Effects of Adrenalectomy and Naloxone Administration on Food Intake, Plasma Insulin and Glucose in Genetically Obese Mice

by

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EFFECTS OF ADRENALECTOMY AND NALOXONE ADMINISTRATION ON FOOD INTAKE, PLASMA INSULIN AND GLUCOSE IN GENETICALLY OBESE MICE

BY

KATHLEEN MARIE FELDKIRCHER

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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Abstract

Genetically obese (ob/ob) mice have higher corticosterone, adrenocorticotrophin (ACTH), and β-endorphin levels than lean (+/?) mice. Removal of circulating corticosterone by adrenalectomy (ADX) ameliorates many of the symptoms characteristic of the obese syndrome; however, body weight, plasma insulin, and plasma glucose may not reach lean (+/?) levels. Moreover, ADX exacerbates even further the elevated ACTH and β-endorphin levels. Because opiate receptor antagonists have been found to selectively decrease food intake and suppress plasma insulin secretion, it was hypothesized that these responses might be enhanced in ob/ob mice that were given the antagonist naloxone (NLX) injections. Male ob/ob (n=50) and +/? (n=55) mice were adrenalectomized or sham-adrenalectomized at approximately 5 weeks of age. Two weeks following surgery mice were food deprived for 6 h and then weighed and injected intraperitoneally with either 0.15 M saline, 0.5 mg/Kg BW NLX, or 2.0 mg/Kg BW NLX. Food intake measurements were recorded every 30 min for 2-h. Two days following the re-feeding test, mice were food deprived for 6 h, weighed and injected with the appropriate dose of either saline or NLX. Thirty min after injection the animals were sacrificed and plasma extracted for later determination of corticosterone, insulin, and glucose. Results indicated that ADX had an effect on body weight and food intake in obese but not in lean mice prior to the re-feeding test. Naloxone decreased food consumption in a dose-dependent manner in both obese and lean mice during the re-feeding test. Naloxone's anorectic effects were longer-lasting in obese mice than in lean mice. Plasma insulin and glucose concentrations were normalized to lean control values in obese mice by surgery alone. Naloxone did not exert an additional effect on these plasma levels in ADX obese mice. These findings support the permissive roles of both corticosterone and β-endorphin in the control of feeding in ob/ob mice.

Introduction

Adults living in Canada and the United States have higher rates of obesity compared to those residing in the United Kingdom, Australia or other European countries (Garrow, 1981). Despite today's preoccupation with dieting, the prevalence of obesity is rising, with 27% of women and 24% of men (i.e., more than 34 million adults) in the United States being assessed as obese (Kuczmarski, 1992; Matz, 1987). A criterion of 20% or more above desirable weight according to the Metropolitan Height/Weight Tables or a body mass index (weight in kilograms divided by height in meters squared) greater than 27.3 for women or 27.8 for men are typically used to judge obesity (Kuczmarski, 1992; Matz, 1987). Storage of energy in the form of fat is often associated with an increased risk of hypertension, cardiovascular disease, non-insulin-dependent diabetes mellitus, hypercholesterolaemia, reproductive problems, and early mortality (Pi-Sunyer, 1991; Vital and Health Statistics, 1983). In addition to these potential medical complications, obese individuals may be at a psychosocial disadvantage. Staffieri (1967) found that children as young as 6 years of age used pejorative words (e. g., stupid, ugly, dirty, lazy, cheats, and lies) to characterize silhouettes of an obese child.

Moreover, obese adults have been denied jobs, promotions, educational opportunities, and even the right to adopt a child unless they lose weight (Stunkard & Wadden, 1992; Wadden & Stunkard, 1985).

The causes and permissive factors associated with obesity are still unknown. Higher rates of obesity may be partly accounted for by increased dietary fat consumption, changes in eating habits, greater availability of a wider variety of palatable foods, decreased daily energy expenditure, and a multiplication of stressors; all of which might be linked to societal advances in technology (Brownell & Wadden, 1992). In addition to these social contributors, studies have demonstrated that biological factors are also involved in the etiology of obesity. Body weight, body fat distribution, resting metabolic rate, fat cell number, as well as psychological status are believed to be influenced by genetic components (Bouchard, et al., 1990; Bouchard, et al., 1989; Garner & Wooley, 1992; Marcus, et al., 1990; Stunkard, Harris, Pedersen, & McClearn, 1990; Wadden & Bell, 1990). It seems likely that obesity is multiply determined by genetic, metabolic, endocrine and psychosocial factors.

Because obesity is a leading public health problem, Brownell & Wadden (1992) contend that future research should be directed towards investigating the etiology of weight gain, which might then lead to promising advances in the treatment of obesity. The present study addressed this issue using an animal model of human obesity and human type II diabetes, the obese-hyperglycemic (C57BL/6J, ob/ob) mouse.

Genetically Transmitted Obesity: The Obese (ob/ob) Mouse

A first step toward identifying effective solutions to obesity is the identification of major causes and permissive factors associated with obesity. One approach to this issue is through the use of different animal models of obesity (Sclafani, 1984). One model which has been widely studied is the Bar Harbor genetically obese (ob/ob) mouse. In this model, obesity is inherited as an autosomal recessive mutation (gene symbol ob, on Chromosome 6, linkage group XI), and obesity is visually recognizable after weaning (Ingalls, Dickie, & Snell, 1950). Although the primary defect that produces phenotypic alterations is still under investigation (Johnson, Greenwood, Horwitz, & Stern, 1991), numerous biobehavioral characteristics have been associated with the development of the obese syndrome in this strain. These include hyperphagia, gross adiposity, hypoactivity, hyperglycemia, hyperinsulinemia, hypothermia, impaired oxygen consumption, reduced muscle mass, impaired fertility, and endocrine abnormalities (Bray & York, 1979). Additionally, obese mice have altered neuroanatomical organization and neurotransmitter functions (Bereiter & Jeanrenaud, 1979; Lorden, Oltmans, & Margules, 1976; Margules, Moisset, Lewis, Shibuya, & Pert, 1978; Schouten, Jenks, & Van der Kroon, 1982).

Although the obese phenotype is not detectable by visual inspection until Postnatal Days 25-28, characteristics of the obese genotype are present early in development. Two of the earliest biological abnormalities found in preobese mice are hypothermia and decreased oxygen consumption. Decreased core temperature has been observed as early as 10-14 days of age for preobese mice subjected to either cold exposure (12-14 °C) or normal laboratory temperatures (21-25 °C), and a 1.5-2.5 °C difference in core temperature has been observed between adult obese and lean mice housed under similar laboratory conditions (Boissonneault, Hornshuh, Romsos, & Leveille, 1976; Smith & Romsos, 1984; Trayhurn & James, 1978). The lowered core temperature suggests that the metabolic rates in obese mice may be lower than in lean animals. These observations are consistent with those of Van der Kroon, van Vroonhoven and Douglas (1977), who found oxygen consumption reduced in preobese mice by 5 days postpartum.

Food intake and growth patterns have also been investigated in obese and lean mice. Hyperphagia increases dramatically during the dynamic phase of obesity (1st - 6th month) in the ob/ob (Bray & York, 1979), with adult obese mice having a 44% higher food intake than lean mice (Joosten & Van der Kroon, 1974). Obesity in the ob/ob mouse may only, in part, be the result of hyperphagia because pair-feeding obese with lean animals does not prevent the development of obesity. Obese mice utilize dietary energy more efficiently than their lean littermates, with reduced energy expenditure for thermogenesis providing a partial explanation for the increased energy efficiency of obese mice (Smith & Romsos, 1984; Thurlby & Trayhurn, 1979).

In addition to thermogenic and behavioral abnormalities, ob/obs have a wide range of endocrine defects. For example, between Postnatal Days 17-21, ob/obs serum insulin increases and glucose decreases (hypoglycemia) (Dubuc, 1977). The increase in insulin results from both hypertrophy and hyperplasia of the beta cells of the pancreas (Bray & York, 1979). Although the underlying mechanism of hyperinsulinemia remains unclear, Beloff-Chain (1979) has suggested that there may be an excessive production of pituitary factors that stimulate insulin secretion in the obese mouse. While the serum insulin levels increase, there is a transition from hypoglycemia to hyperglycemia.

Obese mice have lower pituitary prolactin (PRL) (Larson, Sinha, & Vanderlaan, 1976; Sinha, Salocks, & Vanderlaan, 1975) and luteinizing hormone (LH) levels (Swerdloff, Batt, & Bray, 1976) and elevated growth hormone (GH) and follicle-stimulating hormone (FSH) levels compared to lean controls (Naeser, 1974). On the other hand, serum levels of PRL, LH, GH, and FSH are lower in obese mice compared to lean littermates (Sinha et al., 1975; Swerdloff et al., 1976). The discrepancy in pituitary and serum levels of these hormones suggests that the ob/ob might have deficits in the synthesis and release of pituitary hormones (Lorden & Oltmans, 1977). Serum corticosterone is elevated around Day 17 (Dubuc, 1977; Naeser, 1974) and remains significantly elevated in obese mice throughout their life span and across diurnal fluctuations compared with lean controls (Saito & Bray, 1983). Elevated adrenocorticotrophic hormone (ACTH) levels have also been found in the ob/ob (Edwardson & Hough, 1975).

The Adrenal Gland

The larger adrenal glands (Naeser, 1975) and higher circulating levels of corticosterone in ob/ob's compared to lean controls (Dubuc, 1977; Naeser, 1974), suggests that the ob/ob's hyperadrenocorticism may play a major role in the development and/or maintenance of its obesity. Several lines of evidence have linked adrenal cortical hormones to feeding behavior, insulin secretion, and obesity. First, hyperphagic rats with ventromedial hypothalamic (VMH) lesions and genetically obese rats (fa/fa) and mice (ob/ob) have exaggerated basal corticosterone levels. Increasing adrenal cortical activity by implanting ACTH-secreting tumors produces hyperphagia, hyperinsulinemia, and obesity in lean mice (Hausberger, 1961). Similarly, administering corticosteroid to patients frequently leads to rapid gain in body weight and the development of obesity (Royal College of Physicians, 1983).

Second, bilateral adrenalectomy ameliorates certain components of energy imbalance observed in rodent models of obesity. For example, adrenalectomy of VMH-damaged animals and in genetically obese rodents reduces their hyperphagia which, in turn, suppresses their rapid rate of body weight gain (e.g., Bruce, King, Phelps, & Veitia, 1982; Debons, Tse, Zurek, Abrahamsen, & Maayan, 1986; Toyukama & Himms-Hagen, 1989; Vander Tuig, Ohshirna, Yoshida, Romsos, & Bray, 1984). Adrenalectomy also lowers body energy density (kcal/g carcass) in obese mice more than could be attributed to decreased food intake (Vander Tuig, et al., 1984). Thus, the ob/ob's high circulating levels of glucocorticoids might serve to sustain their hyperphagia and lower energy expenditure.

Third, there is some indication that the effects of adrenalectomy can be abolished with glucocorticoid replacement therapy. Bruce et al. (1982) found that corticosterone replacement in adrenalectomized, VMH-lesioned animals markedly potentiated their rate of weight gain, while no replacement was followed by weight loss. Similarly, corticosterone replacement therapy in adrenalectomized fa/fa rats and ob/ob mice increased food intake, body weight, and serum insulin (Castonguay, Dallman, & Stern, 1986; Freedman, Horwitz, & Stern, 1986; Tokuyama & Himms-Hagen, 1987). These data suggest that corticosterone may contribute to the maintenance of experimentally and genetically transmitted obesity in rodents.

Although adrenalectomy is the only surgical/physiological manipulation identified thus far that will normalize most of the components of energy balance and prevent the development of obesity in ob/ob mice, its effectiveness appears to be diet-dependent. Unlike adrenalectomized ob/ob mice fed a pelleted stock diet, animals fed a semipurified high-fat diet (Grogan, Kim, & Romsos, 1987) or a glucose-based semipurified diet (Warwick & Romsos, 1988) still exhibited the full obesity syndrome.

To briefly summarize, many abnormalities of the _ob/ob_ are partially ameliorated by adrenalectomy and reinstated by chronic treatment with glucocorticoids. Interestingly, although plasma insulin levels in adrenalectomized obese mice approach lean control values, they remain slightly elevated. The possibility exists that the obesity in the adrenalectomized _ob/ob_ is in part attributable to the remaining moderate hyperinsulinemia coupled with reduced energy expenditure due to persistent thermoregulation at a lower than normal body temperature (Holt & York, 1984; Saito & Bray, 1984). The persistent hyperinsulinemia may reflect the expected high concentrations of ACTH and β-endorphin, an endogenous opiate, in adrenalectomized mice (Tokuyama & Himms-Hagen, 1989).

Endogenous Opiates

The discovery of stereospecific opiate receptors that mediate opiate activity was followed by the identification of the endogenous opioid peptides (Hughes, 1975). The first report of the presence of two pentapeptides in the brain (i.e., leucine (leu)- and methionine (met)-enkephalin) with opiate action on smooth muscle launched numerous investigations on the physiological role of endogenous opioids (Hughes, Smith, Kosterlitz, Morgan, & Morris, 1975). Margules (1979) has speculated that an endogenous opioid-mediated regulatory system (endorphinergic system) and a system antagonistic to its action (endoloxonergic system) conceptually can be considered subdivisions of the autonomic nervous system. He contended that the endorphinergic division employs endogenous opioids to increase the influx of energy and decrease its efflux; whereas, the endoloxonergic division employs endogenous naloxone-like substances to decrease the influx of energy and to increase its expenditure. Because β-endorphin stimulates feeding behavior when administered peripherally or centrally and because genetically obese mice and rats display both hyperphagia and elevated pituitary and plasma β-endorphin levels, Margules proposed that the overeating of these rodents is a preparation for impending famine that causes obesity. When famine is expected, the organism will be stimulated to build up energy stores by increasing its food intake. Pre-famine feeding is associated with hyperinsulinemia that is stimulated by an increase of β-endorphin and ACTH from the anterior pituitary gland. The β-endorphin release also reduces overall energy expenditure by reducing thyrotrophin release.

In support of Margules' theory, strong evidence has linked endorphins to feeding behavior and obesity.

(a) For example, in satiated rats microinjections of β-endorphin directly into either the paraventricular nucleus (PVN) (Leibowitz & Hor, 1980) or the ventromedial nucleus of the hypothalamus (Grandison & Guidotti, 1977) elicits food consumption. Such enhancement of food intake by intracerebral β-endorphin may reflect its effect on regional opiate receptors. In this regard, Morley, Levine, Gosnell, and Billington (1984) have provided evidence for a β-endorphin-epsilon receptor system in the PVN, which modulates food intake.

(b) Genetically obese rodents have elevated pituitary, brain, and plasma β-endorphin levels (Garthwaite, Martinson, Tseng, Hagen, & Menahan, 1980; Govoni & Yang, 1981; Khawaja, Bailey, & Green, 1989; Khawaja, Chattopadhyay, & Green, 1991; Margules, Moisset, Lewis, Shibuya, & Pert, 1978; Morley, Levine, Yim, & Lowy, 1983; Recant, Voyles, Luciano, & Pert, 1980; Recant, Voyles, Wade, Awoke, & Bhathena, 1983; Rossier, Rogers, Shibasaki, Guillemin, & Bloom, 1979; Timmers, Voyles, Zalenski, Wilkins, & Recant, 1986). In addition, Davis, Lowy, Yim, Lamb, and Malven (1983) observed plasma β-endorphin levels elevated in rats during conditions that can induce opiate-related hyperphagias (i.e., 2-deoxy-D-glucose, food deprivation, and darkness), thereby demonstrating that a peripheral component may be physiologically relevant to opiate-induced feeding (Yim & Lowy, 1984).

(c) Opiate antagonists (such as naloxone and naltrexone) suppress spontaneous food intake and weight gain in rats (Brands, Thornhill, Hirst, & Gowdy, 1979) and food intake in food-deprived rats and mice (Brown & Holtzman, 1979). Naloxone, a highly specific antagonist at μ-opiate receptors, in 1.0-10.0 mg/kg body weight (BW) doses, reduced food consumption in food-deprived rats (Holtzman, 1974). In later work, Holtzman (1979) showed that 0.3-10.0 mg/kg BW doses of naloxone suppressed eating and drinking in rats that had been food deprived for 48 h or water deprived for 24 h. Intracerebral naloxone or naltrexone injections into the VMH and naloxone injections into the lateral hypothalamus (LH) decreased 90-min food intake in food-deprived (20-h) rats, as did subcutaneous naloxone injections (Thornhill & Sauders, 1984). Because naloxone suppression occurs after either central or peripheral administration, it is likely that both central and peripheral opiate receptors are involved in feeding regulation, although some recent research emphasizes central mediation of its action on energy intake (Gilson, 1989; Gilson & Wilson, 1989).

(d) Opiate antagonists also suppress food intake in obese rodents (Ferguson-Segall, Flynn, Walker, & Margules, 1982). Margules, Moisset, Lewis, Shibuya, and Pert (1978) found that small doses of the opiate antagonist naloxone selectively abolished overeating in 20-h food-deprived genetically obese mice (ob/ob) and rats (fa/fa). A dose of naloxone as small as 0.25 mg/kg BW selectively depressed food intake in these obese animals by 30%, with no effect on lean mice. At higher doses of naloxone both obese and lean animals displayed a dose-dependent reduction in food intake. However, the obese mice were 10 times more sensitive to the suppressant effects of naloxone than the lean mice. Similarly, Atkinson (1982) observed that a bolus dose of 15 mg of naloxone suppressed food intake of massively obese human subjects by 29%, but had no effect on lean human subjects.

In conclusion, β-endorphin stimulates feeding behavior when administered peripherally or centrally. Because the ob/ob has elevated central and plasma β-endorphin levels and enhanced sensitivity to the anorectic effects of opiate antagonists (Margules et al., 1978), the hyperactive opiate system in the obese mouse may be an important contributor to its overeating.

β-endorphin and ACTH

The polypeptides ACTH and β-endorphin have been shown to be part of a much larger precursor glycoprotein, pre-proopiomelancortin, 31,000 daltons, or 31K-precursor (Levine, Morley, Gosnell, Billington, & Bartness, 1985; Mains, Eipper, & Ling, 1977). Early immunocytochemical studies of normal pituitary tissue observed that ACTH, β-lipotrophin (the immediate precursor to β-endorphin), β-endorphin, and α-endorphin were present in the same cells of the anterior and intermediate lobes of the pituitary gland (Bloom, Battenberg, Rossier, Ling, Leppaluto, Vargo, & Guillemin, 1977). Moreover, the adenohypophysis secretes ACTH and β-endorphin simultaneously in increased amounts in male Holtzman rats in response to acute stress, adrenalectomy, and during in vitro response to corticotrophin releasing factor (CRF). On the other hand, subcutaneous injections of the synthetic glucocorticoid dexamethasone inhibited the secretion of both ACTH and β-endorphin (Guillemin, Vargo, Rossier, Minick, Ling, Rivier, Vale, & Bloom, 1977).

Glucocorticoid hormone secretion from the adrenal gland is under the regulation of the hypothalamo-adenohypophyseal axis. Corticotropin-releasing factor is a potent stimulator of ACTH and β-endorphin secretion. The release of ACTH from pituitary cells increases the secretion of glucocorticoids, which, in turn, exert negative feedback effects on both the hypothalamus and adenohypophysis (Liposits, Oht, Harrison, Gibbs, Paull, & Bohn, 1987). Thus, both ACTH and β-endorphin share a common regulatory mechanism (i. e., hypothalamic releasing factor, or feedback by glucocorticoids) which would control their biosynthesis and secretion.

A human correlate to increased activity of ACTH and β-endorphin is Cushing's disease. This syndrome was first described in 1932 and is characterized by specific adipose tissue accumulation on the face, upper back, trunk, and girdle areas. Other symptoms include protein wasting of the skin, muscle and bones; impaired glucose tolerance leading to diabetes mellitus; hypertension and cardiovascular disease (Cushing, 1932). It is believed that the excess ACTH stimulates the release of cortisol which promotes protein-wasting and enhanced gluconeogenesis. The β-endorphin that is co-released with the ACTH activity stimulates the release of insulin from the pancreas, which, in turn, promotes lipogenesis in the white adipose tissue.

Margules (1979) has postulated that increased protein-wasting, gluconeogenesis, and lipogenesis produced by abnormally high ACTH and β-endorphin activity may contribute to the hyperphagia and obesity associated with middle age and thus similar to a non-tumorous form of Cushing's syndrome. Because the ob/ob mouse has excess pituitary ACTH, increased plasma, pituitary, and brain β-endorphin levels, and excess glucocorticoid production, Margules (1979) proposed that the ob/ob also suffers from a non-tumorous form of Cushing's disease.

Consistent with this speculation, a decrease in body weight, plasma glucose, plasma insulin, and a restoration of the normal feeding response to a fasting challenge and glucose load occurs in adrenalectomized (2 month-old) genetically ob/ob mice (Naeser, 1973). Based on these data, a large part of the ob/ob 's problem may be due to excessive adrenal secretion (i. e., high levels of circulating corticosterone).

However, adrenal hyperfunction can not be the only permissive factor because body weight, serum insulin, and serum glucose are not necessarily restored to the levels of lean controls in obese adrenalectomized mice. Margules (1979) contended that these failures are explained by high circulating levels of β-endorphin that exist in obese mice. β-endorphin would stimulate insulin secretion in the pancreas, in addition to the feeding response in these rodents. He further stated that both of these actions should survive the surgical manipulation and may indeed be enhanced by it because adrenalectomy increases the β-endorphin content in the pituitary (Margules, 1979).

Morphine and β-endorphin also produce hyperglycemia (Feldberg & Shaligram, 1972) and stimulate insulin release from isolated pancreatic islets (Green, Perrin, Pedley, Leslie, & Pyke, 1980). Bailey & Flatt (1987) observed the opiate receptor antagonist naloxone (1.0 mg/kg BW, IP) produced a fast latency, transient elevation in glucose and suppression of insulin concentrations in lean mice, and produced qualitatively similar but more prolonged responses in 12-14-week-old Ashton ob/ob mice. Conversely, selective stimulation of μ- and δ-opiate receptors using the enkephalin analogues Tyr-D-Ala-Gly-MePhe-NH(CH2)2OH (1 mg/kg BW, IP) and Tyr-D-Ala-Gly-Phe-D-Leu (10 mg/kg BW, IP), respectively, rapidly and transiently increased glucose and insulin concentrations in lean and ob/ob mice. However, the obese mice exhibited greater glucose and insulin responses to these analogues. Bailey & Flatt (1987) concluded that increased responsiveness to μ- and δ-opiate receptor stimulation may contribute to the hyperglycaemia and hyperinsulinemia of obese-diabetic mice.

In an attempt to understand the roles of adrenal corticosteroids in modulating the feeding response to morphine, Bhakthavatsalem & Leibowitz (1986) administered morphine (IP or into the PVN) to male

Sprague-Dawley rats who were either adrenalectomized or sham-operated. They observed that adrenalectomy lessened morphine-induced feeding in rats (who had access to a single diet of chow, milk, and sugar or were tested in a self-selection feeding paradigm) and that a single injection of corticosterone rapidly and reliably restored feeding. Their findings emphasize the dependency of morphine-elicited feeding on circulating corticosterone in non-pathological animal models. Interestingly, this opiate-glucocorticoid linkage has not been studied in pathological animal models such as genetically obese rodents or animals with VMH damage.

Statement of the Research Problem

The present study was designed to assess the relative contributions of corticosterone and endorphins on food intake, plasma insulin secretion, and plasma glucose levels in approximately 7-week-old male genetically ob/ob and lean (+/?) mice. The intact obese mouse has higher levels of corticosterone, ACTH and β-endorphin. Adrenalectomy eliminates corticosterone thereby further increasing ACTH and beta-endorphin levels (Guillemin, 1977). Although adrenalectomy ameliorates many of the symptoms characteristic of the obese syndrome, body weight, plasma glucose, and plasma insulin may not reach lean control levels. It has been hypothesized that increased circulating β-endorphin may account for these failures (Margules, 1979). Because the opiate receptor antagonists naloxone and naltrexone have been found to selectively decrease their food intake (Margules, 1978) and suppress their plasma insulin secretion (Bailey & Flatt, 1987; Recant et al., 1980), it was thought that these responses might be enhanced in adrenalectomized obese mice who were given naloxone injections.

Overview of Design

Male ob/ob and +/? mice were adrenalectomized or sham-operated at approximately 5 weeks of age and maintained ad lib on standard chow and 0.9% saline solution. On the food intake test day mice were weighed and then food deprived for 6 h. Mice were reweighed after the deprivation period and injected with either saline, 0.5 mg/kg BW naloxone, or 2.0 mg/kg BW naloxone intraperitoneally. Food intake was recorded 30 min, 1 h, 1.5 h, and 2 h after injection. After testing, animals were housed individually in clean cages and maintained ad lib on standard lab chow. Two days later mice were weighed and food deprived for 6 h. Mice were reweighed after the deprivation period and injected with the appropriate dose of saline or naloxone. Thirty min after injection mice were sacrificed and samples collected for the determination of corticosterone, glucose, and insulin. It was expected that adrenalectomized obese mice who were given naloxone would not consume as much chow as adrenalectomized saline controls or sham-adrenalectomized animals. Moreover, it was anticipated that plasma glucose and plasma insulin levels would be further reduced in these animals (adrenalectomized, naloxone-treated obese mice).

The independent variables were phenotype (ob/ob, +/?), surgery (adrenalectomy vs. sham-operated), and drug dose (saline, 0.5, 2.0 mg/kg body weight). The dependent measures were body weight (g), food intake (g), plasma corticosterone (µg/dL), plasma insulin (ng/ml), and plasma glucose (mg/dL). These procedures yielded a 2 x 2 x 3 (Phenotype x Surgery x Drug Dose) experimental design. Data were analyzed using a univariate analysis of variance (ANOVA) and a multivariate analysis of variance (MANOVA). In addition, a priori pair-wise group comparisons were analyzed.

Method

Subjects

Obese (ob/ob) male (n=60) and lean (+/?) male (n=60) mice (Mus musculus, C57BL/6J) were obtained from The Jackson Laboratory, Bar Harbor, ME. Twelve ob/ob and 12 +/? mice were shipped weekly for five consecutive weeks at weaning (4 weeks ± 3 days of age) and upon arrival were housed individually in clean polypropylene nesting cages (27.3 cm x 16.5 cm x 12.7 cm) with sufficient wood-chip bedding, in a mouse colony room cycled on a 12-h light/dark cycle (lights on at 0700 h). Additional mice were obtained from our breeding colony of C57BL/6J ob/+ mice. Room temperature and humidity were maintained between 23-25 °C and 30-50%, respectively. All mice were maintained ad lib on water and standard lab chow (Rodent Blox, protein 24.0%; fat 6.5%; crude fiber 3.7%; ash 7.9%; carbohydrate, 45.4% [nitrogen-free extract]; moisture 12.5%; to yield a calculated metabolizable energy of 3.1 Kcal/g, Wayne Pet Food Division, Chicago, IL).

Apparatus and Procedure

Obese and lean mice were randomly assigned to one of two surgical treatment conditions at approximately 5 weeks of age: (1) bilateral adrenalectomy (ADX, n=55, 26 obese and 29 lean) and fed ad lib postoperatively until the days of testing; and (2) sham adrenalectomy (SHAM, n=50, 24 obese and 26 lean) and fed ad lib postoperatively until the days of testing. At approximately 7 weeks of age, ADX and SHAM animals were food-deprived for 6-h pretest and randomly assigned to one of three test drug conditions: (a) intraperitoneal (IP) saline injection and ad lib refeeding for 2 h (n=38); (b) 0.5 mg/kg body weight dose of naloxone (IP) and fed ad lib for 2 h (n=34); (c) 2.0 mg/kg body weight dose of naloxone (IP) and fed ad lib for 2 h (n=33). The drug doses were selected on a combined basis of both the current literature as well as pilot studies.

Adrenalectomy. Prior to surgery animals were weighed to the nearest 0.1 g on a Sartorius digital balance (Model #FF4742). All surgeries were performed under light ether anesthesia on weekdays between 1000-1500 h. Surgery consisted of dorsolateral incisions just posterior to the diaphragm. The adrenal glands were gently lifted to the opening of the incision with tissue forceps and a sterile 6-in. cotton-tipped applicator (Harwood Products Company). Each gland was excised with a small amount of adhering adipose tissue, by curved-tipped scissors. Sham operations followed a similar procedure of lifting and exposing the adrenals, but only a small amount of periadrenal adipose tissue was removed with the forceps before the adrenals were replaced in the peritoneum. Incisions were closed with stainless steel wound clips (7.5 mm, Michel, Germany). A total of 149 mice underwent either bilateral adrenalectomy or a sham-operation. Thirty-three subjects died following surgery, therfore, yielding a 77 % surgical success rate. Immediately following surgery, mice were weighed and housed individually in clean cages with a sufficient amount of wood-chip bedding for a 2-week recovery period. Food was made available continuously to all animals. Adrenalectomized mice's drinking water was replaced with a 0.9% sodium chloride solution for the duration of the study; while all sham-operated groups continued on tap water.

The general health of all animals was monitored daily until the days of testing (i. e., 7 weeks of age). Additionally, body weights were recorded immediately after surgery, 1 week postoperatively, on the food intake test day (i. e., 2 weeks postoperatively), and on the plasma test day (i. e., 2 weeks + 2 days postoperatively). Three days prior to the food intake test, a representative sample of mice ($\underline{n}$=86) were placed in clean cages with a small amount of wood-chip bedding, a piece of paper towel bedding and a preweighed quantity of food. On the test day, these polypropylene cages were stored in a room adjacent to the mouse colony room, and food spillage retrieved, weighed, and figured into the mean daily pretest food intake measures.

On the food intake test day, 7-week-old mice were weighed to the nearest 0.1 g on a Sartorius digital balance and then food deprived for 6 h (between 0900-1500 h). Following the food deprivation period, body weights were again recorded, and IP injections of the appropriate drug dose were administered in volumes of 1 cc/100 g body weight (BW) through a 5/8-in. (1.59 cm) 25-ga. needle attached to a microliter syringe. All solutions were prepared fresh on the day of testing. The appropriate dose of naloxone hydrochloride (Sigma Chemical Company, MO, USA) was weighed on a Sartorius analytical balance and dissolved in 0.15 M saline solution. Immediately following IP injections of saline (0.9%) or naloxone, animals were placed individually in clean polypropylene cages with a small piece of paper towel bedding and a preweighed quantity of standard lab chow (which was placed in a lid container that was securely attached to the floor of the cage with adhesive tape) for a 2-h test period. Food intake measurements (i. e., the amount of lab chow consumed) were recorded 30 min, 1 h, 1.5 h, and 2 h after injection. Mice were weighed after the re-feeding test and then returned to their home cages and maintained ad lib on standard lab chow.

On the plasma test day mice were weighed to the nearest 0.1 g on a Sartorius digital balance and then food deprived for 6 h (between 0900-1500 h). Body weights were recorded prior to treating the mice with the appropriate dose of either saline or naloxone. Thirty min after injection (peak plasma levels) the mice were sacrificed by decapitation (at 1500-1600 h). Core blood was collected into heparinized plastic beakers and transferred to labeled 1.5 ml Eppendorf polypropylene micro test tubes (Brinkman Instruments Company) and then centrifuged for 5 min (Eppendorf Centrifuge Model # 5412 7090/02, Brinkman Instruments Company). Plasma was extracted and placed into clean, labeled 1.5 ml Eppendorf micro test tubes and stored at -20 °C for later determination of corticosterone, insulin, and glucose.

Postmortem Assays

Corticosterone radioimmunoassay procedure. Plasma corticosterone was determined by radioimmunoassay (Endocrine Sciences, Tarzana, CA) and used to verify successful adrenalectomy (< 1.0 µg/dl). Reagents included the following:

(1) Borate Buffer 0.05 M, pH 8.0. Reagent grade boric acid crystals (2 g) were dissolved in 500 ml

distilled water containing 0.30 ml of 10 N sodium hydroxide.

(2) Bovine Serum Albumin (Schwarz/Mann No. 751). Bovine serum albumin (1 g) was dissolved in 10 ml of 0.05 M borate buffer (pH 8.0) containing 0.1% sodium azide.

(3) Bovine Gamma Globulin (Schwarz/Mann No. 3004). Bovine gamma globulin powder (250 mg) was dissolved in 10 ml of 0.9% sodium chloride containing 0.1 % sodium azide. This solution was stored at 4 °C.

(4) Stock 1,2-^{3}H-Corticosterone (New England Nuclear No. NET-182). Labeled corticosterone (250 Mc) was diluted in methanol (5.0 ml) and stored at 4 °C.

(5) Corticosterone Standards. Stock standards were prepared in redistilled ethanol. Working standards of 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, and 10.0 ng/0.10 ml were prepared in redistilled methanol from the stock solution and stored at 4 °C.

(6) Ammonium Sulfate. A saturated solution of reagent grade salt in distilled water was prepared and confirmed by excess crystals after 2 h.

(7) Scintillation Fluid. PPO (10 g) was dissolved in 2 L toluene containing 40 ml methanol.

(8) Stock Antiserum. The antiserum was stored at -10 °C.

(9) Dilute Antiserum. This solution was made

just prior to use and consisted of 8.0 ml borate buffer, 0.02 ml 1,2-^{3}H-corticosterone, 0.20 ml 10% bovine serum albumin, 0.20 ml of 2.5% bovine gamma globulin, and 0.067 ml antibody.

Incubation with the antibody. Eppendorf (1.5 ml) micro test tubes were labeled in duplicate with the working corticosterone concentrations (i. e., 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10.0 ng/0.10 ml). Then, 0.05 ml of each concentration were pipetted into the appropriate Eppendorf tube, while 0.05 ml alcohol was pipetted into the 0 concentration tube, as well as the non-specific binding tube. Plasma samples were thawed; 0.025 ml plasma transferred to a labeled Eppendorf tube, and 0.025 ml of 10% BSA added. The samples were capped and heated in a 60 °C water bath for 30 min. Absolute alcohol (0.20 ml) was added to each sample. The contents were thoroughly mixed on a vortex mixer (Thermolyne Maxi Mix), allowed to stand for 5 min, mixed again, allowed to stand for an additional 5 min, mixed again, and then centrifuged for 5 min. The supernatant (0.20 ml) was extracted and 0.05 ml transferred to labeled Eppendorf tubes. The diluted plasma samples were also run in duplicate.

The solvents were evaporated to dryness in a vacuum oven at 45 °C (10-20 min with drying time varying according to the number of samples). Bovine serum albumin (0.25%, 0.05 ml) was pipetted into each tube and mixed on a vortex. Dilute antiserum (0.20 ml) was added to each tube, mixed on a vortex mixer, and incubated at 37 °C in a water bath for 45 min. Note that 0.20 ml borate buffer solution (without antiserum) was added to the non-specific binding tube. The samples were then incubated at room temperature for 2 h.

Separation of free and bound steroid. Saturated ammonium sulfate solution (0.25 ml) was added to each tube. The contents were mixed thoroughly on a vortex mixer and then centrifuged for 5 min. The supernatant (0.40 ml) was carefully transferred to labeled liquid scintillation vials (Research Products International Corporation).

Scintillation counting. Scintillation fluid (5.0 ml) was added, and the vials capped tightly and shaken on a mechanical shaker (Eberbach Corporation, Ann Arbor, MI) for 15 min. The samples stood in a dark room for 3 h before being counted on a Beckman Liquid Scintillation System (# LS-3133 P, Beckman Instruments, Inc., Irvine, CA). Each sample was counted for 10 min. Note that 0.20 ml of borate buffer solution was added to a total counts scintillation vial before scintillation fluid was added.

Calculations. In the assay procedure used, the steroid is incubated with the antiserum for 2 h at room temperature, and ammonium sulfate precipitation is used to separate free and bound steroid. Standard curves were constructed by plotting the percentage of unbound 1,2-^{3}H-corticosterone as a function of the unlabeled corticosterone content. The percentage unbound =(x) / 0.8(y)(z) X 100, where x = cpm in 0.40 ml of supernatant after ammonium sulfate, and y = cpm in 0.20 ml dilute antiserum, and z = appropriate unbound fraction from non-specific binding check. It should be noted that non-specific binding has been demonstrated (e. g., sticking to the glassware) and in the absence of antibody, the percentage unbound label should be greater than 95%.

Insulin assay procedure. Plasma insulin concentrations were measured using the enzyme-linked immunosorbent assay (ELISA) procedure (Kekow, Ulrichs, Muller-Ruchholtz, & Gross, 1988). Reagents included the following:

(1) Coating buffer 0.05 M, pH 9.6. Sodium carbonate (1.59 g), 2.93 g sodium bicarbonate, and 0.20 g of sodium azide were dissolved in 1 L of distilled water.

(2) Incubation buffer for insulin antibody (FAM). Disodium phosphate (5.77 g), 1.05 g of monosodium phosphate, 1.00 g of bovine serum albumin, and 0.24 g

of sodium merthiolate were dissolved in 1.0 L of distilled water.

(3) Incubation buffer for insulin standards and samples (sodium FAM). Sodium chloride (0.6 g) and 5.9 g of bovine serum albumin were added to 100 ml of the FAM buffer [which is described in (2) above].

(4) Washing buffer, 0.15 phosphate-buffered saline, pH 7.2. Sodium chloride (8.0 g), 0.2 g potassium chloride, 1.15 g disodium phosphate, 0.2 g potassium phosphate, and 0.5 ml of Tween 20 were added to 1 L of distilled water.

(5) 2,2'-Azinobis(3-ethylbenzthiazolinesulfonic Acid (ABTS) solution. One ml ABTS solution (0.02 g ABTS dissolved in 5 ml of distilled water) was added to 11 ml of citrate buffer (9.6 g citric acid monohydrate dissolved in 500 ml of distilled water) and 4 μL of hydrogen peroxide.

(6) Stop solution. Citric acid monohydrate (31.5 g) and 0.5 g of sodium azide were dissolved in 500 ml of distilled water.

Incubation with coating antibody. Polystyrene microliter plates with 96 round-bottomed wells were coated with 150 μl of rabbit anti-guinea pig antibody (EY Laboratories, San Mateo, CA). The plate was covered with plastic wrap and allowed to stand for 4 days at 4 °C.

Incubation with insulin antibody. Each well was rinsed with 250 µl of washing buffer and then the supernatant was suctioned off (Miniwash, Dynatech Product Laboratories, Inc., Serial #1058, Model B). The plates were washed two additional times and then 100 µl of insulin antibody (Novo, Bagsvaerd, Denmark; antibody M 8309) was added to each well, and the plate was placed in 4 °C for 2 days.

Incubation with insulin (unlabeled). Each well was rinsed with 250 µl of washing buffer and then the supernatant was suctioned off. This washing step was repeated two additional times. Appropriate standards were prepared that ranged from 0 ng/ml insulin to 10 ng/ml insulin (i. e., 0 ng/ml, 0.125 ng/ml, 0.25 ng/ml, 0.50 ng/ml, 1.0 ng/ml, 2.5 ng/ml, 5.0 ng/ml, 10 ng/ml). The standards and plasma samples were all diluted appropriately with sodium FAM and used in triplicate when possible (100 µl each/well). The plates were covered in plastic wrap and placed in a 37 °C oven for 45 min.

Incubation with peroxidase-labeled insulin. Peroxidase conjugate-labeled insulin (Sigma, St. Louis, MO) was added to each well (100 µl/well). The plate was covered in plastic wrap and incubated at 37 °C for 35 min.

Measurement of substrate degradation after removal of unbound insulin. Each well was rinsed with 250 µl of washing buffer and the supernatant was suctioned off. This washing step was repeated two additional times and then ABTS solution (100 µl) was added to each well and the uncovered plate incubated at room temperature for approximately 1 h. The enzyme reaction was terminated by adding 100 µl of stop solution per well and the optical density was measured (Mini reader II, Dynatech Product Laboratories, Inc., Serial #2949; Series 2 General Applications Program).

Calculations. ELISA is characterized by the principle of competitive saturation of an insulin antibody with either unlabeled (plasma samples and standards) or peroxidase-labeled insulin. Standard curves were constructed by plotting optical density (OD) as a function of insulin content. The relationship obtained from the insulin standards was used to calculate the insulin content in the plasma samples.

Glucose assay procedure. Plasma glucose was determined using Glucose GOD-PAP Reagent Set (Boehringer Mannheim Diagnostics, Indianapolis, IN). Reagents included the following: Working Glucose Reagent (Buffer/Enzymes/4-Aminophenazone) which was reconstituted with 100 ml distilled water and Phenol which was added and gently mixed until dissolved. The

Working Glucose Reagent was stored in an amber bottle at 2-8 °C until ready for use.

Testing. Distilled water (0.01 mL), standard (0.01 mL) and the plasma specimen (0.01 mL) were pipetted into cuvettes (Fisher Scientific Company). Working Glucose Reagent (2.0 mL) was added to the blank, standard, and specimen mixed well and incubated at 23-25 °C for 25 min. The end color was read against a reagent blank within the following 15 min using a micro sample spectrophotometer (wavelength capability 480-520 nm, Gilford Instruments).

Calculations. In the assay procedure used, glucose was oxidized by glucose oxidase (GOD) in an aqueous solution to gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts in the presence of peroxidase (POD) with phenol and 4-aminophenazone forming a red dye. The intensity of color formed is proportional to the glucose concentration and can be measured photometrically between 480 and 520 nm. The absorbance of all standards and specimens was measured against a reagent blank. The concentration of glucose was calculated as follows: Absorbance of Specimen/Absorbance of Standard x Concentration of Standard = mg/dL glucose.

Statistical Analyses

Results were analyzed using a 2 x 2 x 3 (Phenotype x Surgery x Drug Dose) univariate analysis of variance (ANOVA) as well as a multivariate analysis of variance (MANOVA). The ANOVAs and MANOVAs were performed on the variables body weight, mean daily food intake pretest, food intake during the refeeding test, cumulative intake during the refeeding test, plasma insulin, and plasma glucose using a Statistical Analysis System (SAS) general linear model (GLM) package (SAS, 1989). In addition, a priori pair-wise mean group comparisons were analyzed using a SAS linear contrasts program (SAS, 1989). It was expected that ADX, naloxone-treated obese mice (ADX-NLX-OBs) would consume less food than ADX, saline-treated obese mice (ADX-SAL-OBs) and SHAM, naloxone-treated obese mice (SHAM-NLX-OBs). It was also anticipated that ADX-NLX-OBs would ingest less chow than ADX, naloxone-treated lean mice (ADX-NLX-LEAN), ADX, saline-treated lean mice (ADX-SAL-LEAN) and all sham-treated lean animals (SHAM-NLX-LEAN, SHAM-SAL-LEAN). Moreover, it was expected that plasma insulin levels for ADX-NLX-OBs would be less than ADX-SAL-OBs and SHAM-NLX-OBs and would reach control lean values. Similarly, plasma glucose levels for ADX-NLX-OBs were expected to be less than SHAM-NLX-OBs. Linear contrasts were performed on group mean differences (SAS, 1989). The alpha value for all comparisons was set at $p < 0.05$.

Results

Body Weight

Mean absolute body weight. Phenotype affected body weight immediately following surgery, F (1, 104) = 158.73, $p < 0.0001$, one week postoperatively, F (1, 104) = 160.45, $p < 0.0001$, on the refeeding test day, F (1, 104) = 160.36, $p < 0.0001$, and on the plasma test day, F (1, 104) = 170.18, $p < 0.0001$, with obese mice being heavier than leans (see Table 1). Although Surgery did not influence body weight immediately postoperatively, by 1 week postoperatively ADX mice (M = 21.24) weighed less than SHAM controls, F (1, 104) = 46.08, $p < 0.0001$, an effect which persisted on the refeeding test day, F (1, 104) = 45.65, $p < 0.0001$, and the plasma test day, F (1, 104) = 55.75, $p < 0.0001$.

Table 1

Mean (±SD) Body Weight (g) as a Function of Phenotype, Surgery, and Postoperative Sampling Time

		Postoperative Sampling Time			
Phenotype	Surgery	Immediate	1 Wk	2 Wks	2 Wks +
Obese	ADX	25.6(3.2)	23.9(3.1)	24.7(3.8)	25.0(3.8)
	Sham	24.7(3.2)	29.1(3.5) [bc]	31.2(3.1) [bc]	32.6(3.2) [bc]
Lean	ADX	18.8(2.0) [a]	18.9(2.2) [a]	20.7(2.4) [a]	21.1(2.5) [a]
	Sham	18.8(1.5) [a]	20.7(1.6) [abc]	21.4(1.4) [ab]	21.8(1.4) [ab]

a = $p < 0.05$ for comparisons between phenotypes

b = $p < 0.05$ for comparisons between surgery

c = $p < 0.05$ for phenotype x surgery comparisons

A Phenotype x Surgery interaction, F (1, 104) = 10.84, $p < 0.0015$, one week postoperatively showed that adrenalectomy lowered obese mice's body weights compared to their SHAM controls, but not to the same level as ADX leans, which exhibited lower weights than their SHAM controls. However, by two weeks postoperatively the Phenotype x Surgery interaction, F (1, 104) = 28.90, $p < 0.0001$, revealed that although ADX obese mice continued to have lower body weights than their SHAM controls, ADX lean mice's weights did not differ from those of SHAM lean controls. Both ADX obese and SHAM obese mice weighed significantly more than either lean group. Similar profiles occurred two days later on the plasma test day, Phenotype x Surgery, F (1, 104) = 38.52, $p < 0.0001$. Body weights were reduced in ADX obese compared to SHAM obese controls, but higher in ADX obese than ADX leans or SHAM lean controls.

Mean percentage body weight gain. Body weight (g) was converted into a percentage change from postoperative body weight to better assess the relative growth rate. Percentage body weight gain was significantly affected by Surgery one week, F (1, 104) = 105.29, $p < 0.0001$, two weeks, F (1, 104) = 83.55, $p < 0.0001$, and two weeks plus two days, F (1, 104) =

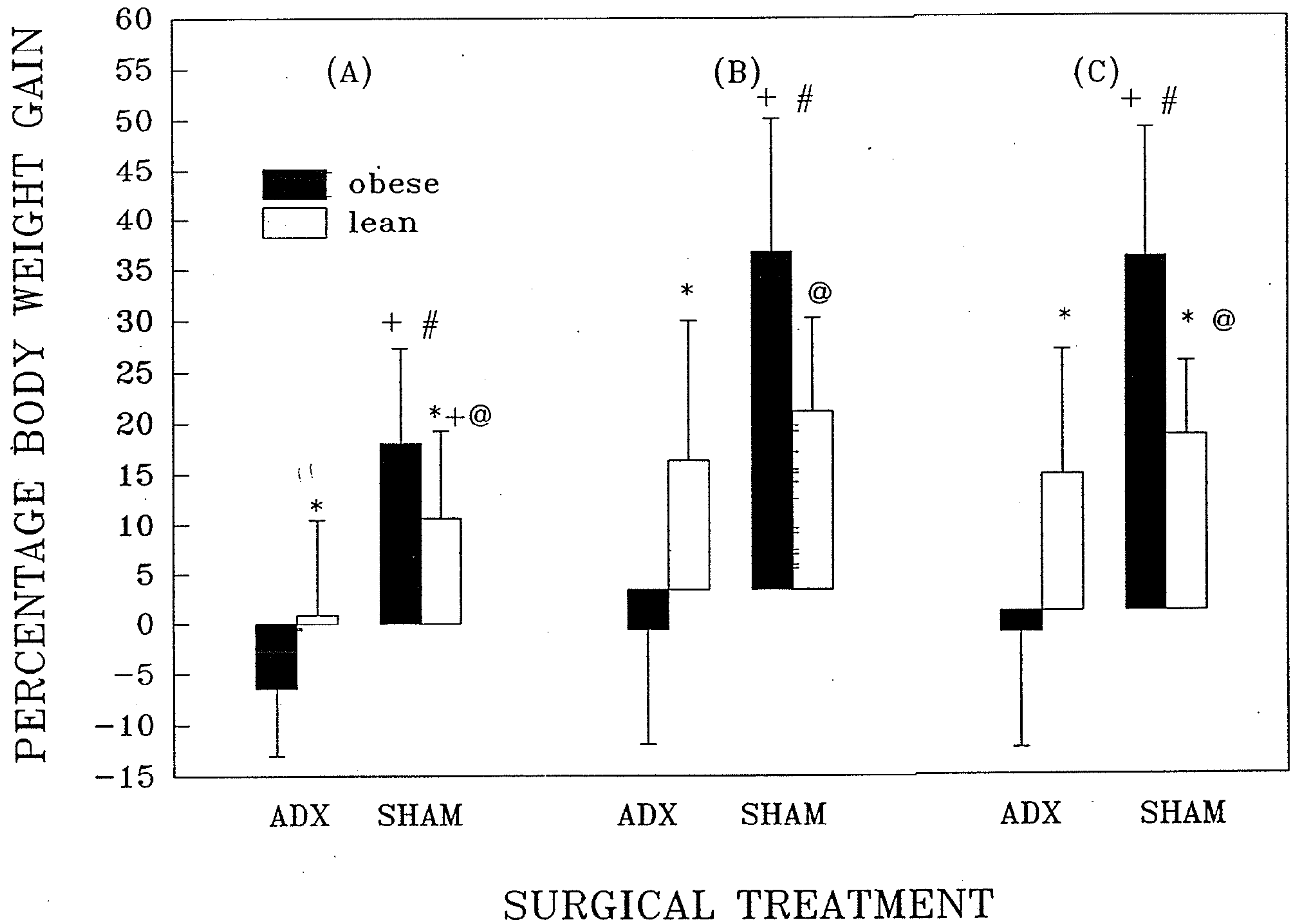
92.71, $p < 0.001$ postoperatively, with ADX mice gaining less than SHAM mice at each time ($M = -2.57$ % vs. $M = 14.26$ %; $M = 3.95$ % vs. $M = 20.44$ %; $M = 5.82$ % vs. $M = 24.17$ %, respectively). Figure 1 depicts Phenotype x Surgery interactions at each of those times. In Panel A, $F(1, 104) = 18.68$, $p < 0.0001$, ADX obese mice had a decreased percentage body weight gain compared to SHAM obese, ADX lean, and SHAM lean mice one week postoperatively. ADX lean mice had a smaller percentage body weight gain compared to SHAM lean mice, and SHAM obese mice had a larger percentage body weight gain compared to either lean group. The profiles in Panel B, $F(1, 104) = 47.71$, $p < 0.0001$, and Panel C, $F(1, 104) = 58.70$, $p < 0.0001$, are similar to observations one week postoperatively, except no significant differences were found between ADX and SHAM lean mice. ADX obese mice had a decreased percentage body weight gain compared to SHAM obese, ADX lean, and SHAM lean; and SHAM obese mice had a larger percentage body weight gain compared to either lean group on the refeeding test day and the plasma test day.

Food Intake

Mean daily food intake prior to the refeeding test. The mean daily food intake (g/day) calculated for three consecutive days prior to the food intake test situation was significantly affected by Surgery, F

Figure Caption

Figure 1. Effects of adrenalectomy on percentage body weight gain (+ SD) in genetically obese and lean mice. (* $p < 0.05$ for between phenotype comparisons; + $p < 0.05$ for between surgical treatment comparisons; # $p < 0.05$ for comparisons between SHAM OBESE & ADX-LEAN mice; and @ $p < 0.05$ for comparisons between ADX-OBESE & SHAM-LEAN mice).


PERCENTAGE BODY WEIGHT GAIN
60
55
50
45
40
35
30
25
20
15
10
5
0
−5
−10
−15
(A)
(B)
(C)
obese
lean
+ #
*+@
*
@
* @
ADX
SHAM
SURGICAL TREATMENT

(1, 85) = 69.28, $p < 0.0001$, and Phenotype, F (1, 85) = 3.99, $p < 0.0362$. ADX mice ($M = 3.40$) consumed less food compared to SHAM mice ($M = 4.44$), and obese mice ($M = 4.00$) consumed more daily food compared to lean mice ($M = 3.79$). A Surgery x Phenotype interaction effect, F (1, 85) = 52.24, $p < 0.0001$, revealed that SHAM obese mice ($M = 5.08$) consumed more food than ADX obese ($M = 3.05$), ADX lean ($M = 3.72$), and SHAM lean mice ($M = 3.87$). In addition, ADX obese mice ingested less than ADX lean and SHAM lean mice.

Mean food intake during the refeeding test. Surgery, F (1, 104) = 42.83, $p < 0.0001$, Phenotype, F (1, 104) = 74.22, $p < 0.0001$, and Drug Dose, F (2, 104) = 48.41, $p < 0.0001$, main effects were found on food consumed (g) during the first 1/2 h of re-feeding. Overall, ADX mice ($M = 0.06$) consumed less than SHAM mice ($M = 0.13$), and obese mice consumed more ($M = 0.14$) than lean mice (0.05). At the highest dose of naloxone all mice ($M = 0.03$) consumed less food compared to the lowest dose group ($M = 0.09$) and the saline group ($M = 0.15$). Mice in the lowest drug dose condition consumed less than saline-treated mice.

Surgery x Phenotype, F (1, 104) = 58.61, $p < 0.0001$, Phenotype x Drug Dose, F (2, 104) = 7.96, $p < 0.0006$, and Surgery x Drug Dose interaction effects, F (1, 104) = 6.04, $p < 0.0034$, were also found. ADX obese ($\underline{M}$ = 0.07) consumed less than SHAM obese ($\underline{M}$ = 0.22) mice, but SHAM obese consumed more than SHAM lean ($\underline{M}$ = 0.04) mice. No differences were found between ADX obese and ADX lean mice ($\underline{M}$ = 0.06), and between ADX lean and SHAM lean mice. Obese mice consumed more ($\underline{M}$ = 0.23) than lean mice ($\underline{M}$ = 0.09) in the saline condition and obese mice ($\underline{M}$ = 0.13) ingested more than lean mice ($\underline{M}$ = 0.05) at the lowest dose of naloxone. Obese mice ($\underline{M}$ = 0.05) ate less at the highest dose of naloxone compared to obese mice ($\underline{M}$ = 0.13) in the lowest dose of naloxone condition, and saline-treated obese mice ($\underline{M}$ = 0.23). Lean mice ($\underline{M}$ = 0.01) ingested less at the highest drug dose compared to saline-treated lean mice ($\underline{M}$ = 0.09), however, no differences were found between lean mice in the highest drug dose compared to lean mice in the lowest dose of naloxone condition.

A Surgery x Phenotype x Drug Dose interaction effect, $\underline{F}$ (2, 104) = 5.00, $\underline{p}$ < 0.0087, is illustrated in Figure 2. Linear contrasts showed that SHAM obese saline-treated mice consumed more food than ADX obese saline-treated, ADX lean saline-treated, and SHAM lean saline-treated mice. SHAM lean saline-treated mice ingested less chow than ADX obese and ADX lean saline-treated mice. At the lowest drug dose, SHAM obese mice again consumed more food than ADX obese 0.5 NLX, ADX lean 0.5 NLX, and SHAM lean 0.5 NLX mice. No significant differences were found between the ADX obese, ADX lean, and SHAM lean mice at this drug dose level. At the highest drug dose, SHAM obese mice ingested more than ADX obese 2.0 NLX, ADX lean 2.0 NLX, and SHAM lean 2.0 NLX mice. As was observed at the lowest drug dose, no significant differences were observed between ADX obese, ADX lean, and SHAM lean mice in the 2.0 NLX condition. Drug dose differences were found within each Surgery x Phenotype condition. ADX obese saline-treated mice consumed more than ADX obese 0.5 NLX mice and ADX obese 2.0 NLX mice. Similarly, ADX lean saline-treated mice ingested more than ADX lean 0.5 NLX mice and ADX lean 2.0 NLX mice. SHAM lean mice ate less chow at the highest drug dose level compared to SHAM lean 0.5 NLX mice. SHAM obese mice consumed less food at the highest drug dose level compared to SHAM obese 0.5 NLX mice and SHAM obese saline-treated mice. In addition, SHAM obese saline-treated mice ate more than SHAM obese 0.5 NLX mice.

Food ingested during the second 1/2 h of re-feeding was significantly affected by Phenotype, $\underline{F}$ (1, 104) = 4.91, $\underline{p}$ < 0.0291, and Drug Dose, $\underline{F}$ (2, 104) = 5.75, $\underline{p}$ < 0.0044, with, overall, obese mice consuming more ($\underline{M}$ = 0.06) than lean mice ($\underline{M}$ = 0.04) and saline-treated mice ($\underline{M}$ = 0.07) eating more than 2.0 NLX mice ($\underline{M}$ = 0.02). Neither a Surgery main effect, $\underline{F}$ (1, 104) = 2.37, $\underline{p}$ <

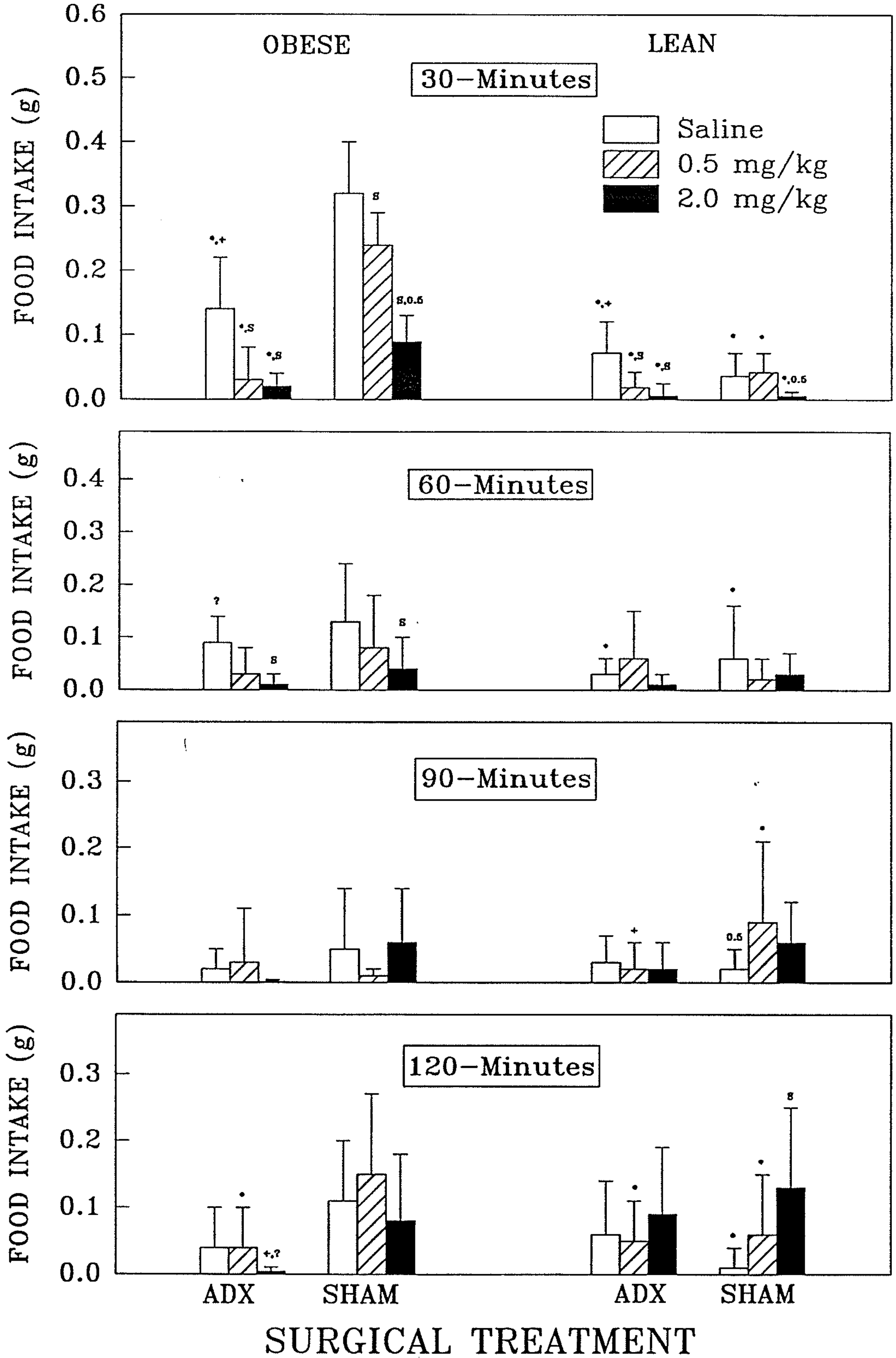
0.13, nor any interaction effects (e. g., Surgery x Phenotype x Drug Dose interaction effect F (2, 104) = 1.12, $p < 0.33$) were found. As depicted in Figure 2, linear contrasts revealed that ADX obese saline-treated mice ingested more food than ADX lean saline-treated mice. SHAM obese saline-treated mice consumed more food than SHAM lean saline-treated mice. ADX obese 2.0 NLX mice consumed less than ADX obese saline-treated mice. Similarly, SHAM obese 2.0 NLX mice consumed less than SHAM obese saline-treated.

Food consumed during the third 1/2 h of re-feeding was significantly affected by Surgery, F (1, 104) = 4.30, $p < 0.0410$. Overall, ADX mice (M = 0.02) consumed less food than SHAM mice (M = 0.04). Linear contrasts also found that ADX mice at the highest drug dose level (M = 0.01) ate less than SHAM mice at the highest drug dose level (M = 0.06).

A Surgery x Phenotype x Drug Dose interaction effect, F (2, 104) = 3.55, $p < 0.0326$, was found. As shown in Figure 2, ADX lean 0.5 NLX mice consumed less food than SHAM lean 0.5 NLX. Similarly, SHAM obese 0.5 NLX ate less than SHAM lean 0.5 NLX mice. SHAM lean saline-treated mice consumed less than SHAM lean 0.5 NLX mice.

Figure Caption

Figure 2. Effects of intraperitoneal naloxone administration on mean food intake (+ SD) 30 min, 60 min, 90 min, and 120 min postinjection in adrenalectomized and sham-adrenalectomized genetically lean and obese mice. Superscripts over each histogram represent significant ($p < .05$) mean differences (* if different than SHAM OBESE mice within a drug condition; + if different than SHAM LEAN mice within a drug condition; ? if different than ADX LEAN mice within a drug condition; and s (saline) and 0.5 (0.5 mg/kg) naloxone dose if different within each phenotype x surgery treatment condition.


OBESE
LEAN
30–Minutes
Saline
0.5 mg/kg
2.0 mg/kg
60–Minutes
90–Minutes
120–Minutes
FOOD INTAKE (g)
ADX
SHAM
ADX
SHAM
SURGICAL TREATMENT

Food ingested during the last 1/2 h of re-feeding was significantly affected by Surgery, F (1, 104) = 7.44, $p < 0.0076$, with ADX mice (M = 0.05) consuming less than SHAM mice (M = 0.09). A Surgery x Phenotype interaction effect, F (1, 104) = 7.21, $p < 0.0086$, and a Phenotype x Drug Dose interaction effect, F (2, 104) = 5.01, $p < 0.0086$, were also found. ADX obese (M = 0.03) consumed less than SHAM obese (M = 0.11) mice, and SHAM obese were found to consume more than SHAM lean (M = 0.06) mice. Overall, ADX obese mice (M = 0.03) ate less than ADX lean mice (M = 0.07), ($p <$ 0.08), suggesting that ADX eliminated intake differences between obese and lean mice. Obese mice (M = 0.04) consumed less than lean mice (M = 0.11) at the highest drug dose level. Saline-treated lean mice (M = 0.04) consumed less than lean mice treated at the highest drug dose level (M = 0.11). No other significant Phenotype x Drug Dose comparisons were found.

A Surgery x Phenotype x Drug Dose interaction effect was not found. As illustrated in Figure 2, linear contrasts showed that ADX obese 2.0 NLX mice ingested less than both ADX lean 2.0 NLX mice and SHAM lean 2.0 NLX mice. In addition, it was found that 30 % of ADX obese mice in the 2.0 NLX condition did not ingest anything in the 2-h refeeding test; whereas, all mice in all other treatment conditions consumed some food during refeeding. ADX obese 0.5 NLX mice, ADX lean 0.5 NLX mice, and SHAM lean 0.5 NLX mice ate less than SHAM obese 0.5 NLX mice. SHAM obese saline-treated mice ingested more than SHAM lean saline-treated. SHAM lean 2.0 NLX mice consumed more than SHAM lean saline-treated mice.

<u>Cumulative food intake during the refeeding test.</u> Cumulative food intake during the first 1/2 h of re-feeding is found previously in the 'Mean food intake during the refeeding test' section (see p. 40). Cumulative food intake during the first hour of re-feeding was significantly affected by Surgery, F (1, 104) = 37.07, $p < 0.0001$, Phenotype, F (1, 104) = 66.95, $p < 0.0001$, and Drug Dose (2, 104) = 50.96, $p < 0.0001$. Linear contrasts found that overall, ADX mice ($M = 0.10$) ingested less than SHAM mice ($M = 0.19$); obese mice ($M = 0.20$) consumed more than lean mice ($M = 0.09$); and saline-treated mice ($M = 0.22$) ingested more than either 0.5 NLX-treated mice ($M = 0.14$) or 2.0 NLX-treated mice ($M = 0.05$). Mice in the highest drug dose condition consumed less chow than mice in the lowest drug dose condition.

A Surgery x Phenotype interaction effect, F (1, 104) = 44.62, $p < 0.0001$, and a Phenotype x Drug Dose interaction effect, F (2,104) = 10.94, $p < 0.0001$, were also found. ADX obese mice (M = 0.11) ingested less than SHAM obese mice (M = 0.30), and SHAM obese consumed more than SHAM lean (M = 0.08). No differences were found between ADX obese and ADX lean (M = 0.09) mice, or between ADX lean and SHAM lean mice. Moreover, obese (M = 0.34) consumed more than lean mice (M = 0.13) in the saline condition, and 0.5 NLX-treated obese (M = 0.19) ingested more than 0.5 NLX-treated lean mice (M = 0.09). Further, saline-treated obese mice consumed more than both 0.5 NLX-treated obese mice, and 2.0 NLX-treated obese mice (M =0.08), and obese mice at the highest drug dose ate less than obese mice at the lowest drug dose. Similarly, lean 2.0 NLX-treated mice (M = 0.03) consumed less than lean saline-treated (M = 0.13) mice.

A Surgery x Phenotype x Drug Dose interaction effect, F (2, 104) = 4.13, $p < 0.0190$, was also found. As illustrated in Figure 3, ADX obese saline-treated mice consumed more than ADX lean saline-treated and SHAM lean saline-treated mice. SHAM obese saline-treated mice ingested more than ADX obese, ADX lean, and SHAM lean mice who were in the saline treatment condition. At the lowest drug dose (0.5 NLX), SHAM obese mice ate more than ADX obese, ADX lean, and SHAM lean mice. In addition, no significant differences were found between ADX obese, ADX lean, and SHAM lean mice at this level. Similarly, at the highest drug dose (2.0 NLX), SHAM obese mice ingested more than ADX obese, ADX lean, and SHAM lean mice. Again, no significant differences were found between ADX obese, ADX lean, and SHAM lean mice at the highest drug dose level. Drug dose differences were found within each Surgery x Phenotype condition. ADX obese saline-treated mice consumed more than both ADX 0.5 NLX mice and ADX 2.0 NLX mice. ADX lean 2.0 NLX mice consumed less than both ADX lean 0.5 NLX mice and ADX lean saline-treated mice. SHAM obese mice consumed less food at the highest drug dose compared to SHAM obese 0.5 NLX mice and SHAM obese saline-treated mice. In addition, SHAM obese saline-treated mice ate more than SHAM obese 0.5 NLX mice. SHAM lean 2.0 NLX mice consumed less than SHAM lean saline-treated mice.

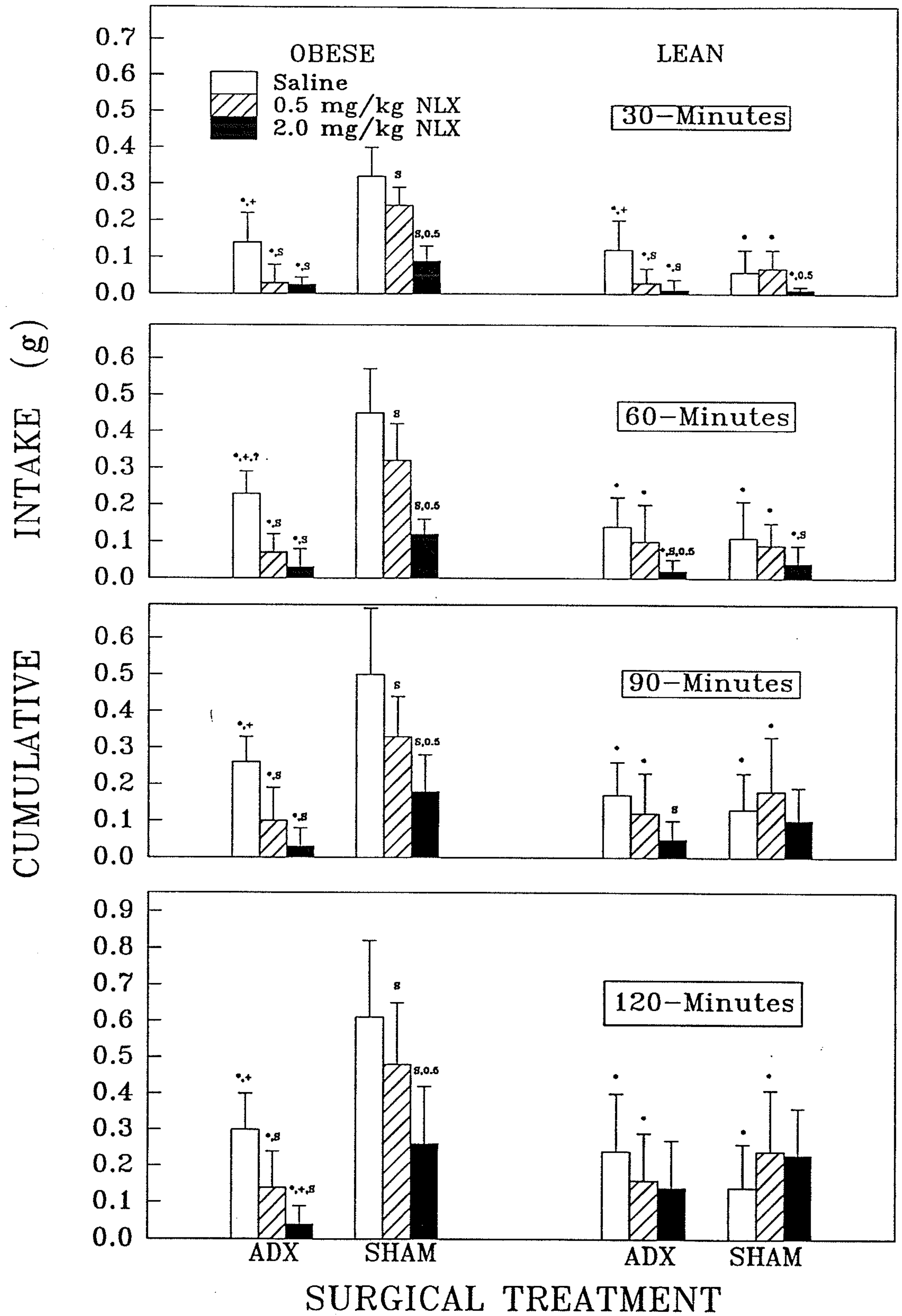
Cumulative food intake after 1-1/2 h of re-feeding was significantly affected by Surgery, $\underline{F}$ (1, 104) = 31.54, $\underline{p} < 0.0001$, Phenotype, $\underline{F}$ (1, 104) = 29.45, $\underline{p} < 0.0001$, and Drug Dose, $\underline{F}$ (2, 104) = 25.08, $\underline{p} < 0.0001$. Overall, ADX mice ($\underline{M}$ = 0.12) ingested less than SHAM mice ($\underline{M}$ = 0.23), and obese mice ($\underline{M}$ = 0.23) consumed more than lean mice ($\underline{M}$ = 0.12). A dose-dependent effect was observed, in that mice consumed less at both the 0.5 NLX drug dose ($\underline{M}$ = 0.18) and the 2.0 NLX drug dose (M = 0.09) compared to the saline condition (M = 0.25). Mice at the highest drug dose ingested less than mice at the lowest drug dose.

A Surgery x Phenotype interaction effect, F (1, 104) = 20.60, p < 0.0001, and a Phenotype x Drug Dose interaction effect, F (2, 104) = 8.83, p < 0.0003, were also found. Contrasts showed that ADX obese (M = 0.13) consumed less than SHAM obese (M = 0.34), and SHAM obese consumed more than SHAM lean (M = 0.13). No differences in food intake were found between ADX obese, ADX lean (M = 0.12), and SHAM lean mice. Obese (M = 0.37) consumed more than lean (M = 0.15) mice in the saline condition. Obese ingested less at both the highest drug dose (M = 0.11) and the lowest drug dose (M = 0.21) compared to the saline-treated mice (M = 0.37). In addition, obese 2.0 NLX-treated mice consumed less than obese 0.5 NLX-treated mice. Similarly, lean 2.0 NLX-treated mice (M = 0.07) ate less than lean saline-treated mice (M = 0.15). No differences were found between either lean saline-treated mice and lean 0.5 NLX-treated mice.

Although a Surgery x Phenotype x Drug Dose interaction effect was not found, F (2, 104) = 2.02, p < 0.14, linear contrasts as illustrated in Figure 3, found that ADX obese saline-treated mice consumed more

Figure Caption

Figure 3. Effects of intraperitoneal naloxone administration on mean cumulative food intake (+ SD) 30 min, 60 min, 90 min, and 120 min postinjection in adrenalectomized genetically obese and lean mice. Superscripts over each histogram represent significant ($p < .05$) mean differences (* if different than SHAM OBESE mice; + if different than SHAM LEAN mice; ? if different than ADX LEAN mice; and s (saline) and 0.5 (0.5 mg/kg) naloxone dose if different within each phenotype x surgery treatment condition.



OBESE
LEAN
Saline
0.5 mg/kg NLX
2.0 mg/kg NLX
30–Minutes
60–Minutes
90–Minutes
120–Minutes
CUMULATIVE INTAKE (g)
ADX
SHAM
ADX
SHAM
SURGICAL TREATMENT

than SHAM lean saline-treated mice. No significant differences were found between ADX obese and ADX lean saline-treated animals. SHAM obese saline-treated mice ingested more than ADX obese saline-treated, ADX lean saline-treated, and SHAM lean saline-treated mice. At the lowest drug dose (0.5 NLX), SHAM obese mice consumed more than ADX obese, ADX lean, and SHAM lean mice. Similarly, at the highest drug dose (2.0 NLX), SHAM obese mice consumed more than ADX obese mice. No significant differences were found between ADX obese, ADX lean, and SHAM lean mice at the highest drug dose level. Drug dose differences were found within each Surgery x Phenotype condition. ADX obese saline-treated mice consumed more than both ADX obese 0.5 NLX mice and ADX obese 2.0 NLX mice. Similarly, ADX lean saline-treated mice ingested more than ADX lean 2.0 NLX mice. SHAM obese mice consumed less food at the highest drug dose level compared to SHAM obese 0.5 NLX and SHAM obese saline-treated mice. In addition, SHAM obese saline-treated mice ate more than SHAM obese 0.5 NLX mice, who, in turn, ingested more than SHAM obese 2.0 NLX mice. Naloxone did not influence differential intake in SHAM lean mice.

Cumulative food intake after 2 h of re-feeding was significantly affected by Surgery, F (1, 104) = 32.54, $p < 0.0001$, Phenotype, F (1, 104) = 16.40, $p < 0.0001$, and Drug Dose, F (2, 104) = 11.03, $p < 0.0001$. Overall, ADX mice (M = 0.17) consumed less than SHAM mice (M = 0.32), and obese mice (M = 0.30) ate more than lean mice (M = 0.19). Mice at the highest drug dose (M = 0.16) ingested less than both 0.5 NLX-treated mice (M = 0.25) and saline-treated mice (M = 0.30).

A Surgery x Phenotype interaction effect, F (1, 104) = 23.86, $p < 0.0001$, and a Phenotype x Drug Dose interaction effect, F (2, 104) = 9.87, $p < 0.0001$, were also found. ADX obese mice (M = 0.16) consumed less than SHAM obese mice (M = 0.45), and SHAM obese consumed more than SHAM lean (M = 0.20) and ADX lean (M = 0.18). No differences were found between ADX obese and ADX lean mice. Obese saline-treated mice (M = 0.44) ate more than lean saline-treated mice (M = 0.19). Obese mice at the highest drug dose (M = 0.15) ingested less than both obese mice at the lowest drug dose (M = 0.30), and saline-treated obese mice (M = 0.44). Obese 0.5 NLX-treated mice consumed less than obese saline-treated mice.

Although a Surgery x Phenotype x Drug Dose interaction effect (F (2, 104) = 2.24, $p < 0.11$) was not found, linear contrasts as depicted in Figure 3, revealed that ADX obese saline-treated mice consumed more food than SHAM lean saline-treated mice. No significant differences were found between ADX obese and ADX lean saline-treated mice, and between ADX lean and SHAM lean saline-treated mice. ADX obese 2.0 NLX mice ingested less chow than SHAM lean 2.0 NLX mice, however, no differences were found between ADX obese and ADX lean 2.0 NLX mice, and between ADX lean and SHAM lean 2.0 NLX-treated mice. In addition, it was found that 30 % of ADX obese mice in the 2.0 naloxone condition consumed absolutely nothing during the 2 h refeeding test. All mice in the other treatment conditions ate something during the re-feeding test. SHAM obese saline-treated mice consumed more than ADX obese saline-treated mice, ADX lean saline-treated mice, and SHAM lean saline-treated mice. At the lowest drug dose (0.5 NLX), SHAM obese mice ingested more than ADX obese, ADX lean, and SHAM lean mice. At the highest drug dose (2.0 NLX), SHAM obese mice ate more than ADX obese mice. Drug dose differences were found for ADX obese mice and SHAM obese mice. ADX obese saline-treated mice consumed more than both ADX obese 0.5 NLX mice, and ADX obese 2.0 NLX mice. SHAM obese mice consumed less food at the highest drug dose compared to SHAM obese 0.5 NLX mice and SHAM obese saline-treated mice. In addition, SHAM obese saline-treated mice ate more than SHAM obese 0.5 NLX mice, who, in turn, ingested more than SHAM obese 2.0 NLX mice. Naloxone did not influence differential intake in SHAM lean mice.

The results of MANOVAs on the dependent variables related to food intake mirrored those of the univariate analysis. All Wilks' Lambda values were significant at the $p \leq 0.05$ level. Therefore, the multivariate analysis provided no unique information than that provided by the univariate analysis.

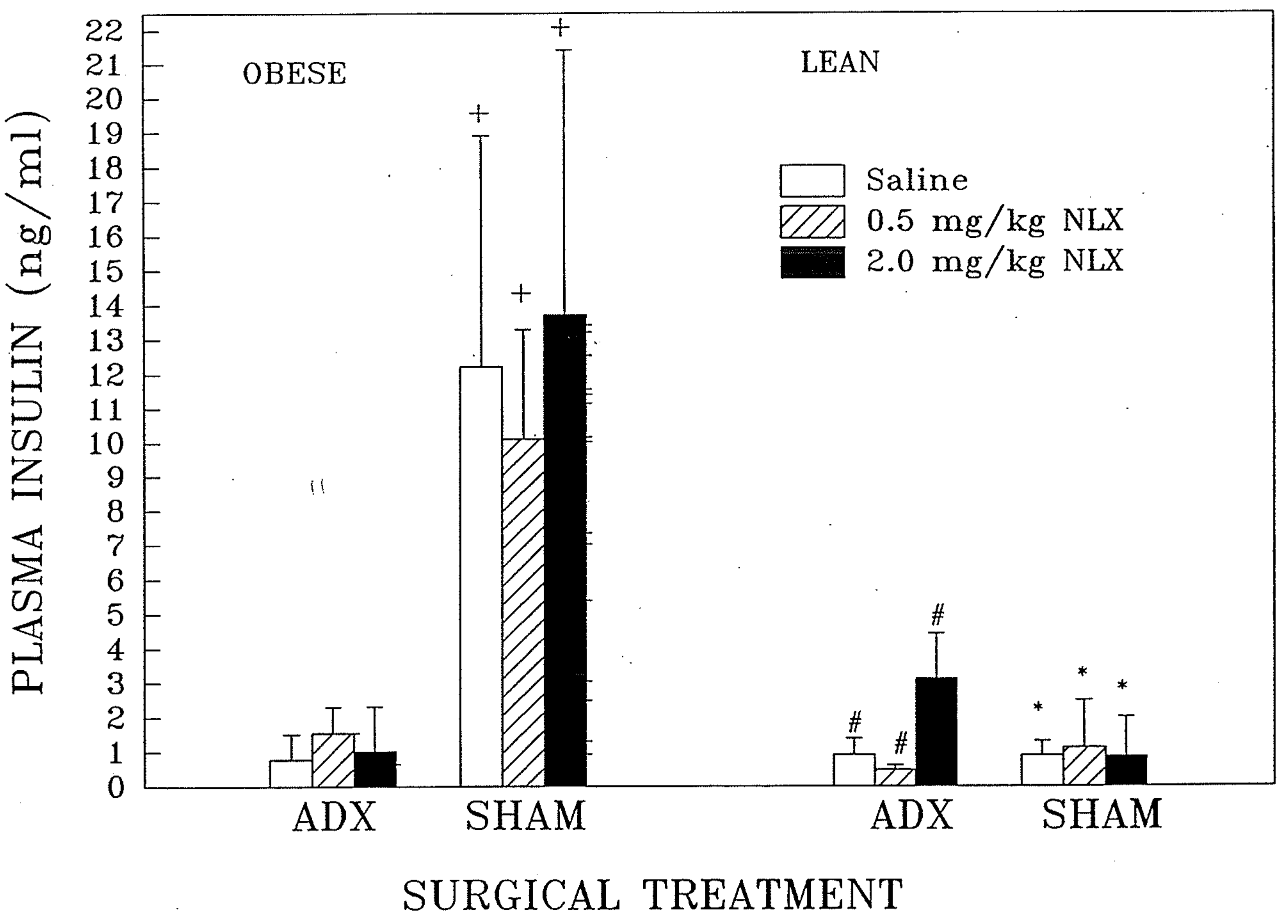
Plasma Corticosterone, Insulin and Glucose Assays

Verification of successful adrenalectomy was measured using a radioimmunoassay procedure. Mice that were adrenalectomized and had less than 1.0 µg/dl of corticosterone in their plasma were used in the present study. It was found that of those mice who survived the surgical procedure, 83 % had an acceptable range of plasma corticosterone. Plasma corticosterone (µg/dl) was lower in ADX obese ($M = 0.7 \pm 0.1$), ADX lean ($M = 0.8 \pm 0.1$), and SHAM lean ($M = 3 \pm 0.6$) mice compared to SHAM obese ($M = 17 \pm 1.8$) mice.

Plasma insulin (ng/ml) was significantly affected by Surgery, $F(1, 90) = 56.88$, $p < 0.0001$, and Phenotype, $F(1, 90) = 60.56$, $p < 0.0001$. ADX mice ($M = 1.38$) had lower plasma insulin levels compared to SHAM mice ($M = 6.57$), and obese mice ($M = 6.41$) had higher levels compared to lean mice ($M = 1.30$). As Figure 4 shows, a Surgery x Phenotype interaction effect, $F(1, 90) = 69.52$, $p < 0.0001$, revealed that

Figure Caption

Figure 4. Effects of naloxone administration on plasma insulin levels (+ SD) in adrenalectomized and sham-adrenalectomized genetically obese and lean mice. (* $p < 0.05$ for between phenotype comparisons; + $p < 0.05$ for between surgery comparisons; # for comparisons between SHAM-OBESE and ADX-LEAN mice).



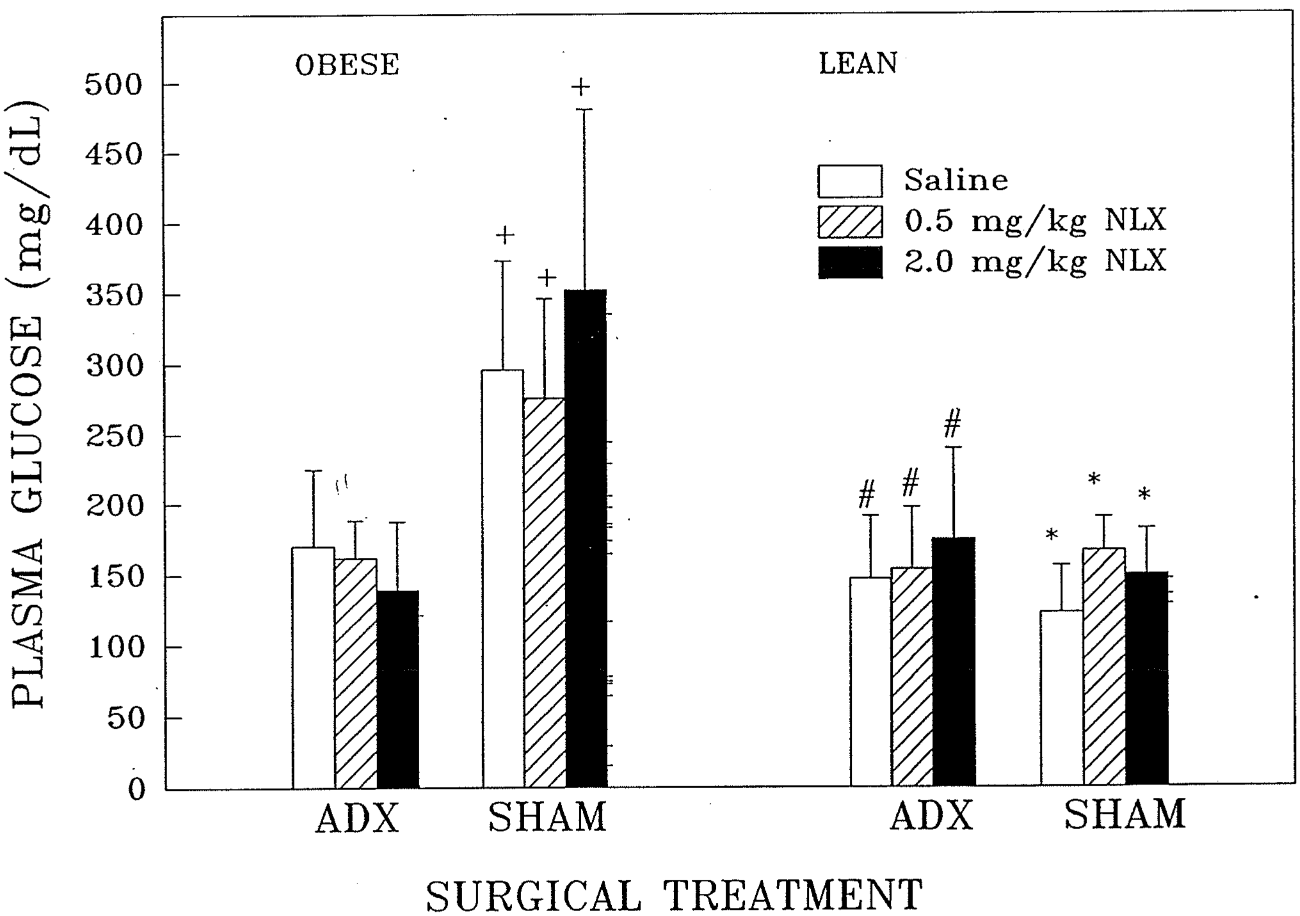
PLASMA INSULIN (ng/ml)
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
OBESE
LEAN
Saline
0.5 mg/kg NLX
2.0 mg/kg NLX
+
#
*
ADX
SHAM
ADX
SHAM
SURGICAL TREATMENT

SHAM obese mice (M = 12.19) had higher insulin levels than ADX obese mice (M = 1.12), ADX lean mice (M = 1.64), and SHAM lean mice (M = 0.95). Insulin levels did not differ between the latter three groups. Furthermore, naloxone administered 30 min presampling did not alter this profile (Phenotype x Surgery x Drug Dose: F (2, 90) = 2.14, $p < 0.12$). Linear contrasts showed that at every drug dose, SHAM obese mice had significantly elevated insulin concentrations compared to ADX obese, ADX lean, and SHAM lean mice.

Plasma glucose levels (mg/dL) were significantly affected by Surgery, F (1, 89) = 28.05, $p < 0.0001$, and Phenotype, F (1, 89) = 37.65, $p < 0.0001$. Adrenalectomy (M = 158.29) lowered glucose levels from SHAM values (M = 224.91), although, overall, obese mice (M = 234.18) maintained higher glucose levels than lean mice (M = 150.87). A Surgery x Phenotype interaction effect, F (1, 89) = 38.95, $p < 0.0001$, (see Figure 5) was also found. SHAM obese mice (M = 310.73) had greater glucose levels compared to ADX obese mice (M = 157.64), ADX lean mice (M = 158.91), and SHAM lean mice (M = 142.83). Glucose levels did not differ among the latter three groups. Although a Surgery x Phenotype x Drug Dose interaction effect (F (2, 89) = 2.24, $p <$ 0.11) was not found, linear contrasts revealed that at every drug dose, SHAM obese mice had significantly

Figure Caption

Figure 5. Effects of naloxone administration on plasma glucose levels (+SD) in adrenalectomized and sham-adrenalectomized genetically obese and lean mice. (* $p < 0.05$ for between phenotype comparisons; + $p < 0.05$ for between surgery comparisons; and # $p < 0.05$ for comparisons between SHAM-OBESE and ADX-LEAN mice).


OBESE
LEAN
Saline
0.5 mg/kg NLX
2.0 mg/kg NLX
PLASMA GLUCOSE (mg/dL)
500
450
400
350
300
250
200
150
100
50
0
+
#
*
ADX
SHAM
ADX
SHAM
SURGICAL TREATMENT

greater glucose concentrations than ADX obese, ADX lean, and SHAM lean mice.

The results of the MANOVAs on the dependent variables plasma glucose and plasma insulin mirrored those of the univariate analysis. All Wilks' Lambda values were significant at the $p \leq 0.05$ level. Therefore, MANOVAs provided no unique information other than that provided in the univariate analysis.

Discussion

Effect of Adrenalectomy on Baseline Body Weight and Food Intake

An increased level of corticosterone in rodents and an excessive endogenous production of cortisol in humans (i. e., Cushing's syndrome) are linked with overeating (especially diets rich in carbohydrates and fats) and obesity (Leibowitz, 1992; Sarker, Thompson, McLeod, 1990). Genetically obese (ob/ob) mice have significantly elevated serum corticosterone levels compared with lean littermates as early as Postnatal Day 17 (Dubuc, 1977; Naeser, 1974). Lowering corticosterone by adrenalectomy ameliorates many aspects of the obese syndrome, including hyperphagia, and body weight gain (Bailey, Day, Bray, Lipson, & Flatt, 1986; Herberg & Kley, 1975; Naeser, 1973; Saito & Bray, 1984; Smith & Romsos, 1985). Treatment with cortisone in adrenalectomized ob/ob mice significantly increases body weight gain and food intake in a dose-related manner with no effect on weight gain in adrenalectomized lean mice, at any dose (Shimura, Bray, & Lee, 1987). These data suggest that the ob/ob's hyperadrenocortism may play a role in the development and/or maintenance of its obesity.

Consistent with previous reports, the present study found that adrenalectomy reduces body weight and daily food intake in obese (ob/ob) mice but not in lean mice. Adrenalectomized obese mice weigh less than SHAM obese mice, as early as one week postoperatively, but more than ADX lean and SHAM lean mice at all times measured postoperatively. Moreover, in comparison to ADX lean, SHAM lean, and SHAM obese mice, ADX obese mice exhibit a negative percentage body weight gain one week after surgery, on the re-feeding test day, and on the plasma test day. For the adrenalectomized obese mice this growth rate may, in part, be accounted for by their lower food consumption that occurs subsequent to surgery. SHAM obese mice continue to grow, as do ADX lean and SHAM lean mice.

Effect of Adrenalectomy and Naloxone Administration During the Re-feeding Test

In addition to elevated corticosterone levels, an abnormal opioid status exists in genetically obese rodents and is thought to contribute to their hyperphagia and obesity. β-endorphin content in the brain, pituitary, gastrointestinal tract, adrenal gland, pancreas, and plasma is significantly higher in several obese strains of mice and rats (Khawaja, Bailey, & Green, 1989; Margules, et al., 1978; Recant, Voyles, Timmers, Awoke, Bhathena, & Wells, 1984). Administration of the opiate antagonist naloxone suppresses food consumption in genetically obese mice

(Levine, Morley, Brown, Handwerger, 1981; Margules et al., 1978; Shimomura, Oku, Glick, & Bray, 1982) Zucker obese (fa/fa) rats (McLaughlin & Baile, 1984) and cafeteria-fed obese rats (Mandenoff, Fumeron, Apfelbaum, & Margules, 1982). At low doses, genetically obese rodents are more sensitive to the suppressive effects of naloxone compared to lean littermate controls. Furthermore, it appears that this difference in sensitivity to threshold doses of naloxone is present before the visual appearance of obesity in genetically obese rodents and thus β-endorphin is likely to be involved in the development and progression of obesity rather than a consequence of obesity (McLaughlin & Baile, 1984; McLaughlin & Baile, 1985).

Opiate agonists (morphine) increase adrenal cortical activity (corticosterone) and pituitary ACTH activity in both rats and humans (Meites, 1984). It has been suggested that the adrenal glands are important in modulating the feeding response to opiate agonists and antagonists. Bhakthavatsalem & Leibowitz (1986) observed that adrenalectomy reduces morphine-induced feeding in male Sprague-Dawley rats and that a single injection of corticosterone restores feeding. Other researchers report that exogenous opiates enhance feeding responses and opiate antagonists (naloxone) attenuate the anorectic effects in adrenalectomized rats (Levine & Morley, 1983; McLean & Hoebel, 1983). In contrast, Wallace, Fraser, Clements, & Funder (1981) found no effect of adrenalectomy on baseline feeding or on the anorectic effect of naloxone in rats. Similarly, Cooper, Jackson, Kirkham, & Turkish (1988) reported that adrenalectomy did not alter the anorectic effect of naltrexone (0.3-3.0 mg/kg) or diprenorphine (0.3-3.0 mg/kg) in non-deprived rats in a 30-min feeding test situation. Both adrenalectomized and sham-adrenalectomized rats consume less palatable food at all drug doses in comparison to saline control rats. Although these results are equivocal, it appears that in some experiments adrenalectomy alters the effects of opiate agonists and antagonists on feeding in non-pathological animal models. Methodological differences might partially explain the contradictory results observed in these studies. Variables such as time of the feeding test (i. e., nocturnal versus the light phase of the diurnal cycle), length of the feeding test situation (i. e., 30 min or longer), and nutritional state of the animal (i. e., fed versus food-deprived) might contribute to these disparate findings.

Removal of circulating glucocorticoids by adrenalectomy increases both ACTH and β-endorphin levels (Guillemin, 1977); and these polypeptides are thought to be raised to even higher abnormal levels in *ob/ob* mice as a consequence of this surgical manipulation (Margules, 1979). Because opiate receptor antagonists selectively decrease food intake in genetically obese mice, it was postulated that the feeding reponse in adrenalectomized naloxone-treated obese mice would be attenuated compared to sham adrenalectomized naloxone-treated obese mice, as well as sham lean and adrenalectomized lean naloxone-treated mice.

Results from the present study demonstrate that naloxone decreases cumulative food consumption in all mice (both obese and lean) at all times measured (i. e., 1/2 h, 1 h, 1-1/2 h, and 2 h) compared to saline-treated mice. As was anticipated, saline-treated SHAM obese mice ate more chow (cumulative intake) than ADX obese, ADX lean, and SHAM lean saline-treated mice at all measurable times during re-feeding. Similarly, at the lowest drug dose of naloxone (0.5 mg/kg), SHAM obese mice consume more cumulative food at all selected times during the 2-h re-feeding test than ADX obese, ADX lean, and SHAM lean mice in the lowest dose of naloxone condition. At the highest drug dose of naloxone (2.0 mg/kg), SHAM obese mice ingest more cumulative food at 1/2 h and 1 h compared to ADX obese, ADX lean, and SHAM lean mice. SHAM obese 2.0 naloxone-treated mice have consumed more chow by 1-1/2 h and by 2 h than ADX obese mice in the highest dose of naloxone condition. By 2 h, the cumulative food intake of SHAM obese 2.0 naloxone-treated mice was equivalent to similarly dosed ADX lean and SHAM lean mice.

No significant differences were observed in cumulative food consumption between ADX obese, ADX lean, and SHAM lean mice at both the lowest and highest drug doses by 1/2 h and 1 h of re-feeding. By 2 h, ADX obese mice in the highest naloxone condition had ingested less food than SHAM lean mice in this drug dose condition, but an equivalent amount compared to ADX lean mice.

As the dose of naloxone increases, the amount of food consumed decreases in comparison to saline-treated mice. ADX obese mice consumed less food at either the 0.5 or 2.0 mg/kg BW dose of naloxone compared to saline-treated ADX obese mice at all times measured during the 2-h re-feeding test. Similarly, SHAM obese mice treated with the highest dose of naloxone ingested less food than if treated with either saline or the lowest dose of naloxone at all times measured during the 2-h re-feeding test. SHAM obese saline-treated mice ate more than SHAM obese 0.5 naloxone-treated mice, who, in turn, ingested more than SHAM obese 2.0 naloxone-treated mice at all times measured in the study.

Similarly, ADX lean mice ingested less food when given the highest dose of naloxone than observed in the saline condition at 1/2 h, 1 h, and 1-1/2 h. ADX lean mice in the 0.5 mg/kg BW condition consumed less chow than ADX lean mice in the saline condition during the first 30 min of re-feeding. No effect was obtained for the low dose after 30 minutes. For the SHAM lean mice the highest drug dose decreased food intake relative to that obtained for the 0.5 mg/kg BW dose after 30 minutes, and relative to the saline condition after 1 h. No drug dose differences were found with SHAM lean mice after 1 h.

Interestingly, naloxone exerted an effect on cumulative food intake in ADX and SHAM obese mice throughout the entire 2-h re-feeding test, but were no longer detectable in lean mice by the end of testing. Drug effects are expected to be observed early in the re-feeding test because naloxone is a short acting opioid antagonist. Naloxone (5 mg/kg) is fully circulated within 5-min post-injection (intravenous) or 15-min post-injection (subcutaneous) and reaches peak efficacy (serum half-life) by 30-40 min post-injection (Berkowitz, Ngai, Hempstead, & Spector, 1975; Ngai, Berkowitz, Yang, Hempstead, & Spector, 1976). The extra fatty tissue that is present in the obese condition may affect the distribution, metabolism or pharmacodynamics of naloxone. One method of circumventing the problem of interpreting effects of opiate antagonists in obese animals is to test them before their obese condition develops (Cooper et al., 1988). The present experiment attempted to control for these effects by adrenalectomizing obese mice at a young age (5 weeks old) and testing them two weeks later. Results suggest that there is a difference in the endorphinergic activity between obese and lean mice, and naloxone is capable of reducing food intake in both adrenalectomized obese and sham obese mice for a longer period of time compared to lean controls.

Effect of Adrenalectomy and Naloxone Administration on Plasma Glucose and Insulin Levels

In addition to an enhanced feeding response, systemic administration of β-endorphin induces hyperglycemia in humans (Feldman, Kiser, Unger, & Li, 1983) and rats (Matsumura, Fukushima, Saito, & Saito, 1984) and can increase plasma insulin concentrations in humans (Giugliano, Cozzolino, Salvatore, Ceriello, & Torella, 1987). There is some indication that hypersecretion of endogenous opioid peptides and/or altered sensitivity of the pancreatic beta cells to β-endorphin may be important factors in the pathogenesis of obesity and non-insulin-dependent diabetes mellitus.

For example, plasma β-endorphin is higher in non-insulin-dependent diabetics (Vermes, Steinmetz, Schoorl, Van der Veen, & Tilders, 1985) and obese subjects (Genazzani, Facchinetti, Petraglia, Pintor, & Corda, 1986; Givens, Wiedmann, Andersen, & Kitabchi, 1980). Similarly, β-endorphin and enkephalin content are elevated in the brain, pituitary, pancreas and plasma of genetically obese mice (Khawaja et al., 1989; Recant et al., 1984). In fact, peripheral administration of β-endorphin (1 mg/kg BW, IP) induces a naltrexone reversible increase in plasma insulin levels within 30 min in ob/ob mice who are 13-15 weeks of age but has no effect on lean controls (Khawaja & Green, 1991). In addition, β-endorphin promotes a naloxone reversible release of insulin from isolated ob/ob and lean mouse islets incubated in a medium containing 6 mM glucose (Khawaja & Green, 1991).

Dynorphin content (an endogenous ligand for kappa-type receptors and a potent appetite stimulant) is raised in the pituitary, as well as the VMH and PVN in ob/ob mice (Ferguson-Segall et al., 1982; Khawaja et al., 1989). These are areas of the hypothalamus in which a microinjection of β-endorphin stimulates feeding. Moreover, an increased number of κ-receptor binding sites have been found in the brain of ob/ob mice compared with lean mice (Khawaja, Bailey, & Green, 1989). Administration of κ-opiate agonists (U 50488h and dynorphin A 1-13) to ob/ob mice raises plasma insulin and glucose levels, and these effects are blocked by simultaneuos administration of naloxone (10 mg/kg). Lean mice also show an increase in plasma glucose, but their response is weaker and is only observed at a higher drug dose. Plasma insulin levels in lean mice are raised transiently by U 50488h and not at all by dynorphin (Khawaja, Green, Thorpe, & Bailey, 1990). These studies demonstrate that κ-agonists can further increase plasma insulin and glucose levels in obese mice to a greater extent than in lean controls.

Researchers have reported that adrenalectomy in obese mice lowers their plasma glucose levels to values observed in lean controls; however, ob/ob's plasma insulin values, although reduced, still remain higher than those of their lean littermates (Bailey et al., 1986; Herberg & Kley, 1975; Naeser, 1973; Smith & Romsos, 1985; Solomon et al., 1977). Because adrenalectomy further elevates β-endorphin levels, which, in turn, raise insulin levels, and opiate receptor antagonists decrease plasma insulin secretion in obese mice, I hypothesized that plasma insulin secretions in adrenalectomized ob/ob's would be reduced to lean control levels after naloxone administration.

Results from this experiment show that

adrenalectomy had an effect on plasma insulin and plasma glucose levels in obese but not in lean mice. ADX obese mice had significantly lower plasma insulin and plasma glucose levels than SHAM obese mice, and equivalent values compared to ADX lean and SHAM lean mice at all drug doses. Naloxone did not exert an additional decrease in plasma insulin or plasma glucose in ADX obese mice at least, at the 30 min postinjection assay conducted in this research. The discrepancies in insulin values in this study and those reported earlier may be explained by differences in age of adrenalectomy and also methods used to verify successful removal of the adrenal glands. Mice in the present study were adrenalectomized at 5 weeks of age, and plasma corticosterone levels were measured using a radioimmunoassay procedure with the criterion of success being less than 1.0 μg/dl corticosterone. Other studies have adrenalectomized mice at a later age and/or have used alternative and less objective methods to access successful adrenalectomy (i. e., visual inspection under magnification of the excised adrenal gland or microscopic inspection of residual adrenal tissue in the body cavity) (Bailey et al., 1986). Smith & Romsos (1985) reported that when obese mice are adrenalectomized at 3 weeks of age plasma insulin values are reduced to those of lean controls when measured at 6 weeks of age. However, when obese mice are adrenalectomized at 6 weeks of age, plasma insulin values 3 weeks later (i. e., 9 weeks old) are four times higher than lean mice. Thus, age at the time of surgery may be a critical factor with respect to insulin values. In this regard, the adrenalectomized obese mice in the present study might have had higher insulin values if they had been sacrificed at 9 rather than 7 weeks of age. An interesting future project would be to adrenalectomize obese mice at 5 weeks of age and then sacrifice the animals at 7 weeks, 8 weeks and 9 weeks of age to determine the exact time when plasma insulin values rise postoperatively, if in fact values become higher than lean controls.

It is indeed curious that naloxone exerted an effect on food consumption but had no impact on plasma insulin levels. Based on these data, naloxone may have exerted its effects on food intake more centrally than peripherally. Another interpretation, however, focuses on the times at which food intake and plasma variables were assessed. Mice were sacrificed 30 min postinjection on the plasma test day - a time corresponding to reported peak systemic drug concentrations in non-obese rodents. It is possible that the combined impact of drug and surgical treatments only became apparent at later times during the 2-h feeding test - times at which these plasma variables were not assayed. Furthermore, this interpretation may help understand the prolonged impact of naloxone on ADX-obese mice, such as the significant suppression of cumulative food intake after 2 h by the higher dose of naloxone beyond its effect on SHAM-obese controls. Future studies using only centrally acting opiate antagonists in adrenalectomized obese mice and studying both the feeding reponse and plasma chemical profiles would be valuable additions to this work.

Conclusions

The results of the current study indicate that naloxone elicits a dose-dependent decrease in feeding (of a pelleted stock diet) for 2 h in food-deprived adrenalectomized obese mice. Research studies report that the benefits of adrenalectomy in obese mice are diet-dependent. Adrenalectomized ob/ob mice exhibit the full obese syndrome when given a high carbohydrate or high fat diet (Grogan, et al., 1987; Warwick & Romsos, 1988). Central or peripheral injections of morphine in rats increases their intake of fat and protein (Bhakthavatslam & Leibowitz, 1986). Similarly, chronic infusion of morphine in 4-month-old Long-Evans rats for 22 consecutive days results in a greater selection of fat compared to carbohydrate or protein (Ottavianii & Riley, 1984). Opiate antagonists reduce fat consumption in normal food-deprived rats (Marks-Kaufman, Plager, & Kanarek, 1985). Gilson & Wilson (1989) found that naloxone not only preferentially reduced total food consumption in obese mice in a dose-dependent manner, but also specifically decreased fat and protein intake. Future studies addressing the role of opiate antagonists in adrenalectomized obese mice on specific macronutrient selection would be of interest.

Additionally, age of adrenalectomy appears to be an important consideration when looking at plasma chemical profiles. The present study found that adrenalectomy at 5 weeks of age reduced plasma insulin and glucose values to those of lean controls. Although body weight, food intake, insulin levels, and carcass energy are significantly reduced in obese mice who are 3-months-old at the time of adrenalectomy, values are still higher than in lean control mice (Feldkircher & Romsos, 1991). Future investigations with both younger and older adrenalectomized obese mice using chronic subcutaneous infusions of naloxone via minipumps to study these variables would shed more light on the opioid-glucocorticoid linkage in this animal model of obesity and non-insulin-dependent diabetes.

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