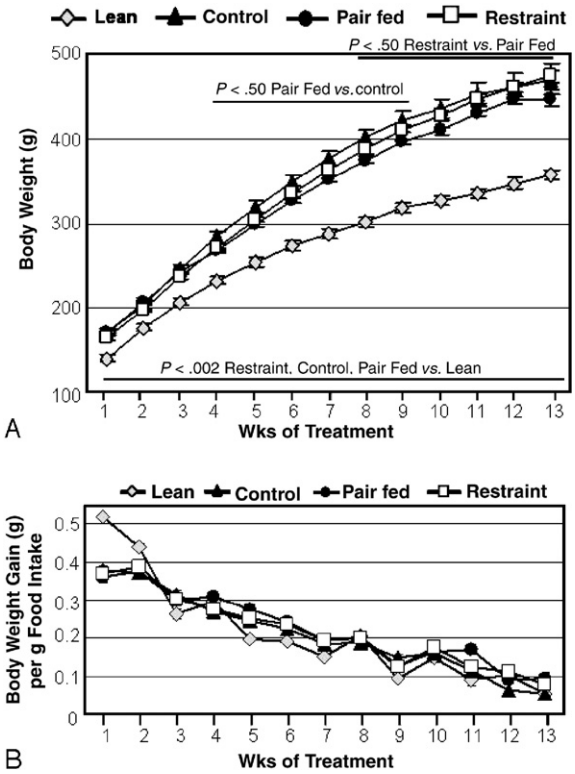

Fig. 3. Recurrent intermittent restraint does not affect body weight in ZDF rats. (A) Mean weekly body weight. Pair feeding ($P < .01$) lowers body weight compared with control and restrained rats, whereas recurrent restraint does not affect body weight. (B) Body weight gain per gram food intake. Body weight gain per gram of food eaten is similar between control, pair fed, and recurrently restrained rats, suggesting differences in body weight are caused by differences in food intake.

and pair feeding lowered morning glucose (treatment-time interaction, $P < .001$; main effect of treatment $P < .0001$; post hoc, $P < .01$). Interestingly, by week 13, morning glucose was 50% lower in restrained rats than in controls ($P < .05$), whereas it was only 30% lower in pair-fed rats ($P < .05$). A slight divergence in mean morning glucose between restrained and pair-fed rats became apparent after 11 weeks of treatment, and a RM ANOVA between them revealed a tendency for restraint to lower morning glucose compared to pair feeding (treatment-time interaction, $P < .08$). Restrained rats consistently maintained lower morning glucose levels compared with control rats from 8 weeks of treatment onward, whereas pair-fed rats consistently maintained lower glucose levels at an earlier time from 5 to 10 weeks of treatment, and then rose in weeks 11 and 12 to levels that were not significantly lower than in control rats. More importantly, because at this time of the study pair-fed rats ate a greater proportion of their food during the day and lower proportion of their food at night, "fed" glucose levels may closer reflect fasting glucose levels in pair-fed rats. Thus, compared with controls, recurrent intermittent restraint maintains lower morning-fed glycemia longer than pair feeding.

3.2.2. Fasting glucose

Fasting glucose was improved over 13 weeks of restraint and pair feeding (treatment-time interaction, $P < .0001$; main effect of treatment, $P < .00001$; post hoc, $P < .006$). In week 13, restraint lowered fasting glucose by approximately 50% ($P < .05$), whereas pair feeding only tended to lower it by approximately 35% ($P = .05$) compared with controls. In week 13, a tendency for a divergence in fasting glucose

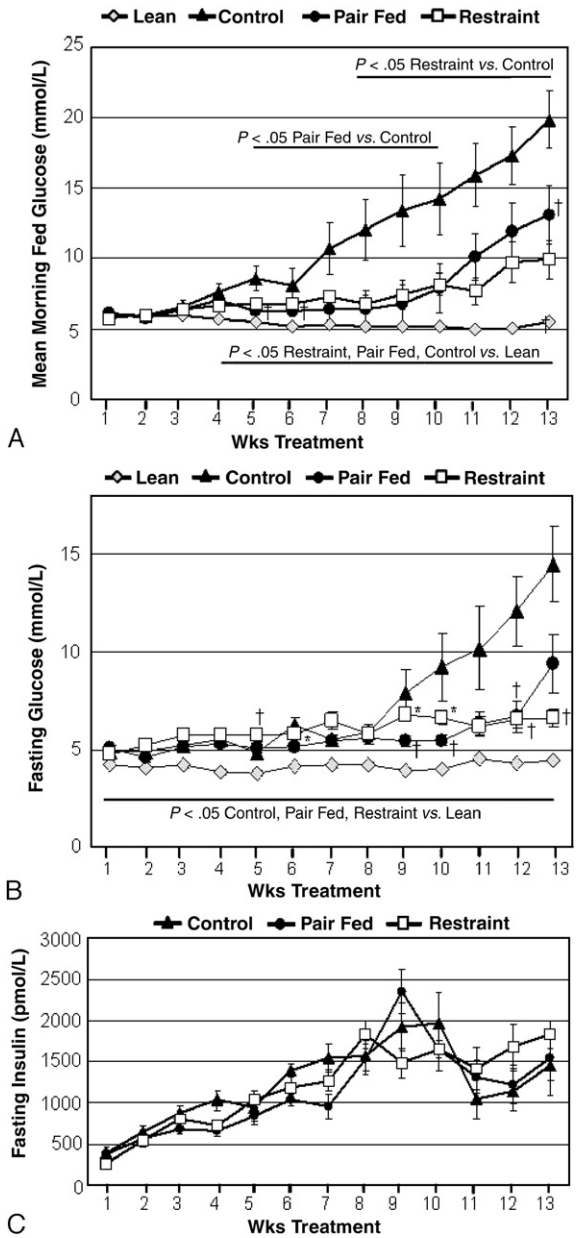

Fig. 4. Effect of recurrent intermittent restraint on development of hyperglycemia. (A) Mean weekly morning fed glycemia. Recurrent restraint ($P < .01$) and pair feeding ($P < .01$) delay fed hyperglycemia. (B) Weekly fasting glycemia. Recurrent restraint ($P < .006$) and pair feeding ($P < .002$) delay fasting hyperglycemia. (C) Weekly fasting insulin. Neither recurrent restraint nor pair feeding affect fasting insulin. $^{\dagger}P < .05$ vs control, $^{*}P < .05$ vs pair fed.

between restrained and pair-fed rats became apparent ($P = .09$) and a RM ANOVA between them showed a significant interaction ($P < .001$). Thus, compared with controls, recurrent intermittent restraint maintains lower fasting glycemia longer than pair feeding. The delay in the development of fasting compared with fed hyperglycemia likely explains why more dramatic differences in fasting glucose between restrained and pair-fed rats were not statistically apparent by RM ANOVA in the time frame of our study. Fasting insulin increased over time in all obese rats (main effect of time, $P < .0001$), but was not different between groups (Fig. 4C). At dilutions necessary for direct comparison of obese ZDF rats, fasting insulin levels in lean rats was below detection and could not be directly compared at lower dilutions because of nonlinearity of dilution.

3.2.3. Glucose tolerance

After 9 weeks of treatment, all rats had impaired glucose tolerance compared with lean rats as demonstrated by both the glucose tolerance curve (main effect of treatment, $P < .00001$; post hoc, $P < .01$) (Fig. 5A) and incremental glucose AUC ($P < .005$) (Fig. 5B). Glucose levels during the glucose tolerance test were improved by pair feeding ($P < .05$) (Fig. 5A), although when calculated from baseline, glucose tolerance was similar among control, pair-fed, and restrained groups (Fig. 5B). After 13 weeks of treatment, all rats again had impaired glucose tolerance compared with lean rats (main effect of treatment, $P < .00001$; post hoc, $P < .005$; $P < .02$ for incremental glucose AUC) (Fig. 5A and B). Glucose levels during the glucose tolerance test were improved by both pair feeding (main effect, $P < .00001$; post hoc, $P < .01$) and restraint (post hoc, $P < .0001$) compared with controls (Fig. 5A). Furthermore, restraint improved glucose levels compared with pair feeding (main effect, $P < .05$) and lowered 90- and 120-minute glucose levels by 30% ($P < .05$) compared with pair feeding. When calculated from baseline, however, the glucose AUC was similar among control, pair-fed, and restrained rats (Fig. 5B), suggesting that the improved glucose tolerance curve between the groups was mainly due to improved fasting glucose levels. More importantly, the 2-hour postload glucose level was significantly closer to baseline levels in restrained compared with pair-fed rats (6.5 ± 1.1 vs 9.4 ± 0.8 mmol/L higher than baseline, restraint vs pair fed, $P < .05$), illustrating improved glycemic control with restraint. Insulin responses during the IPGTT were similar to controls in pair-fed and restrained rats (data not shown).

3.3. Plasma corticosterone and growth hormone

Pre- and posttreatment corticosterone were measured weekly between 0900 h and 1200 h (noon). Pretreatment corticosterone was sampled 30 to 60 minutes after

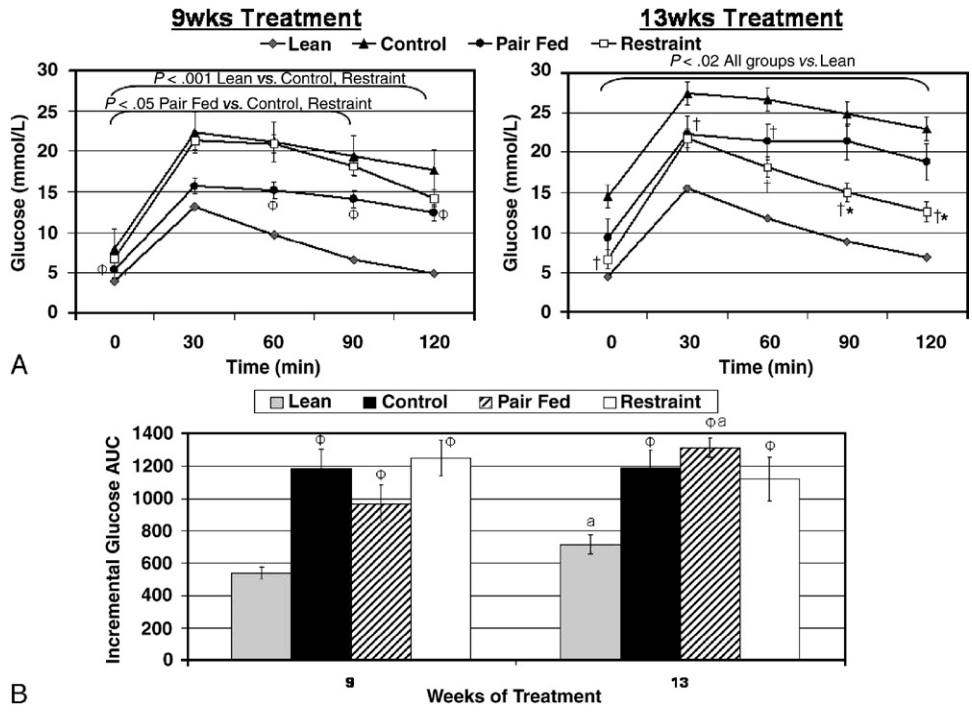

Fig. 5. Effect of recurrent intermittent restraint on glucose tolerance. (A) Glucose responses to IPGTT after 9 and 13 weeks of treatment. After 9 weeks of treatment, pair feeding ($P < .05$), but not restraint, lowers glucose levels during an IPGTT, whereas after 13 weeks of treatment, both recurrent restraint ($P < .0001$) and pair feeding ($P < .01$) improve glucose levels, and restraint further improves glucose levels more than pair feeding ($P < .05$) (A). (B) Incremental AUC for glucose responses to an IPGTT. All obese groups have impaired glucose tolerance after 9 and 13 weeks of treatment ($P < .05$ vs lean) and glucose tolerance declines from 9 to 13 weeks of treatment in lean and pair-fed rats. $^{\Phi}P < .05$ vs lean, $^{\dagger}P < .05$ vs control, $^{*}P < .05$ vs pair fed, $^{a}P < .05$ vs same treatment after 9 weeks.

transportation to the treatment room. Basal corticosterone and growth hormone were obtained at euthanasia between 0900 and 1300 hours without any prior transportation and thus represent basal, nonstressed levels. Pre- and posttreatment corticosterone levels are not shown for lean rats because of differences in daily transportation to the treatment room.

Recurrent restraint did not affect pretreatment corticosterone compared with controls (Fig. 6A). Surprisingly, pretreatment corticosterone was higher in pair-fed than restrained rats (main effect, $P < .01$; post hoc, $P < .005$) and also tended to be higher than in controls ($P = .056$). Using AUC from 0, pair-fed rats had higher pretreatment corticosterone than both control and restrained rats ($P < .05$).

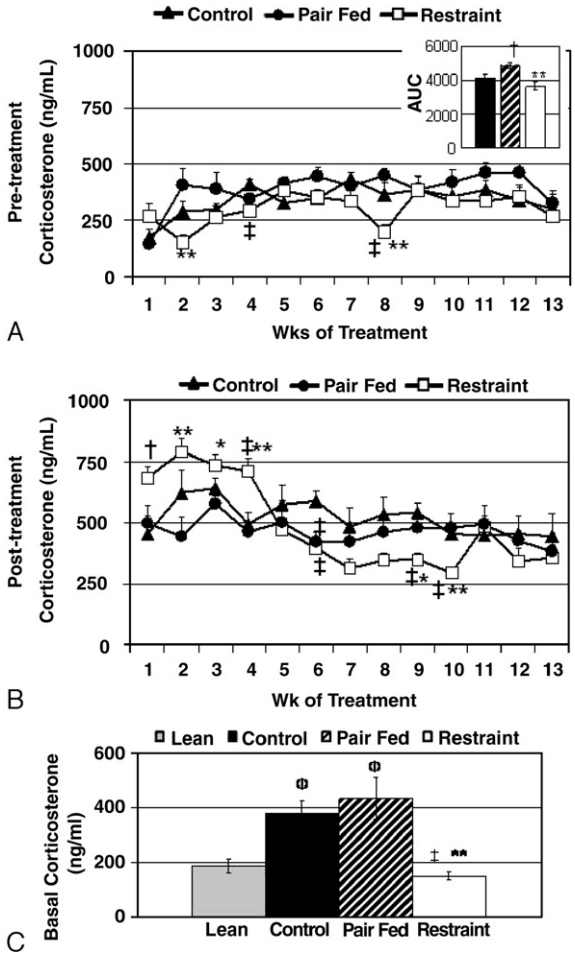

Fig. 6. Effect of recurrent intermittent restraint on plasma corticosterone levels. (A) Pretreatment corticosterone sampled 30 to 60 minutes after transportation to the treatment room. Thirteen weeks of stress does not affect pretreatment corticosterone, although it is increased with pair feeding ($P < .05$, AUC). (B) Posttreatment corticosterone sampled after treatment. Stress leads to adaptation of corticosterone responses because it increases posttreatment corticosterone for the first 4 weeks ($P < .01$) and lowers it from 5 weeks onward ($P < .05$). (C) Basal Corticosterone sampled within 1 minute of agitating the rat at euthanasia. Intermittent restraint stress but not pair feeding normalizes basal corticosterone levels. $^{\Phi}P < .05$ vs Lean, $^{\dagger}P < .05$ vs control, $^{\ddagger}P < .01$ vs control, $^{*}P < .05$ vs pair fed, $^{**}P < .01$ vs pair fed.

Restraint affected posttreatment corticosterone over the 13 weeks (treatment-time interaction, $P < .00001$) such that it was higher in restrained than in pair-fed and control rats for the first 4 weeks (main effect, weeks 1-4, $P < .01$ vs control and pair fed) and lower than in pair-fed and control rats from 5 weeks onward (main effect, weeks 5-13, $P < .05$ vs control and pair fed) (Fig. 6B). Thus, recurrent intermittent restraint activated corticosterone stress responses for the first 4 weeks, but adaptation of the response occurred thereafter. Posttreatment corticosterone in pair-fed rats was similar to controls.

Control rats had markedly elevated basal corticosterone compared with lean rats ($P < .01$) (Fig. 6C). Remarkably, 13 weeks of restraint, but not pair feeding, normalized basal corticosterone such that it was lowered more than 60% compared with control and pair-fed rats ($P < .01$).

Growth hormone is lowered under conditions of obesity [48] and chronic stress [49]. All obese groups had greater than 3-fold lower growth hormone than lean rats ($P < .052$) (Table 1). Restraint and pair feeding further lowered growth hormone approximately 80% compared with controls ($P < .05$).

In summary, recurrent intermittent restraint caused a significant corticosterone response for the first 4 weeks, after which there was adaptation of this response. Intermittently restrained rats showed a normalization of basal corticosterone levels in contrast to pair-fed rats and prevented the increase in pretreatment corticosterone observed with pair feeding.

3.4. Adrenal and spleen weights

Adrenal hypertrophy and atrophy of the spleen can be used as indices of chronic stress. Restrained, control, and pair-fed rats had similar total absolute adrenal weights, which were larger than in lean rats ($P < .05$) (lean, 0.034 ± 0.001; control, 0.053 ± 0.004; pair fed, 0.045 ± 0.003; restraint, 0.050 ± 0.003 g). When normalized to body weight, controls had higher relative adrenal weights than lean rats ($P < .05$) but no other differences were noted (data not shown).

When normalized to body weight, control, pair-fed, and restrained rats had lower spleen weights than lean rats ($P < .05$) (lean, 1.55 ± 0.02; control, 1.32 ± 0.03; pair fed, 1.28 ± 0.04; restraint, 1.29 ± 0.04 mg/kg body weight) although no differences were observed between obese groups.

3.5. Plasma hormone and metabolite profile

Plasma hormone and metabolite levels were obtained in the fed state at euthanasia without prior transportation and thus represent basal, nonstressed levels (Table 1).

3.5.1. Glucose, insulin, and glucagon

All groups had higher glucose and insulin than lean rats ($P < .01$). Restrained ($P < .001$) and pair-fed rats ($P < .05$) had lower glucose than control rats, but only restrained rats had statistically higher insulin ($P < .05$). Pair-fed rats did, however, show a tendency ($P = .07$) for higher fed insulin than controls, and both restrained ($P < .01$) and pair-fed ($P < .05$) rats had higher insulin-glucose ratios than

Table 1
Plasma metabolite, hormone, and adipokine levels after 13 weeks of recurrent intermittent restraint

	Lean (n = 10)	Control (n = 9)	Pair fed (n = 9)	Restraint (n = 9)
Glucose (mmol/L)	5.5 ± 0.2	21.0 ± 1.2 *	14.8 ± 2.3 *,†	12.2 ± 5.4 *,†
Insulin (pmol/L)	309 ± 27	2782 ± 578 *	4892 ± 887 *	4502 ± 470 *,†
Glucagon (ng/L)	68.6 ± 6.8	100.1 ± 5.2 *	86.7 ± 5.9	98.3 ± 5.6 *
FFA (mEq/L)	0.24 ± 0.01	0.68 ± 0.06 *	0.86 ± 0.09 *	0.98 ± 0.10 *,†
Triglycerides (mmol/L)	1.7 ± 0.2	10.1 ± 1.0 *	10.1 ± 1.2 *	11.3 ± 0.9 *
Growth hormone (μg/L)	16.1 ± 5.7	4.8 ± 1.5	0.6 ± 0.04 *,†	1.1 ± 0.5 *,†
Adiponectin (μg/mL)	4.9 ± 0.2	3.6 ± 0.2 *	4.2 ± 0.4	4.0 ± 0.4 *
Leptin (ng/mL)	4.5 ± 0.3	28.5 ± 2.6 *	38.7 ± 3.2 *	33.2 ± 1.7 *

Values are expressed as mean ± SEM.
* $P < .05$ vs lean.
† $P < .05$ vs control.

controls (lean, 55.7 ± 4.7; control, 134.1 ± 32.0; pair fed, 470.8 ± 137.9; restraint, 476.6 ± 92.2 pmol insulin per millimole glucose) suggesting both groups had better beta-cell function. Control and restrained rats had higher glucagon than lean rats ($P < .01$), and pair-fed rats also showed a tendency for increased glucagon ($P = .06$). No other differences in glucagon were noted.

3.5.2. Plasma lipids, leptin, and adiponectin

All groups had higher FFA and triglycerides than lean rats ($P < .05$). Interestingly, restraint, but not pair feeding, increased FFA ($P < .02$), whereas no effects on triglycerides were observed. All obese rats had markedly higher leptin than lean rats ($P < .000001$). Recurrent restraint did not affect leptin, although pair feeding increased leptin compared with controls ($P < .05$). After 13 weeks, control ($P < .0001$) and restrained ($P < .05$) rats had lower adiponectin than lean rats, although adiponectin was not different between obese groups.

3.5.3. Multiple regression analysis

A forward, stepwise, multiple regression was performed on the 25 ZDF rats for whom complete information was available to determine the extent to which fed glucose at euthanasia is affected by the independent variables insulin, FFA, triglycerides, corticosterone, glucagon, growth hormone, leptin, and adiponectin. A significant negative association of fed glucose was demonstrated with insulin ($\beta = -.356$, $P = .0029$) and adiponectin ($\beta = -.449$, $P = .0002$), and positive association with corticosterone ($\beta = .451$, $P = .0002$) in this order of importance, which contributed significantly to the overall R^2 of 0.81861 ($F_{4,20} = 22.5655$). Thus, insulin, adiponectin, and corticosterone contributed to 40%, 21%, and 19% of the variance in fed glucose levels (contribution to R^2 of 0.405, 0.207, and 0.193, respectively).

4. Discussion

Stress is suggested to deteriorate glycemia and accelerate the development of human [3,6,7] and rodent [5] T2DM. However, we show that both recurrent intermittent restraint and food restriction (pair feeding) delay development of hyperglycemia in ZDF rats. Despite restrained rats having slightly higher food intake and body weights than pair-fed rats, recurrent restraint appears to delay fed and fasting hyperglycemia longer than pair feeding and improve the glycemic decline to baseline during glucose tolerance tests compared with pair feeding. More importantly, we suppose that had food intake and body weights been similar between pair-fed and repeatedly restrained rats, the differences in glycemia would have been more dramatic. These effects of recurrent restraint were accompanied by eventual normalization of basal corticosterone and adaptation of corticosterone responses to restraint. At the end of the 13 weeks of treatment, these basal corticosterone levels explained approximately 20% of the variation in fed glucose levels, as demonstrated by multiple regression analysis. Thus, we conclude that the adaptations to recurrent intermittent restraint delayed diabetes in ZDF rats in part through inhibition of food intake, but also by mechanisms in addition to the stress-induced lowering of food intake, perhaps involving normalization of corticosterone.

Our findings substantiate studies of repeated stress in other rodent models of obesity or prediabetic T2DM. Repeated immobilization (but not short-term immobilization) of OLETF rats decreased body weight, improved hemoglobin A_{1c} and glucose tolerance, and prevented development of diabetes in 80% of the rats [33]. Similarly, repeated restraint of high-fat–fed rats reduced body weight, improved insulin sensitivity, and increased hepatic glucose uptake [34,35,50]. More importantly, the proposed mechanism for these improvements was lowering of food intake and/or body mass.

However, we show that in ZDF rats, recurrent intermittent restraint lowers food intake, but not body weight. This inconsistency is not surprising considering the marked hyperglycemia and probable glucosuria in controls and their ensuing drop in weight gain despite hyperphagia. Stress lowered food intake, which is the reason for inclusion of a pair-fed group. Pair-fed rats were fed daily the equivalent amount of food that their paired stressed rat ate on that day of

the study. Food was supplied in wire lid food hoppers and was easily accessible for the rat even at low levels. We speculate that food intake may have been slightly lower in pair-fed rats because they learned that if they ate all of their food, they would be hungry for the remainder of the day, a circumstance that innately hyperphagic rats may avoid. This is reflected in Fig. 2A where pair-fed and restrained rats had similar food intake until the third week, when it is possible that pair-fed rats increased hoarding behavior (the gathering or accumulation of food) as has been shown with food deprivation/fasting [51,52]. More importantly, stress delayed fed and fasting hyperglycemia longer than pair feeding and improved glucose regulation during the glucose tolerance test despite restrained animals having slightly higher food intake and body weights. Thus, to our knowledge we are the first to show that recurrent stress may delay hyperglycemia through additional mechanisms to inhibition of food intake.

Restrained rats had a significant corticosterone response to restraint (sampled 0900-1200 [noon] hours) for the first 4 weeks, followed by adaptation of the response (Fig. 6B). Adaptation of corticosterone responses to chronic stressors are well characterized [53] and is not suggestive of a lack of stress per se, only an adaptation to protect the organism from hypercorticosteronemia. Other indices of chronic stress that were measured were adrenal hypertrophy, atrophy of the spleen, inhibition of growth hormone, and inhibition of food intake. Catecholamine levels were not measured because of the large blood volume required, necessitating highly stressful cannulation surgeries. However, adaptation of epinephrine and norepinephrine responses also occurs after 3½ weeks of recurrent restraint [54], a time frame similar to that observed for adaptation of corticosterone responses to recurrent restraint in our repeatedly restrained ZDF rats. We did not observe atrophy of the spleen or adrenal hypertrophy with recurrent restraint. Adrenal hypertrophy in response to chronic intermittent stress decreases over time [55], and both atrophy of the spleen and adrenal hypertrophy are dependent on hypersecretion of corticosterone, which was not observed by the end of the study in intermittently restrained rats. In addition, we [56] and others [57] observed adrenal hypertrophy in streptozotocin-diabetic rats, which is not further increased with 21 days of recurrent restraint [58]. In ZDF rats, 13 weeks of daily forced swim stress also does not lead to adrenal hypertrophy [45]. Thus, diabetes in control rats may mask any effects of recurrent restraint on adrenal hypertrophy. However, indices of chronic stress mediated at the central level were consistent with our recurrently restrained rats being chronically stressed, including inhibition of food intake (Fig. 2) and growth hormone (Table 1) with intermittent restraint [35,55]. These contradictions between different indices of chronic stress illustrate the difficulties in defining "stress." Thus, although peripheral corticosterone levels and indices of chronic stress that depend on hypersecretion of corticosterone (adrenal hypertrophy, atrophy of spleen) do not reflect a "stressed" state, the centrally mediated indices (food intake, growth hormone) suggest that repeatedly restrained rats continue to be in a state of chronic stress. To our knowledge, there are no comparable studies that measure these parameters using recurrent intermittent restraint for as long duration (13 weeks).

Because of varying intensities, types, and durations of stressors, intermittent stressors sometimes [59,60] but not always [61] elevate basal corticosterone levels. Basal corticosterone levels (obtained at euthanasia) were elevated in control compared with lean rats, as expected in Zucker rats [62], and surprisingly recurrent restraint normalized basal corticosterone levels (Fig. 6C). In contrast with effects on basal corticosterone, pair feeding elevated pretreatment corticosterone (levels obtained after transportation to the treatment room) compared with control and restrained rats (Fig. 6A). This suggests that food restriction alone, which increases activation of CRH neurons and CRH messenger RNA in the paraventricular nucleus of Zucker rats [63] and increases basal corticosterone secretion [64], is also a "stressor." Thus, food restriction may be a more "chronic stressor" and differentially affect whole-body physiology as compared with a habituating stressor such as recurrent restraint. Because adrenalectomy can ameliorate the phenotype of Zucker rats [13], our results suggest that the further delay of hyperglycemia in recurrently restrained compared with food-restricted ZDF rats may result from lower overall corticosterone exposure as the rats adapt to recurrent restraint. Indeed, as shown by multiple regression, approximately 20% of the variance in fed glucose can be explained by increases in basal corticosterone levels. We postulate that changes in basal corticosterone levels affect glycemic control greater than posttreatment (stress induced) corticosterone levels, because rats are exposed to basal levels of corticosterone for most of the day. Thus, the return of posttreatment corticosterone to similar levels as in control rats at week 11 does not affect our hypothesis.

Because of the novelty of our finding that recurrent restraint delays diabetes in ZDF rats, numerous mechanisms responsible for these improvements are possible. During the IPGTT done after 13 weeks of treatment, the improved decline of glucose levels to baseline in repeatedly restrained compared with pair-fed rats, despite similar insulin levels, suggest that repeatedly restrained rats may have better insulin sensitivity than pair-fed rats. This, however, cannot be concluded based on these relationships alone. Fed insulin levels were shown by multiple regression analysis to explain approximately 40% of the variability in fed glucose levels after the 13 weeks of treatment. Indeed, both restraint and pair feeding increased the fed insulin-glucose ratio compared with control rats, suggesting these groups have better beta-cell function and that this may be the mechanism for improved glycemia with reduced food intake. However, because no differences were noted between recurrently restrained and pair-fed rats in terms of insulinemia, it is unlikely that the additional glycemic improvements with restraint are caused by improved beta-cell function.

Lipids are implicated in development of diabetes in ZDF rats [65]. Recent data from our laboratory shows that epididymal fat pad weights are similar to controls after 13 weeks of intermittent restraint or pair feeding (Bates et al, unpublished observations). Similarly, in the current study we did not observe decreases in plasma triglycerides or FFAs, or differences in adiponectin or leptin between obese groups. Thus, a lowering of plasma lipids or fat mass is not responsible for the food restriction–induced or recurrent restraint–induced delay of hyperglycemia. Although adiponectin showed a significant association with fed glucose during multiple regression analysis, it was not different between groups and thus cannot explain the effects of food restriction or repeated restraint.

In summary, we show that both recurrent intermittent restraint and food restriction delay development of fasting and fed hyperglycemia in ZDF rats. However, recurrent restraint delays fed and fasting hyperglycemia longer than pair feeding and improves the return of glucose to baseline levels during a glucose tolerance test, despite restrained rats having slightly higher food intake and body weights than pair-fed rats. Thus, recurrent restraint delays hyperglycemia by mechanisms that cannot be fully explained by restraint-induced inhibition of food intake. Intermittent restraint normalized basal corticosterone to one third that in control and pair-fed rats, prevented an increase in pretreatment corticosterone as seen with pair feeding, and led to habituation of restraint-induced corticosterone responses. In addition, basal corticosterone levels could explain approximately 20% of the variation in fed glucose levels as demonstrated by multiple regression analysis. Thus, we speculate that intermittent restraint leads to adaptations that lower corticosterone exposure and thus improve glycemia. Cortisol is increased in T2DM, and patients with hypercortisolemia show deteriorations in glycemic control [10,11] and higher risk of diabetes complications [12]. Thus, adaptation of cortisol to a repetitive, predictable stress may be more beneficial for glucoregulation than food restriction alone. It remains to be determined whether similar benefits occur with other forms of intermittent stress or whether it is unique to habituating, repetitive stressors such as intermittent restraint. Indeed, positive life stress such as a job promotion or a wedding is associated with improved glycemic control in type 1 diabetic patients, whereas negative stressors are not [8,9]. This study suggests that some intermittent stress and its associated adaptations, as is seen during normal daily life, may be beneficial for delay of T2DM.

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