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Role of glucocorticoids in mediating effects of fasting and diabetes on hypothalamic gene expression

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Published: 09 July 2003

Received: 29 January 2003

BMC Physiology 2003, 3:5

Accepted: 09 July 2003

This article is available from: <http://www.biomedcentral.com/1472-6793/3/5>

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Abstract

Background: Fasting and diabetes are characterized by elevated glucocorticoids and reduced insulin, leptin, elevated hypothalamic AGRP and NPY mRNA, and reduced hypothalamic POMC mRNA. Although leptin replacement can reverse changes in hypothalamic gene expression associated with fasting and diabetes, leptin also normalizes corticosterone; therefore the extent to which the elevated corticosterone contributes to the regulation of hypothalamic gene expression in fasting and diabetes remains unclear. To address if elevated corticosterone is necessary for hypothalamic responses to fasting and diabetes, we assessed the effects of adrenalectomy on hypothalamic gene expression in 48-hour-fasted or diabetic mice. To assess if elevated corticosterone is sufficient for the hypothalamic responses to fasting and diabetes, we assessed the effect of corticosterone pellets implanted for 48 hours on hypothalamic gene expression.

Results: Fasting and streptozotocin-induced diabetes elevated plasma glucocorticoid levels and reduced serum insulin and leptin levels. Adrenalectomy prevented the rise in plasma glucocorticoids associated with fasting and diabetes, but not the associated reductions in insulin or leptin. Adrenalectomy blocked the effects of fasting and diabetes on hypothalamic AGRP, NPY, and POMC expression. Conversely, corticosterone implants induced both AGRP and POMC mRNA (with a non-significant trend toward induction of NPY mRNA), accompanied by elevated insulin and leptin (with no change in food intake or body weight).

Conclusion: These data suggest that elevated plasma corticosterone mediate some effects of fasting and diabetes on hypothalamic gene expression. Specifically, elevated plasma corticosterone is necessary for the induction of NPY mRNA with fasting and diabetes; since corticosterone implants only produced a non-significant trend in NPY mRNA, it remains uncertain if a rise in corticosterone may be sufficient to induce NPY mRNA. A rise in corticosterone is necessary to reduce hypothalamic POMC mRNA with fasting and diabetes, but not sufficient for the reduction of hypothalamic POMC mRNA. Finally, elevated plasma corticosterone is both necessary and sufficient for the induction of hypothalamic AGRP mRNA with fasting and diabetes.

Background

Reduction in hypothalamic melanocortin tone, caused by reduction in proopiomelanocortin (POMC) mRNA and elevation in agouti-related peptide (AGRP) mRNA, is thought to play a key role in mediating nutritional effects on neuroendocrine function [1,2]. Thus fasting [3,4], insulin deficiency [5], and leptin deficiency [4] are all associated with reduced hypothalamic melanocortin tone. However, factors that regulate hypothalamic melanocortin tone are not fully elucidated. Since both fasting and insulin deficiency are associated with reduced plasma leptin [5], and leptin replacement reverses effects of fasting [6] and also effects of insulin deficiency on food intake and body weight [7], it has been suggested that the reduction in leptin accounts for the reduced hypothalamic melanocortin tone in these conditions. However, fasting can influence hypothalamic gene expression in insulin-deficient mice independent of changes in plasma leptin or insulin [5], as well as in leptin resistant *db/db* mice [3]. These data suggest that other factors in addition to leptin may mediate effects of fasting. Since fasting, insulin deficiency, and leptin insufficiency also lead to an elevation in plasma glucocorticoids [8], and adrenalectomy of leptin-deficient *ob/ob* mice largely normalizes hypothalamic gene expression in these mice [9], it is plausible that elevated glucocorticoids may mediate some effects of nutritional state on hypothalamic gene expression. This hypothesis is particularly compelling since recent data suggest that aberrant glucocorticoid activity may play a key role in the development of the metabolic syndrome [10].

Glucocorticoids have previously been reported to increase body weight set point, as determined by hoarding behavior in rats [11], while adrenalectomy has been reported to lower body weight set point in rats [12]. In addition, central infusion of glucocorticoids leads to increases in body weight and food intake with a concomitant induction of hypothalamic NPY [13]. Nevertheless, it remains unclear if an elevation in glucocorticoid concentration contributes to the effect of fasting on hypothalamic gene expression. Adrenalectomy has been reported to block elevations in NPY mRNA in response to fasting [14]. On the other hand, it has been reported that elevation of plasma corticosterone levels is not required for the induction of NPY mRNA by fasting [15]. In addition, the extent to which hypothalamic AGRP and POMC mRNA depends on elevated glucocorticoid levels in fasting and insulin deficiency has not been determined to date. Therefore in the present study we assessed if an elevation of plasma corticosterone is necessary and sufficient to mediate effects of fasting and insulin deficiency on hypothalamic melanocortin tone, as indicated by expression of hypothalamic AGRP and POMC mRNA.

Results and Discussion

Study 1: Effects of adrenalectomy on hypothalamic responses to fasting

Fasting reduced body weight and adipose weight in both the sham-operated and adrenalectomized groups as expected (Table 1). Blood glucose was also decreased by fasting in both the sham-operated group and adrenalectomized group (Table 1). Serum corticosterone concentration increased with fasting as expected in the sham-operated mice (Fig. 1A); adrenalectomy blocked this fasting-induced elevation of corticosterone (Fig. 1A). Therefore the adrenalectomized fasted mice exhibited similar levels of corticosterone as both the adrenalectomized ad lib fed mice and the sham-operated ad lib fed mice (Fig. 1A). As we noted previously, a basal level of corticosterone was detectable in the adrenalectomized mice after surgery [9]. In contrast to corticosterone, other humoral factors were not significantly affected by adrenalectomy. Serum insulin and serum leptin both exhibited the same decreases in the adrenalectomized fasted and sham-operated fasted animals compared to the adrenalectomized ad lib fed and sham-operated ad lib fed mice (Fig. 1B and 1C); therefore the levels of these humoral factors were the same in the sham-operated fasted mice and the adrenalectomized fasted mice.

Fasting increased hypothalamic AGRP mRNA as expected in the sham-operated fasted group, and adrenalectomy completely blocked the induction of AGRP by fasting (Fig. 2A). Adrenalectomy also produced a trend to decrease AGRP mRNA in the ad-lib fed mice but this effect was not significant (Fig. 2A). Fasting decreased hypothalamic POMC mRNA as expected in the sham-operated fasted group, but fasting had no effect on POMC mRNA in the adrenalectomized animals (Fig. 2B). As with AGRP, NPY mRNA was also elevated after fasting, and this effect of fasting on NPY mRNA was also completely blocked by adrenalectomy (Fig 2C). Adrenalectomy therefore completely blocked the effects of fasting on hypothalamic AGRP, POMC and NPY mRNAs.

Study 2: Effects of adrenalectomy on hypothalamic responses to insulin deficiency and fasting

Streptozotocin injection induced diabetes in sham-operated mice (Table 2). Interestingly, adrenalectomy attenuated the rise in blood glucose in streptozotocin-injected mice (Table 2). Fasting reduced blood glucose in the ADX-STZ-Fast group below even that of control mice (Table 2). Streptozotocin injection led to reduced body weight and adiposity in sham-operated animals as previously described [5,17] (Table 2), whereas adrenalectomy attenuated the loss of both body weight and adiposity associated with insulin deficiency (Table 2). Streptozotocin injection also led to significant hyperphagia, typical of

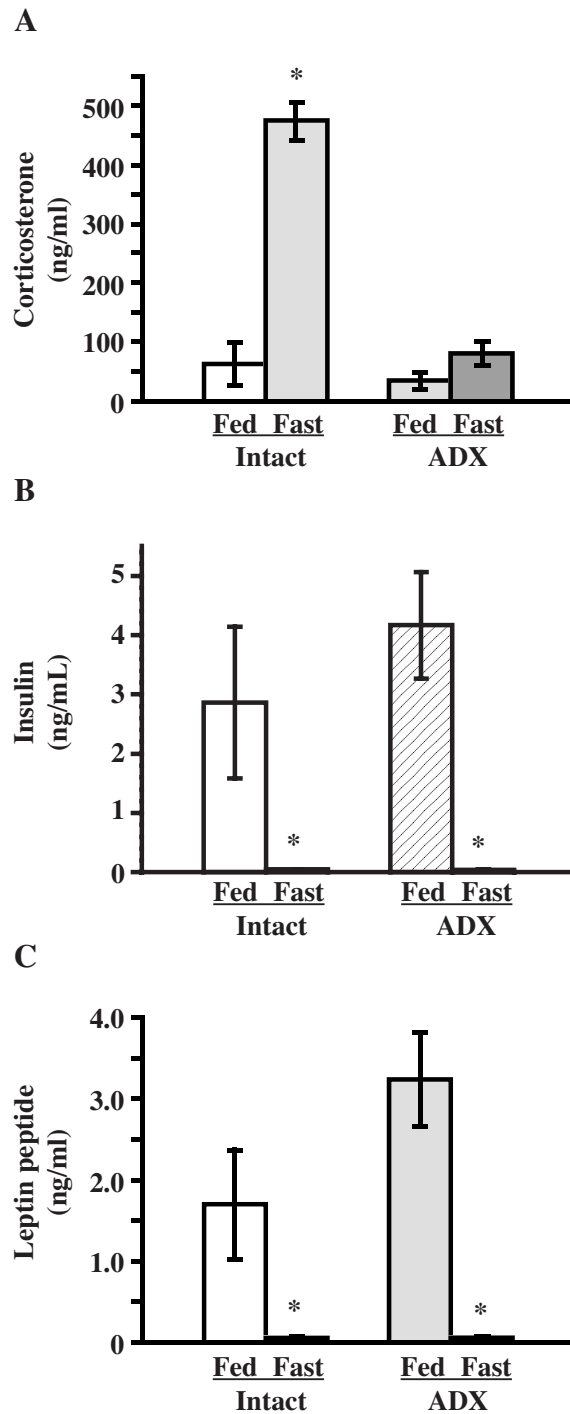


Figure 1

Effects of fasting on blood hormones in intact and adrenalectomized mice. (A) Fasting induces serum corticosterone in intact mice but not in adrenalectomized mice. Fasting decreases both serum insulin (B), and serum leptin (C) to the same levels in intact and adrenalectomized mice. Data are expressed as mean \pm SEM. Groups with different letters are statistically different ($p < 0.05$), reflecting ANOVA followed by Tukey-Kramer post hoc tests comparing every group to every other group. Thus groups with the same letter do not differ from each other at a $p < 0.05$ level.

Table 1: Effects of fasting on body weight, adipose weight and glucose in intact and adrenalectomized mice.

Group	n	Body weight (g)	Adipose weight (g)	Blood glucose (mg/dl)
Intact/Ad lib fed	6	22.8 ± 0.8 ^a	0.14 ± 0.04 ^a	108.8 ± 15.1 ^a
Intact/Fasted	6	16.1 ± 0.4 ^b	0.02 ± 0.002 ^b	60.3 ± 4.3 ^b
ADX/Ad lib fed	10	23.6 ± 0.4 ^a	0.22 ± 0.02 ^a	124.1 ± 6.6 ^a
ADX/Fasted	9	19.5 ± 0.7 ^b	0.04 ± 0.006 ^b	52.6 ± 4.8 ^b

Data are expressed as mean ± SEM. Groups with different letters are statistically different ($p < 0.05$), reflecting ANOVA followed by Tukey-Kramer post hoc tests comparing every group to every other group. Thus groups with the same letter do not differ from each other at a $p < 0.05$ level.

diabetic mice [17] (Table 2), and adrenalectomy also ameliorated this response to insulin deficiency (Table 2).

Serum corticosterone was elevated in the Sham-STZ group and was decreased in both ADX-STZ and ADX-STZ-Fast groups (Fig. 3A). Adrenalectomy did not result in complete elimination of corticosterone but instead resulted in low but detectable levels of serum corticosterone as previously noted [9] and described above. Serum insulin was reduced to the same extent in Sham-STZ, ADX-STZ, and in ADX-STZ-Fast groups, compared to control mice, as would be expected with streptozotocin injection (Fig. 3B). Serum leptin was also reduced to the same levels in all experimental groups compared to control mice (Fig. 3C).

Hypothalamic AGRP mRNA was significantly elevated in sham-operated diabetic (vs. sham-operated euglycemic) mice as expected [5,17] (Fig. 4A). However, adrenalectomy completely blocked the elevation of AGRP mRNA in diabetic mice whether or not they were fasted (Fig. 4A). Hypothalamic POMC mRNA was reduced in diabetic mice as expected [5,17] (Fig. 4B). Adrenalectomy blocked this reduction in POMC mRNA in diabetic mice whether or not they were fasted (Fig. 4B). As with AGRP mRNA, NPY mRNA was elevated in diabetic mice, and adrenalectomy blocked this induction of NPY mRNA in diabetic mice whether or not they were fasted (Fig. 4C). In summary, hypothalamic AGRP, POMC and NPY mRNA all exhibited the expected changes in association with streptozotocin-induced diabetes [5,17] (Fig. 4A,4B,4C), and adrenalectomy largely or completely blocked all these responses to insulin deficiency whether or not the mice were fasted (Fig 4A,4B,4C).

Study 3: Effects of corticosterone implants on hypothalamic gene expression

Implantation of corticosterone implants for 48 hours significantly increased serum corticosterone, compared to placebo-implanted mice, as expected (Fig. 5A). Interestingly, the corticosterone implants also significantly increased serum insulin and leptin (Fig. 5B and 5C). In contrast, the corticosterone implants did not significantly influence body weight or food intake compared to pla-

cebo-implanted mice (body weight of 24.5 ± 0.6 g for the placebo group compared to 24.4 ± 0.6 g for the corticosterone implant group; food intake of 4.3 ± 0.1 g/day for the placebo group compared to 4.1 ± 0.5 g/day for the corticosterone implant group), although epididymal adipose tissue depot was slightly smaller in the corticosterone implant group (0.19 ± 0.01 g for the placebo group compared to 0.15 ± 0.01 g for the corticosterone implant group; $p < 0.05$). Corticosterone implants also had no influence on blood glucose (134.8 ± 10.9 mg/dl for the placebo group compared to 128.8 ± 4.3 mg/dl for the corticosterone implant group).

Implantation of corticosterone implants for 48 hours significantly elevated hypothalamic AGRP expression compared to the placebo group (Fig. 6A). Hypothalamic POMC mRNA was also significantly elevated in the corticosterone implant group (Fig. 6B). Hypothalamic NPY mRNA exhibited a trend toward an increase although the effect was not significant ($p = 0.14$; Fig. 6C). Therefore hypothalamic AGRP and POMC mRNAs were both induced by the glucocorticoid implant, whereas the NPY mRNA was not significantly influenced.

Conclusions

In the present study, adrenalectomy and corticosterone implants were used to address the role of elevated corticosterone in mediating the effects of fasting and insulin deficiency on hypothalamic gene expression, resulting in several novel observations. First, we have demonstrated that adrenalectomy can block the elevation of AGRP mRNA and the reduction in POMC mRNA with fasting, consistent with previous findings by Ponsalle et al. concerning NPY [14], and suggesting that increases in corticosterone mediate fasting-induced changes in melanocortin tone. Second, we have also shown that hypothalamic responses to insulin deficiency require elevation in corticosterone. Third, we have demonstrated using corticosterone implants, that elevated corticosterone is sufficient to enhance hypothalamic AGRP expression, but not to reduce hypothalamic POMC mRNA. Together these results suggest that an elevation of corticosterone is necessary for reduction in melanocortin tone

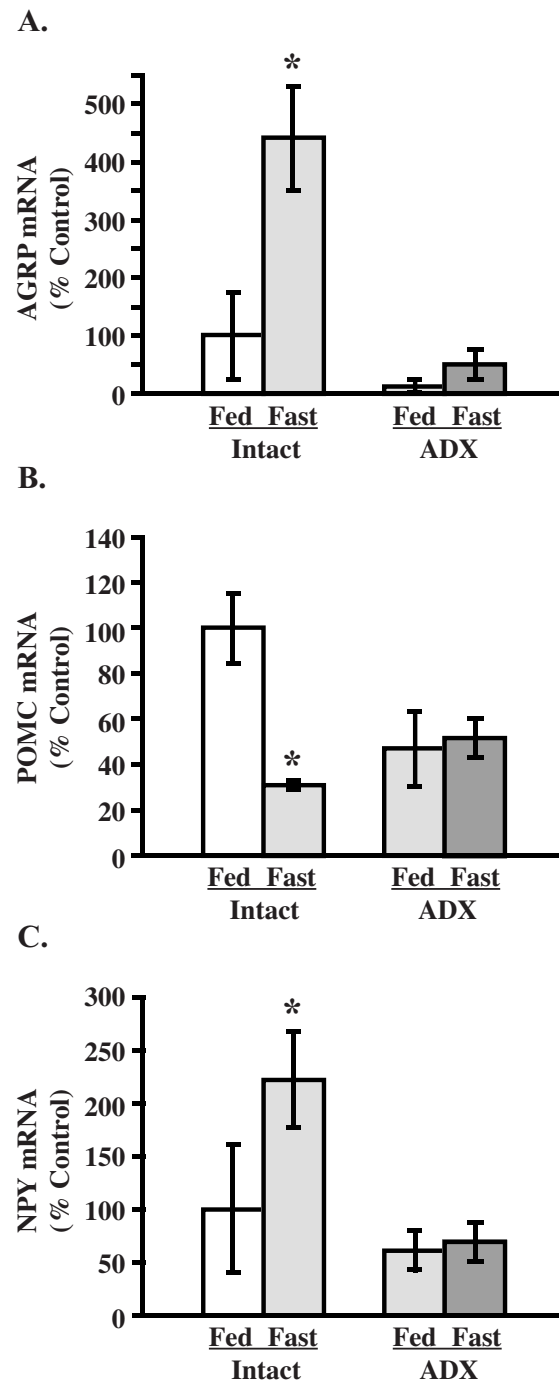


Figure 2

Effects of fasting on hypothalamic gene expression in intact and fasted mice. (A) Hypothalamic AGRP is induced by fasting in intact mice however the fasting associated induction is blocked by adrenalectomy. (B) Fasting reduces hypothalamic POMC mRNA in intact mice but not in adrenalectomized mice. (C) Similar to AGRP, hypothalamic NPY mRNA is induced in intact mice but not in adrenalectomized mice. Data are expressed as mean \pm SEM. Groups with different letters are statistically different ($p < 0.05$), reflecting ANOVA followed by Tukey-Kramer post hoc tests comparing every group to every other group. Thus groups with the same letter do not differ from each other at a $p < 0.05$ level.

Table 2: Effects of adrenalectomy, diabetes and fasting on body weight, adipose weight, food intake and glucose.

Group	n	Body weight (g)	Adipose weight (g)	Food intake (g/day)	Glucose (mg/dl)
Control	9	24.3 ± 0.5 ^a	0.20 ± 0.01 ^a	4.3 ± 0.1 ^a	138.1 ± 6.5 ^a
Sham/STZ	8	17.8 ± 0.8 ^b	0.02 ± 0.01 ^b	6.1 ± 0.6 ^b	578.5 ± 17.1 ^b
ADX/STZ	11	21.5 ± 0.9 ^c	0.07 ± 0.02 ^c	4.8 ± 0.1 ^c	368.8 ± 20.4 ^c
ADX/STZ/Fast	5	19.0 ± 0.3 ^b	0.03 ± 0.01 ^c	N/A	44.8 ± 13.3 ^d

Data are expressed as mean ± SEM. Groups with different letters are statistically different ($p < 0.05$), reflecting ANOVA followed by Tukey-Kramer post hoc tests comparing every group to every other group. Thus groups with the same letter do not differ from each other at a $p < 0.05$ level.

during fasting and with diabetes and is sufficient to induce hypothalamic AGRP mRNA but not to reduce POMC expression.

Although a reduction in plasma leptin plays an important role in mediating neuroendocrine responses to fasting [6] and diabetes [17], we have previously demonstrated that fasting can regulate hypothalamic melanocortin tone independently of both leptin and insulin [5]. The present studies suggest that corticosterone may play a role in mediating these leptin-independent effects. Fasting was associated with elevated corticosterone (Fig. 1A) and reduced insulin and leptin (Fig. 1B and 1C) as well as melanocortin tone (Fig. 2A and 2B) as expected. However, preventing the elevation of corticosterone completely blocked the effect of fasting on hypothalamic AGRP and POMC mRNA (Fig. 2A and 2B), without preventing effects of fasting on plasma leptin or insulin. Adrenalectomy also blocked the effects of fasting on hypothalamic NPY mRNA (Fig. 2C), confirming the findings of Ponsalle et al. [14]. These data suggest that leptin's effect to reverse effects of fasting on melanocortin tone may require and be mediated by a reduction in glucocorticoids.

Glucocorticoids were also elevated, as expected, in insulin-deficient diabetes (Fig. 3A), and serum insulin, leptin and melanocortin tone were also reduced in this condition (Fig. 3B and 3C, 4A and 4B). As with fasting, the reduction in plasma leptin could largely be a reflection of the reduction in adipose weight, as well as plasma insulin. In turn, as with fasting, the reduction of plasma leptin could be the cause of the elevation of hypothalamic AGRP and NPY mRNA, as well as the reduction of hypothalamic POMC mRNA. On the other hand, blocking the rise in corticosterone by adrenalectomy prevented the reduction in hypothalamic POMC mRNA and the induction of AGRP mRNA, without reversing either the reduced leptin or the reduced adiposity, characteristic of insulin-deficient diabetic mice (Fig. 4A and 4B). These data suggest that reversal of effects of insulin deficiency by leptin [7] may require and be mediated by a reduction in glucocorticoids. We had previously shown that fasting could regulate hypothalamic gene expression in insulin-deficient

mice even though in these mice fasting did not influence plasma leptin or insulin [5]. Since adrenalectomy blocked the fasting-induced reduction in melanocortin tone in diabetic mice (as it had in euglycemic mice in the first study), these results suggest that effects of fasting on melanocortin tone in diabetic mice may be mediated at least in part by a rise in plasma corticosterone (itself possibly secondary to the reduction in plasma leptin).

Implanting corticosterone implants for 48 hours led to an elevation in both hypothalamic AGRP and POMC mRNA (Fig. 6B). Since adrenalectomy blocked the elevation of AGRP mRNA by fasting and insulin deficiency, elevation of corticosterone would be expected to increase hypothalamic AGRP mRNA, as was indeed observed. However, the corticosterone implants produced the unexpected effect of elevating hypothalamic POMC mRNA, a rare if not unique circumstance in which hypothalamic AGRP and POMC mRNA are regulated in the same direction. The basis of this unexpected phenomenon is not yet clear. However, since leptin and insulin both stimulate hypothalamic POMC [18], the elevation of insulin and leptin in the corticosterone-implanted mice (Fig. 5B and 5C), the former possibly mediated by glucocorticoid-induced insulin resistance and the latter possibly mediated by the resulting hyperinsulinemia, might mediate the observed effect of corticosterone implants on hypothalamic POMC in these implanted mice. On the other hand, leptin inhibits hypothalamic AGRP mRNA [4], yet AGRP mRNA was nevertheless elevated in the face of elevated insulin and leptin. These data suggest that in fasting and insulin-deficient diabetes, as in leptin-deficient *ob/ob* mice [9,19], glucocorticoids dominate leptin and insulin levels in determining AGRP expression, whereas insulin and leptin dominate glucocorticoids in determining hypothalamic POMC expression. These data are also consistent with the observation that in streptozotocin-induced diabetic rats, replacement of insulin is not sufficient to normalize elevated serum corticosterone or the elevated hypothalamic AGRP mRNA while it does partially normalize plasma leptin and hypothalamic POMC mRNA [17].

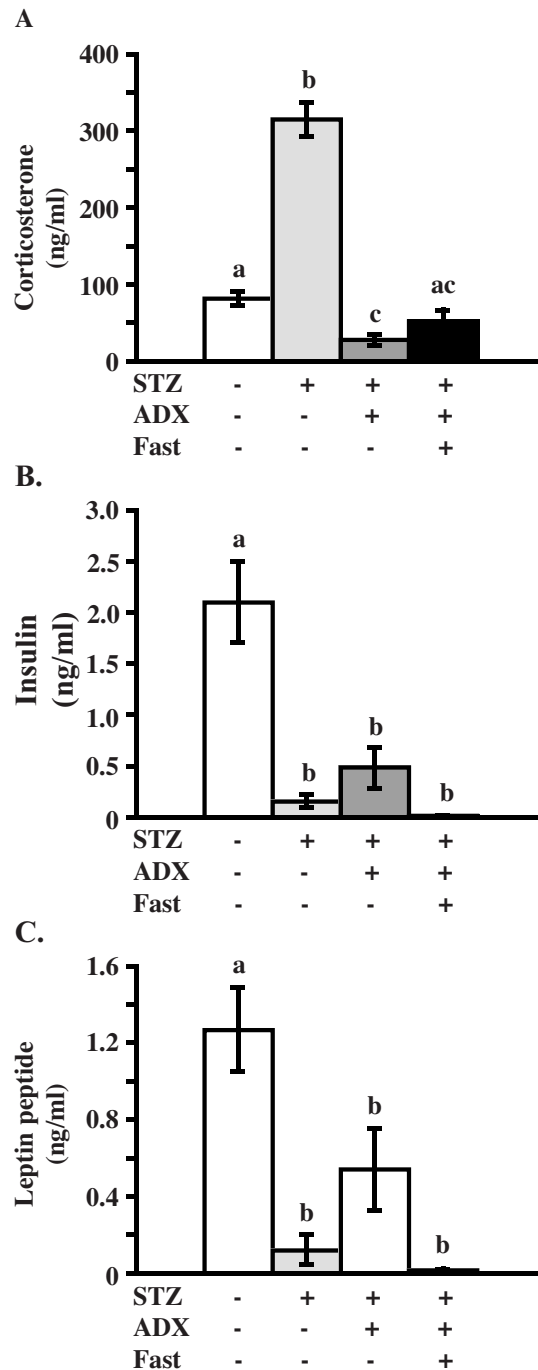


Figure 3

Effects of adrenalectomy, streptozotocin injection and fasting on levels of blood hormones. (A) Streptozotocin injection elevates levels of serum corticosterone however adrenalectomy prevents the induction associated with streptozotocin. Both serum insulin (B), and serum leptin (C) are reduced to the same extent in all experimental conditions. Data are expressed as mean \pm SEM. Groups with different letters are statistically different ($p < 0.05$), reflecting ANOVA followed by Tukey-Kramer post hoc tests comparing every group to every other group. Thus groups with the same letter do not differ from each other at a $p < 0.05$ level.

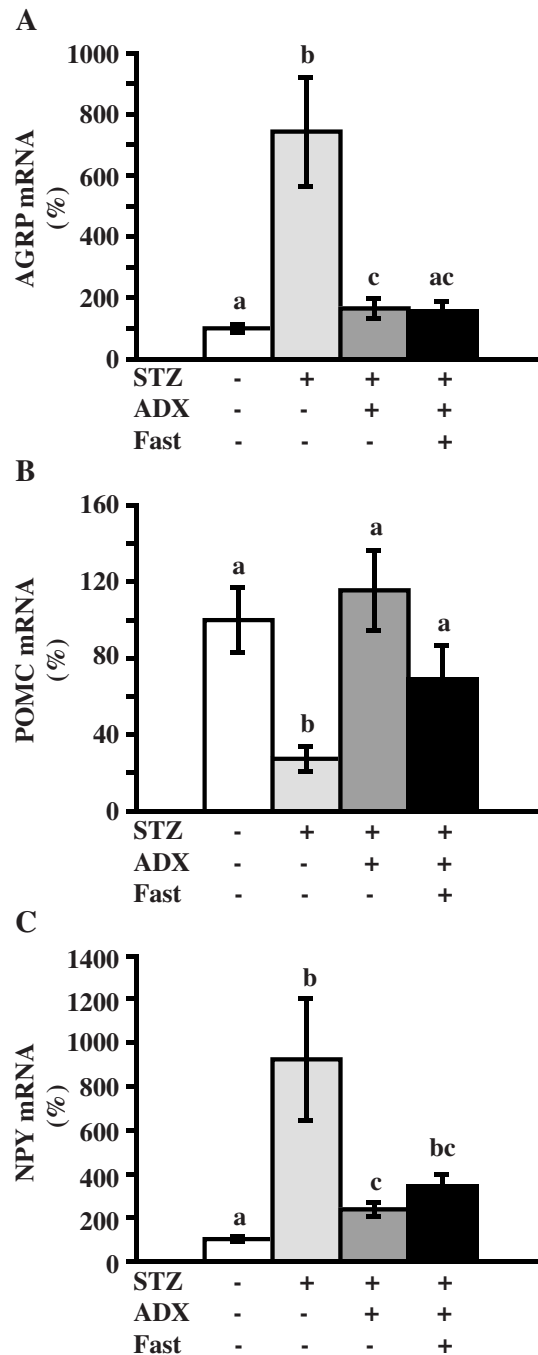


Figure 4
Effects of adrenalectomy, streptozotocin injection and fasting on levels of hypothalamic gene expression. (A) Hypothalamic AGRP mRNA is induced by streptozotocin injection, however the induction is partially blocked by adrenalectomy. (B) Hypothalamic POMC mRNA is reduced by streptozotocin injection, but adrenalectomy completely blocks the reduction associated with streptozotocin. (C) Similar to AGRP, NPY mRNA is induced by streptozotocin injection and partially blocked by adrenalectomy. Data are expressed as mean percentage \pm SEM of the control group. Data are expressed as mean \pm SEM. Groups with different letters are statistically different ($p < 0.05$), reflecting ANOVA followed by Tukey-Kramer post hoc tests comparing every group to every other group. Thus groups with the same letter do not differ from each other at a $p < 0.05$ level.

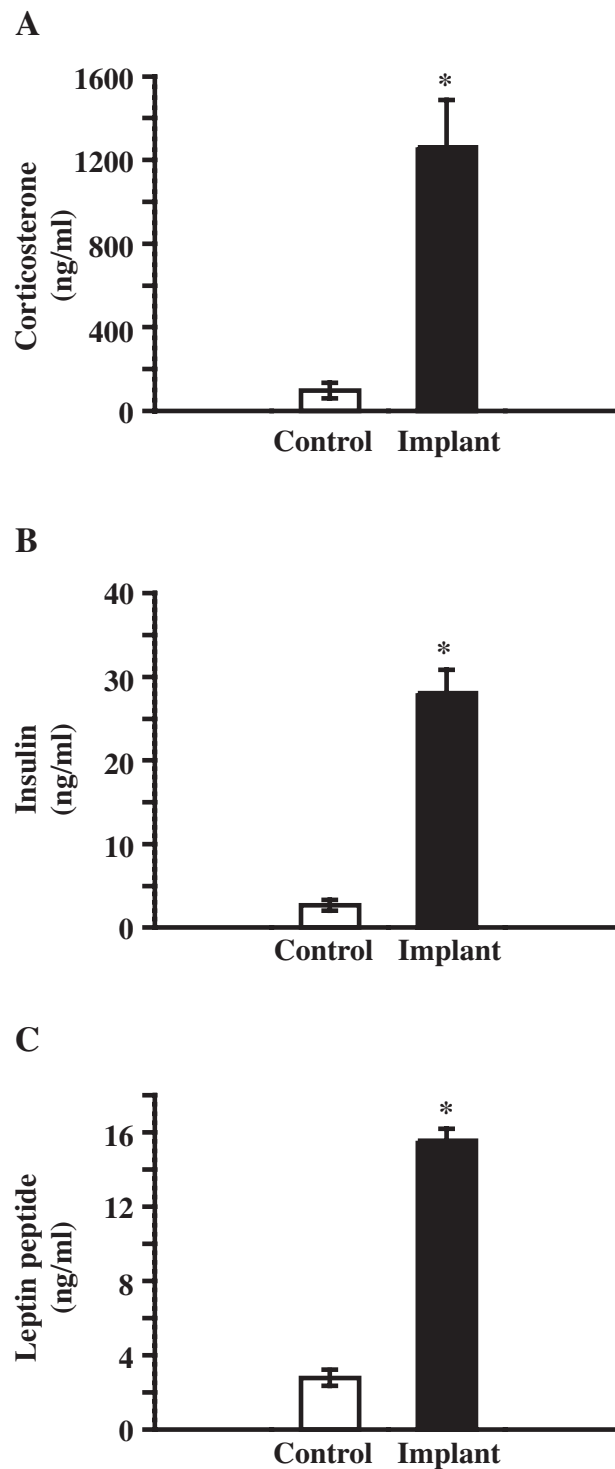


Figure 5
Effects of corticosterone implants on levels of blood hormones. Corticosterone implants elevates levels of serum corticosterone (A), insulin (B), and leptin (C). Statistical significance was determined by unpaired student's t-test ($p < 0.05$) and is denoted by *.

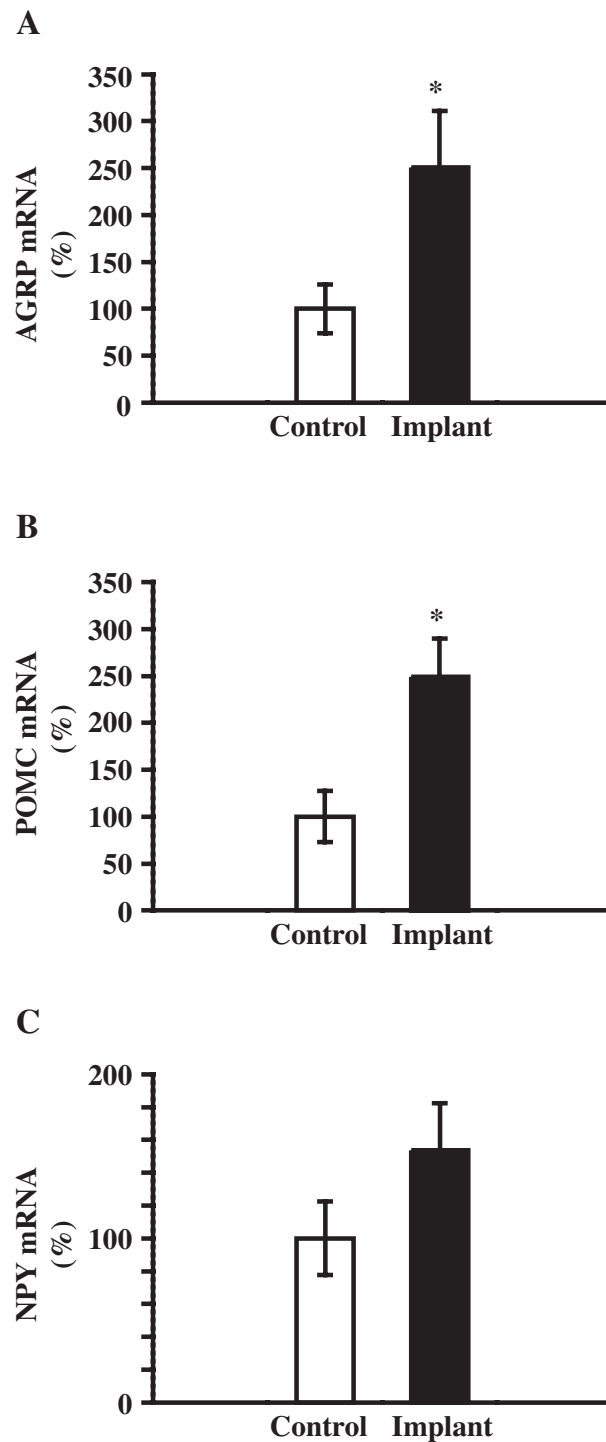


Figure 6
Effects of corticosterone implants on hypothalamic gene expression. Corticosterone implants induces both hypothalamic AGRP (A) and POMC (B) mRNA. (C) NPY mRNA is also elevated by corticosterone implants but the effect did not reach statistical significance. Data are expressed as a mean percentage \pm SEM of placebo implant group. Statistical significance was determined by unpaired student's t-test ($p < 0.05$) and is denoted by *.

After the present paper was submitted for review, we became aware of a recent study by Savontaus et al. examining the role of glucocorticoids in the regulation of hypothalamic gene expression in rats [20], using a roughly similar experimental design as in the present study. In several respects the conclusions from this study are similar to the present study (for example, corticosterone implants increased hypothalamic AGRP and POMC mRNA, and adrenalectomy blocked the effect of fasting on hypothalamic POMC mRNA). However, in one key respect the results reported by Savontaus et al. differed from those in the present paper: adrenalectomy failed to block the effects of fasting on hypothalamic AGRP and NPY, even though corticosterone implants were observed to induce AGRP mRNA. Since we observed that adrenalectomy also blocks the effects of diabetes on hypothalamic gene expression in the present study, we suspect the difference between the present study and that of Savontaus et al. is probably due to different regulatory requirements in mice and rats. For example, it is clear that the regulation of AGRP differs in rats and mice, since in mice hypothalamic AGRP mRNA is elevated by a deficiency in leptin receptor function [4] but in rats this is not true [21]. However, hypothalamic POMC is regulated similarly in rats and mice, and this is probably the more important element regulating metabolic phenotype.

Taken together, these data suggest that elevated glucocorticoids are sufficient to drive the increase in hypothalamic AGRP mRNA during fasting and diabetes but not the reduction in POMC mRNA. Interestingly, at least with respect to food intake and body weight over this 48-hour period, the elevation of POMC appears to compensate for the elevation in AGRP to produce no net functional effect on body weight homeostasis in the presence of significantly elevated plasma corticosterone. After more chronic exposure, this delicate balance could plausibly become disrupted, leading to glucocorticoid-mediated development of the metabolic syndrome [10]. Thus interactions between insulin, leptin, and glucocorticoids, and the neuropeptide responses they generate within the melanocortin system, allow complex metabolic responses to nutritional status but may also allow the development of metabolic pathologies.

Methods

Animals

The appropriate Institutional Animal Review Board had approved all studies.

To study the effect of adrenalectomy on fasting-induced changes in hypothalamic gene expression (Study 1), two month-old male C57Bl/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were individually housed with free access to food and water under

12:12 h light-dark cycle (lights on at 07:00 h). Of the 31 mice, 19 mice were adrenalectomized and the rest underwent a sham operation. Adrenalectomy was performed, as described previously, by bilateral flank incision under anesthesia (2.5% 2,2,2-tribromoethanol, 0.015–0.017 ml/g body weight, i.p.) [9]. Sham adrenalectomy entailed the same procedure as adrenalectomy, except that the adrenal glands were grasped but not removed. After the surgery all mice were given one injection of dexamethasone (45 mg/kg b.w. i.p.) to facilitate recovery from surgery. The drinking water was replaced with normal saline (0.9% NaCl) to compensate for the loss of mineralocorticoids. Body weight was monitored for one week following the operation at which time it was verified that the animals had recovered initial body weight as expected. All mice were placed in fresh cages with new bedding, and food was removed from half of the adrenalectomized mice and half of the sham operated mice for 48 hours. This resulted in four groups; sham ad lib fed ($n = 6$), sham 48 hour fast ($n = 6$), adrenalectomized ad lib fed ($n = 10$) and adrenalectomized 48 hour fast ($n = 9$).

To study the effects of adrenalectomy, insulin deficiency and fasting (Study 2) 33 male C57Bl/6J mice were obtained at 2 months of age from The Jackson Laboratory (Bar Harbor, ME) and were individually housed with free access to feed and water under 12:12 h light-dark cycle as described above. Mice were treated with daily injections of dexamethasone (45 mg/kg body weight i.p.) for three weeks during which time they were either adrenalectomized or sham operated as described above. Half the sham-operated and all the adrenalectomized mice were injected with streptozotocin 10 days after the surgery (160 mg/kg body weight; freshly purchased from Sigma, St. Louis, MO and dissolved in 25 mM Citric Acid Buffer pH = 4.5) while the rest received buffer alone by tail vein injection 10 days after surgery. Dexamethasone treatment continued until four days after the streptozotocin injection. Mice were then monitored for two weeks without the dexamethasone injection at which point half the adrenalectomized-STZ treated group were fasted while the rest of the mice were ad lib fed. This resulted in four separate groups: sham-operated-buffer-injected-ad lib fed (control; $n = 9$), sham-operated-streptozotocin-injected-ad lib fed (sham-STZ; $n = 8$), adrenalectomized-streptozotocin-injected-ad lib fed (ADX-STZ; $n = 11$) and adrenalectomized-streptozotocin-injected-fasted mice (ADX-STZ-Fast; $n = 5$).

To study the direct effects of corticosterone (Study 3), a corticosterone implant study was conducted with 16 C57Bl/6J retired breeders also obtained from The Jackson Laboratories (Bar Harbor, ME). As with the first and second studies, mice were individually housed with free access to food and water under 12:12 h light-dark cycle

Table 3: Brief anesthesia with carbon dioxide before sacrifice does not increase plasma corticosterone.

Method of Sacrifice		
Treatment groups	n	Corticosterone (ng/ml)
Mice with CO ₂	8	85.25 ± 12.47
Mice without CO ₂	5	96.4 ± 23.22
Rat with CO ₂	5	106 ± 30.79
Rat without CO ₂	5	203 ± 79.12

Mice or rats were either sacrificed with carbon dioxide as described in the Methods section, or decapitated immediately after removing from cage. Data are expressed as mean ± SEM. By either paired or unpaired t-test, there was no effect of method of sacrifice ($p > 0.05$).

(lights on at 07:00 h). Mice were divided into two groups: one received corticosterone implants of 30 mg (Innovative Research of America, Sarasota, FL) ($n = 8$), while the other mice received placebo implants (Innovative Research of America, Sarasota, FL) ($n = 8$). Body weight and food intake were monitored for 48 hours after which time all the mice were sacrificed.

For all studies, mice were sacrificed following a balanced design towards the end of the light period (between 17:00 and 18:30 h). Mice were decapitated after a brief (approximately 45 second) exposure to carbon dioxide; we have verified in pilot studies that the brief exposure to carbon dioxide does not significantly affect the plasma corticosterone levels under these conditions (Table 3). Adrenalectomy was verified in each individual by visual inspection to assure that no visible traces of the adrenal glands were observed. Brains for Northern blot analysis were quickly removed and the hypothalamus was dissected out, frozen on dry ice, and stored at -70°C until use. Epididymal white adipose tissue was also removed, weighed, and frozen on dry ice, and stored at -70°C until use.

Blood chemistry

Blood glucose was measured by a Lifescan One-Touch II glucose meter (Johnson & Johnson, Mountain View, CA). Serum insulin and leptin were assayed by an enzyme-linked immunosorbent assay (ELISA) with commercial kits (CRYSTAL CHEM INC., Chicago, IL) and serum corticosterone was assayed by radioimmunoassay (RIA) with commercial kits (ICN Biomedicals Inc., Costa Mesa CA).

RNA analysis

Total RNA was extracted from tissue using TRIzol (GIBCO BRL, Gaithersburg, MD). Five micrograms of total RNA from hypothalamus was subjected to Northern blot analysis, as described previously, to measure POMC, AGRP and NPY mRNA [3–5,9]. Seven micrograms of total RNA from adipose tissue was also subjected to Northern blot analysis to measure leptin mRNA. The blots were probed

with single-stranded internally labeled DNA probes as described previously [16]. To monitor RNA loading, membranes were re-probed and hybridized with a ^{32}P -labeled probe encoding 18S ribosomal RNA. The total integrated densities of hybridization signals were determined by phosphorimager (STORM 860, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

For the first study, statistical analysis entailed a two-way analysis of variance (ANOVA) followed, when indicated by appropriate p-values ($p < 0.05$), by Tukey-Kramer post-hoc test, using the JMP statistical package implemented on the Macintosh operating system. For the second study a one-way analysis of variance was performed and followed, when indicated by appropriate p-values ($p < 0.05$), by Tukey-Kramer post-hoc test using the JMP statistical package. For the third study unpaired t-tests were carried out using the JMP statistical package. A p-value of less than 0.05 was considered significant.

Authors' contributions

Author 1 (HM) carried out the animal work, the mRNA analysis, and the statistical analysis and drafted the manuscript. Author 2 (TMM) assisted in animal surgeries. Author 3 (FI) carried out the tail vein injections in the 2nd study. Author 4 (JB) carried out all the hormones assays. Author 5 (JHS) contributed to the hormone assays. Author 6 (CMV) originally conceived of the study, participated in its design and coordination and edited the manuscript.

All authors read and approved the final manuscript.

Acknowledgments

These studies were supported by the NIH (NS41183-01 and AG19934-01).

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