Enhanced leptin sensitivity and improved glucose homeostasis in mice lacking suppressor of cytokine signaling-3 in POMC-expressing cells

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Summary

Suppressor of cytokine signaling-3 (Socs-3) negatively regulates the action of various cytokines, as well as the metabolic hormones leptin and insulin. Mice with haploinsufficiency of Socs-3, or those with neuronal deletion of Socs-3, are lean and more leptin and insulin sensitive. To examine the role of Socs-3 within specific neurons critical to energy balance, we created mice with selective deletion of Socs-3 within pro-opiomelanocortin (POMC)-expressing cells. These mice had enhanced leptin sensitivity, measured by weight loss and food intake after leptin infusion. On chow diet, glucose homeostasis was improved despite normal weight gain. On a high-fat diet, the rate of weight gain was reduced, due to increased energy expenditure rather than decreased food intake; glucose homeostasis and insulin sensitivity were substantially improved. These studies demonstrate that Socs-3 within POMC neurons regulates leptin sensitivity and glucose homeostasis, and plays a key role in linking high-fat diet to disordered metabolism.

Introduction

Leptin is an adipocyte-derived hormone whose actions are required for normal energy homeostasis (Flier, 2004; Friedman, 2000; Zhang et al., 1994). This is best illustrated by loss of function mutations in genes encoding leptin or the leptin receptor, which result in severe obesity in both rodents and humans (Chen et al., 1996; Chua et al., 1996; Clement et al., 1998; Farooqi et al., 2002; Montague et al., 1997; Zhang et al., 1994). In common forms of human obesity and acquired obesity in rodents secondary to ingestion of high-fat diets, leptin levels are increased and the response to exogenous leptin is reduced, reflecting a leptin resistant state in the absence of identified mutations (Considine et al., 1996; Frederich et al., 1995; Heymsfield et al., 1999). The mechanism for such leptin resistance has been explored in mice with high-fat diet-induced obesity (DIO), and reduction in the ability of exogenous leptin to activate leptin signaling in the hypothalamus after such diets has been observed (El-Haschimi et al., 2000). Two key questions in the field include identification of the molecular mechanism for the defect in leptin signaling, and the precise sites where this altered signaling occurs.

Leptin exerts its effects on body weight primarily through activation of the long form of the leptin receptor, and subsequent activation of the JAK-STAT3 pathway in specific hypothalamic neurons (Elmquist et al., 1997; Myers, 2004; Vaisse et al., 1996). Previous studies in our laboratory identified Socs-3 as a potential mediator of central leptin resistance (Bjorbaek et al., 1998) and subsequent experiments with Socs-3 haploinsufficient mice confirmed this, as these mice displayed attenuated diet-induced obesity and an improvement in both leptin and insulin sensitivity (Howard et al., 2004). Further, studies using mice lacking Socs-3 selectively in neurons showed that diet-induced obesity, as well as leptin and insulin sensitivity are importantly influenced by the level of neuronal Socs-3 expression (Mori et al., 2004). Although these studies clearly demonstrate that Socs-3 in general and neuronal Socs-3 in particular plays a major role in the capacity to develop leptin resistance, the identity of specific neurons that contribute to Socs-3 mediated leptin resistance remains unknown.

Leptin receptors are widely expressed in hypothalamic and extra-hypothalamic sites (Cheung et al., 1997; Elmquist et al., 1998; Fei et al., 1997; Mercer et al., 1996; Schwartz et al., 1996). However, the arcuate nucleus of the hypothalamus is a key target of leptin. A recent study demonstrated that in DIO, leptin resistance as assessed by reduced activation of STAT3 phosphorylation in response to leptin is disproportionately evident in the arcuate nucleus (Munzberg et al., 2004). Further, this study demonstrated that the inhibitor of leptin signaling, suppressor of cytokine signaling-3 (Socs-3) was induced specifically in this area (Munzberg et al., 2004). Within the arcuate nucleus, leptin directly inhibits orexigenic NPY and AgRP expressing neurons and simultaneously activates anorexigenic POMC containing neurons (Cowley et al., 2001; Elias et al., 1999; Mizuno et al., 1998; Mizuno and Mobbs, 1999; Schwartz et al., 1997; van den Top et al., 2004). Recently, a study by Balthasar et al. (Balthasar et al., 2004) demonstrated that deletion of leptin receptors specifically from POMC-expressing cells resulted in hyperleptinemia and a modest increase in fat mass, reflecting the specific role of leptin action on these neurons. In addition, in a leptin receptor null background, re-expression of leptin receptors in the arcuate nucleus resulted in a modest decrease in body weight,

and an even more striking improvement in glucose homeostasis (Coppari et al., 2005).

Taken together, these observations support an important role for leptin signaling in the arcuate nucleus, and POMC neurons in particular, in energy homeostasis and metabolic regulation. Given this fact, and the evidence that Socs-3 is a molecular mediator of leptin resistance, we wondered to what extent expression of Socs-3 in POMC neurons was required for setting the level of leptin sensitivity as well as sensitivity to high-fat diet-induced obesity and altered glucose homeostasis. To that end, we created mice specifically lacking Socs-3 in POMC expressing cells by crossing animals expressing Cre recombinase under control of the POMC promoter (Balthasar et al., 2004) with mice in which the Socs-3 gene is flanked by LoxP sites (Mori et al., 2004; Yasukawa et al., 2003).

Results

Generation of mice lacking Socs3 in POMC-expressing cells

To generate mice lacking Socs-3 in POMC expressing cells, we crossed mice expressing Cre recombinase under control of the POMC promoter (POMC-Cre) to mice with both alleles of Socs-3 flanked by LoxP sites (Socs-3$^{lox/lox}$). To verify that Socs-3 was deleted from POMC neurons in the hypothalamus, we performed double in situ hybridization on brains from animals acutely treated with leptin, using a digoxigenin labeled POMC probe and a ^{35}S labeled Socs-3 probe. The ability of leptin to stimulate Socs-3 mRNA in POMC neurons has been described previously (Elias et al., 1999). In leptin-treated Socs-3$^{lox/lox}$; POMC-Cre animals there was a clear reduction in the number of neurons in which Socs-3 transcript was coexpressed with POMC transcript (Figures 1A and 1B). As seen in previous studies (Balthasar et al., 2004), a high percentage of POMC neurons responded in the Socs-3$^{lox/lox}$ mice to leptin treatment. Approximately, 56% of POMC cells displayed leptin-induced Socs-3 mRNA in the Socs-3$^{lox/lox}$ mice when stringent criteria for coexpression were employed (5X background) (Figure 1C). In the Socs-3$^{lox/lox}$; POMC-Cre mice however leptin-induced Socs-3 mRNA was found in only 4.7% of the population (Figure 1C). These findings demonstrate that the mice selectively lack Socs-3 transcript in POMC neurons. To verify that Socs-3 mRNA levels were not altered in other tissues, we examined the level of Socs-3 mRNA in liver and pituitary and found no difference in levels between the two genotypes (Figure 6F and data not shown).

Mice lacking Socs3 in POMC-expressing cells are more sensitive to leptin

As previous studies from our laboratory and others have shown (Bjorbaek et al., 1998, 2000), Socs-3 is a negative regulator of leptin signaling. To test whether reduced Socs-3 expression in POMC-expressing cells results in increased sensitivity to exogenous leptin in vivo, we implanted minipumps containing a low dose of leptin into Socs-3$^{lox/lox}$; POMC-Cre mice (n = 6) and Socs-3$^{lox/lox}$ littermates (n = 7). Although all animals lost weight in response to the surgery, the Socs-3$^{lox/lox}$; POMC-Cre animals demonstrated greater weight loss over time compared to the Socs-3$^{lox/lox}$ animals (Figure 2A). Further, food intake was significantly decreased over the 14 day period in mice lacking Socs-3 in POMC-expressing cells (Figure 2B). We measured the leptin concentration at the termination of the infusion and found that both groups of animals had similar final leptin levels, suggesting that the decrease in body weight and food intake was not due to a difference in leptin concentration, but instead was due to increased leptin sensitivity (Figure 2C). Indeed, terminal leptin levels were inversely correlated with the loss in body weight in the Socs-3$^{lox/lox}$; POMC-Cre animals, a relation not observed in the control littermates (Figure 2D). Based on the decrease in body weight and food intake, expression of the leptin-regulated neuropeptides AgRP, POMC, and NPY were investigated in hypothalami upon termination of the infusion experiment (Figure 2E). Significant genotype-specific changes were observed, as POMC mRNA was increased and NPY mRNA was

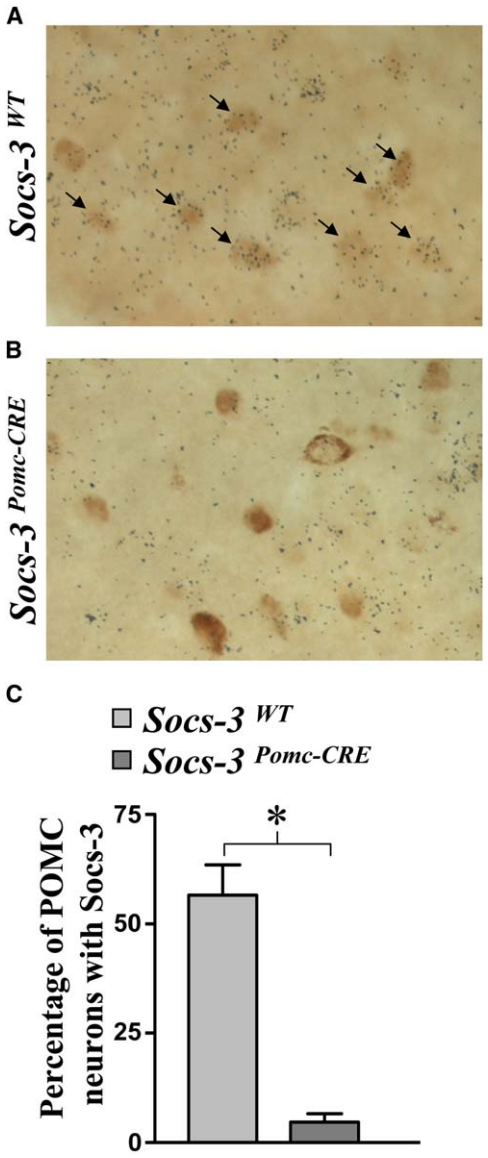

Figure 1. Deletion of Socs-3 from POMC-expressing neurons

A and B) Double in situ hybridization for POMC (digoxigenin labeled, brown stain) and Socs-3 (^{35}S labeled, silver grains) in leptin treated Socs-3$^{lox/lox}$ (*Socs-3 ^{WT}*) (**A**) and Socs-3$^{lox/lox}$; POMC-Cre (*Socs-3 $^{POMC\text{-}Cre}$*) mice (**B**) (n = 3).

C) Percentage of POMC labeled neurons that were positive for Socs-3. Neurons were counted positive when the silver grain content exceeded 5× over background. *$p < 0.001$ by Student's t test.

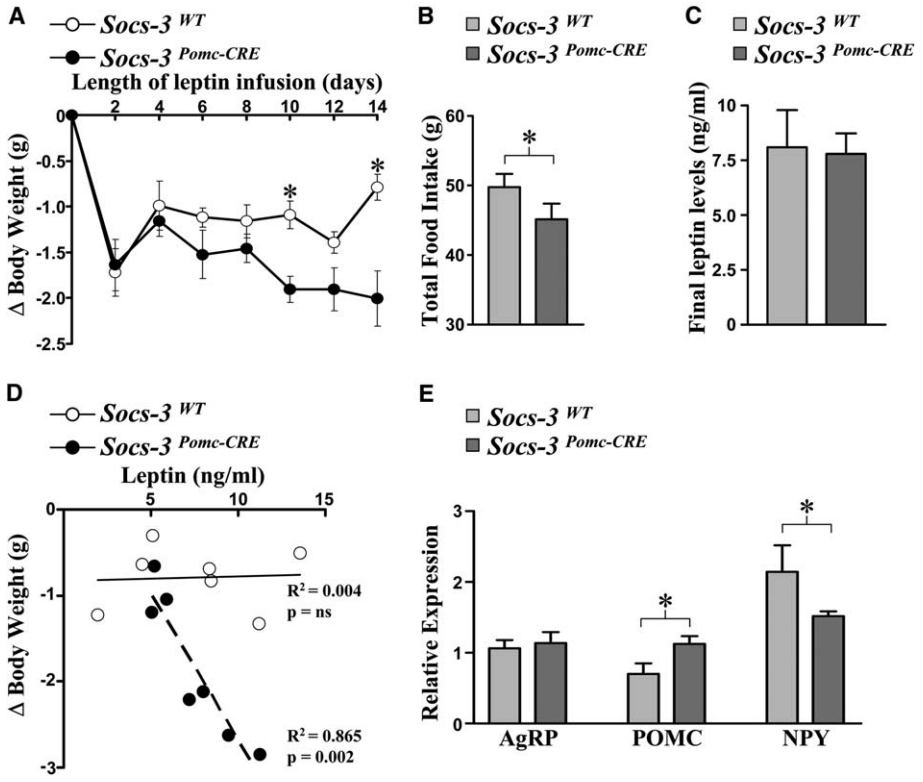

Figure 2. Socs-3$^{lox/lox}$; POMC-Cre mice are more sensitive to exogenous leptin

A) The change in body weight over 14 days in response to implantation of a osmotic minipump delivering 0.3 μg leptin per hour in Socs-3$^{lox/lox}$ (*Socs-3* ^{WT}) (n = 7) and Socs-3$^{lox/lox}$; POMC-Cre mice (*Socs-3* $^{POMC\text{-}Cre}$) (n = 6).

B) Total food intake (g) over 14 days.

C) Final leptin levels (ng/ml).

D) Linear regression of the body weight loss correlated to terminal leptin levels.

E) Hypothalamic expression of POMC, NPY, and AgRP, relative to total 18S RNA content.

*p < 0.05 by Student's t test.

decreased in mice lacking Socs-3 in POMC-expressing cells in response to leptin, and correlated very well with the observation that the Socs-3$^{lox/lox}$; POMC-Cre mice had an exaggerated decrease in food intake. There was no change in AgRP mRNA levels. Thus, by multiple criteria, the Socs-3$^{lox/lox}$; POMC-Cre animals are more sensitive to exogenous leptin.

Mice lacking Socs3 in POMC-expressing cells have improved glucose homeostasis on chow diet

Since mice lacking leptin receptors on POMC neurons display a modest increase in body weight (Balthasar et al., 2004), we compared the weights of Socs-3$^{lox/lox}$ (n = 9) and Socs-3$^{lox/lox}$; POMC-Cre (n = 9) animals over time while on chow diets. Interestingly, although the mice lacking Socs-3 in POMC-expressing cells are more sensitive than controls to low doses of infused leptin, body weight (Figure 3A) and food intake (Figure 3B) of these animals were not significantly different between genotypes while on chow diet. This suggests that in animals fed regular chow, the lack of Socs-3 in POMC-expressing cells is not by itself sufficient to alter body weight homeostasis. However, despite no change in body weight, blood glucose levels in the Socs-3$^{lox/lox}$; POMC-Cre animals were significantly lower (Figure 3C). To further examine whether the Socs-3$^{lox/lox}$; POMC-Cre animals have altered glucose homeostasis, a glucose tolerance test was performed. Socs-3$^{lox/lox}$; POMC-Cre animals had improved glucose tolerance compared to weight and age matched littermate controls (Figure 3D). An insulin tolerance test (ITT) demonstrated that both groups were equally sensitive to insulin (Figure 3E). Glucose homeostasis was improved despite finding no significant difference in adiposity between the genotypes as measured by fat pad weights either individually or collectively (data not shown). Leptin levels were slightly lower in the Socs-3$^{lox/lox}$; POMC-Cre animals in both the fed (31.6 ± 6.9 versus 22.1 ± 2.6) and fasted states (10.3 ± 1.5 versus 7.92 ± 1.6; data not shown), although the differences were not significant. These results suggest that loss of Socs-3 expression in POMC-expressing cells has little or no effect on body weight under chow fed conditions, but under these conditions Socs-3 in POMC-expressing cells exerts effects on glucose homeostasis.

High-fat-fed mice lacking Socs3 in POMC-expressing cells have attenuated weight gain and improved glucose homeostasis

To determine whether Socs-3 in POMC-expressing cells plays a greater role in energy homeostasis by contributing to leptin resistance induced by high-fat diet, we next fed Socs-3$^{lox/lox}$; POMC-Cre (n = 10) and control littermates (n = 8) a high-fat, high-sucrose diet. Previous experiments have shown that mice placed on such a diet develop obesity, hyperleptinemia and hyperinsulinemia (El-Haschimi et al., 2000; Lin et al., 2000). Further, other studies have demonstrated that in response to DIO Socs-3 expression levels in the arcuate nucleus are increased (Bjorbaek et al., 1998; Munzberg et al., 2004). The mice were placed on the diet at 8 weeks of age, and a difference in the rate of increase in body weight was evident immediately, with mice lacking Socs3 in POMC-expressing cells gaining less weight (Figure 4A). With increasing duration of diet however, the rate of body weight increase stabilized and the difference in body weight between the two groups became insignificant. The total food intake over the experiment did not differ between the groups, whether measured as the total amount over the experimental time frame (Figure 4B) or on a weekly basis (data not shown). Leptin levels were significantly decreased in the Socs-3$^{lox/lox}$; POMC-Cre animals on a HFD in both the fed and fasted state (Figure 4C). Since

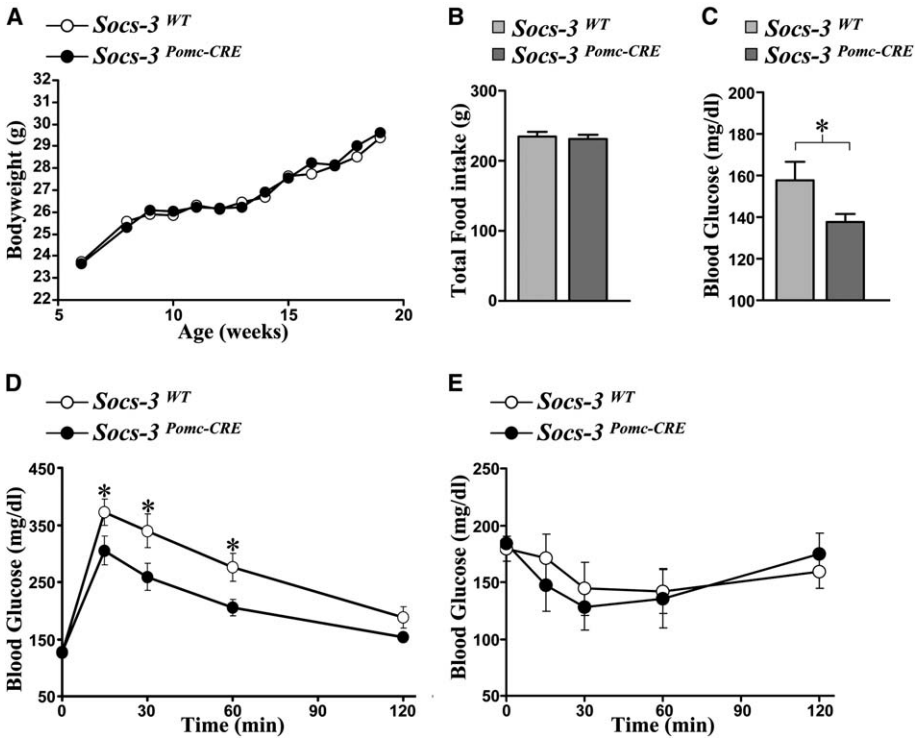

Figure 3. Improved glucose tolerance in Socs-3$^{lox/lox}$; POMC-Cre mice

A) Body weight curves of Socs-3$^{lox/lox}$ (*Socs-3* ^{WT}) (n = 9) and Socs-3$^{lox/lox}$; POMC-Cre mice (*Socs-3* $^{POMC\text{-}Cre}$) (n = 9).

B) Total food intake over 11 weeks and terminal blood glucose levels (26 weeks of age).

C) Blood glucose (fed state) levels at the end of the study.

D) Glucose tolerance test at 23 weeks of age (1.5 mg glucose/g body weight).

E) Insulin tolerance test at 22 weeks of age (1 mU Humulin R/g body weight).

*p < 0.05 by Student's t test.

the Socs-3$^{lox/lox}$; POMC-Cre mice had a reduced rate of weight gain while on high-fat diet, yet had no alteration in feeding, the rate of energy expenditure was determined. Over a 24 hr period, the Socs-3$^{lox/lox}$; POMC-Cre mice displayed a slight but significant increase in VO_2 and respiratory exchange ratio (RER) (Figures 4D and 4E), but no difference in the amount of heat generated was detected (Figure 4G). There also appeared to be an increase in activity in the Socs-3$^{lox/lox}$; POMC-Cre mice, however this increase was not statistically significant (Figure 4F).

To examine the effects of Socs-3 deletion from POMC-expressing cells on glucose homeostasis we performed glucose and insulin tolerance tests after 12 and 13 weeks on a high-fat diet. Similar to chow-fed animals, glucose tolerance was greatly improved in Socs-3$^{lox/lox}$; POMC-Cre animals compared to control littermates (Figure 5A). Furthermore, the Socs-3$^{lox/lox}$; POMC-Cre mice also displayed a higher sensitivity to insulin in the ITT compared to littermates (Figure 5B). As has been described before, a high-fat diet results in an increase of both

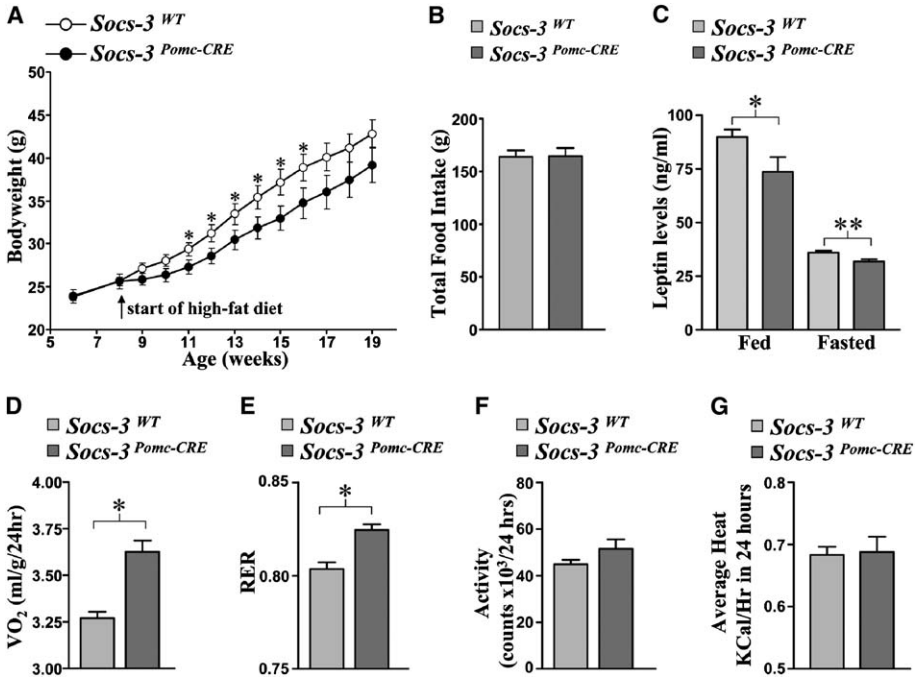

Figure 4. Socs-3$^{lox/lox}$; POMC-Cre mice have attenuated weight increase on a high-fat diet

A) Body weight curves of Socs-3$^{lox/lox}$ (*Socs-3* ^{WT}) (n = 8) and Socs-3$^{lox/lox}$; POMC-Cre mice (*Socs-3* $^{POMC\text{-}Cre}$) (n = 10) on a high-fat, high-carbohydrate diet. Diet started at 8 weeks of age. *p < 0.05 between genotypes for given week. Body weights at week 19 are 39.1 ± 2.0 and 42.8 ± 1.6.

B) Total food intake over 11 weeks on a HFD.

C) Leptin levels in either the fed or fasted state.

D) Oxygen consumption averaged over a 24 hr period.

E) Respiratory exchange ratio (VO_2/VCO_2) averaged over a 24 hr period.

F) Activity averaged over a 24 hr period.

G) Average heat generated over a 24 hr period.

*p < 0.001 by Student's t test.

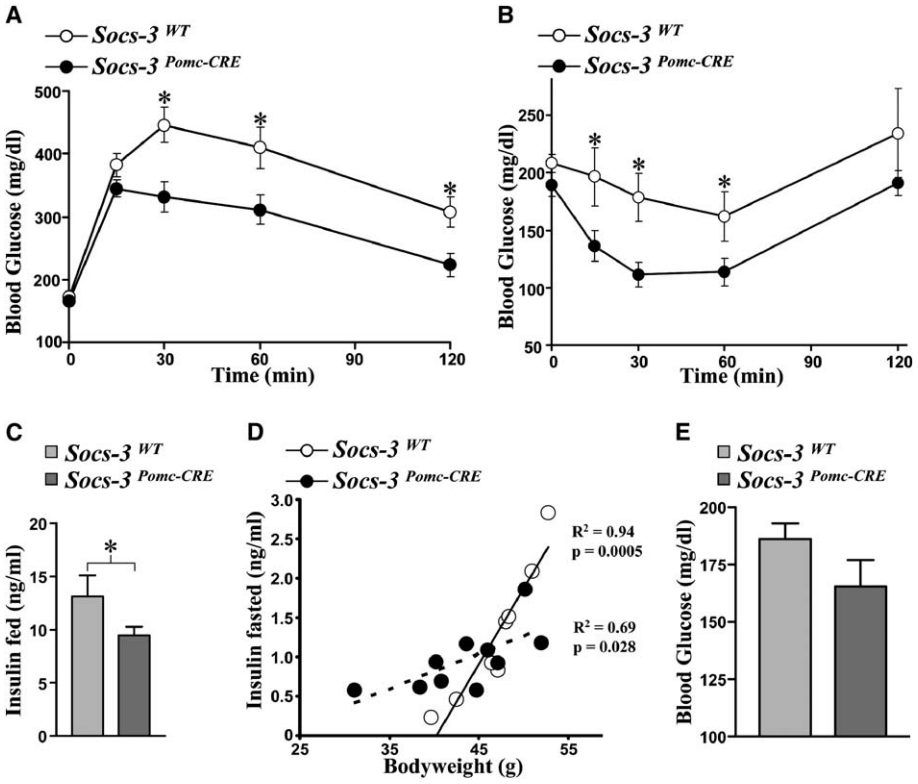

Figure 5. Socs-3$^{lox/lox}$; POMC-Cre mice have an improved glucose homeostasis on HFD

A) Glucose tolerance test at 23 weeks of age (1.5 mg glucose/g body weight) in Socs-3$^{lox/lox}$ (*Socs-3* ^{WT}) (n = 8) and Socs-3$^{lox/lox}$; POMC-Cre mice (*Socs-3* $^{POMC\text{-}Cre}$) (n = 10).

B) Insulin tolerance test at 22 weeks of age (1 mU Humulin R/g body weight).

C) Terminal insulin levels of fed mice.

D) Correlation between fasted insulin levels and body weights between the groups.

E) Terminal blood glucose levels in fed mice.

*p < 0.05 by Student's t test.

insulin and leptin levels. Socs-3$^{lox/lox}$; POMC-Cre mice displayed significant lower levels of insulin in the fed state compared to control littermates (Figure 5C). Further, when the insulin levels in the fasted state were plotted in relationship to body weight we noted a strong positive correlation between the body weight and the levels of insulin (Figure 5D) in control Socs-3$^{lox/lox}$ mice. In comparison, in the Socs-3$^{lox/lox}$; POMC-Cre mice, insulin levels did not rise as strongly with an increase in body weight. In addition, as was seen in animals fed a regular-chow diet, the Socs-3$^{lox/lox}$; POMC-Cre animals displayed lower blood glucose levels in the fed state (Figure 5E). These results therefore demonstrate that expression of Socs-3 in POMC-expressing cells plays an important role in linking high-fat diets to changes in glucose tolerance and insulin sensitivity. Deletion of Socs-3 from POMC-expressing cells results in increased insulin sensitivity in the context of a high-fat diet.

Mice lacking Socs3 in POMC-expressing cells are protected from hepatic steatosis

Exposure to high-fat diet increased the mass of all fat depots as assessed at the termination of the experiment (when body weights no longer differed between genotypes), and there were no significant differences between the two genotypes in this regard (Table 1). We also determined the total liver weight at the termination of the study. In the littermate controls, the HFD resulted in a significant increase of the liver weight (expressed either as grams or as percentage of BW) (data not shown, Figure 6A) when compared to age-matched animals that were fed a chow diet. A similar change in liver weight, predominantly the result of the formation of a fatty liver, has been seen in other studies (Gregoire et al., 2002). The Socs-3$^{lox/lox}$; POMC-Cre mice did not display this increase in liver weight on HFD, and liver weight as percentage of total body weight was also similar to age-matched mice fed a chow diet (Figure 6A). Recent studies have shown that the enzyme SCD-1 is a crucial regulator of the formation of fatty liver (Asilmaz et al., 2004). Further, an increase in SCD-1 levels correlates with the development of fatty liver in response to a high-fat diet (Biddinger et al., 2005). Consistent with these findings, we noted an increase in SCD-1 mRNA levels in HFD control mice, but no change in SCD-1 was observed in Socs-3$^{lox/lox}$; POMC-Cre mice (Figure 6B). We also measured other genes involved in the regulation of glucose homeostasis and fatty acid metabolism and found that a similar pattern was observed for the mRNA levels of PGC-1α, PEPCK, and Socs-3 (Figures 6C, 6E, and 6F). We did not find a change in the regulation of SREBP-1 (Figure 6D). These results suggests that POMC-expressing cells have the capacity to influence the formation of a fatty liver in response to high-fat diets, and expression of Socs-3 in these cells is a major regulator of this circuit.

Discussion

Socs-3 is a leptin inducible inhibitor of leptin signaling in vitro and in vivo. Its expression is notably increased in the arcuate nucleus in response to leptin and HFD, and deletion of just one Socs-3 allele results in haploinsufficiency, accompanied by increased leptin sensitivity and resistance to HFD (Bjorbaek et al., 1998; Howard et al., 2004; Munzberg et al., 2004). A similar phenotype is seen when Socs-3 is deleted selectively in neurons, but the role played by Socs-3 in specific neurons involved in regulation of energy balance and metabolism is as yet unknown (Mori et al., 2004).

Table 1. Individual fat pad weights after 18 weeks on HFD

Adipose tissue depot	Socs-3$^{lox/lox}$	Socs-3$^{lox/lox}$; POMC-Cre
perirenal plus retroperitoneal	0.960 ± 0.1	1.09 ± 0.1
perigonadal	1.97 ± 0.2	2.25 ± 0.2
mesenteric	1.47 ± 0.1	1.16 ± 0.1
subcutaneous	3.06 ± 0.3	2.58 ± 0.2
brown	0.45 ± 0.05	0.50 ± 0.05

At the termination of the HFD study, individual fat pads were carefully dissected and weighed. After analysis, there were no significant differences in fat pad weight between any of the groups when tested by Student's t test.

In these experiments, we demonstrate that the ability of Socs-3 to regulate in vivo leptin sensitive energy balance is mediated in part via Socs-3 within POMC neurons. Socs-3$^{lox/lox}$; POMC-Cre animals were more sensitive to low dose exogenous leptin with respect to body weight, food intake, and neuropeptide expression, and had lower leptin levels when placed on a high-fat diet. Further, loss of Socs-3 in POMC-expressing cells reduced the rate of weight gain in response to a high-fat diet, although the degree of protection against DIO in Socs-3$^{lox/lox}$; POMC-Cre mice was less than that observed in Socs-3 haploinsufficient animals or mice in which Socs-3 was removed from all neurons (Howard et al., 2004; Mori et al., 2004). One factor to be considered with our animal cohorts was the fact that they were on a mixed genetic background, with approximately even contributions from C57BL/6, FVB and BALB/C inbred mouse lines. Certain strains are more sensitive to HFD and this mixed background likely resulted in a greater variation in the weight gain. Nonetheless, the Socs-3$^{lox/lox}$; POMC-Cre had reduced rates of body weight gain during the course of HFD, though the difference became insignificant by the end of the study. A recent study demonstrated that Socs-3 levels were significantly upregulated specifically in the arcuate nucleus as early as 4 weeks on a HFD (Munzberg et al., 2004). This is in contrast to another suggested inhibitor of leptin signaling, PTP-1B, expression of which is not upregulated with HFD. Therefore, the decreased rate of body weight gain in the Socs-3$^{lox/lox}$; POMC-Cre mice in the earlier weeks is likely explained by lack of Socs-3 and enhanced leptin signaling in POMC-expressing cells. However, as the rate of body weight gain between the two genotypes became more similar in the later weeks on a HFD, factors apart from Socs-3 in the POMC neuron, such as Socs-3 in other locations or PTP-1B, likely contribute more importantly to the regulation of body weight in this period. Also, Socs-3 deletion from all neurons resulted in a greater body weight decrease in response to a HFD (Mori et al., 2004), suggesting that Socs-3 also regulates energy balance in neurons apart from POMC neurons. This is consistent with data suggesting that leptin regulates body weight through actions in multiple sites (Balthasar et al., 2004; Boston et al., 1997; Coppari et al., 2005; Grill et al., 2002).

Although Socs-3 in POMC neurons influences energy balance in response to leptin and high-fat diet, Socs-3 in POMC neurons has an even greater influence over glucose metabolism. Removal of Socs-3 from POMC-expressing cells resulted in greatly improved glucose homeostasis when animals were placed on a high-fat diet. While it is possible that this improvement in glucose homeostasis is in part due to the decreased rate of body weight gain in the earlier weeks, several other possibilities should be considered. First, in mice fed normal chow at a relatively young age (7 and 9 weeks), glucose levels were consistently lower in Socs-3$^{lox/lox}$; POMC-Cre mice (data not shown), a finding that was consistent over the course of the study. Second, blood glucose measurements taken at the end of both chow feeding and HFD studies demonstrated reduced glucose levels on the Socs-3 deficient genotype. Indeed, Socs-3$^{lox/lox}$; POMC-Cre mice fed a chow diet had improved glucose tolerance,

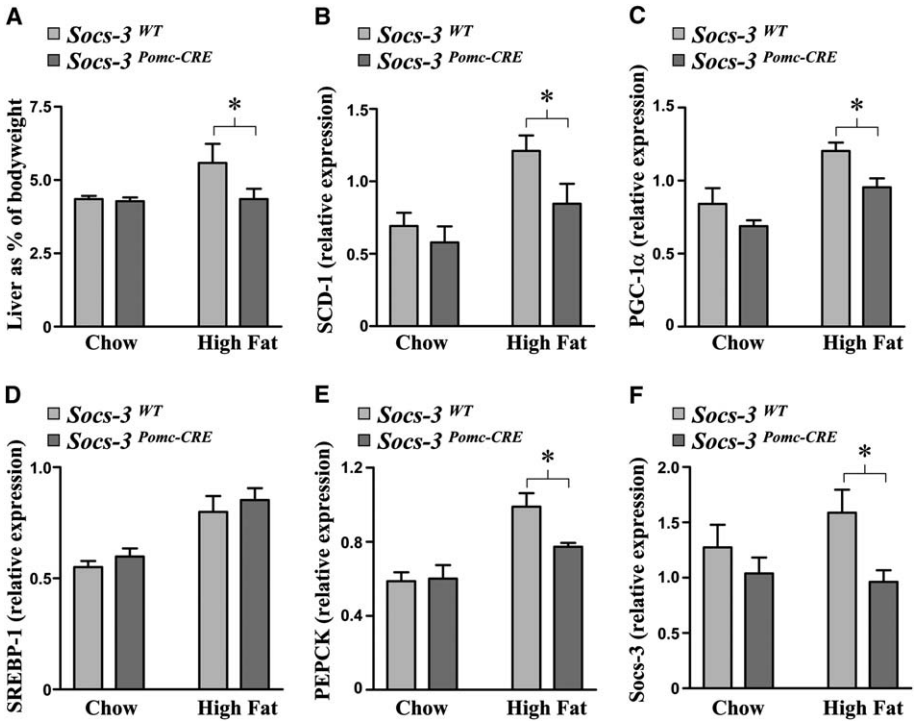

Figure 6. Socs-3$^{lox/lox}$; POMC-Cre mice are protected from development of enlarged liver and have reduced levels of SCD-1

A) Liver weight expressed as a percentage of body weight in Socs-3$^{lox/lox}$ (*Socs-3* ^{WT}) (n = 9) and Socs-3$^{lox/lox}$; POMC-Cre mice (*Socs-3* $^{POMC\text{-}Cre}$) (n = 9) on regular-chow diet or Socs-3$^{lox/lox}$ (*Socs-3* ^{WT}) (n = 8) and Socs-3$^{lox/lox}$; POMC-Cre mice (*Socs-3* $^{POMC\text{-}Cre}$) (n = 10) on a HFD.

B–F) Gene expression analysis using real-time PCR of SCD-1 (**B**), PGC-1a (**C**), SREBP-1 (**D**), PEPCK (**E**), and Socs-3 (**F**).

*$p < 0.05$ by Student's t test.

suggesting that even under these conditions of unaltered body weight and food intake, the lack of Socs-3 in POMC-expressing cells results in improved glucose tolerance. Whether this improvement in peripheral metabolism due to lack of Socs-3 in POMC-expressing cells is due to increased leptin signaling in POMC neurons, or to changes in other Socs-3 regulated pathways in POMC-expressing cells cannot be determined by these studies. In this regard, Socs-3 has been implicated quite strongly as a factor that can suppress insulin signaling in several cell types (Senn et al., 2003; Shi et al., 2004; Ueki et al., 2004). Recent studies suggest that insulin action on central pathways can exert potent effect upon peripheral glucose metabolism including hepatic glucose production (Obici et al., 2001, 2002). It is therefore possible that enhanced insulin signaling in POMC neurons by removal of Socs-3 results in a lower hepatic glucose production, which could produce an improved GTT. It is also possible that enhanced glucose metabolism in mice lacking Socs-3 in POMC neurons is due to neuronal actions of leptin (Pocai et al., 2005). This is consistent with recent data demonstrating that restoration of leptin receptors in the arcuate nucleus significantly reduces blood glucose levels in leptin receptor null animals with only a modest decrease in body weight (Coppari et al., 2005).

Several studies have provided data that the central melanocortin system can regulate glucose homeostasis independent of body weight changes (Mizuno et al., 2003; Obici et al., 2001). In leptin-deficient mice (ob/ob), transgenic overexpression of the POMC gene resulted in lower blood glucose levels, improved glucose tolerance and lowered the weight of the liver to the level of wild-type control animals (Mizuno et al., 2003). These studies are in agreement with our findings, further supporting the role of the POMC neuron, and POMC itself, in the regulation of glucose homeostasis. Interestingly, in response to a low dose of leptin, mice lacking Socs-3 in POMC cells have higher POMC expression in the hypothalamus than control animals, suggesting that POMC itself could be responsible for the improved glucose homeostasis. However, removal of leptin receptors from POMC neurons lowered levels of POMC in the hypothalamus, yet no effect on glucose homeostasis was observed in animals on a chow diet (Balthasar et al., 2004). Therefore, it is possible that a factor within POMC neurons other than POMC, such as CART, could play a role in regulating glucose homeostasis. Since insulin and glucose tolerance tests are poor means for assessing changes in hepatic glucose output, further studies will be required to determine whether Socs-$3^{lox/lox}$; POMC-Cre mice have altered hepatic glucose production, and/or enhanced insulin sensitivity in other peripheral tissues when Socs-3 is deleted from POMC-expressing cells. It will also be of great interest to determine whether these effects are due to enhanced signaling in POMC neurons by leptin or insulin, or both of these hormones.

It is of interest to note that although leptin infusion lowered food intake to a greater extent in Socs-$3^{lox/lox}$; POMC-Cre mice, we detected no difference in food intake between the genotypes fed low-fat or high-fat diets. Of course, similar food intake was maintained despite lower leptin levels in Socs-$3^{lox/lox}$; POMC-Cre mice, suggesting that the animals have reached a new equilibrium in leptin sensitivity. In addition, the Socs-$3^{lox/lox}$; POMC-Cre mice had a greater increase in energy expenditure in response to a HFD, partially compensating for the increase in caloric intake on HFD. A recent study by Coppari et al. demonstrated that re-activation of endogenous leptin receptors specifically in the arcuate nucleus resulted in an increase in locomotor activity (Coppari et al., 2005). Therefore, it is reasonable to hypothesize that enhanced leptin action on POMC neurons is contributing to the observed increase in energy expenditure, whether through changes in locomotor activity or through other mechanisms.

One very interesting difference between the two genotypes was the weight of the liver. Gross anatomical comparisons between the livers of Socs-$3^{lox/lox}$; POMC-Cre and control littermates displayed a difference in both color and size. The total weight of the liver of control mice was 0.75 grams higher than the Socs-$3^{lox/lox}$; POMC-Cre mice on a HFD. When the data was expressed as percentage of body weight (to correct for a slight increase in total body weight and body length), it became evident that the livers of Socs-$3^{lox/lox}$; POMC-Cre mice on a HFD had a similar ratio of liver weight: body weight as the mice on a chow diet. The control littermates however had an increased ratio, a common occurrence in mice that are fed a HFD, suggesting that enhanced leptin signaling in the POMC neurons could protect against the development of fatty liver. Indeed, gene expression analysis demonstrated that the Socs-$3^{lox/lox}$; POMC-Cre mice had significantly lower levels of Stearoyl-CoA Desaturase 1 (SCD-1), PGC-1α, Socs-3 and PEPCK in response to HFD. Previous studies have demonstrated that increases in PGC-1α and PEPCK increase hepatic gluconeogenesis (Sun et al., 2002; Yoon et al., 2001). For instance, adenoviral-mediated overexpression of PGC-1α in vivo in the liver resulted in higher blood glucose levels, elevated insulin levels and elevated PEPCK levels (Yoon et al., 2001). These results support our hypothesis that hepatic gluconeogenesis is increased in HFD, and that genetic removal of Socs-3 from POMC-expressing cells results in an improved glucose homeostasis at least in part by actions to reduce hepatic gluconeogenesis. The exact mechanisms by which changes in activity of POMC neurons due to Socs-3 deletion alters hepatic gene expression remains to be investigated, although previous studies have suggested that the melanocortin pathway is involved (Mizuno et al., 2003; Obici et al., 2001). In addition, leptin has been shown to downregulate SCD-1 through a mechanism that involves the CNS (Asilmaz et al., 2004). Further, a genetic cross of asebia mice, which lack functional SCD-1, and ob/ob mice, which lack the leptin gene and have massive obesity and fatty liver, resulted in complete reversal of the fatty liver phenotype (Cohen et al., 2002). These observations suggest that lower levels of Socs-3 in POMC-expressing cells enhance leptin's ability to suppress SCD-1 and thus prevent the formation of a fatty liver.

A major goal of current research is to use mouse genetics to assess the role of specific molecular pathways within identified neurons in the control of energy balance and metabolism. It is known from prior work that POMC neurons and the signaling modulator Socs-3 are important components of energy balance and metabolic circuitry. Here we provide definitive genetic evidence that Socs-3 modulates leptin's ability to regulate energy balance. In addition, Socs-3 in POMC-expressing cells is shown to importantly modulate peripheral glucose metabolism, in part independent of body weight or obesity. Whether these metabolic consequences of Socs-3 loss in POMC-expressing cells are due to altered signaling by leptin, insulin, or both in these cells will be the subject of future work.

Experimental procedures

Generation of Socs-3$^{lox/lox}$; POMC-Cre mice

Socs-3 mice were obtained from Dr. Yoshimura and are described previously (Yasukawa et al., 2003). Briefly, a targeting vector was constructed that contained LoxP sites around the 2nd exon of the Socs-3 gene. C57BL/6 ES cells were transfected, selected for homologous recombination and injected into BALB/C blastocysts. Chimeras were bred with C57BL/6 mice to generate mice heterozygotes for the conditional allele. POMC-Cre mice were described previously (Balthasar et al., 2004). Briefly, these mice were generated by inserting the Cre recombinase coding region into the ATG of the POMC transcript using homologous recombination in bacteria and a bacterial artificial chromosome containing the POMC gene. POMC-Cre BAC DNA was then injected into fertilized one-cell stage embryos of FVB mice. To generate Socs-3$^{lox/lox}$; POMC-Cre, mice we first crossed mice heterozygous for the conditional Socs-3 allele (BALB/C and C57BL/6 mixed background) with mice carrying the POMC-Cre BAC (FVB and C57BL/6 mixed background). Subsequent crosses were performed to achieve a colony of Socs-3$^{lox/lox}$ and Socs-3$^{lox/lox}$; POMC-Cre mice. During breeding no mice of pure background were introduced, so the total population was on a mixed background containing C57BL/6, BALB/C, and FVB strains. Only animals from these crosses were used in experiments, and at all times littermates were compared. All animal protocols were approved by the institutional animal care and use committee (IACUC). Mice were housed in a temperature-controlled environment (22°C–24°C) with a 14 hr light/10 hr dark cycle and provided ad libitum water and chow (Harlan Teklad, diet 8664).

Double in situ hybridization of POMC and Socs-3 mRNA

Socs-3$^{lox/lox}$ (n = 3) and Socs-3$^{lox/lox}$; POMC-Cre mice (n = 3) were fasted overnight and given an intraperitoneal injection with leptin (5 μg/g body weight; A.F. Parlow, National Hormone and Peptide Program). After 2 hr, animals were perfused with 10% buffered formalin (Sigma-Aldrich), sectioned at 25 μm on a microtome, and double in situ was performed for Socs-3 and POMC as previously described (Elias et al., 1999). Silver grains were counted on several fields of vision within the arcuate nucleus for three animals in each group. The number of neurons containing both POMC and Socs-3 were estimated by assessing the silver grains overlying POMC cells. The cells were classified as double-labeled when the density of silver grains was at least 5-fold higher as the background levels (Balthasar et al., 2004; Elias et al., 1999).

Leptin minipump study

Eighteen-week-old Socs-3$^{lox/lox}$ (n = 7) and Socs-3$^{lox/lox}$; POMC-Cre (n = 6) mice were anesthetized with isoflurane and implanted with subcutaneous osmotic minipumps. The reservoir allowed for a 14 day continuous delivery of murine leptin (0.3 μg per hour). Animals were singly housed and monitored every other day for body weight changes and food intake. Fourteen days after implantation, animals were sacrificed and blood was collected via cardiac puncture. Hypothalami were dissected, flash-frozen in liquid nitrogen, and stored at −80°C for RNA analysis.

RNA extraction and real-time PCR

Total RNA was isolated from tissues with an RNeasy lipid tissue kit from Qiagen. One microgram of RNA was used for generation of cDNA using random hexamers (Superscript, Ambion). Quantitative real-time PCR was performed on a MX3000 Stratagene system, utilizing Taqman based probes for NPY, AgRP and POMC peptides (Balthasar et al., 2004) and Taqman universal PCR master mix (Applied Biosystems). Primer/probe sets for Socs-3, PGC-1α, SREBP-1, PEPCK and SCD-1 were purchased from Applied Biosystems (assays on demand). All samples were analyzed as a multiplex reaction measuring both the gene of interest and 18S RNA content as an internal control. All reactions were performed in triplicate.

Insulin and glucose tolerance tests

Insulin and glucose tolerance test were performed on Socs-3$^{lox/lox}$ and Socs-3$^{lox/lox}$; POMC-Cre mice. For the insulin tolerance test, insulin (humulin R, Eli Lilly) was injected intraperitoneally (1 mU/g body weight) and blood glucose levels were measured through a tail-nick at 0, 15, 30, 60, and 120 min. For the glucose tolerance test, animals were injected with glucose (1.5 mg/g body weight) and blood glucose was measured as described above.

High-fat diet studies

Socs-3$^{lox/lox}$ (n = 8) and Socs-3$^{lox/lox}$; POMC-Cre (n = 10) mice 8 weeks of age were placed on a diet containing 58% kCal from fat and 25.5% kcal from carbohydrates (Research Diets, D12331). Animals were singly housed and food intake and body weight were measured weekly. Blood glucose measurements were performed at weeks 7, 9, and 18 of age. Oxygen consumption and activity were measured at week 19 using a comprehensive lab animal monitoring system (CLAMS, Columbia instruments). Glucose tolerance tests were performed at week 21 and 23, and insulin tolerance was examined at week 22. On week 25, animals were fasted overnight and blood was collected from a tail-nick. At week 26, animals were sacrificed by CO2 inhalation, blood was collected by cardiac puncture, and tissues were removed and stored at -80C for further analysis. All animals were sacrificed over a 4 day timeframe between the hours of 10:00 and 12:00.

Statistical analysis

All results are expressed means ± SEM. Comparisons between groups were tested using an unpaired Student's t test in Graphpad Prism (Graphpad, San Diego) or a Mann-Whitney U test when appropriate. A p value of less than 0.05 was considered to be statistically significant. Regression curves were calculated with Microsoft Excel.

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