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A rodent model of rapid-onset diabetes induced by glucocorticoids and high-fat feeding

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SUMMARY

Glucocorticoids (GCs) are potent pharmacological agents used to treat a number of immune conditions. GCs are also naturally occurring steroid hormones (e.g. cortisol, corticosterone) produced in response to stressful conditions that are thought to increase the preference for calorie dense 'comfort' foods. If chronically elevated, GCs can contribute to the development of type 2 diabetes mellitus (T2DM), although the mechanisms for the diabetogenic effects are not entirely clear. The present study proposes a new rodent model to investigate the combined metabolic effects of elevated GCs and high-fat feeding on ectopic fat deposition and diabetes development. Male Sprague-Dawley rats (aged 7-8 weeks) received exogenous corticosterone or wax (placebo) pellets, implanted subcutaneously, and were fed either a standard chow diet (SD) or a 60% high-fat diet (HFD) for 16 days. Animals given corticosterone and a HFD (cort-HFD) had lower body weight and smaller relative glycolytic muscle mass, but increased relative epididymal mass, compared with controls (placebo-SD). Cort-HFD rats exhibited severe hepatic steatosis and increased muscle lipid deposition compared with placebo-SD animals. Moreover, cort-HFD animals were found to exhibit severe fasting hyperglycemia (60% increase), hyperinsulinemia (80% increase), insulin resistance (60% increase) and impaired β-cell response to oral glucose load (20% decrease) compared with placebo-SD animals. Thus, a metabolic syndrome or T2DM phenotype can be rapidly induced in young Sprague-Dawley rats by using exogenous GCs if a HFD is consumed. This finding might be valuable in examining the physiological and molecular mechanisms of GC-induced metabolic disease.

INTRODUCTION

Presently, there are a number of available rodent models used to represent both the pre-diabetic or insulin-resistant disease (Aleixandre de Artinano and Miguel Castro, 2009; Kennedy et al., 2010) and hyperglycemic conditions associated with the development of type 2 diabetes mellitus (T2DM) (Islam and Loots, 2009). These animal models are important because they allow for examination of the disease state at the tissue and cellular level, and thus might help to develop new treatments and therapies for patients. Ideally, a good rodent model of T2DM should represent a similar metabolic profile and pathogenesis to humans with pre-diabetes or T2DM. However, few models fully capture all characteristics of the disease pathophysiology (ectopic fat deposition, insulin resistance and hyperglycemia), and sometimes the models used do not reflect what typically occurs in humans, perhaps because genetic models or chemical agents are used to induce hyperglycemia, rather than attempting to examine the established risk factors for diabetes (Herder et al., 2011). For example, the Zucker diabetic fatty (ZDF) rat, first established in the early 1980s (Clark et al., 1983), is one of the most commonly used models of T2DM because it develops severe insulin resistance, β-cell compensation then decompensation, and eventually overt hyperglycemia by ~8-10 weeks of age (Finegood et al., 2001). Although ZDF rats are leaner than the original Zucker fatty rat strain, the former express higher basal glucose and insulin levels and excessive ectopic fat deposition because of leptin receptor deficiency, which causes hyperphagia and altered substrate metabolism (Clark et al., 1983). One major limitation of the ZDF model is that it does not mimic the vast majority of humans who develop T2DM with an intact leptin receptor gene (Rolland et al., 1998), not to mention that it is a costly and genetically spontaneous model that is not easily manipulated. Indeed, non-genetic lifestyle-induced models of T2DM are scarce because most rodent species are relatively resistant to prolonged periods of high-fat feeding and/or physical inactivity and might not develop the hyperglycemic phenotype desired by the researcher (Islam and Loots, 2009).

Considerable evidence from human studies (Anagnostis et al., 2009; Rosmond, 2005) and various rodent models (Chan et al., 2003; Matthews and Hanley, 2011; Wang et al., 2011) suggest that elevations in glucocorticoids (GCs) are tightly linked with diabetes development, either at the circulating or cellular level. At the cellular level, the pre-receptor enzyme 11-β-hydroxysteroid dehydrogenase type 1 (11-βHSD-1) and the glucocorticoid receptor (GR) act to modulate intracellular GC action, which can dramatically influence tissue function (liver, muscle, adipose tissue and brain) to promote metabolic syndrome (Stewart, 1996). The activity of 11-βHSD-1 has also been closely followed in those with diabetes and is suspected to be involved in the pathogenesis of the disease (Bujalska et al., 2005). Interestingly, elevations in GCs alter food intake, energy expenditure and ectopic fat deposition, and promote widespread whole body insulin resistance (Peckett et al., 2011). In particular, elevation of GC levels in rodents (Dallman, 2010), or even increases in stress levels in humans (Tomiyama et al., 2011), promote the consumption of energy dense 'comfort' foods. These associations between GCs and altered metabolism suggest a possible role of

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these stress hormones in the etiology of T2DM development. Indeed, exogenous GCs are potent diabetogenic agents that, when prescribed for the treatment of many acute and/or chronic conditions, increase whole body insulin resistance and plasma glucose levels and cause muscle proteolysis, all features of T2DM development. In one study, a striking tenfold increased risk of diabetes development was calculated for patients receiving hydrocortisone at a dose of ~120 mg/day (Gurwitz et al., 1994).

Generally, it is believed that the transition from an insulin resistant or pre-diabetic state into overt T2DM occurs when the secretory capacity of the pancreatic β-cells is no longer able to maintain euglycemia to combat insulin resistance. It is plausible that the chronic upregulation of GCs could act directly on pancreatic islets, inducing insulin resistance (van Raalte et al., 2009). However, it is also possible that increased circulating free fatty acid levels and excessive ectopic lipid deposition, which clearly induce whole body insulin resistance, in addition to elevations in GCs themselves, are the combined driving forces behind disease development (Campbell et al., 2010; Eckel et al., 2011; Reed et al., 2000).

Surprisingly, very little research has been done on the collective effect of GCs and high-fat feeding, although each stressor on its own can result in insulin resistance (Aleixandre de Artinano and Miguel Castro, 2009; Karatsoreos et al., 2010). The purpose of this present study is to develop a cost-effective, GC- and high-fat-fed-induced rodent model of diabetes mellitus. We believe that this model of rapid-onset diabetes (ROD) mimics many of the symptoms observed in patients with T2DM (Kennedy et al., 2010), as well as provides evidence that elevated GCs induce diabetes advancement if susceptible individuals do not consume a balanced diet.

RESULTS

Corticosterone treatment results in attenuated body mass gains despite hyperphagia, but increases relative central adipose mass and muscle atrophy

At day 11 of treatment, all corticosterone-treated rats exhibited attenuation in body mass growth and less gross caloric intake compared with placebo-treated rats (Table 1). Caloric intake (expressed relative to whole animal body mass) was ~twofold higher in animals given corticosterone and a high-fat diet (cort-HFD), compared with all other treatment groups (Table 1). Cort-HFD rats had more than a twofold greater relative epididymal fat pad mass when compared with controls [placebo-treated rats fed a standard chow diet (placebo-SD); 9.81±1.65 versus 4.56±0.54 g/kg body mass, $P<0.05$, main effect of diet, $P<0.01$]. Epididymal fat pad mass was not significantly elevated in either cort-SD- or placebo-HFD-treated rats when compared with placebo-SD-treated rats (5.82±0.28 versus 5.27±0.57 g/kg, $P=0.06$). Mesenteric fat mass was also visibly more abundant in cort-HFD-treated rats than the other three groups (data not shown). Corticosterone treatment resulted in reduced relative adrenal mass and decreased thymus mass (data not shown), as expected. Cort-HFD rats, in particular, had increased liver and pancreatic mass (all $P<0.05$). Absolute muscle mass was lower in all corticosterone-treated animals (data not shown), although relative mass of the soleus and plantaris was unchanged. Cort-HFD rats had lower relative epitrochlearis muscle mass compared with the placebo-SD group (Table 1; $P<0.01$).

Cort-HFD results in hyperglycemia, hyperinsulinemia, insulin resistance and reduced insulin tolerance

Cort-HFD animals demonstrated dramatically elevated fasted blood glucose levels (i.e. fasting hyperglycemia) compared with all other groups (14.0±1.9 mmol/l in cort-HFD compared with 4.2±0.2 mmol/l in the placebo-SD group; $P<0.05$; Fig. 1A). Neither the cort-SD nor placebo-HFD groups demonstrated fasting hyperglycemia. Moreover, following an oral glucose gavage on day 13, only cort-HFD animals had sustained hyperglycemia throughout the 120 minutes of the glucose tolerance test ($P<0.05$; Fig. 1A). By contrast, the cort-SD group had mildly elevated blood glucose levels, compared with placebo-SD, but only at 30 and 60 minutes post oral glucose gavage ($P<0.05$; Fig. 1A). The placebo-HFD group failed to demonstrate elevated glucose levels at any time during the glucose tolerance test, as expected based on the short timeframe of treatment. Fasted insulin level in the cort-HFD group was

Table 1. Body composition and caloric intake of placebo- and corticosterone-treated rats

	Placebo-SD	Placebo-HFD	Cort-SD	Cort-HFD	Main effect of corticosterone	Main effect of diet	Interaction
Gross body mass (g)	358.9±5.6	350.8±4.5	265.9±7.4$^{\#}$	226.1±9.1$^{\#}$	$P<0.001$	$P<0.01$	$P<0.05$
Caloric intake (kcal/kg body mass × day)	0.28±0.01	0.42±0.01$^{\#}$	0.33±0.01$^{\#}$	0.61±0.02$^{\#}$	$P<0.001$	$P<0.001$	$P<0.001$
Left adrenal mass (g/kg body mass)	0.1±0.01	0.1±0.01	0.031±0.01	0.05±0.06	$P<0.001$	–	–
Epitrochlearis mass (g/kg body mass)	0.14±0.01	0.15±0.01	0.18±0.012	0.09±0.02$^{\#}$	–	$P<0.01$	$P<0.01$
Soleus mass (g/kg body mass)	0.42±0.01	0.40±0.02	0.42±0.02	0.49±0.06	–	–	–
Epididymal fat pad mass (g/kg body mass)	4.56±0.54	5.82±0.28	5.27±0.57	9.81±1.65$^{\#}$	$P<0.05$	$P<0.01$	$P=0.06$
Liver mass (g/kg body mass)	39.90±1.44	36.16±1.21	58.35±3.83†	68.62±3.20†	$P<0.001$	–	$P<0.05$
Pancreas mass (g/kg body mass)	2.48±0.13	2.61±0.08	3.92±0.46	3.40±0.52	$P<0.01$	–	–

†, significance from the respective placebo group ($P<0.05$); $^{\#}$, significance versus all other treatment groups ($P<0.05$). All values are means ± s.e.m.; $n=7$-10.

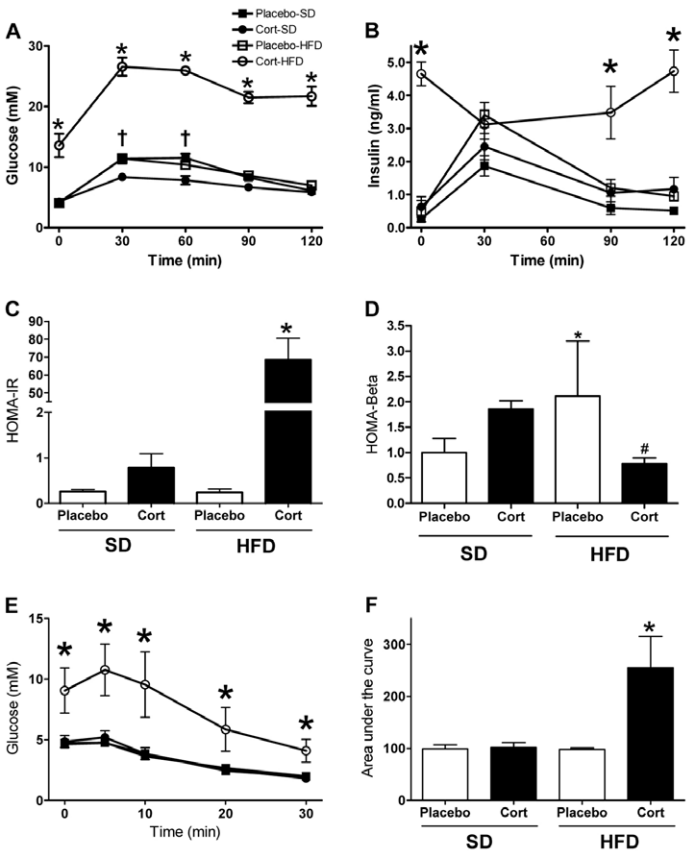

Fig. 1. Corticosterone treatment increases glucose intolerance and insulin sensitivity. (A) Glucose concentrations (mmol/l) were measured every 30 minutes post oral glucose load (1.5 g/kg). (B) Insulin concentrations (ng/ml) during the oral glucose tolerance test (OGTT). (C) HOMA-IR, as calculated by the formula: [insulin (μU/ml) × glucose (mmol/l)]/22.5. (D) HOMA-β, as calculated by the formula: [20 × insulin (μ units/l)]/[glucose (mmol/l) – 3.5]. This calculation indicates the functional response of the pancreatic β-cells to a bolus of glucose. A higher HOMA-β value suggests a greater response of the β-cells to glucose, indicating higher β-cell function. (E) Glucose concentrations during the insulin tolerance test. (F) Insulin resistance, as measured by the AUC for glucose levels following the insulin tolerance test. All values are means ± s.e.m., *n*=5-6. *, Statistical significance from all other treatment groups, *P*<0.05. #, significance from cort-SD, *P*<0.05, using a two-way ANOVA. †, cort-SD significantly different from placebo-SD, *P*<0.05.

elevated ~threefold compared with all other groups (*P*<0.05; Fig. 1B). No differences in fasting insulin concentrations were found among the cort-SD, placebo-SD and placebo-HFD groups.

The homeostasis model assessment for insulin resistance (HOMA-IR) was used to determine insulin resistance on the basis of fasted insulin and glucose levels. The cort-SD group had a twofold higher HOMA-IR than the placebo-SD group, whereas the cort-HFD group had a 15-fold increase compared with placebo-SD animals (*P*<0.05; Fig. 1C). The HOMA-β index, commonly used as an index of β-cell function, showed cort-SD animals to have an ~twofold increase in β-cell function relative to the placebo-SD group, whereas the cort-HFD group demonstrated reduced HOMA-β levels relative to all other groups (*P*<0.05 compared with the cort-SD group). Moreover, cort-HFD animals had a 20% decrease in HOMA-IR levels and no differences were found in placebo-HFD compared with placebo-SD animals (*P*<0.05; Fig. 1D).

An insulin tolerance test was also carried out in all treatment groups on day 15 to further characterize whole body insulin sensitivity. As expected, cort-HFD animals had higher plasma glucose levels prior to and after the insulin injection, compared with all other treatment groups (*P*<0.05; Fig. 1E). The cort-HFD group also demonstrated a 2.5-fold higher glucose area under the curve (AUC) following insulin injection, illustrating impaired insulin sensitivity (*P*<0.05; Fig. 1F). There were no differences found in glucose response to insulin injection among the other three groups.

Cort-HFD treatment impairs normal corticosterone circadian rhythm, as well as increasing fasting insulin, leptin and triglycerides levels

All treatment groups demonstrated a normal circadian rhythm of corticosterone secretion prior to pellet surgery, as measured by trough and peak corticosterone levels measured in the early evening (20:00 hours) and in the morning (08:00 hours) (data not shown). However, 7 days after pellet implantation, cort-SD- and cort-HFD-treated animals both had significantly elevated corticosterone levels at the 08:00 hour measurement only (*P*<0.05; Table 2). On day 10, the cort-HFD group demonstrated more than a 17-fold rise in fasting plasma insulin, a >fivefold elevation in plasma leptin levels and >threefold elevation in plasma triglyceride levels compared with placebo-SD animals (*P*<0.05; Table 2).

Corticosterone treatment causes islet hyperplasia and increases ectopic fat deposition, effects that were further exacerbated with high-fat feeding

To demonstrate altered islet morphology, pancreas sections were collected and stained with hemotoxylin and eosin. As shown, there were no observed differences between placebo-SD and placebo-HFD islet area (Fig. 2A). However, corticosterone treatment increased islet area, with the greatest increase observed in the cort-HFD group (Fig. 2A). In addition to elevations in visceral adiposity, Oil red O staining of liver and tibialis anterior muscle sections revealed increased ectopic lipid accumulation in both corticosterone-treated groups, effects that were exacerbated with the HFD (Fig. 2B,C). HFD feeding alone caused a much less pronounced effect on ectopic lipid deposition.

Cort-HFD significantly augments adipose tissue 11-βHSD-1 expression, whereas cort-SD treatment increases GC receptor expression

Cellular GC activity and the genomic effects of GCs within tissues are largely determined by the presence of active and inactive hormone in the circulation as well as the cellular expression of the pre-receptor enzyme 11-βHSD-1 and GR. As previously mentioned, 11-βHSD-1 acts in various target tissues to convert inactive GCs (11-dehydrocorticosterone) into their active form (corticosterone) (Stewart, 1996). To determine the effects of corticosterone and HFD treatment on cellular mediators of GC action, we performed real-time PCR (RT-PCR) to analyze mRNA expression of *GR* and 11-βHSD-1 in visceral (epididymal fat pads) and subcutaneous adipose tissue because we hypothesized that corticosterone treatment would increase the expression of 11-βHSD-1 in visceral tissue but also exacerbate its effects with high-fat feeding. In epididymal fat depots,

Table 2. Plasma composition of corticosterone, fasted insulin, leptin, triglycerides and non-esterified fatty acids in placebo-SD, cort-SD, placebo-HFD and cort-HFD animal groups

	Placebo-SD	Placebo-HFD	Cort-SD	Cort-HFD	Main effect of corticosterone	Main effect of diet	Interaction
Peak corticosterone (ng/ml)	270.3±51.3	303.2±140.7	295.2±74.4	585.6±126.9	-	-	-
Trough corticosterone (ng/ml)	45.6±12.8	19.8±3.8	310.0±60.3#	595.9±122.0#	$P<0.001$	-	$P<0.05$
Insulin (ng/ml)	0.27±0.05	0.50±0.32	0.63±0.29	4.65±0.36*	$P<0.001$	$P<0.001$	$P<0.001$
Leptin (ng/ml)	1.20±0.19	3.08±0.55	4.59±0.72	6.23±0.42	$P<0.001$	$P<0.001$	-
Triglycerides (mM)	0.13±0.01	0.19±0.02	0.24±0.04	0.40±0.13	$P<0.05$	$P<0.05$	-
NEFAs (mM)	0.92±0.01	0.79±0.16	0.62±0.05	0.62±0.06	-	-	-

NEFAs, non-esterified fatty acids; *, statistical significance from placebo group ($P<0.05$); #, significance versus all other treatment groups ($P<0.05$). All values are ± s.e.m.; n=5-6 per group.

corticosterone treatment significantly increased *11-βHSD-1* mRNA expression, regardless of diet type. By contrast, GR expression was only elevated in cort-SD compared with placebo-SD animals ($P<0.05$; Fig. 3A,B). In subcutaneous adipose depots, *11-βHSD-1* mRNA expression was elevated twofold in both corticosterone-treated groups; however, this increase was greatly augmented by the HFD ($P<0.05$; Fig. 3C). Compared with the placebo-SD group, *GR* mRNA expression tended to be elevated in the cort-SD group and was significantly reduced in the placebo-HFD group ($P<0.05$; Fig. 3D).

DISCUSSION

T2DM is a complex disease and the mechanisms for its development are probably varied. However, it is generally held that whole body insulin resistance is related to increased ectopic lipid accumulation in insulin-sensitive tissues (Unger et al., 2010) and that β-cell dysfunction occurs via lipid and glucose toxicity of the islets (Giacca et al., 2011; Poitout et al., 2010). One possible mediator of T2DM development is an elevation in circulating and/or tissue levels of GCs (Reynolds, 2010; Rose et al., 2010). Indeed, exogenous GC treatment dramatically increases the odds ratio of developing T2DM in humans (Lansang and Hustak, 2011). The goal of the present study was to develop a new non-genetic model of GC-induced diabetes that is cost effective and rapidly inducible. The main finding of this study is that the simple combination of exogenous GCs with a HFD rapidly induces excessive ectopic fat deposition, hyperinsulinemia and/or insulin resistance, and severe hyperglycemia in young Sprague-Dawley rats, effectively mimicking GC-induced diabetes mellitus.

Strengths and limitations of the corticosterone- and HFD-induced diabetic model

Rodent models of metabolic syndrome or diabetes should largely reflect the biochemical profile and pathogenesis that occurs clinically in humans with pre-diabetes or T2DM. The rodent model presented here, what we term as the 'rapid onset of diabetes' (ROD) model, clearly exaggerates the fundamental physiological parameters that are typically observed in individuals with T2DM who might have chronically elevated GC levels and

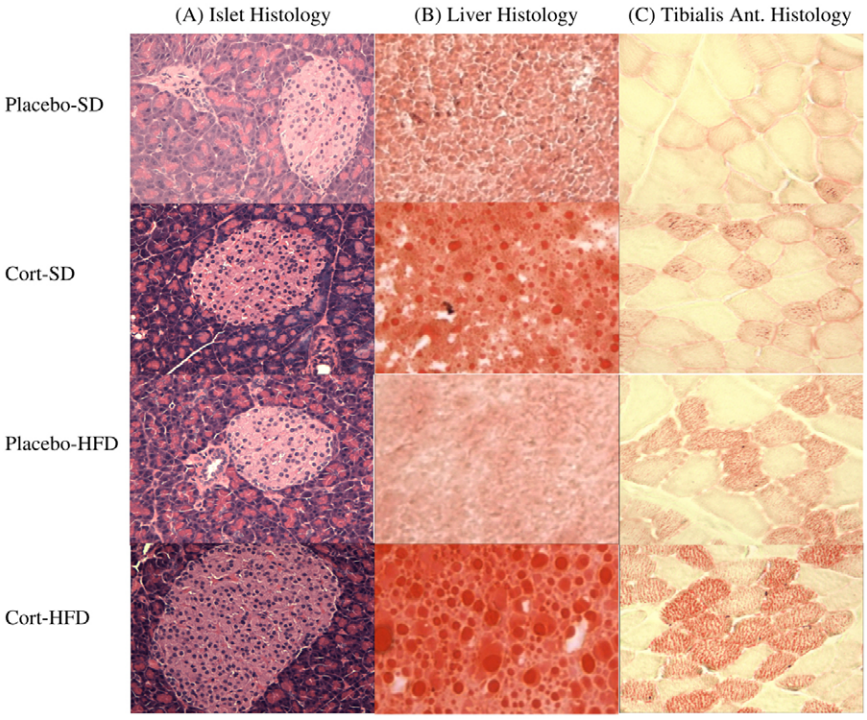

Fig. 2. Histochemistry staining analysis. (A) Pancreatic islet hemotoxylin and eosin staining, indicating pancreas islet areas. (B) Liver Oil Red O staining, indicating liver total lipid content. (C) Tibialis anterior Oil Red O staining, indicating muscle total lipid content in placebo-SD, cort-SD, placebo-HFD and cort-HFD animals (20× magnifications).

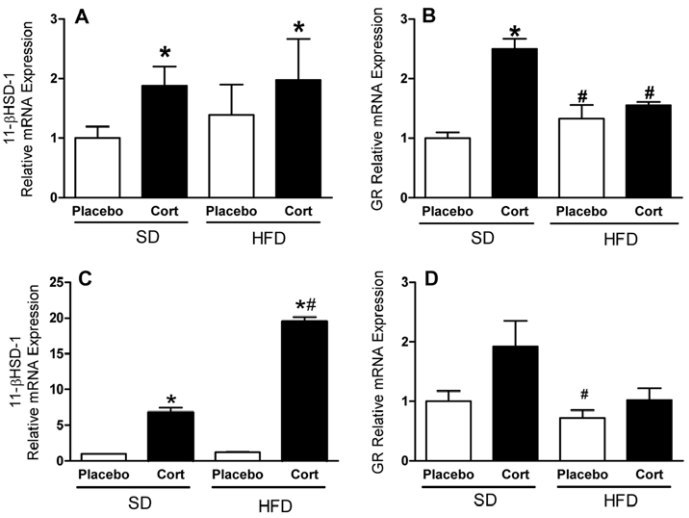

Fig. 3. Tissue determination of corticosterone action in adipose tissue. (A) Relative *11-βHSD-1* and (B) *GR* mRNA expression in epididymal fat deposits. (C) *11-βHSD-1* and (D) *GR* mRNA expression in subcutaneous fat deposits in placebo-SD, cort-SD, placebo-HFD and cort-HFD rats. All protein was standardized to β-actin expression. *n*=3-5. *, *P*<0.05 from placebo-SD; #, significance from cort-SD. All values are ± s.e.m.

who may consume an energy dense 'westernized' HFD. Here, we clearly demonstrate that the combination of elevated GCs and a HFD in otherwise healthy Sprague-Dawley rats induces a diabetic phenotype including glucose intolerance, insulin resistance, hyperglycemia and hyperinsulinemia as early as 1 week postintervention and produces a blood biochemical profile that is strikingly similar to T2DM individuals. Interestingly, HOMA-IR and HOMA-β scores indicate extremely elevated levels of insulin resistance and β-cell dysfunction in this model, despite major increases in β-cell compensation, as measured by increased islet areas in the cort-HFD group. It is important to note that neither corticosterone alone nor HFD alone promoted hyperinsulinemia or hyperglycemia in this short timeframe of study, thereby suggesting that these two treatments (i.e. GCs and HFD) have a synergistic effect on diabetes development. Moreover, cort-HFD animals were found to have increased levels of hepatic and muscle fat accumulation (Fig. 2B,C), which was not observed when corticosterone or a HFD was tested alone. Importantly, prolonged corticosterone treatment alone, in similarly aged Sprague-Dawley rats over a period of 6-8 weeks, failed to promote any observable increases in fasting glucose or impaired glucose tolerance (our unpublished observations). Taken together, the exposure to elevated GCs, in combination with a HFD, rapidly induces an obvious T2DM phenotype in young Sprague-Dawley rats, which are otherwise very resistant to the development of hyperglycemia.

One limitation of this model is that the procedure does require some surgical expertise, general anesthesia and possibly an increased risk for infection because of immune system suppression. An alternative to this procedure would be to provide these animals corticosterone in their drinking water, as has been previously established in a murine model of metabolic syndrome (Karatsoreos et al., 2010). However, the surgical implantation of corticosterone provides a more steady state drug exposure that more effectively abolishes the diurnal GC rhythm, compared with providing it in the drinking water, because animal water consumption tends to be primarily in the early evening (Karatsoreos et al., 2010).

We acknowledge that our model does affect the immune system and the endogenous hypothalamic-pituitary-adrenal (HPA) axis, because ROD animals display decreases in thymus and adrenal weights. However, these results have been shown in various other models of corticosterone administration, i.e. spiking rodent drinking water with high amounts of corticosterone (Karatsoreos et al., 2010). We also realize that immune suppression is not characteristic of the diabetic phenotype (type 1 or type 2), nor is the endogenous HPA axis downregulated, unless diabetic individuals are on exogenous GCs.

The physiological relevance of the amount of corticosterone that was administered to the animals in our study also warrants discussion. Because the corticosteroid-treated animals in this study received ~20 mg/day/kg body weight (~6.25 mg/day in rats that weighed ~0.3 kg), we estimate that our treatment might be fivefold higher in corticosterone dose (assuming that there was no loss in drug potency with the procedure used to manufacture the pellets) than in humans treated with exogenous GCs that are known to increase the risk of diabetes development (Panthakalam et al., 2004). It was decided that, for the purpose of this study, we wished to treat the animals with the 'most tolerable dose' of corticosterone as a proof of principle to rapidly induce hyperglycemia and hyperinsulinemia.

Prevalence of GC-induced diabetes in humans

The prevalence of GC-induced diabetes in clinical populations, or in individuals exposed to chronic stress, is not clear. According to Lansang and Hustak (Lansang and Hustak, 2011), GC-induced hyperglycemia might be quite common. In humans with rheumatoid arthritis who are receiving corticosteroid therapy, ~10% develop diabetes in the first 2 years of drug commencement (Panthakalam et al., 2004). In a case control report, the incidence of initiating an oral hypoglycemic agent or exogenous insulin (i.e. indicative of diabetes development) increased dramatically with GC treatment and was positively correlated with the amount of drug that the patients were receiving. More specifically, a striking tenfold increased risk (i.e. 10.34 odds ratio compared with no treatment) was calculated for patients receiving a hydrocortisone-equivalent dose of ≥120 mg/day (Gurwitz et al., 1994). Moreover, studies of a single intravenous dexamethasone injection of just 10 mg, which is equivalent to ~260 mg of hydrocortisone (Lansang and Hustak, 2011), causes sustained hyperglycemia within ~4 hours in healthy humans (Hans et al., 2006; Pasternak et al., 2004). Taken together, it is clear that exogenous GCs in modest to high doses promote hyperglycemia in otherwise healthy humans.

Effects of corticosterone treatment on food intake, body mass and body composition

Elevations in GC levels have widely been cited as promoting hyperphagia, particularly the increased consumption of energy dense 'comfort' foods (Dallman, 2003; Tomiyama et al., 2011). Despite increased food intake, at least expressed relative to body mass, the corticosterone-treated animals in this study clearly

demonstrate significantly lower body weight (Table 1). Although the mechanism for attenuated growth is unclear, GCs are well known to reduce overall body mass and body length, probably by promoting muscle proteolysis and by lowering the growth hormone (GH)–IGF-1 axis in young growing mammals (Peckett et al., 2011). Indeed, Dong et al. demonstrated that attenuation in body weight gain with elevations in GCs is associated with a reduction in overall protein synthesis but not with reduced energy intake (Dong et al., 2007). More specifically, GCs are thought to reduce skeletal muscle protein synthesis and elevate proteolysis specifically in muscles containing a high proportion of fast twitch type II fibers (Falduto et al., 1990). In support of this, the cort-HFD-treated animals in this study showed a significant reduction in epitrochlearis (primarily composed of type II fibers) but not in soleus (primarily type I) muscle mass, suggesting that this treatment attenuates growth primarily in glycolytic muscle fibers. The observed muscle atrophy coincides with evidence from individuals with T2DM who experience reduced muscle strength and atrophy particularly when glycemic control is poor (Andersen et al., 2004; Huang et al., 2010; LeBrasseur et al., 2011).

As expected, we found that body mass gain was dramatically attenuated with corticosterone treatment but, surprisingly, a HFD did not increase body mass in corticosterone-treated animals. It is well established that the development of T2DM from a state of pre-diabetes is often associated with reductions in body mass, perhaps because a large number of calories are lost to glycosuria. In contrast to our observations that increased corticosterone exposure attenuates body mass gains in rodents, Karatsoreos et al. showed that C57/BL6 mice exposed to high amounts of corticosterone (100 μg/ml) in their drinking water for 4 weeks had elevated body mass gains, despite initial reductions in mass, compared with placebo treatment (Karatsoreos et al., 2010). However, C57/BL6 mice are genetically engineered to rapidly develop insulin resistance when they are metabolically challenged (Coleman and Hummel, 1973; Coleman, 1978) and the study by Karatsoreos et al. (Karatsoreos et al., 2010) used mature mice that were well past their adolescent growth phase when the GC-induced inhibition of the GH–IGF-1 axis would have less influence on body mass gains. It is also crucial to note that, although the C57/BL6 mice that were provided corticosterone in their drinking water did develop insulin resistance, they did not develop hyperglycemia (i.e. T2DM). Thus, the body mass gain in that study might have been more dramatic than what was observed in our study because the cort-HFD animals would have had substantial caloric loss due to glycosuria.

One of the hallmarks of the development of metabolic syndrome and T2DM is the elevation in ectopic lipid accumulation in non-subcutaneous adipose stores (increased central adiposity, muscle and liver lipid accumulation). In this new ROD model, we observed dramatic increases in ectopic lipid deposition with corticosterone treatment, particularly in those animals fed a HFD (Table 1). Corticosterone is known to stimulate an increase in fat accumulation within central adipose stores because of an increased number of adipocytes as well as via adipose cell hypertrophy (Gounarides et al., 2008; Peckett et al., 2011). Increased visceral adiposity is characterized as an important factor in the development of T2DM in human subjects (Bays et al., 2008; Bray et al., 2008). Similarly, cort-HFD-treated rats did have increases in visceral adiposity, which might have played a role in the progression of their insulin resistance. Indeed, intracellular lipid accumulations in endocrine pancreas, liver and skeletal muscle cells have all been previously shown to contribute to the pathogenesis of impaired insulin secretion and insulin resistance (Dong et al., 2007; Unger, 2003). In support of the notion that ectopic fat accumulation promoted the diabetes phenotype, ROD animals exhibited significant increases in ectopic fat deposition in the liver and muscle compared with all other animals (Fig. 2), which coincided with their deteriorated insulin sensitivity. Numerous studies have demonstrated the link between ectopic fat deposition and insulin resistance in persons with overt T2DM (Lara-Castro and Garvey, 2008; van der Zijl et al., 2011). Future studies are needed, however, to determine whether the increase in lipid end products associated with increased ectopic fat deposition is a driving force behind the development of T2DM in this rodent model, as has been proposed in humans with excessive liver and muscle lipid deposition (Samuel et al., 2010).

Glucose tolerance and insulin sensitivity

Compared with all other treatment groups, cort-HFD rats demonstrated deteriorating glycemic control and severe insulin resistance, as measured by both oral glucose and insulin tolerance tests (Fig. 1). Cort-HFD animals exhibited a threefold elevation in plasma glucose concentration and a 17-fold increase in insulin level compared with placebo-SD animals. Moreover, following oral gavage of glucose, cort-HFD animals had sustained elevations in glucose and insulin concentrations, thereby demonstrating similar characteristics to individuals with T2DM (Luijf et al., 2011; Pour and Dagogo-Jack, 2011). Measurement of HOMA-IR revealed almost a 70-fold increase in whole body insulin resistance in cort-HFD rats compared with the placebo-SD and placebo-HFD groups. Moreover, higher HOMA-β index values in cort-SD animals indicate a higher pancreatic β-cell function compared with placebo-treated animals. Importantly, however, the HOMA-β value was significantly lower in cort-HFD-treated animals compared with cort-SD animals despite the finding that cort-HFD animals had larger islet areas than all other groups (Fig. 1). These results suggest that the combination of elevated corticosterone with a HFD might initially increase β-cell compensation, possibly as a result of elevated insulin resistance, but that these β-cells have insufficient capacity to secrete enough insulin to normalize glycemia in response to oral glucose stimulation. In other words, although cort-HFD animals have increased islet areas and elevated insulin concentrations (in both the basal and stimulated states) compared with all other groups, these animals remain extremely glucose intolerant because of insufficient insulin signaling within various insulin target tissues.

Intracellular GC activation and other plasma markers of insulin resistance or T2DM

Our ROD model exhibits hyperglucocorticoidemia, exaggerated GC tissue reactivation and increased diabetes-related blood markers, all of which are present in humans and rodents with overt T2DM. Indeed, our cort-HFD animals show elevations in plasma GC levels beyond those observed with corticosterone treatment alone (Table 2), thereby suggesting that a HFD might exaggerate hyperglucocorticoidemia. Importantly, hyperglucocorticoidemia is frequently observed in both humans and rodent models of

diabetes (Campbell et al., 2010; Chan et al., 2003; Kawano et al., 1992; Roy et al., 1990). It has been previously proposed that heightened levels of plasma GCs in animals exhibiting metabolic syndrome might be a result of increased 11-βHSD-1 activity in liver, muscle and adipose tissue (Gathercole and Stewart, 2010). From our data, we can conclude that our corticosterone-treated animals have an increase in 11-βHSD-1 activity within adipose tissue (Fig. 3). This finding is more exaggerated in corticosterone-treated animals given a HFD, which might help to explain the higher circulating levels of corticosterone.

Finally, it is important to note that the ROD model has other metabolic similarities to diabetes development, including hyperleptinemia and hyperlipidemia. Leptin is secreted by the adipocytes in response to increased adiposity and is thought to play a role in the development of peripheral insulin resistance and perhaps in the progression of T2DM, although the role of this adipokine in disease development is controversial (Ceddia, 2005). We confirm here that elevation in plasma leptin levels occurs in corticosterone-treated animals and that these increases are dramatically enhanced by a HFD (Table 2). Moreover, ROD animals experience hypertriglyceridemia similar to rodent models of and individuals with T2DM (Clark et al., 1983; Kumar et al., 2010), perhaps due to increased fat intake and/or dramatic increases in insulin resistance caused by corticosterone and high-fat feeding. Further research is required, however, to establish the mechanisms of hyperglycemia development in this model (e.g. defective hepatic and muscle insulin signaling, glucose turnover and/or β-cell function).

Conclusion

In conclusion, the present study demonstrates a new cost effective rapidly inducible rodent model of GC-induced T2DM, which has a similar anthropometric, biochemical and pathogenic profile as patients with severe insulin resistance or metabolic disease. This ROD model can be considered a new important tool for determining the complex multi-organ and multi-tissue connections between elevated GC levels and the development of metabolic syndrome and T2DM.

METHODS

Ethics statement

All experiments were approved by the York University Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines.

Animals

Forty young (~age 7-8 weeks; initial weight 200-250 g) male Sprague-Dawley rats were purchased from Charles River Laboratories (Montreal, QC, Canada). Only male rats were selected for this study because female rats have differences in reproductive hormones throughout their ovulatory cycle that could affect glucose metabolism (Gustavsson et al., 2011). All animals had a 7 day habituation period to a 12-hour light-dark cycle (lights on at 08:00 hours and lights off at 20:00 hours) in a temperature (22-23°C)- and humidity (50-60%)-controlled room. The animals (body mass 200-250 g) were then randomly assigned to one of four groups: placebo-treated and fed a rodent standard diet (placebo-SD); placebo-treated and fed a high-fat diet (placebo-HFD); corticosterone-treated and fed a standard diet (cort-SD) and corticosterone-treated and fed a high-fat diet (cort-HFD) (n=8-10 per group; all individually housed). A timeline of the treatment protocol is presented in Fig. 4.

Surgical procedures and design

Following habituation, all placebo-treated animals had 4×100 mg wax pellets (~0.2 cm in diameter each) implanted subcutaneously between their scapulae under aseptic conditions (termed day 0), whereas glucocorticoid-treated animals had 4×100 mg of corticosterone (C2505, Sigma-Aldrich, Oakville, Ontario, Canada) pellets surgically implanted in an identical surgical procedure (see below). Pilot data from our laboratory found that a 200 mg corticosterone implant failed to significantly increase peak corticosterone levels over a 2 week period (see supplementary material Fig. S1). In addition, these rats did not develop fasting hyperglycemia when given with a HFD. Pellets were created by similar protocols to those published by Meyer et al. (Meyer et al., 1979). Briefly, this involves the subcutaneous implantation of solid corticosterone pellets formed from the molten hormone and weighing ~100 mg. Purified corticosterone was carefully melted in a small stainless steel spoon over a low gas flame and then poured into a 6 mm diameter hole drilled in a paraffin block. After the pellet cooled and solidified, it was removed from the wax with the aid of a scalpel and trimmed to the correct weight. Rats were anesthetized at 5% isoflurane and maintained at 2.5% isoflurane for the remainder of the surgery while steroid pellets were implanted in the nape of the neck. After the skin in the area had been shaved and treated with an ethanol-alcohol solution (Betadine, Purdue Products, Stamford, CT), a small incision was made, and the underlying fascia was spread and separated with

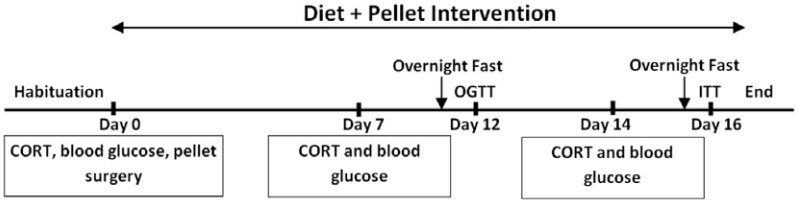

Fig. 4. Treatment protocol timeline. Animals were given 7 days of habituation before pellet surgeries were performed (day 0). Also on this same day, plasma corticosterone levels as well as blood glucose levels were sampled (i.e. basal measurements). On day 7, corticosterone and blood glucose measurements were repeated and animals were fasted overnight on the night of day 11. Animals were then given an oral glucose tolerance test (OGTT) on day 12 and were subsequently allowed a 2-day wash-out period before another plasma corticosterone and blood glucose sample was taken (evening of day 14). Animals were then fasted on the night of day 15 and finally subjected to an insulin tolerance test (ITT) on day 16.

a hemostat and inserted into the opening. The pellet was placed under the skin at least 2 cm caudal to the incision, which was then closed with 9-mm wound clips (VWR, Mississauga, ON, Canada). Rats then recovered in individually housed sterilized, standard rodent cages. Following recovery from surgery, SD-fed animals were given rodent SD (14% fat, 54% carbohydrate, 32% protein and 3.02 calories/g) ad libitum, whereas HFD-fed animals were provided with a 60% HFD (60% fat, 21% carbohydrate, 18% protein and 5.1 calories/g) ad libitum (Cat# TD. 06414, Harlan Laboratories, Madison, WI). The composition of the fat in the HFD was as follows: saturated: 37%; monounsaturated: 47%; polyunsaturated: 16%. Food intake and body weight were measured every other day.

Plasma analyses

Whole blood was collected via the tail-nick procedure and placed in EDTA microvette tubes (~50 µl) at 20:00 hours on day 6 and 14 and at 08:00 hours on day 7 and 15 to determine 'peak' and 'trough' corticosterone levels, as previously described (Campbell et al., 2010), and insulin and leptin levels. Immediately following blood collection, plasma samples were separated from whole blood by centrifugation (90 seconds at 13,000 ***g***) and frozen at −20°C until further analysis. Commercially available kits were used to measure insulin (INSKR020, Crystal Chem, Downers Grove, IL), corticosterone (07-120102, MP Biomedicals, Solon, OH), non-esterified free fatty acids (HR Series NEFA-HR, Wako Chemicals, Richmond, VA), triglycerides (TR0100, Sigma-Aldrich, Oakville, ON, Canada) and leptin (RADPK-81K, Milliplex rat adipokine kit, Millipore, Etobicoke, ON, Canada) at select time points (see below) from blood collected via tail-nick or from trunk blood collected at the end of the experimental period (i.e. decapitation).

Oral glucose tolerance test

On day 12, following an overnight fast, body mass was determined and blood glucose level assessed via tail-nick and a hand-held glucometer (Contour blood glucose meter, Bayer, Toronto, ON, Canada). In addition, ~40 µl of plasma was collected via tail-nick for basal insulin measurements (see above). Following this, an oral gavage of 50% glucose (1.5 g/kg body weight) was performed on each animal (time 0), followed by blood collection for insulin and glucose every 30 minutes. At 120 minutes after oral glucose gavage, animals were returned to their regular housing conditions and respective diets. HOMA-IR values were calculated according to the formula: [insulin (µ units/ml) × glucose (mmol/l)]/22.5. HOMA-β values were calculated according to the formula: [insulin (µ units/ml) × 20]/[glucose (mmol/l) − 3.5]. Values represent fasting glucose and insulin levels (Matthews et al., 1985).

Intraperitoneal insulin tolerance test

On day 15, all animals were fasted overnight with body weights and blood glucose recorded, as stated above. The following morning (day 16), the animals were injected intraperitoneally (IP) with 0.75 units insulin/kg body weight (time 0). Blood glucose levels were measured at 5, 10, 20 and 30 minutes post IP injection. At the end of the 30 minutes, animals were killed via decapitation and trunk blood was collected and separated into plasma as described above. Gastrocnemius, soleus, plantaris and epitrochlearis muscles, left and right adrenals, inguinal subcutaneous and epididymal adipose depots, and liver were removed, weighed and flash frozen in liquid nitrogen. Insulin resistance was measured as the AUC for the glucose concentrations during the insulin tolerance test. All tissues were stored in −80°C until further analysis.

Tissue processing

Pancreas was excised from animals, weighed and cut into ten randomized pieces. Each piece was washed in saline and then placed into 10% buffered formalin for 48 hours. After the incubation period, pancreas pieces were placed into 70% ethanol and prepared for paraffin embedded sectioning. Pancreas pieces were sectioned into 5-µm thick sections and mounted onto microscope slides, stained with eosin and counterstained with hemotoxylin. Representative islets from each animal treatment group were identified at 20× magnification and analyzed for islet areas using the slide scanner software (Aperio Scanscope CS, Vista, CA, scanned at the Advanced Optical Microscopy Facility, University Health Network, Princess Margaret Hospital, Toronto, ON, Canada).

Frozen liver and muscle tissue from each animal were snap frozen, cryosectioned (10-µm thick) and stained with Oil Red O for neutral lipid content as previously described (Koopman et al., 2001). Briefly, muscle and liver sections were fixed in 3.7% formaldehyde at room temperature. During this time, Oil Red O solution consisting of 0.5 g Oil Red O powder (Sigma-Aldrich, Canada) and 100 ml of 60% triethyl phosphate (Sigma-Aldrich, Canada) was combined and filtered. Following fixation in paraformaldehyde, slides were then immersed in filtered Oil Red O solution for 30 minutes. Immediately after, slides underwent three washes with ddH_2O, were allowed to air dry for 10 minutes and were then sealed with Permount (Sigma-Aldrich, Canada). Images were acquired at 10× magnification using a Nikon Eclipse 90i microscope (Nikon, Canada) and Q-Imaging MicroPublisher 3.3 RTV camera with Q-capture software.

mRNA expression

Epididymal and subcutaneous fat pad total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) and Eppendorf spin columns were used to concentrate RNA. Total RNA concentrations were quantified by ultraviolet spectrophotometry at 260 nm, and RNA purity was verified by the 260- to 280-nm ratio (Nanodrop ND-1000; Fisher Scientific, Wilmington, DE). Total RNA was diluted to 1 µg/µl using RNA-free water and the first-strand of cDNA was synthesized with DNase I (Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H-RT (Invitrogen). Duplicate samples of 5 µl of mRNA were quantified using a Bio-Rad MyIQ single color real-time PCR detection system using SYBR green (Bio-Rad, Mississauga, ON, Canada). Each reaction contained 12.5 µl SYBR green mix, 1 µl of each forward and reverse primer (5 µM), and 5.5 µl RNase-free H_2O. The PCR protocol conditions consisted of an initial denaturation for 3 minutes at 95°C, followed by 30 cycles at 95°C for 15 seconds, annealing at 60°C for 45 seconds and extending at 72°C for 30 seconds. Analysis was conducted using the 2^^ method. All samples were normalized to the expression level of the housekeeping gene *β-actin*, which did not significantly change among experimental treatments. Primer sets were as follows: 11-βHSD-1 Forward: 5′-GGGAGGCCATGTGGTATTGAC-3′, Reverse: 5′-CGAGTTCA-

TRANSLATIONAL IMPACT

Clinical issue

The increasing occurrence of individuals developing metabolic syndrome and type 2 diabetes mellitus (T2DM) has become a major worldwide health concern. This disease is associated with excessive body adiposity caused by over-nutrition, physical inactivity and perhaps increased levels of psychosocial stressors. T2DM is also associated with increased levels of stress hormones such as glucocorticoids (GCs), both at the circulating and cellular level. In addition, patients receiving GCs as a treatment for various conditions have an increased risk of developing T2DM. However, it is unclear whether and how GCs contribute to T2DM development.

Results

This study reports a simple, cost-effective rodent model of rapid onset of diabetes (ROD), involving GC treatment. The authors show that ROD is induced in normal 7- to 8-week-old Sprague-Dawley rats when fed a high-fat diet (HFD) and treated with corticosterone, a naturally occurring GC, for 16 days. Although treated animals have a significantly lower body weight and muscle mass compared with animals receiving either HFD or GCs alone, their relative food intake is increased, and they show severe ectopic fat deposition (central adiposity, intramyocellular lipid deposition and hepatic steatosis). Moreover, animals receiving both GCs and a HFD exhibit severe hyperglycemia, hyperinsulinemia, insulin resistance and an impaired β-cell response to oral glucose challenge compared with all other treatment groups.

Implications and future directions

This rodent model of ROD involving GC treatment is a new and cost-effective way to recapitulate many of the fundamental phenotypic and biochemical characteristics observed in individuals with T2DM. This model also provides evidence that increased levels of GCs can promote ROD when combined with a HFD. Future analyses are needed to determine the mechanisms by which the combination of GCs and HFD promote the disease. Studying the capacity of various therapeutic approaches (e.g. pharmacologic, dietary strategies, exercise) to improve the metabolic phenotype observed in this model should help to better understand which potential therapeutic approaches will be promising in humans with GC-associated T2DM.

AGGCAGCGACAC-3′. Glucocorticoid Receptor (GR) Forward: 5′-CCAAAACTCACTCGGATGCA-3′, Reverse: 5′-AGGTGCTTTGGTCTGTGGGATA-3′. Beta Actin Forward: 5′-TGTGGCATCCATGAAACTAC-3′, Reverse: 5′-CTCAGGAGGAGCAATGATCT-3′.

Statistical analyses

For all experiments, the appropriate one-or two-way ANOVA was performed as required to identify significant differences between treatment groups using Statistica 6.0 software (StatSoft, Tulsa, OK), with significance found when $P<0.05$. If significant differences were found by a one- or two-way ANOVA, then the Tukey's honestly significant difference post-hoc test was performed. Data are presented as mean ± s.e.m.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

Y.S., J.L.B., A.D., J.E.C. and M.C.R. conceived and designed the experiments. Y.S., J.E.C., J.L.B. and A.D. performed the experiments. Y.S., J.L.B., A.D. and A.P. analyzed the data. Y.S. and J.L.B. wrote the paper, and M.C.R. edited the paper.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.008912/-/DC1

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