Neuroendocrine Perturbations in Human Obesity

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“Rideo ergo sum”

Contents

Chapter 1

General Introduction

Most individuals match energy intake, expenditure and storage with great precision (1-3). This phenomenon reflects an active regulatory process, which is termed energy homeostasis. Energy homeostasis promotes stability in body weight and in the amount of body energy stored in the form of fat. The ability of animals to conserve energy in the form of adipose tissue and the timed process of body fattening can be considered as an evolutionary advantage to survive periods of food shortage (4). For example, body fat stores provide energy for hibernation, migration or pregnancy. However, nowadays the abundance of highly palatable energy dense foods combined with minimal requirement for physical activity (increased industrialization, urbanization and mechanization) strongly promotes the expansion of adipose tissue mass towards levels at which the risk of morbidities and mortality are severely increased.

Obesity - Definition and Classification

In medical terms, the excessive accumulation of body fat is called "obesity". "Obesity" originates from the Latin word "Obesus" that means fat, plump or swollen and its past principle "Obedere" means to eat upon or to eat away. A rough measurement for the diagnosis and the classification of obesity is the body mass index (BMI), which is calculated as follows: weight (kg)/(length (m)) 2. A BMI of 25-30 kg/m^2 is considered as overweight and a BMI > 30 kg/m^2 indicates obesity (5). The classification of obesity according to the WHO guidelines, using the BMI is given in Table 1.

Table 1.

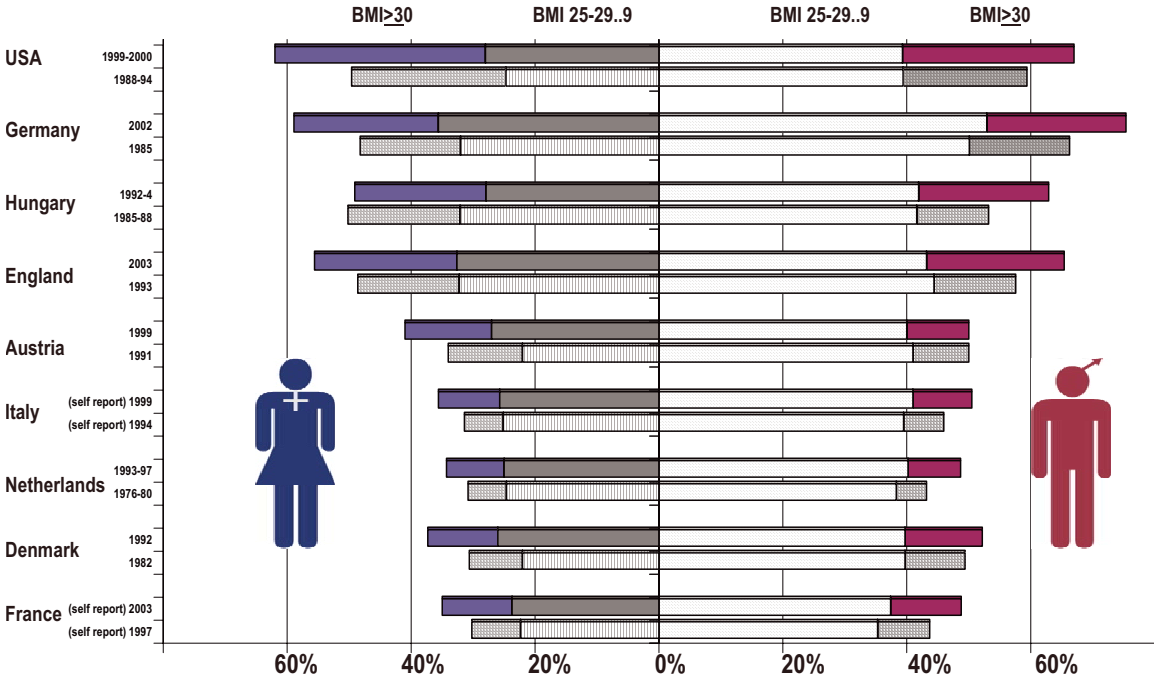
Classification	BMI (kg/m^2)	Risk co-morbidity	Action level & Consequences
Normal	18.5-24.9	Medium	
Overweight	25-29.9	Slightly increased	1: Prevention weight gain
Obesity	≥ 30		2: Weight reduction (10-15%)
Level I	30-34.9	Increased	and stabilisation body with
Level II	35-39.9	Severely increased	professional care
Level III	≥ 40	Highly increased	

Derived from: Meinders AE, Fogteloo J. NTvG 2003 Sep 20; 147(38):1847-51 and Guidelines WHO Tech Rep Ser 894, 2000

Obesity - Epidemiology

The overall prevalence of obesity has risen dramatically over time. Globally there are more than 1 milliard overweight adults and at least 300 million of them are obese (World Health Organization). The obesity epidemic is not restricted to industrialized societies; obesity often co-exists with under-nutrition in developing countries and the increasing prevalence of obesity in these countries is often faster than in the developed world (5). Furthermore, obese adults of developing countries, who were undernourished in early life, tend to develop hypertension, cardiovascular disease and diabetes at earlier age and in more severe form than those who were never undernourished. Figure 1 shows the increasing prevalence of adult overweight and obesity in the USA and Europe.

Figure 1.



Derived from: European Obesity Task Force EU Platform Briefing Paper March 2005

In the Netherlands the prevalence of adult obesity has risen from 4.9 to 8.5% in men and from 6.2 to 9.3% in women between the late 1970s and mid- 1990s. Table 2 represents prevalence of overweight and obesity among adults in the Netherlands based on data collected from studies between 1998 and 2002.

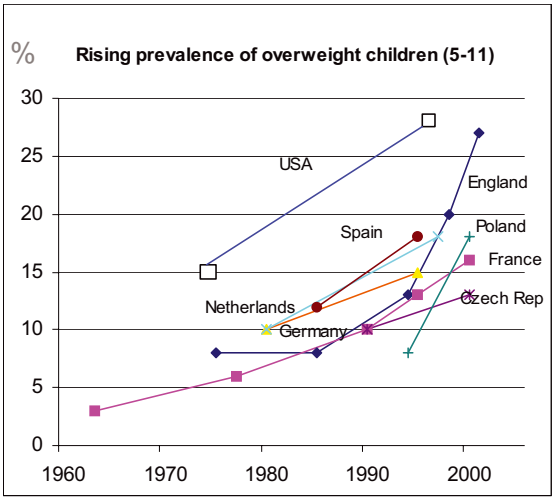
Table 2.

Prevalence in the Netherlands	% BMI 25-29.9	% BMI ≥ 30	% BMI ≥25
Male	43.5	10.4	53.9
Female	28.5	10.1	38.6

Derived from: European Obesity Task Force EU Platform Briefing Paper March 2005

The global obesity epidemic affects individuals of all ages and the rising prevalence and incidence of overweight and obesity among youngsters is a rapidly growing problem in many countries. This rapid increase is especially worrisome because of the well described association between childhood obesity and increased cardiovascular risk and mortality in adulthood (6;7). Furthermore, persistent obesity in adulthood after childhood obesity is associated to a higher degree of adverse health consequences compared to the pattern of adversity of obesity confined to adult life (8). The cut off point for excess fatness of overweight or obesity in children and adolescents is based on the sex-specific "percentile of BMI for age". Overweight is defined as ≥ 85th percentile of BMI for age and obesity ≥ 95th percentile of BMI for age (9). According to global estimates of the WHO, world wide about 22 million children under the age of five are overweight. Figures of the International Obesity Task Force show that one in 10 school-age children (age 5-17 years old) is overweight, which includes a total of 155 million children world wide. 30-45 Million children within that figure are classified as obese. Although the rates of increase vary among different countries, a rapidly rise of childhood overweight and obesity has been observed in the USA and Europe (Figure 2).

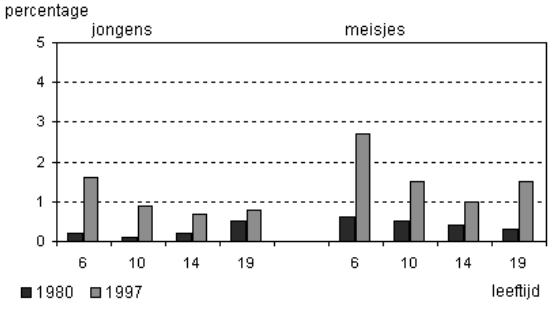
Figure 2.



Derived from: European Obesity Task Force EU Platform Briefing Paper March 2005

Figure 3 shows the severely increased prevalence of overweight and obesity among boys and girls in the Netherlands between 1980 and 1997.

Figure 3.



Derived from: Hirasing RA, et al. NTvG 2001; 145(20);1303-4)

Finally, a cynical remark could be made that the wide spread occurrence of obesity not only affects human beings but also the domestic animals they take care of. This is well illustrated by the increasing incidence and prevalence of obesity among cats and dogs and treating co-morbidities such as diabetes mellitus type 2 of obese pets is one of the growing common activities for veterinary clinicians (10;11). Thus, the abundance of highly palatable energy dense foods in the civilized society even badly affects man's best friend.

Obesity-Health Consequences

Obesity is associated with numerous metabolic disturbances, such as insulin resistance, diabetes mellitus type 2, dyslipidemia and hypertension (12-15). The development of type 2 diabetes is not only confined to older adults with increased body fatness but also affects obese children even before puberty. Conversely, 85% of people with type 2 diabetes are overweight (16). Furthermore, obesity leads to several other health problems including disturbances of the respiratory and musculoskeletal system (for example sleep apnoea and osteoarthritis), gallbladder disease, skin difficulties and infertility (17;18). Obesity is also associated with increased risks of cancer, in particular cancer of the breast, colon, prostate, endometrium, kidney and gallbladder (19-22). Epidemiologic studies in primarily white populations have shown a strong linkage between obesity and increased mortality rates. This increase begins to rise slowly at a BMI > 25 kg/m^2 and steeply increases at a BMI>30 kg/m^2, towards a 1.5-2.0 fold excess independent risk of mortality compared to individuals with a BMI < 25 kg/m^2 (23).

Obesity - Visceral Adiposity

Excess of fat deposition within the abdomen, or so called visceral adiposity, confers an independent risk for metabolic (diabetes mellitus type 2) and cardiovascular complications than does adipose accumulation elsewhere (24-27). This close relation between excess body fat in the visceral depot and metabolic disturbances or cardiovascular disease might be explained by the specific endocrine features of the visceral fat depot and/or its unique anatomical relation to the hepatic portal circulation (thereby releasing adipokines and free fatty acids directly into the portal venous system in stead of the peripheral systemic circulation).

Waist circumference appears to be a reliable index of intra-abdominal fat mass (27). Changes in waist circumference reflect changes in risk factors for cardiovascular disease and other co-morbidities associated with visceral obesity (28). Therefore, waist circumference can be used as a simple additional tool to assess health risks associated with visceral obesity. Table 3 shows the cut off point of sex-specific waist circumferences that denote increased risk for metabolic complications associated with obesity in the Caucasian population (27).

Table 3.

	Risk metabolic complications	
	Increased	Substantially increased
Male	≥ 94 cm	≥ 102 cm
Female	≥ 80 cm	≥ 88 cm

Obesity-Treatment

The fundamental approach to reverse the obesity epidemic is effective weight management for obese individuals and groups at risk for developing obesity (5;29). Most of the putative strategies in order to achieve weight reduction include life style management focused on dietary intervention and increased physical activity, with the use of a variety of pharmacological agents such as orlistat and sibutramine. In the most severe cases invasive techniques (bariatric surgery) might be used in order to achieve weight loss. However, obesity remains a medical condition which is difficult to manage and weight regain is among the greatest challenges related to a weight loss intervention (30).

Obesity - Pathophysiology

The pathophysiologic mechanism of obesity remains elusive and explanations are still lacking why some individuals are more likely to suffer from obesity than others in the same environment. Various factors such as genetic, social, behavioural and physiological cues are responsible for the development of obesity. As obesity runs in families it has been recognized and appreciated that a genetic component is involved its development (31). So far, a number of families with rare pleiotropic obesity syndromes have been studied by linkage analysis and some chromosomal loci for obesity syndromes have been recently described (see Online Mendelian Inheritance in Man (OMIM) http://www.ncbi.nlm.nih.gov/omim/). The Prader-

Willi syndrome is the most common syndromal cause of human obesity (estimated prevalence of about 1 in 25.000) caused by deletion or disruption of the paternal segment 15q11.2-q12. Other syndromes in which obesity is a recognized part of the phenotype are for example Albright hereditary osteodystrophy, Fragile X syndrome or the Bardet Biedl Syndrome. Single gene mutations, such as either the dominant (yellow, Ay/a) or recessive (ob/ob, db/db, fa/fa, tb/tb) gene defects, are known to cause genetic and experimental syndromes of obesity in rodents (32-35). Based on these studies it has been hypothesized that mono genetic defects can lead to disorders of energy balance and obesity in humans. Indeed, these candidate gene mutations have also been identified in obese humans. For example, mutations of the leptin gene (leading to congenital leptin deficiency) and leptin receptor genes are linked to early onset, severe obesity (36;37). Additionally, mutations of the gene encoding pro-opiomelanocortin (POMC), which is known as one of the anorexigenic hypothalamic neuropeptides, are associated with early onset childhood obesity (38). Also, loss of function mutations of other signalling molecules (Prohormone Convertase 1 deficiency) or receptors (Melanocortin 4 Receptor deficiency) of the melanocortin system which is involved in the regulation of body weight in humans, lead to severe childhood obesity (39). Finally, de novo mutations in the neurotrophin receptor TrkB and missense mutations in the cocaine- and amphetamine-regulated transcript (CART) induce severe obese phenotypes through physiological disturbances in the regulation appetite and energy intake (40). Although a strong linkage has been described between genetics and obesity, in general, the development of obesity is not simply due to single gene mutations (41). A comprehensive and updated reference for all association studies in obesity genetics is available in the form of the obesity gene map established by Bouchard, Chagnon, Perusse and colleagues at The Pennington Biomedical Research Centre (link to http://www.obesite.chaire.ulaval.ca/genemap.html).

Obesity-Neuroendocrinology

From a biological point of view, obesity might be explained by differences in the regulation of energy homeostasis between obese and lean individuals. Energy homeostasis is achieved by variable effects on energy intake, expenditure and storage, coordinated through the central nervous system (42;43). Signals related to either short term nutrient availability (e.g. nutrients and gastro intestinal peptides) or the amount of energy consumed over a more prolonged time period and proportion of body adiposity (the so called “long term” signals) emanate from adipose, endocrine, gastro-intestinal and neuronal systems. These efferent signals are received and integrated in the hypothalamus. On its turn, this specific brain area exerts homeostatic control over energy intake, expenditure and storage through modulation of various processes, including food intake, physical activity and neuroendocrine secretion.

The neuroendocrine system provides a source of humoral messengers many of which can modulate energy homeostasis in target cells of different organ systems. As neuroendocrine factors are involved in the regulation of energy homeostasis, alterations of the endocrine environment might contribute to the development or maintenance of excess adipose tissue mass and the obese phenotype. This thesis will focus on changes of the neuroendocrine environment in obese women. The hormonal systems studied in the obese women, will be shortly introduced in the next paragraph.

Growth Hormone

Growth Hormone (GH) is an anabolic hormone which has several effects on glucose, lipid and protein metabolism. GH increases plasma glucose concentrations through stimulation of endogenous glucose production of the liver and GH reduces peripheral glucose uptake (diabetogenic hormone)(44). Furthermore, GH stimulates protein synthesis, whereas GH inhibits protein breakdown and amino acid oxidation (45-47). Finally, GH has a profound impact on fat storage; it enhances adipose tissue lipolysis through stimulation of lipolytic enzymes and the inhibition of lipogenic enzymes (48-50) and GH facilitates lipolytic actions of epinephrine (51). Although most of the GH deficient patients are not clinically obese, they show an increased amount of body fat, with a predominant visceral adiposity (52;53). GH replacement reduces their body fat with the largest decrease in visceral fat mass independently of changes in body weight (54;55). However, it is not known whether these changes of body fat deposition observed in GH deficient patients are primarily due to the consequential loss of the lipolytic and anabolic GH actions per se. Nevertheless, it has been invariably observed that both spontaneous pulsatile GH secretion as well as the GH response to various provocative exogenous stimuli are markedly blunted in obese individuals (56). Thus, obesity and in particular visceral obesity (57), is associated with hyposomatotropism. As it has been found that

hyposomatotropism is not compensated by increased adipose tissue responsiveness to GH (58), the reduced circulating plasma GH might contribute to enlarged adipose tissue mass in obese humans.

Corticotroph Axis

The hypothalamic-pituitary-adrenal (HPA) axis is essential for the response to stress and survival. However, the HPA hormonal ensemble also regulates lipid metabolism and body fat distribution. Changes in circulating glucocorticoid levels are associated with alterations of energy homeostasis. For example, it has been shown that removal of the adrenals reduces energy intake and adipose tissue weights in rodents, which is reversed by glucocorticoid replacement (29;59-65). Furthermore, glucocorticoid administration promotes body weight gain in rodents and humans (66-68). Hypercortisolism in patients with Cushing's syndrome leads to excess of fat in the visceral depot. Lowering of plasma cortisol levels in these patients returns body fat accumulation back to normal (69;70). Although cortisol is considered to be the main messenger conveying HPA signals to target tissues, adipocytes express ACTH receptors and ACTH poses lipolytic actions in some animal species (71). Corticotrophin releasing hormone (CRH), which stimulates ACTH secretion in the pituitary gland, reduces both food intake (acting as a satiety factor at hypothalamic level) and body weight. Furthermore, CRH simultaneously increases energy expenditure in normal weight and obese rodents (72;73). Several studies suggest that the hypothalamo-pituitary-adrenal (HPA) axis is hyperactive in obese animals and humans. Experimental studies in genetically obese rodents show that these animals have high levels of glucocorticoids (74-79). Clinical studies report that both plasma ACTH and cortisol concentrations rise to higher levels in response to CRH administration alone or in combination with arginine vasopressin (AVP) in obese humans compared to normal weight controls (80-82). Moreover, the cortisol response to ACTH is exaggerated in obese volunteers (83-85) and it has been reported that stress induced cortisol secretion is increased in abdominally obese women (86). A few previous papers reported that diurnal plasma ACTH concentrations are higher in obese individuals, while circulating cortisol levels are similar to those in lean controls (87;88). Furthermore, urinary free cortisol excretion appears to be elevated in abdominally obese humans (83;84), while suppression of plasma cortisol levels by dexamethasone (89) or hydrocortisone is blunted (89-91). Recently it has been published that tissue specific changes in cortisol metabolism are associated with obesity. At tissue level, the conversion of cortisone into active cortisol is catalysed by the enzyme 11βHSD type 1, which in turn stimulates adipocyte differentiation of stromal cells to mature adipocytes. Both experimental animal studies as well as clinical studies have shown that 11βHSD type 1 is increased in the liver and visceral adipose tissue in obesity (92). Furthermore, urine analysis of cortisol and cortisone metabolites show that cortisol/cortisone ratios are significantly lower in patients with obesity, which might indicate enhanced 11βHSD type1 activity in obese individuals (93). Finally, transgenic 11βHSD type 1 over expressing mice are characterized by visceral obesity while its circulating corticosterone levels are normal (94). These genetically mutated mice were also hyperglycaemic, hyperinsulinemic and glucose intolerant. These findings suggest that increased production of active glucocorticoids in adipose tissue through 11βHSD type 1 over expression, leads to visceral obesity and its associated metabolic perturbations. It has been suggested that this phenomenon possibly reflects a tissue specific (visceral) Cushing's syndrome in obese humans (95). Taken together, previous data implicate that changes of the HPA axis might be involved the development or maintenance of the (upper body) obese phenotype.

Prolactin

Prolactin (PRL) is a versatile hormone that, among many other biological actions, affects energy balance and food metabolism. Exogenous PRL administration increases fat storage in animals when injected at certain times of the day (96;97). PRL influences body fattening directly or indirectly through stimulation of food intake and multiple metabolic routes (98-100). PRL augments activity of the key enzyme for lipid accumulation (lipoprotein lipase) in bird adipocyte tissue and in the liver of rats (101;102). Furthermore, PRL also modulates adipocyte differentiation (103). Indeed, PRL receptor gene knockout mice have considerably reduced fat mass and primarily visceral fat is diminished (104). PRL has been reported to influence carbohydrate metabolism through its direct effects on pancreatic functioning. For example, PRL increases pancreatic insulin secretion and decreases the glucose threshold for insulin secretion through increasing glucokinase and glucose transporter 2 (factors involved in organ specific glucose disposal) (60;105-108). However, insufficient data exists about the direct impact of PRL on peripheral glucose metabolism in humans. Increased body weight or a recent history of weight gain is

frequently observed in hyperprolactinemic men and women (109), whereas these patients lose weight once treated effectively with dopaminergic agents (dopamine 2 receptor agonists), which decrease PRL secretion. Variable abnormalities of plasma PRL concentrations have been observed in obese humans. Several papers report that both basal (single measurement) as well as 24 h (hourly measured) integrated plasma PRL levels are similar in obese and normal weight humans, whereas the PRL release in response to a number of secretagogues was blunted in obese individuals (110-118). Thus, PRL can be considered as another humoral messenger being causally involved in or maintaining the obese state.

Thyrotroph Axis

The hypothalamic pituitary thyroid (HPT) hormonal ensemble orchestrates a variety of metabolic processes, including thermogenesis and energy expenditure, thereby affecting energy balance (119-121). Hypothyroidism is associated with a moderate increase in body weight and decreased appetite, whereas weight loss with normal or increased food intake is a hallmark of thyrotoxicosis. Numerous studies have evaluated the HPT axis status in obese humans even when they were clinically and biochemically euthyroid and the results were conflicting. The majority of these studies suggests that there is no substantial change in basal thyroid hormone concentrations, although a few papers document serum triiodothyronine (T_3) elevation in obese subjects (122-125). The basal serum TSH concentration in a single plasma sample was similar in obese and non-obese subjects in some studies (126;127), whereas others documented higher basal TSH concentrations in obese humans (123;126-129). Also, a larger rise of plasma TSH in response to TRH stimulation is found in obese subjects, while other studies revealed normal or reduced TSH responses (111;113;123;126-134). Synthetic thyroid hormones as well as various other thyroid hormone preparations have been and are still used as adjunctive measures to induce or facilitate weight loss. However, as triiodothyronine treatment enhances mostly body protein loss and only to a small extent loss of body fat (135), thyroid hormone supplements are not recommended in the treatment of obesity.

Leptin

The adipocyte is well recognized as a bona fide endocrine cell and several adipocyte derived hormones, or adipokines have been recently discovered (136;137). Leptin is among these adipocyte derived hormones and is one of the afferent signals informing the brain of adipose tissue energy reserves (fat stores). There exists a positive correlation between the amount of fat cell mass and leptin secretion. The effects of leptin are achieved by its interaction with specific leptin receptors, which are both located in peripheral tissues and within the central nervous system. Leptin is transported across the blood-brain barrier and it binds to specific receptors on appetite modulating neurons, most notably but not exclusively in the hypothalamic arcuate nucleus. Leptin promotes negative energy balance (inhibition food intake and stimulation energy expenditure) in order to maintain body weight homeostasis (138-141). Next to its effect on energy balance and food intake, many other activities of leptin have been described. For example, leptin affects bone formation, functioning of the immune system, the gonadal system and modulates fertility (142). Leptin deficient animals and humans are hyperphagic, obese and infertile (36;143), whereas exogenous leptin administration reverses obesity in leptin deficiency. However, leptin deficiency and leptin receptor deficiency is an extremely rare cause of human obesity. In fact, the majority of obese humans have high circulating leptin concentrations and this hyperleptinemic state is accompanied by a relatively low ratio of leptin CSF to serum levels compared to lean individuals (144). Therefore, it has been proposed that obese humans are leptin resistant. This leptin resistance might result from defects in transport across the blood-brain barrier or might be due to impaired leptin signalling. Thus, changes in leptin are associated with obesity and this neuroendocrine perturbation might be involved in the generation or the persistence of the obese state.

Effect of weight loss on neuroendrocrine perturbations associated with obesity

Caloric restriction and weight loss ameliorates the metabolic profile and affects energy expenditure in obese individuals. Also, changes of different hormonal systems after weight loss have been described in literature. For example, it has been invariably observed that reduced GH secretion and secretion reversed to nearly normal levels after substantial weight loss in obese humans (145;146). Furthermore, weight loss is also associated with a profound decrease in circulating leptin levels in obese humans (147). Variable effects of weight reduction on the HPA hormonal ensemble in obese humans has been

described literature. Some studies reported that weight loss reduced single measurements of cortisol concentrations (148;149), whereas others found increased (150) or unchanged (151) plasma cortisol levels after weight loss in obese individuals. Furthermore, some authors reported that plasma ACTH concentrations in response to CRH administration increased towards similar levels before and after weight loss in obese humans, whereas the cortisol response to CRH was either blunted or unaltered (81;82;152;153). Weight loss appeared to have no effect on suppression of plasma cortisol levels by dexamethasone (152;153) and 24 h urinary cortisol concentrations were decreased or unaltered after weight loss (152;154). Previous clinical studies concerning the impact of body weight loss on the thyrotroph and lactotroph endocrine systems have shown variable results. Some studies reported that the serum PRL response to TRH injection is blunted after a four week period of caloric restriction (320 kCal/day) or a 36 hour fast in obese subjects (155;156), whereas others found no impact of a 3-9 week period of total fasting on TRH induced PRL release in obese males (157). Prolonged fasting (no caloric intake) during twelve days significantly increased hourly integrated (spontaneous) PRL concentrations in six obese women compared to normal controls (six women, one man)(158), whereas others found no changes in basal serum PRL levels during caloric restriction in obese females (155). Furthermore, most studies have shown that weight loss reduces TSH concentrations and the TSH response to TRH, whereas others report unchanged plasma TSH or TRH induced TSH responses in obese individuals after weight loss (157;159-162). As plasma PRL and TSH concentrations are characterized by circadian fluctuations, adequate appreciation of the impact of body weight loss on PRL/TSH release requires frequent measurement of these hormones over time. However, the impact of weight loss on diurnal PRL/TSH concentration patterns and secretion rates has not been studied before. Therefore, the impact of body weight loss on spontaneous diurnal concentrations/ secretion rates of the thyrotroph and lactotroph hormonal systems will be studied in this thesis.

Factors involved in neuroendrocrine perturbations associated with obesity

The cause of the neuroendocrine perturbations associated with obesity remains elusive and numerous physiological cues may be involved in the altered hormonal milieu in obese humans. The impact of two different factors are studied in this thesis:

1. Free Fatty Acids

Free Fatty Acids (FFAs) are released from the fat cell into the blood. Obesity is associated with high circulating FFA concentrations (163;164). Previous studies in animals have shown that circulating FFAs inhibit GH secretion (165-168). Therefore, it has been hypothesized that the increased amount of circulating FFAs might be among the physiological factors involved with the hyposomatotropism.

Excess fat can be stored in various adipose depots and it appears that neuroendocrine alterations particularly occur in viscerally obese patients (145;169-171). Visceral fat is morphologically and functionally distinct from subcutaneous fat (171;172). Venous output of visceral fat drains directly into the portal system of the liver, while FFAs from subcutaneous fat enter the systemic circulation. Moreover, cellularity and FFA turnover are higher per unit adipose tissue. FFA infusion into the portal vein enhances pituitary-adrenal axis and sympathetic nervous system activity, whereas systemic FFA infusion does not exert appreciable effects on these neuroendocrine systems (173-175). Therefore, it has been hypothesized that the high portal FFA flux, brought about by excess visceral fat, may particularly modulate hormonal secretion of the exceedingly active hypothalamo-pituitary-adrenal (HPA) axis of obese individuals.

2. Dopamine

Dopamine is among the neurotransmitters involved in the central adjustment of food metabolism and hormonal secretion (176-178). Dopamine exerts its effect through activation of the dopamine D2 receptor (D2R), which is located on the cell membrane of its target cells. A myriad of experimental and clinical studies suggests that reduced dopamine 2 receptor (D2R) mediated neurotransmission is associated with the metabolic syndrome, the cluster of clinical features including insulin resistance, hyper insulinemia, dyslipidemia, visceral obesity and hypertension (179). It has been reported that central dopamine 2 receptor expression is reduced in obese individuals (180). Based on previous studies, one might postulate that deficit D2R dopaminergic transmission might be involved in the metabolic and neuroendocrine perturbations in obese humans.

Methods for investigating neuroendocrine changes in obesity

In most of the previous studies investigating hormonal systems in obesity, only single plasma hormone measurements were performed or exogenously stimulated hormone response peaks were studied. However, the majority of plasma hormone concentrations fluctuate over the day. These circadian variations of serum hormone concentrations appear to be important for their biological function (4;181). Furthermore, hormonal secretion into the blood often occurs in a pulsatile fashion. Frequent blood sampling at short time intervals is required to adequately detect these high frequency variations.

Evaluating hormone secretion is different from primarily inspecting plasma or serum hormone concentrations over time. Circulating hormone concentrations result from combined influences of prior and ongoing hormone secretion, distribution and elimination. Hormone distribution and elimination kinetics associated with metabolism and/or removal of intact hormone from the circulation and calculation of regularity and circadian rhythmicity of hormone concentration time series data provides insight of hormonal release. Various validated computer techniques have been developed to appraise information about hormonal kinetics, secretory parameters, regularity and nyctohemeral rhythmicity, calculated from in vivo measured hormone concentrations (for review see (182)). In the studies of this thesis different mathematical techniques were used to calculate these parameters from the hormone concentration time series data, which is further explained in Appendix B.

Thus, proper appreciation of spontaneous hormonal concentrations requires frequently measured hormone concentrations over 24 hours. During all experiments described in this thesis, blood was sampled for 24 hours at 10 min time intervals, while physiological conditions were standardized and kept constant (sleep-wake cycles, activities, meal schedules).

Aims of the thesis

The spontaneous diurnal plasma concentration patterns and the secretion of the thyrotroph, lactotroph and corticotroph axis have not been studied in obese women before and variable changes have been found in previous studies evaluating these endocrine systems in obesity. Thus, the **first aim** of this thesis is to delineate differences of diurnal spontaneous hormonal concentrations and secretion of the thyrotroph, lactotroph and corticotroph axis in obese and lean premenopausal women.

Both PRL as well as TSH synthesis and secretion is inhibited by dopamine (DA) through dopamine 2 receptor (D2R) activation at the lactotoroph/thyrotroph cell membrane. Dietary restriction/weight loss is associated with increased dopaminergic signalling in animals. This might implicate that weight loss affects diurnal secretion rates of thyrotroph and lactotroph endocrine systems. As the thyrotroph axis regulates energy expenditure, oxygen consumption and fuel metabolism and changes in body weight are accompanied by compensatory changes in energy expenditure, this might also implicate that weight loss is associated with adaptations of the spontaneous diurnal activity of these endocrine systems. Therefore, the **second aim** was to investigate the impact of body weight loss on the altered hormonal secretion of the lactotroph and thyrotroph axis in obese women.

Free Fatty Acids (FFAs) modulate hormonal secretion of the somatotroph and corticotroph axis. It has been postulated that the increased amount of circulating FFAs and in particular the FFAs released from visceral adipose tissue into the portal circulation might be among the pathophysiological cues causing the altered hormonal secretion of somatotroph and corticotroph endocrine systems in obese humans. Therefore, the **third aim** was to study the impact of Acipimox, known as a lipid lowering drug which reduces circulating FFA levels, on the somatotroph and the corticotroph hormonal ensemble in obese premenopausal women.

Hormonal secretion and food metabolism is centrally regulated by the dopaminergic system. Hormonal release by the pituitary is regulated by dopamine through activation of the dopamine D2 receptor (D2R) of its target cells. Obese humans appear to have reduced D2R binding sites in their brain. Therefore, altered central regulation of hormonal secretion by the dopaminergic system might be involved in the neuroendocrine and metabolic perturbations in obese humans. Thus, the **final aim** of this thesis was to study the impact of enhanced dopaminergic signalling on neuroendocrine perturbations and metabolic profiles in obese premenopausal women.

Outline of the thesis

Chapter 1 is the general introduction of the thesis. In **Chapter 2** spontaneous 24 h PRL secretion in obese premenopausal women is compared to PRL release in a control group of similar age and sex and **Chapter 3** evaluates the impact of body weight loss (induced by a very low calorie diet) on PRL release in obese premenopausal women. **Chapter 4** delineates differences between spontaneous 24 h TSH secretion in obese premenopausal women and lean controls and in **Chapter 5** the effect of body weight loss induced by long term caloric restriction on diurnal TSH levels of obese females is studied.
In **Chapter 6** the impact of lowering circulating FFAs by Acipimox, a powerful anti-lipolytic drug, on spontaneous GH release in obese individuals is investigated. **Chapter 7** represents differences of spontaneous diurnal ACTH and cortisol secretion in obese and lean premenopausal women and the effect of Acipimox on the HPA hormonal ensemble in obese individuals. In Chapter 8 the effect of short term treatment with bromocriptine (D2R agonist) on spontaneous diurnal insulin, glucose and lipid plasma concentration time series and resting energy expenditure in obese premenopausal women is shown. In Chapter 9 the effect of short term bromocriptine treatment on spontaneous diurnal leptin concentrations in obese premenopausal women is described. Results of all studies published in Chapter 2 to 9, are discussed and summarized in **Chapter 10**. A Dutch summary of the thesis is given in **Chapter 11**.
Appenix A is the list of abbreviations used in the thesis. **Appendix B** briefly explains the different mathematical methods used in this thesis to analyse diurnal hormonal rhythms.

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Chapter 2

Prolactin Release is Enhanced in Proportion to Excess Visceral Fat in Obese Women.

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Abstract

Prolactin (PRL) promotes (visceral) fat accrual in a variety of animal models. The release of PRL by the pituitary is tonically inhibited by dopamine through activation of the dopamine D2 receptor (D2R) of lactotroph cells and obese humans appear to have reduced D2R binding sites in their brain. Therefore, we hypothesized that spontaneous PRL release is enhanced in obese humans. To evaluate this hypothesis, we measured 24 h plasma PRL concentrations at 10 min intervals in eleven obese premenopausal women (BMI 33.3 ± 0.7 kg/m^2) and ten lean premenopausal women of similar age (BMI 21.2 ± 0.6 kg/m^2). Total body fat was determined using DEXA and subcutaneous and visceral fat area was measured by MRI in ten obese subjects. PRL secretion rate was estimated by deconvolution analysis. All subjects were studied in the early follicular stage of their menstrual cycle. PRL secretion was significantly enhanced in obese women (total daily release 137 ± 8 vs. lean controls 92 ± 8 µg/L/24 h, $P = 0.001$) in proportion to their BMI ($R^2 = 0.55$, $P < 0.001$). Interestingly, PRL release was particularly associated with the size of the visceral fat mass (total PRL secretion vs. visceral fat area $R^2 = 0.64$, $P = 0.006$). These data show that spontaneous PRL release is considerably enhanced in obese women in proportion to the size of their visceral fat mass. Since PRL is inhibited by D2R activation we speculate that elevated PRL secretion may be due to reduced D2R availability in the brain.

Introduction

Prolactin is an extremely versatile hormone that, among many other biological actions, affects energy balance and fuel metabolism. It stimulates food intake and fat deposition in female rats and birds and has lipogenic effects in hepatocytes (for review see (1)). Moreover, PRL receptor knockout mice have considerably reduced fat mass, where visceral fat is particularly diminished (2).

Lactotrophs have a high intrinsic basal secretory activity and tonic inhibition by dopaminergic input via the dopamine D2 receptor (D2R) is required for maintenance of low circulating PRL levels (3). Thus, the D2R is instrumental in the control of PRL secretion. Experimental studies suggest that the number of D2R is reduced in the brain of a variety of obese animal models and D2R activation reduces body weight in these rodents (4). Also, it appears that the availability of D2R binding sites in striatal nuclei of obese humans is considerably reduced in proportion to their BMI (5). Therefore, we hypothesized that spontaneous PRL release is enhanced in obese humans, which then might modulate glucose and lipid metabolism to promote fat accrual. To test this postulate, we measured spontaneous 24 h PRL secretion in obese premenopausal women and compared various features of PRL release (estimated by deconvolution analysis) with those obtained in a control group of similar age and sex.

Subjects and methods

Subjects

Eleven healthy obese premenopausal women (BMI > 30 kg/m^2) and 10 lean (BMI < 25 kg/m^2) controls of similar sex and age were recruited through advertisements in local news papers. The obese subjects were recruited so as to vary widely

with respect to girth, while their BMI was required to fall within a relatively narrow range to be able to specifically judge the effect of body fat distribution on hormone release. All participants were required to have regular menstrual cycles. Smoking and use of medication or oral contraceptives were exclusion criteria. Chronic disease was excluded by medical history, physical examination and routine biochemical/haematological laboratory tests. All subjects gave written acknowledgement of informed consent for participation.

Clinical Protocol

The protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center. All subjects were admitted to the Clinical Research Unit of the Department of General Internal Medicine in the early follicular stage of their menstrual cycle. A cannula for blood sampling was inserted into an antecubital vein and blood samples for basal parameters were withdrawn. The cannula was attached to a 3-way stopcock and kept patent by a continuous saline infusion. Blood samples were taken with S-monovetten (Sarstedt, Etten-Leur, The Netherlands). One hour after admission, 24 h blood sampling started and blood was collected at 10 minute intervals. Subjects remained recumbent, except for bathroom visits. Meals were served according to a fixed time schedule. Vital signs were recorded at regular time intervals during the day. Lights were switched off at 2300 h. We did not register sleeping episodes by EEG during the 24 h blood samplings. However, great care was taken not to disturb patients while sampling blood during their sleep.

Body fat distribution

Total amount and location of excess body fat mass was determined in the obese women only. Total body fat mass (TBFM) was quantified using dual energy X-ray absorptiometry (DEXA) (6). Visceral and subcutaneous adipose tissue areas were assessed in the obese women by MRI as described before, using a multi slice fast spin echo sequence (Gyro scan –T5 whole body scanner 0.5 Tesla, Philips Medical Systems, Best, The Netherlands)(7). Unfortunately, MRI imaging was impossible in one participant because of claustrophobia. MRI images were analysed independently by two observers.

Assays

Each tube, except the serum tubes, was immediately chilled on ice. Samples were centrifuged at 4000r/min at 4 °C during 20 minutes, within 60 min of sampling. Subsequently, plasma was divided into separate aliquots and frozen at -80 °C until assays were performed. Basal free thyroxine (T_4) concentrations were estimated using electrochemoluminescence immunoassay (Roche Diagnostics Nederland BV, Almere, Netherlands) and estradiol was determined by RIA (Diagnostic Systems Laboratory, Webster, TX). Plasma PRL concentrations were measured with a sensitive time- resolved fluoro immunoassay with a detection limit of 0.04 µg/L (Delfia, Wallac Oy, Turku, Finland). The PRL IFMA was calibrated against the 3rd WHO standard: 84/500, 1 ng/ml = 36 mU/L. The intra-assay coefficient of variation varies from 3.0-5.2% and inter-assay coefficient of variation is 3.4-6.2%, in the concentration range from 0.1-250 µg/L.

Calculations and statistics

Cluster

The Cluster program describes various characteristics of pulsatile hormone concentration profiles (8). A concentration peak is defined as a significant increase in the test peak cluster vs. the test nadir cluster. We used a 2 x 1 cluster configuration (2 samples in the test nadir and one in the test peak) and t-statistics of 2.0 for significant up- and downstrokes in PRL levels to constrain the false positive rate of peak identification to less than 5% of signal free noise. The locations and durations of all significant plasma hormone peaks were identified and the following parameters were determined: mean PRL concentration, peak frequency, mean peak height (maximum value attained in the peak), peak amplitude (mean incremental peak height), incremental peak height as a percentage of nadir, mean peak area (above the baseline) and mean inter peak valley concentration (nadir).

Pulse

Deconvolution analysis estimates hormone secretion and clearance rates on the basis of hormone concentration time-series. The Pulse algorithm is a waveform-independent deconvolution method, which can be used for calculation of mean and basal secretion, without specifying shape, number and time of secretory events (9). The technique requires a priori specification of hormonal half-life in plasma. PRL disappearance from plasma is best described by a two-compartment model, characterized by a fast component half-life of 18.4 min and a slow component half-life of 139 min where the fractional contribution of the slow component to the overall decay amounts to 49.5% (10). Pulse quantifies 24 h basal and pulsatile hormone secretion. Total daily production is the sum of basal and pulsatile release.

Approximate Entropy

Approximate Entropy (ApEn) is a scale- and model- independent statistic that
assigns a non- negative number to time series data, reflecting regularity of these data (11). We used normalized ApEn parameters of m = 1, r = 20% and 1000 for the number of runs, to test for regularity in 24 h plasma PRL concentrations, as described previously (12). Hence, this member of the ApEn family is designated ApEn (1, 20%). The ApEn metric evaluates the consistency of recurrent subordinate (non pulsatile) patterns in a time series, and thus yields information distinct from and complementary deconvolution (pulse) analyses (13). Higher absolute ApEn values denote greater relative randomness of hormone patterns. Data are presented as normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series.

Cosinor

Cosinor analysis entails trigonometric regression of a cosine function on the full 24 h plasma hormone concentration profile vs. time. Cosinor analysis was used to define the acrophase (clock time during 24 h at which PRL concentration is maximal) of the plasma PRL concentration profile.

Statistical analysis

Means of PRL secretion parameters of both groups were compared using non paired two-tailed independent Student's t-test. Significance level was set at 0.05. Data is presented as mean ± SEM, unless otherwise specified. Pearson's correlation analysis was used to determine the association between BMI and various features of pulsatile PRL secretion in obese and normal weight women. Univariate analysis was used to describe the relationship between various specific anthropometric measures (% body fat (%BF), subcutaneous fat mass (SFM) and visceral fat mass (VFM)) and PRL secretion parameters in the obese subjects only.

Results

Subjects

Eleven obese and 10 lean subjects were enrolled in this study. Mean age was similar in both groups (obese 38.1 ± 2.1 vs. lean 32.7 ± 2.7 yr, $P = 0.128$) while BMI was significantly different (obese 33.3 ± 0.7 vs. lean 21.2 ± 0.6 kg/m^2, $P < 0.001$).

All subjects were clinically euthyroid and T_4 levels were within the normal range in the obese and lean subjects. We did not find significant differences between mean basal estradiol (E2) levels (obese 169 ± 32 vs. lean 197 ± 55 pmol/L, $P = 0.637$).

Plasma PRL concentration profiles

Mean 24 h PRL concentration, peak amplitude, peak width, peak area and peak height were significantly higher whereas peak frequency was significantly lower in obese subjects compared to lean controls (Table 1).

Regularity of plasma PRL concentration- time series

The regularity of 24 h plasma PRL concentration time series as determined by the ApEn statistic was similar in obese vs. lean subjects (0.47 ± 0.03 vs. 0.50 ± 0.05 respectively, P = 0.616).

Acrophase of plasma PRL concentration times series

The acrophase of the nyctohemeral PRL rhythm, which is characterized by a cosine wave, occurred in the early morning in obese and lean subjects at comparable clock-times (obese 0431 h ± 87 min and lean 0653 h ± 111 min, P = 0.330).

Features of PRL secretion

Both basal and pulsatile prolactin secretion rates were clearly higher in obese subjects, where basal release (as a fraction of total secretion) was particularly enhanced (Table 2). Graphical illustrations of representative plots of 24 h plasma PRL concentration patterns in two obese subjects and age-matched lean controls are shown in Figure 1.

BMI and features of PRL concentration profiles/secretion rates

Both obese and lean subjects were included in correlation analyses of BMI vs. PRL concentration parameters. BMI was positively associated with mean 24 h plasma PRL concentration, peak amplitude, peak width, peak area and maximum peak height, whereas an inverse linear relationship was found between BMI and plasma concentration peak frequency (Table 3). Basal, pulsatile and total PRL secretion rates were also strongly positively correlated with BMI (Table 3 and Figure 2).

Body fat distribution and features of PRL concentration profiles/secretion rates

Pearson's correlations between measures of body fat mass and distribution (% BF, VFM, SFM) and various features of PRL release were estimated in ten obese subjects only (MRI could not be performed in one subject because of her claustrophobia). Univariate analysis revealed that PRL secretion rates were specifically associated with the size of the visceral fat depot (Table 4 and Figure 2), whereas the % BF and subcutaneous fat area were not significantly associated with features of PRL release (Table 4).

Discussion

These data clearly show that PRL release is enhanced in obese premenopausal women in proportion to their BMI. Interestingly, PRL release was particularly associated with the size of the visceral fat area, which accords with experimental data that suggest that PRL directs excess energy primarily towards the visceral fat depot (2).

Several previous papers report that basal (single measurement) PRL levels are similar in obese and normal weight humans, while PRL release in response to a number of secretagogues was blunted in obese individuals compared to lean controls (14-22). Moreover, 24-hour integrated plasma PRL levels (measured hourly) were not significantly different in obese and normal weight humans in one earlier study (23). Our findings are in apparent conflict with these observations. However, as far as we are aware, spontaneous PRL release, as calculated by deconvolution analysis from frequently sampled plasma hormone time series data, has never been quantified in obese humans before. Blood sampling at short time intervals is required to adequately detect high frequency variations of PRL plasma concentrations. Single or even hourly PRL measurements are not likely to accurately reflect spontaneous PRL secretion given the pulsatile nature of its release process. Other factors that may explain the difference between our study and previous ones pertain to the subjects included and the design of the studies. For example, both men and women of varying age were enrolled in the study that measured hourly PRL concentrations, the women were both pre- and postmenopausal and premenopausal women were studied at different stages of their menstrual cycle. In addition, various factors act in concert to orchestrate PRL secretion by the pituitary gland. A number of (putative) positive feed forward signals (e.g. TRH) interact with inhibitory inputs (primarily dopamine via the D2R) and direct negative feedback restraint by PRL itself to generate a pulsatile release profile. In this context, it is quite conceivable that enhanced spontaneous PRL secretion (our study) dampens secretagogue-induced PRL release (14,17-22) via strengthened negative feedback in obese humans.

PRL is an extremely versatile hormone, which plays a role in the regulation of carbohydrate and lipid metabolism in a variety of species. In fish, birds and rodents, PRL promotes fat storage through stimulation of food intake and multiple metabolic routes (1) and knock-out of the PRL receptor gene in mice causes loss of body fat, primarily from the visceral depot (2). The latter observation agrees with our data, in that PRL release in our subjects was particularly associated with the size of their visceral fat area. Humans with prolactinoma tend to be obese and lose weight once treated effectively (with D2R agonists) (24,25). Activation of the D2R is the major route to suppress pituitary PRL release. D2R antagonism in the treatment of schizophrenia enhances circulating PRL levels and causes weight gain in a very high percentage of patients (26-28). Interestingly, Wang et al showed that the number of D2R binding sites in the brain of obese humans is strongly reduced and inversely associated with BMI (5). Collectively, current perceptions are in keeping with the postulate that prolactin may be one of the endocrine messengers that relay reduced D2R mediated dopaminergic neural signals to peripheral tissues to promote (visceral) fat storage. However, it clearly requires further investigation to establish if dopaminergic mechanisms indeed underlie enhanced prolactin release in obese humans, since D2R activity was not addressed directly in this study.

Although the above data provide evidence to the contrary, we cannot rule out that enhanced PRL release was a consequence of obesity in our subjects. For example, circulating leptin levels are increased in obese humans (29,30) and leptin stimulates PRL secretion in vitro in pituitary lactotrophs and has a stimulatory effect on steroid induced and spontaneous PRL secretion in rats (31). Thus, hyperleptinemia (as a corollary of obesity) may promote PRL release in obese humans.

In conclusion, we here show that PRL secretion is enhanced in obese premenopausal women. Total daily release is strongly associated with BMI and with the size of the visceral fat depot in particular. We speculate that enhanced PRL secretion may be a mechanistic link between reduced D2R availability in the brain and (visceral) obesity.

Tables and Figures

Table 1. Features of 24 h plasma PRL concentration profiles in lean and obese premenopausal women

Parameter	Obese (n = 11)	Controls (n = 10)	P-value [a]
Mean 24 h plasma concentration (µg/L)	6.8 ± 0.4	5.1 ± 0.5	0.008
Maximum pulse height (µg/L)	8.6 ± 0.6	5.8 ± 0.5	0.002
Pulse amplitude (µg/L)	3.3 ± 0.3	1.8 ± 0.1	< 0.001
Pulse width (min)	83 ± 7	56 ± 4	0.003
Pulse area (µg/L/min)	229 ± 30	91 ± 13	0.001
Percentage peak increase [b]	167 ± 6	151 ± 6	0.061
Nadir concentration (µg/L)	5.1 ± 0.3	4.0 ± 0.4	0.039
Number of pulses (n/24 h)	13 ± 1	17 ± 1	0.009

Parameters were calculated from 24 h PRL concentration profiles using Cluster analysis.

a) P-value independent Student's t-test lean vs. obese subjects

b) percentage is calculated fraction of mean nadir concentration (µg/L)

Table 2. PRL secretion rates in lean and obese premenopausal women

Parameter	Obese	Controls	P-value [a]
Basal secretion (µg/L/24 h)	49 ± 3	25 ± 2	0.001
Pulsatile secretion (µg/L/24 h)	88 ± 5	67 ± 6	0.014
Total secretion (µg/L/24 h)	137 ± 8	92 ± 8	0.001
% non-pulsatile secretion [b]	36 ± 1	27 ± 1	<0.001

Parameters were calculated from 24 h PRL concentration profiles using the Pulse algorithm, which is a waveform-independent deconvolution method. Total daily production is the sum of basal and pulsatile release.

a) P-value independent Student's t-test lean vs. obese subjects

b) percentage is calculated fraction basal PRL secretion (µg/L/24 h) of total PRL (µg/L/24 h) production.

Table 3. Correlations between BMI and PRL secretion parameters in obese and normal weight subjects

Lean and Obese Subjects (N = 21)	BMI	
Parameter	R-square	P-value
Number of pulses	0.32 [1)]	0.008
Mean 24 h plasma concentration (µg/L)	0.42	0.002
Nadir concentration (µg/L)	0.31	0.009
Peak Area (µg/L/min)	0.48	<0.001
Basal secretion (µg/L/24 h)	0.69	<0.001
Pulsatile secretion(µg/L/24 h)	0.37	0.003
Total secretion(µg/L/24 h)	0.55	<0.001

Pearson's correlation analysis was used to determine the association between BMI and various features of pulsatile PRL secretion in obese and normal weight women.

1) Parameter was negatively correlated with BMI

Table 4. Correlation analyses for %BF/SFM/VFM and PRL Secretion Parameters in obese subjects.

Obese Subjects (N = 10)	%BF		SFM		VFM	
Parameter	R-square	P-value	R-square	P-value	R-square	P-value
Number of pulses	0	0.878	0.12 [1)]	0.316	0.10 [1)]	0.372
Mean 24 h plasma concentration (µg/L)	0	0.908	0.02 [1)]	0.682	0.50	0.022
Nadir concentration (µg/L)	0	0.884	0	0.786	0.64	0.006
Peak Area (µg/L/min)	0.05 [1)]	0.068	0.12	0.32	0.23	0.16
Basal secretion (µg/L/24 h)	0.07 [1)]	0.452	0.06 [1)]	0.512	0.76	<0.001
Pulsatile secretion(µg/L/24 h)	0	0.936	0.03	0.652	0.49	0.024
Total secretion(µg/L/24 h)	0.02 [1)]	0.724	0	0.982	0.64	0.006

Univariate analysis was used to describe the relationship between various specific anthropometric measurements and PRL secretion parameters in the obese subjects only. Total % body fat (%BF) was determined using DEXA and subcutaneous fat mass (SFM) and visceral fat mass (VFM) were estimated with MRI.

1) Parameter was negatively correlated with %BF/SFM/VFM

Figure 1.

Serum PRL concentration time series in two obese subjects (closed symbols) and two control subjects (open symbols). Data reflect sampling of blood every 10 min for 24 h. Sampling starts at 1800 h. The age of the women in the upper panel was 33 yr and the BMI was 20.6 and 31.0 (kg/m^2). The age of the women in the lower panel was 48 and the BMI was 21.5 and 32.3 (kg/m^2).

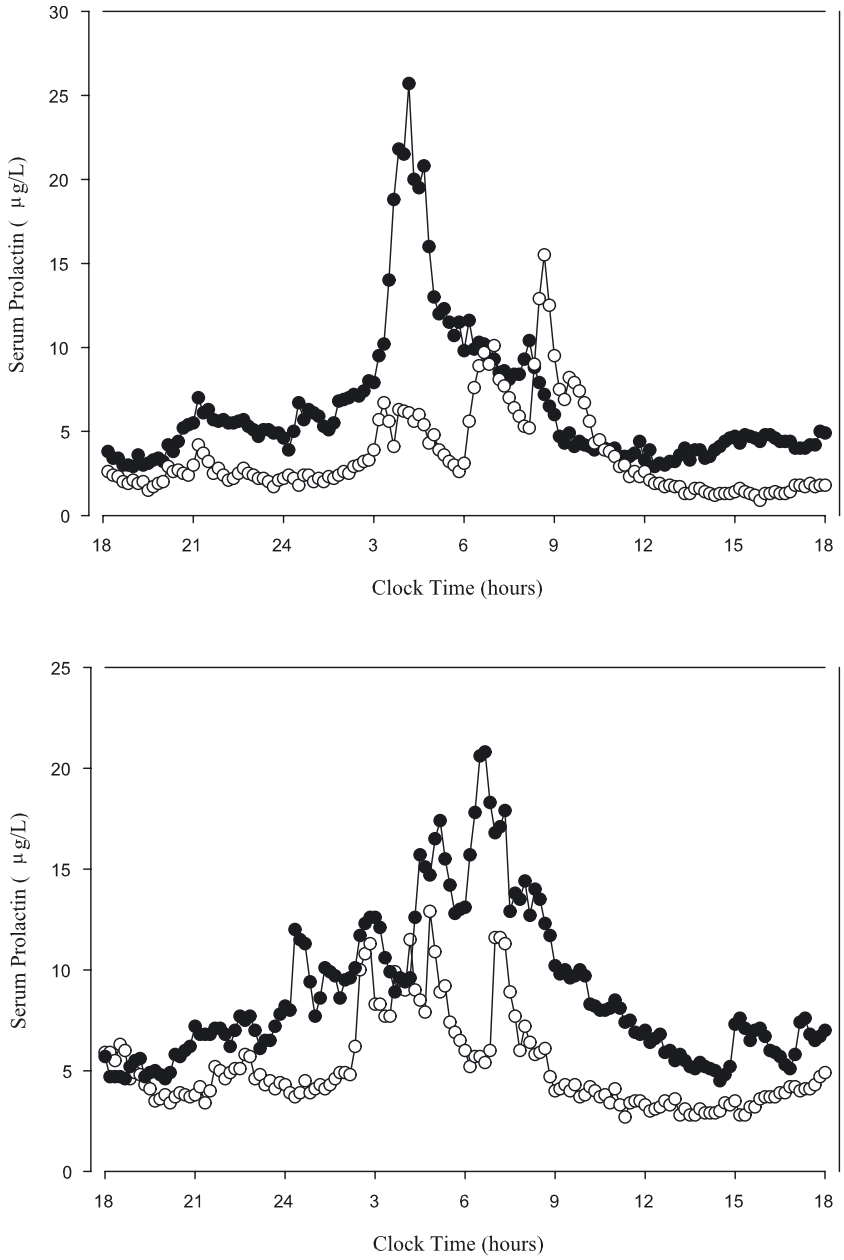
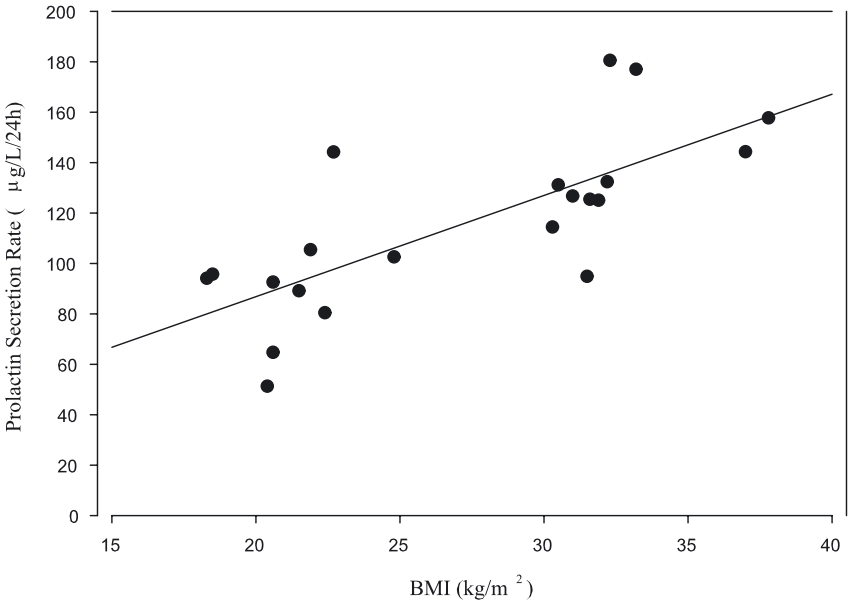
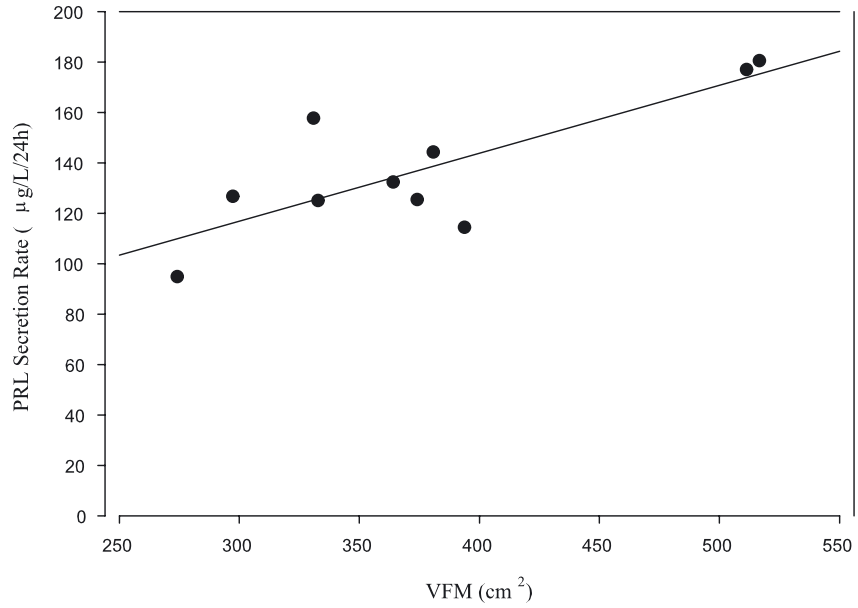


Figure 2.

Upper panel: Linear correlation between body mass index (BMI) and the 24 h PRL secretion rate in 11 obese women and 10 lean women. The PRL secretion rate was calculated by deconvolution analysis of the plasma concentration profile, obtained by 10 min blood sampling during 24 hr.

Lower panel: Linear correlation between visceral fat mass (VFM) and PRL secretion rate in 10 obese subjects.





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Chapter 3

Increased Circadian Prolactin Release is Blunted after Body Weight Loss in Obese Premenopausal Women

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Abstract

We recently showed that PRL release is considerably enhanced in obese women in proportion to the size of their visceral fat mass. PRL release is inhibited by dopamine 2 receptor (D2R) activation and dietary restriction/weight loss is associated with increased dopaminergic signalling in animals. Therefore, we hypothesized that enhanced PRL release in obese humans would be reversed by weight loss. To evaluate this postulate, we measured 24 h plasma PRL concentrations at 10 min intervals in eleven obese premenopausal women (BMI 33.3 ± 0.7 kg/m^2) before and after weight loss (50% reduction of overweight/15% absolute weight loss, using a very low calorie diet) in the follicular phase of their menstrual cycle.

The 24 h PRL concentration profiles were analysed by a peak detection program (Cluster) and a waveform-independent deconvolution technique (Pulse). Spontaneous 24 h PRL secretion was significantly reduced in obese women (mean daily release before 128 ± 24 vs. after weight loss 110 ± 17 µg/V_{dl} x 24 h, P = 0.05). Body weight loss particularly blunted PRL secretory burst mass (Pulse area before 230 ± 28 vs. after weight loss 221 ± 31 µg/V_{dl} x min, P = 0.03), whereas burst frequency was unaffected (Number of pulses before 11 ± 1 vs. after weight loss 12 ± 1 n/24 h, P = 0.69). Thus, elevated PRL secretion rate in obese women is significantly reduced after loss of 50% of overweight. We speculate that amelioration of deficit dopamine D2 receptor mediated neurotransmission and/or diminutions of circulating leptin/estrogen levels might be involved in the physiology of this phenomenon.

Introduction

We recently showed that spontaneous diurnal PRL secretion is considerably enhanced in proportion to the size of the visceral fat mass in obese premenopausal women compared to lean controls of similar age and sex (19). Since prolactin (PRL) has been reported to possess potent lipogenic and diabetogenic effects (for review see (2)), hyperprolactinemia may modulate glucose and lipid metabolism to promote fat accrual in obese humans.

Although dietary restriction is consistently associated with low circulating plasma PRL concentrations in several animal species (10), the results of studies evaluating the effects of caloric restriction and body weight loss on plasma PRL concentrations in humans have been contradictory. Indeed, some studies suggest that the serum PRL response to TRH injection is blunted after a four week period of caloric restriction (320 kCal/day) or a 36 hour fast in obese subjects (20; 34), whereas others found no impact of a 3-9 week period of total fasting on TRH induced PRL release in seven hospitalized obese males (5). Finally, prolonged fasting (no caloric intake) during twelve days significantly increased hourly integrated (spontaneous) PRL concentrations in six obese women compared to normal controls (six women, one man)(8), whereas no changes in basal serum PRL levels were found during caloric restriction in another study of obese females (20). As far as we are aware, the effect of body weight loss per se on spontaneous PRL release, as calculated by deconvolution analysis from frequently sampled plasma hormone time series data, has never been quantified in obese humans before.

PRL synthesis and secretion is inhibited by dopamine (DA) through dopamine 2 receptor (D2R) activation at the lactotoroph cell membrane (1). Studies in rats showed that caloric restriction increases hypothalamic DA levels (13) and retards age associated loss of central dopamine receptors (22). Furthermore, it has been reported that obese humans are refractory to stimulation of PRL release by metoclopramide (MET), which normally increases PRL release by blockade of the dopamine

2 receptor at the pituitary level, whereas short term fasting increased the MET induced PRL response (28). These data suggest that food restriction and body weight loss restore central dopaminergic tone in obese humans, at least to a certain extent. Therefore, we hypothesized that spontaneous PRL release would be reduced after weight loss in obese individuals. To test this postulate, we evaluated 24 h plasma PRL concentrations, measured at 10 min intervals, in eleven obese premenopausal women before and after 50% reduction of their overweight (15% absolute weight loss) by means of a very low calorie diet (500 kCal/day).

Subjects and Methods

Subjects

11 healthy obese premenopausal women (BMI 33.1 ± 1.2 kg/m^2) were enrolled in the study, after given written acknowledgement of informed consent for participation. A historical control group of 10 lean controls (BMI 21.4 ± 0.8 kg/m^2, $P < 0.05$ vs. obese) of similar sex and age (obese 35.8 ± 2.3 vs. lean 36.7 ± 2.4 yr, $P = 0.80$) was included for comparison of PRL secretion data with those in the obese women after weight loss (published data ref (19)). All subjects underwent medical screening, including medical history, physical examination, standard laboratory haematology, blood chemistry and urine tests. Acute or chronic disease, smoking, recent transmeridional flights, night-shift work, weight change prior to the study (> 3 kg in 3 months) and use of medication were exclusion criteria for participation. All participants were required to have regular menstrual cycles and not using oral contraceptives.

Body fat distribution

Specific body fat measurements were obtained in the obese subjects before and after weight loss. Total body fat mass was quantified using bioelectrical impedance analysis (Bodystat 1500, Bodystat Ltd., UK) and was expressed as a percentage of total body weight (23). Visceral and subcutaneous adipose tissue areas were assessed by MRI as described before, using a multi slice fast spin echo sequence (Gyro scan –T5 whole body scanner 0.5 Tesla, Philips Medical Systems, Best, The Netherlands)(21).

Weight Loss Program

Obese subjects were prescribed a liquid very low calorie diet (2MJ/day; 43% proteins, 15% fat, 42% carbohydrates; Modifast, Novartis, Veenendaal, The Netherlands) after the first study occasion, in order to reduce 50% of their overweight. Ideal body weight for height was determined according to the Metropolitan Life Insurance tables (1983). The subjects were instructed to keep their physical activity level constant. All subjects weekly visited the research center for medical screening (medical examination and blood chemistry tests if necessary) by the research physician. Obese subjects reduced their overweight within a mean time period of 4 months.

Clinical Protocol

The protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center. All subjects were studied in the early follicular stage of their menstrual cycle. Identical methodology was used to study spontaneous 24 h PRL secretion in obese and normal weight women. Obese women were studied twice, before and after body weight loss. All subjects used a standard eucalorie diet (1980 kCal/8.3 MJ per day), consisting of Nutridrink (Nutricia, Zoetermeer, The Netherlands) and Modifast, three days prior to each admission until the end of the blood sampling period. Subjects were admitted to the research center at 0800 h, after an overnight fast. A 20-gauge cannula for blood sampling was inserted into an antecubital vein. The cannula was attached to a constant withdrawal pump (Conflo, Carmeda AB, Taeby, Sweden) and tubes were switched every 10 minutes. The iv cannula was kept patent by a continuous 0.9% NaCl and heparin (1 U/ml) infusion (500ml/24 h). One hour after insertion of the iv cannula, blood sampling started. Each tube contained 1.2 ml of blood (totaling 174 ml blood). The plasma prolactin concentration was determined in every 10-minute sample, while leptin levels were measured every 20 minutes. Meals were served according to a fixed time schedule (0930 h breakfast, 1300 h lunch, 1830 h dinner). Vital signs were recorded at regular time intervals. No daytime naps were allowed. Lights were

switched off at 2300 h and switched on at 0730 h and great care was taken not to disturb patients while sampling blood during their sleep (no EEG sleep recording was performed).

Assays

Sampled tubes were immediately chilled on ice. Samples were centrifuged at 4000 r/min at 4 °C during 20 minutes, within 60 min of sampling. Subsequently, plasma was divided into separate aliquots and frozen at -80 °C until assays were performed. Plasma PRL concentrations were measured with a sensitive time-resolved fluoro immunoassay with a detection limit of 0.04 µg/L (Delfia, Wallac Oy, Turku, Finland). The PRL IFMA was calibrated against the 3rd WHO standard: 84/500, 1 ng/ml = 36 mU/L. The intra-assay coefficient of variation varies from 3.0-5.2% and inter-assay coefficient of variation is 3.4-6.2%, in the concentration range from 0.1-250 µg/L. Plasma leptin concentrations were determined by RIA (Linco Research, St. Charles, MO) with a detection limit of 0.5 µg/L and the inter-assay coefficient ranged from 6-7%. Basal free thyroxine (T_4) concentrations were measured using an automated system (Elecsys 2010, Roche Diagnostics Nederland BV, Almere, Netherlands) with a detection limit of 2 pmol/L and the inter-assay coefficient ranged from 3.8-5.6%. Estrogen concentrations were determined by RIA (Orion Diagnostica, Espoo, Finland) with a detection limit of 6 pmol/L and an inter-assay coefficient of 6.8%.

Calculations and statistics

Cluster

The Cluster program describes various characteristics of pulsatile hormone concentration profiles (32). A concentration peak is defined as a significant increase in the test peak cluster vs. the test nadir cluster. We used a 2 x 1 cluster configuration (2 samples in the test nadir and one in the test peak) and t-statistics of 2.0 for significant up- and downstrokes in PRL levels to constrain the false positive rate of peak identification to less than 5% of signal free noise. The locations and durations of all significant plasma hormone peaks were identified and the following parameters were determined: mean PRL concentration, peak frequency, peak width, mean peak height (maximum concentration attained within the peak), mean peak area (above the baseline), overall mean concentration of the inter peak valley (nadir) and the total area under the curve.

Pulse

Deconvolution analysis estimates hormone secretion and clearance rates based on hormone concentration time-series. The Pulse algorithm is a waveform-independent deconvolution method, which can be used for calculation of hormonal secretion, without specifying shape, number and time of secretory events (17). The technique requires a priori specification of hormonal half-life in plasma. PRL disappearance from plasma is best described by a two compartment model, characterized by a fast component half-life of 18.4 min and a slow component half-life of 139 min where the fractional contribution of the slow component to the overall decay amounts to 49.5% (29). Pulse was used to quantify mean 24 h PRL secretion. Secretion rates were calculated per liter distribution volume.

Approximate entropy

Approximate Entropy (ApEn) is a scale- and model- independent statistic that assigns a non- negative number to time series data, reflecting regularity of these data (27). Higher ApEn values denote greater relative randomness of hormone patterns. Normalized ApEn parameters of m = 1 (test range), r = 20% (threshold) and 1000 for the number of runs were used, as described previously (15). Hence, this member of the ApEn family is designated (1, 20%). The ApEn metric evaluates the consistency of recurrent subordinate (non pulsatile) patterns in a time series and thus yields information distinct from and complementary to deconvolution (pulse) analyses (33). Data are presented as normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series. ApEn ratios close to 1.0 express highly irregular (maximum randomness) secretory patterns.

Circadian Rhythmicity

Nyctohemeral characteristics of PRL concentration patterns were determined using a robust curve fitting algorithm (LOWESS analysis, SYSTAT version 11 Systat Inc, Richmond, CA, (4; 7)). The acrophase is the clock time at which the fitted PRL concentration is maximal. The amplitude of the rhythm was defined as half the difference of the nocturnal zenith (maximum) and the day-time nadir (minimum). The relative amplitude was the maximal percentage increase of the mesor value.

Statistics

Data are presented as means ± SEM, unless otherwise specified. The means of PRL concentration and secretion parameters in obese subjects before and after weight loss were statistically analysed using non- parametric Wilcoxon signed-rank test. Means of PRL secretion and concentration parameters between groups (obese vs. lean) were compared using non-parametric Mann Whitney U- test. Non-parametric tests were used because the distribution of data was not normal. Significance level was set at 0.05. Regression analysis was used to determine the correlation between differences of 24 h PRL secretion (before and after weight loss) and changes of body composition parameters in obese subjects. Multiple regression analysis, using body weight, BMI, percentage total body fat, visceral and subcutaneous fat areas and mean 24 h leptin concentrations as independent variables was performed to estimate the correlation between differences of 24 h PRL secretion vs. mean 24 h leptin concentrations and different features of body composition in the obese subjects.

Results

Subjects

Both obese and lean historical subjects were studied in the early follicular phase of their menstrual cycle (Estrogen (E2) levels obese before weight loss 203 ± 22 and lean 190 ± 82 pmol/L, P = 0.84). All subjects were clinically euthyroid (Free thyroxine (free T_4) levels obese before weight loss 15.1 ± 0.5 and lean 16.5 ± 0.6 pmol/L, P = 0.10). Body composition parameters and baseline serum measurements were obtained at each study occasion in the obese subjects. BMI, percentage total body fat as well as sizes of visceral and subcutaneous fat areas were significantly reduced at the end of the weight loss period. No significant loss of lean body mass was seen at the end of the weight loss period. An overview of the subject characteristics and baseline serum measurements is given in Table 1.

PRL concentration and secretion parameters

An overview of PRL concentration- and secretion parameters is shown in Table 2. Different characteristics of 24 h PRL hormone concentration profiles were determined using Cluster. Mean 24 h PRL concentration, mean peak amplitude, (maximum concentration attained within the peak) peak area, and inter peak valley (nadir) were significantly lower, whereas peak frequency and peak width were unaltered in obese subjects after weight loss. Pulse analysis revealed that mean 24 h PRL secretion was significantly reduced by weight loss in the obese women (before 128 ± 24 vs. after weight loss 110 ± 17 $\mu g/V_{dl}$ x 24 h, P = 0.04). After weight loss, all PRL concentration and secretion parameters remained significantly enhanced in the obese women compared to those obtained in the lean historical controls. A graphical illustration of the mean 24 h plasma PRL concentrations in the obese subjects before and after weight loss and those in age-matched lean historical controls vs. clock time is presented in Figure 1. The mean 24 h PRL secretion in obese women before and after weight loss and in lean controls is shown in Figure 2.

Regularity of plasma PRL concentration time series

ApEn ratios of PRL concentration time series data were significantly affected by weight loss in the obese subjects (before 0.46 ± 0.05 vs. after weight loss 0.50 ± 0.05 respectively, P = 0.01) and were similar after weight loss in the obese and normal weight premenopausal women (lean 0.50 ± 0.05, P = 0.97 vs. obese).

Differences of 24 h PRL secretion and body composition

Pearson's correlations between differences in PRL secretion before and after weight loss vs. differences of body fat mass and distribution were estimated in the obese subjects only. PRL secretion was not related to body composition parameters before body weight loss in the obese women. Obese subjects had mean changes of body weight of 13.5 ± 1.7 (4.6-25.2) kg; BMI of 4.8 ± 0.6 (1.4-8.4) kg/m^2; percentage total body fat of 6.9 ± 0.9 (2.9-14.1) %; visceral fat area of 174 ± 29 (94-358) cm^2; and subcutaneous fat area of 697 ± 90 (214-1332) cm^2. Univariate analysis, including differences of body weight, BMI, percentage total body fat, visceral and subcutaneous fat areas as independent variables, revealed that there was a positive (but not significant) correlation between delta body weight, BMI, delta percentage total body fat, delta subcutaneous fat area but not visceral fat area vs. differences in PRL secretion rates before and after weight loss (Table 3).

Leptin and 24 h PRL secretion

Mean 24 h leptin concentrations were significantly reduced in the obese subjects after weight loss (before 37.4 ± 6.7 vs. after weight loss 19.7 ± 4.0 µg/L, $P < 0.01$) but values were still significantly higher than those lean controls (lean 12.8 ± 2.5 µg/L, $P < 0.01$ vs. obese). Multiple regression analysis, including body weight, BMI, percentage total body fat and mean 24 h leptin and estrogen concentrations as independent variables, revealed that differences in 24 h PRL secretion were significantly positively correlated to differences in mean 24 h leptin concentrations ($R^2 = 0.61$, $P < 0.01$, Figure 3), body weight change ($R^2 = 0.34$, $P = 0.01$) and BMI ($R^2 = 0.31$, $P = 0.02$).

Diurnal variation 24 h PRL concentration profiles

Analysis of the diurnal variation in plasma PRL concentrations revealed that the acrophase of the nyctohemeral PRL rhythm occurred at night at similar clock-times before and after weight loss in obese subjects (obese before 0400 h ± 15 min and after 0430 h ± 16 min respectively, $P = 0.46$) and the time points of the acrophase before and after weight loss in the obese subjects were not significantly different from the lean subjects (0530 h ± 01 h 16 min). The mesor (before 11.7 ± 1.9 vs. after 9.9 ± 1.3 µg/L respectively, $P = 0.03$) as well as the amplitude (before 5.4 ± 1.0 vs. after weight loss 4.4 ± 0.8 µg/L respectively, $P = 0.01$) of the rhythm were significantly decreased after weight reduction in obese subjects, whereas the relative increase in PRL concentration was not significantly altered after weight loss in the obese women (before 49.5 ± 4.6 vs. after weight loss 46.6 ± 4.9 % respectively, $P = 0.12$).

Discussion

The present study shows that elevated PRL secretion rates in obese women are significantly reduced after loss of 50% of overweight (15% absolute weight loss). Body weight loss particularly blunted PRL secretory burst mass, whereas burst frequency was unaffected. However, PRL secretion remained significantly higher than that in normal weight controls.

Only a few previous clinical studies evaluated the effect of calorie restriction and weight loss on PRL secretion in obese humans and conflicting results have been reported (5; 8; 20; 34). Although some studies suggest that TRH induced PRL release is not affected by severe calorie restriction, most papers show that the incremental peak of serum PRL in response to TRH is significantly reduced after a four week period of caloric restriction (20; 34), which is in line with the results of the present study. Furthermore, low circulating PRL concentrations were found in food restricted animals (10), which also corroborates our data. To our knowledge, this is the first study to evaluate the effect of body weight loss (and not the effect of the severe calorie restriction since the obese women were studied after using a balanced eucaloric diet for three days after the weight loss period) per se on diurnal spontaneous PRL secretion rates (as estimated by deconvolution analysis) in obese humans.

Dopamine is the major inhibitor of PRL synthesis and secretion (1) and D2R expression is diminished in hypothalamic nuclei of obese Zucker rats and in the striatum of obese humans (35). Dietary restriction and weight loss are accompanied by increased dopaminergic signalling in animals (13; 22), and indirect evidence suggests that calorie restriction also reinforces central dopaminergic tone in obese humans (28). Although dopaminergic neuronal activity was not directly assessed in the present study, it is conceivable that body weight loss enhanced D2R mediated neurotransmission to reduce diurnal PRL secretion rates in our obese subjects. Alternatively, other physiological cues such as leptin or estrogen might

have changed PRL secretion after body weight loss in the present study. Exogenous estrogens raise basal serum PRL levels (11; 37) and estrogens enhance PRL release in response to several exogenous stimuli (3; 18). Estrogen concentrations were significantly reduced after reduction of overweight in the present study, a finding which has been reported previously by other authors (24; 30). However, changes of 24 h PRL secretion in response to weight loss were not related to the decrease of plasma estrogen concentrations. Leptin is one of the various other cues apparently modulating PRL secretion. Leptin administration restores lactation in leptin deficient ob/ob mice (6) and leptin infusion raises plasma PRL concentrations in fasted rats to levels similar to those in fed littermates (36). These findings suggest that leptin plays a role in the control of PRL release. Indeed, a direct stimulatory effect of leptin on PRL secretion has been observed in vitro in anterior pituitary cells (31; 38) and in vivo in rats (14; 16). The reduction of 24 h PRL secretion in response to weight loss in the present study was closely associated with the mean decrease of plasma leptin concentrations, which supports the thesis that both phenomena are related. Thus, the diminution of PRL secretion in response to weight loss may be due to changes in leptin and/or estrogen levels.

Obesity predisposes to the metabolic syndrome, which is a major risk factor for cardiovascular disease and diabetes mellitus type 2. A plethora of data from animal and clinical studies suggests that reduced dopaminergic neurotransmission is involved in the pathogenesis of syndrome X. Furthermore, treatment with D2R antagonists induces obesity and diabetes mellitus type 2, whereas D2R activation ameliorates the metabolic profile in obese nondiabetic and diabetic humans (for review see (26)). Caloric restriction and weight loss tend to restore the metabolic profile to normal in obese individuals (9). In a variety of animal species PRL exerts potent lipogenic and diabetogenic effects. For example, PRL injections promote body fat storage in rats and birds and PRL stimulates lipoprotein lipase activity both in the liver in rats and adipose tissue in birds. Furthermore, PRL activates glycogen phosphorylase-a in hepatocytes and directly stimulates insulin release by the pancreas, thereby affecting carbohydrate metabolism (for review see (2)). The data presented here support the notion that the beneficial effect of weight loss on metabolic parameters in obese individuals may be brought about by amelioration of deficit D2R mediated dopaminergic transmission in hypothalamic nuclei and that PRL serves as a messenger mediating the favourable effects of dopamine on glucose and lipid metabolism in peripheral tissues. We did not measure the effect of weight loss on metabolic parameters (i.e. oral glucose tolerance test, stimulated area under the insulin curve and androgen levels) and dopaminergic tone in the present study. Thus, it clearly requires further investigation to test this postulate. For example, imaging studies assessing D2R availability in the brain of obese humans before and after weight loss are needed and the impact of D2R antagonism on the metabolic benefits and prolactin secretion rate in response to weight loss must be determined.

PRL levels remained higher after weight loss in the obese women compared to normal controls. Our study design does not allow for definitive conclusion as to why this is. Obese subjects may have intrinsic regulatory cues promoting PRL release that are at least partly independent of their weight. Alternatively, PRL levels remained higher because our subjects' body weight did not completely normalize in response to calorie restriction.

It is important to note, that all obese subjects took a standard liquid, eucalorie diet for 3 days prior to each study occasion to "wash out" any potential confounding effect of calorie restriction per se on the PRL secretion rate. Although it is unclear from the literature how long a wash out period is needed exactly to achieve that goal, the secretion rate and/or plasma concentration of various other hormones responds rather quickly (i.e. within hours to days) to changes in nutrient availability. Therefore it is reasonable to assume that the decline of PRL levels we report here is due to weight loss and not to calorie restriction.

In conclusion, body weight loss partly reverses elevated PRL secretion in obese women. Amelioration of deficit D2R dopaminergic transmission and/or reduction of circulating leptin and estrogen levels may all be involved in the physiology of this phenomenon.

Tables and Figures

Table 1. Subject characteristics and fasting basal serum measurements

Parameter	Obese (N = 11)		P-value [a]
	Before Weight loss	After Weight loss	
Weight (kg)	92.7 ± 4.1	79.2 ± 3.2	< 0.01
BMI (kg/m^2)	33.1 ± 1.2	28.2 ± 0.8	< 0.01
WHR	0.85 ± 0.03	0.81 ± 0.02	0.03
Lean Body Mass (kg)	53.0 ± 1.6	50.5 ± 1.6	0.08
Body Fat (%)	41.2 ± 1.8	35.1 ± 1.3	< 0.01
Visceral Fat Mass (cm^2)	432 ± 8	258 ± 5	< 0.01
Subcutaneous Fat Mass (cm^2)	2659 ± 18	1961 ± 13	< 0.01
Estrogen (E2) (pmol/L)	203 ± 22	163 ± 25	< 0.01

Data are presented as means ± SEM

a) P-values were determined by non- parametric Wilcoxon signed-rank test, before vs. after weight loss in obese women

Percentage body fat was estimated by bioelectrical impedance analysis and was calculated as a fraction of total body weight. Visceral and subcutaneous fat areas were determined using MRI.

Table 2. PRL concentration parameters and secretion rates

Parameter	Obese (N = 11)		P-value [a]	Controls (N = 10)	P-value [b]
	Before Weight Loss	After Weight Loss			
Mean 24 h concentration (µg/L)	10.0 ± 1.8	8.6 ± 1.3	0.03	5.1 ± 0.5	< 0.01
Number of pulses (n/24 h)	11 ± 1	12 ± 1	0.69	17 ± 1	0.01
Pulse width (min)	83 ± 7	80 ± 5	0.72	56 ± 4	< 0.01
Pulse amplitude (µg/L)	12.2 ± 2.2	10.2 ± 1.4	0.01	5.8 ± 0.5	< 0.01
Pulse area (µg/Lxmin)	230 ± 28	221 ± 31	0.03	91 ± 13	< 0.01
Nadir concentration (µg/L)	8.4 ± 1.8	6.8 ± 1.1	0.05	4.0 ± 0.4	< 0.01
Total Area (µg/Lx24 h)	14 474 ± 2666	12 354 ± 190	0.02	7 276 ± 644	< 0.01
Mean 24 h secretion (µg/V_{dl} x 24 h)	128 ± 24	110 ± 17	0.04	67 ± 6	< 0.01
ApEn ratios	0.46 ± 0.05	0.50 ± 0.05	0.01	0.50 ± 0.05	0.32

Data are presented as means ± SEM.

Concentration parameters were calculated from 24 h PRL concentration profiles using Cluster analysis. Mean PRL secretion was calculated from 24 h PRL concentration profiles using the Pulse algorithm, which is a waveform-independent deconvolution method. Secretion rates are calculated per liter distribution volume (V_{dl}).

a) P-values were determined by non- parametric Wilcoxon signed-rank test, before vs. after weight loss in obese women

b) P-values were determined by non parametric Mann Whitney U- test, obese after weight loss vs. lean historical women (19)

Table 3. Correlations between differences of 24 h PRL secretion (before and after weight loss) and changes of body composition parameters in obese subjects

Obese Subjects (N = 11)	Delta 24 h PRL secretion (Difference Before-After weight loss)	
Parameter (Difference Before-After weight loss)	R-square	P-value
Delta Body Weight (kg)	0.34	0.06
Delta BMI (kg/m^2)	0.31	0.07
Delta Percentage Total Body Fat[1)] (%)	0.55	0.08
Delta Subcutaneous Fat Area (cm^2)	0.33	0.07
Delta Visceral Fat Area[2)] (cm^2)	0.04	0.57

Pearson's correlation analysis was used to determine the association between differences of 24 h PRL secretion (before and after weight loss) and changes of body composition parameters in the obese subjects

1) Percentage is the calculated fraction of the total body weight

2) Parameter was negatively correlated with Δ 24 h PRL secretion

Figure 1.

Mean serum PRL concentration time series of the obese subjects before (-●-) and after weight loss (-⊕-) and mean serum PRL concentration time series of the historical control subjects (-○-). Data reflect sampling of blood every 10 min for 24 h. Sampling starts at 0900 h. Lights were switched off and subjects went to sleep (lights off) at 2300 h until 0730 h next morning (vertical grey bar). Sleep was not interrupted.

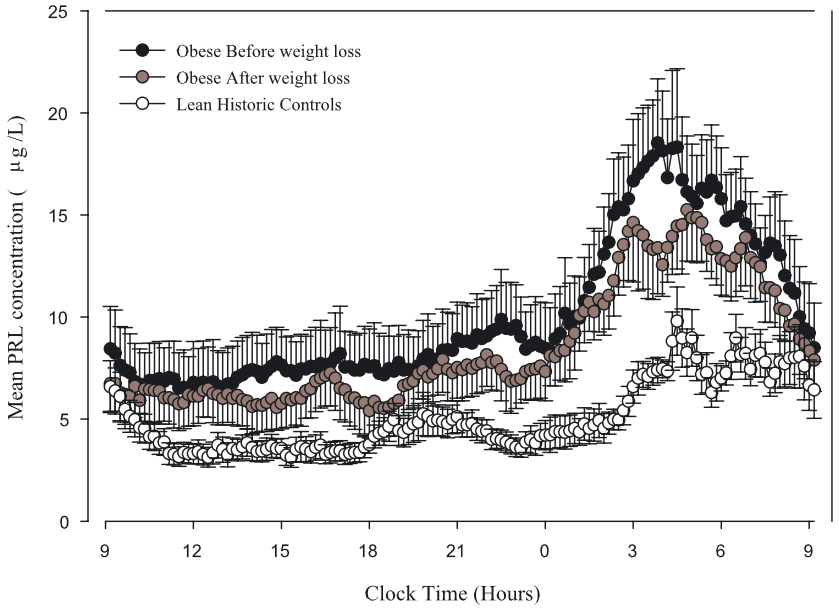


Figure 2.

Diurnal PRL secretion in obese women before (black bars) and after weight loss (grey bars) and in lean historical controls (white bars). Error bars of the box plot represent SEM.

* P < 0.05 Before vs. after weight loss obese women, statistical analysis was performed using non- parametric Wilcoxon signed-rank test

# P < 0.05 Obese vs. lean historical women (19), statistical analysis was performed using non parametric Mann Whitney U- test

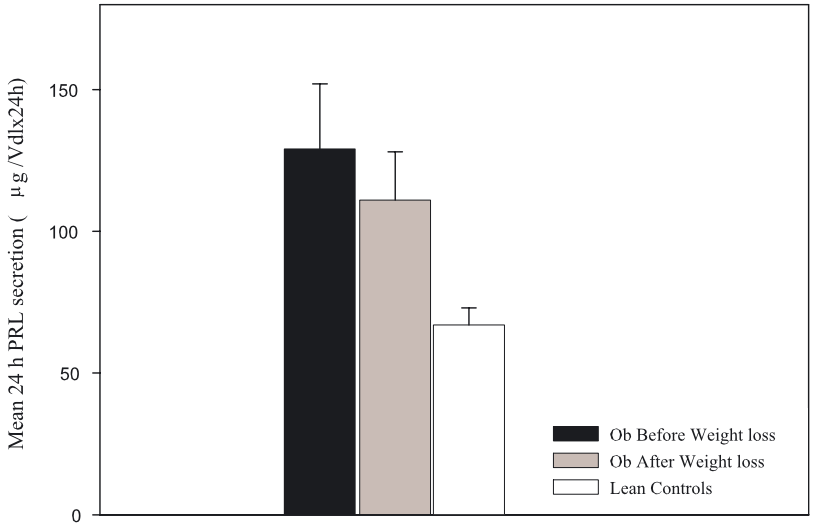
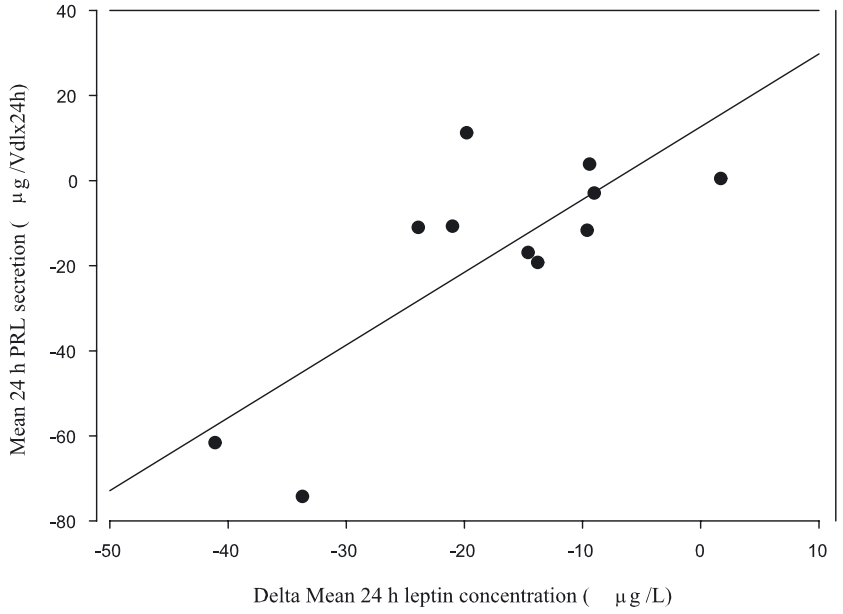


Figure 3.

Correlations between the decrease of 24 h PRL secretion and 24 h leptin concentrations after body weight loss in the obese women. Obese women were included in multiple regression analysis of mean 24 h leptin concentrations and different features of body composition vs. differences of 24 h PRL secretion before and after weight loss. PRL secretion is calculated per liter distribution volume. Differences in PRL secretion were significantly positively related to differences in mean 24 h leptin concentrations ($R^2 = 0.61$, $P < 0.01$).



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Chapter 4

Spontaneous Diurnal TSH Sec0retion is Enhanced in Proportion to Circulating Leptin in Obese Premenopausal Women

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Abstract

1. Context. Recent evidence implicates leptin as an important modulator of thyroid axis activity.
2. Objective. To study spontaneous 24 h TSH secretion and 24 h circulating leptin concentrations in obese and lean women.
3. Design. Prospective parallel study (2004).
4. Setting. Clinical Research Center LUMC.
5. Participants. 12 healthy obese premenopausal women (BMI 33.2 ± 0.9 kg/m^2) and 11 lean controls (BMI 21.4 ± 0.5 kg/m^2) were studied in the follicular phase of their menstrual cycle.
6. Intervention(s). None.
7. Main Outcome Measure(s). Spontaneous 24 h TSH concentrations (10 min time intervals) and secretion, calculated using waveform-independent deconvolution technique (Pulse). 24 h circulating leptin concentrations (20 min time intervals).
8. Results. Mean TSH concentration (obese 1.9 ± 0.2 vs. lean 1.1 ± 0.1 mU/L, P = 0.009) and secretion rate (obese 43.4 ± 5.5 vs. in lean 26.1 ± 2.2 mU/liter distribution volume. 24 h, P = 0.011) were substantially enhanced in obesity, whereas the fasting free thyroxine concentrations were similar (free T_4 in obese 15.4 ± 1.5 vs. in lean 16.4 ± 1.5 pmol/L, P = 0.147). TSH secretion was positively related to 24 h leptin concentrations (R^2 = 0.31, P = 0.007).
9. Conclusions. TSH release is enhanced in the face of normal plasma free thyroxine concentrations in obese premenopausal women and hyperleptinemia may well be involved in this neuroendocrine alteration.

Introduction

The hypothalamic pituitary thyroid (HPT) hormonal ensemble orchestrates a variety of metabolic processes, including thermogenesis and energy expenditure, thereby affecting energy balance (1-3). As obesity is a phenotypic expression of energy imbalance (4), it is conceivable that obese individuals have altered HPT axis activity.

Numerous studies have evaluated HPT axis status in obese humans and the results were conflicting. The majority of these studies suggests that there is no substantial change in basal thyroid hormone concentrations (5), although a few papers document serum triiodothyronine (T_3) elevation in obese subjects (6-8). The basal serum TSH concentration in a single plasma sample was similar in obese and non-obese subjects in some studies (9-11), whereas others documented higher basal TSH concentrations in obese humans (6;12). Also, some papers report a larger rise of plasma TSH in response to TRH stimulation in obese subjects, while other studies revealed normal or reduced TSH responses (9-19). As serum TSH concentrations fluctuate during the day whereas circulating thyroid hormone levels are relatively stable, proper appreciation of HPT axis activity requires measurement of TSH release over 24 hours (20) while thyroid hormone determination in a single sample usually suffices. To our knowledge, spontaneous TSH concentration profiles over 24 hours have not been measured in obese humans. Here we report data delineating 24 hour TSH secretion in obese premenopausal women.

Subjects and Methods

Subjects

Twelve healthy obese premenopausal women (BMI > 30 kg/m^2) and 11 lean (BMI 18-25 kg/m^2) controls of similar sex and age were enrolled in this study, after given written acknowledgement of informed consent for participation. All participants were required to have a regular menstrual cycle and not using oral contraceptives. Subjects were studied in the follicular phase of their menstrual cycle. Chronic disease, depression (present or in history), smoking, recent transmeridional flights, night-shift work, weight change (> 3 kg in 3 months) and use of medication were exclusion criteria. All subjects had an unremarkable medical history and no abnormalities were found during physical examination, standard laboratory haematology, blood chemistry and urine tests.

Body fat distribution

Total amount and location of excess body fat mass was determined in the obese women only. Total body fat mass was expressed as a percentage of total body weight and was quantified using dual energy X-ray absorptiometry (DEXA, Hologic QDR4500) (21). Visceral and subcutaneous adipose tissue areas were assessed in the obese women by MRI as described before, using a multi slice fast spin echo sequence (Gyroscan –T5 whole body scanner 0.5 Tesla, Philips Medical Systems, Best, The Netherlands)(22). MRI images were independently analysed by two observers.

Clinical Protocol

The protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center. Subjects were admitted to the Clinical Research Unit of the Department of General Internal Medicine in the early follicular stage of their menstrual cycle. A cannula for blood sampling was inserted into an antecubital vein. The cannula was attached to a 3-way stopcock and kept patent by a continuous saline infusion. Blood samples were taken with S-monovette (Sarstedt, Etten-Leur, The Netherlands) at 10 min intervals for determination of plasma TSH concentrations and at 20 min intervals for the determination of leptin concentrations. Subjects remained recumbent, except for bathroom visits. No daytime naps were allowed. Meals were served according to a fixed time schedule. Lights were switched off at 2300 h. Vital signs were recorded at regular time intervals and great care was taken not to disturb patients while sampling blood during their sleep (no EEG sleep recording was performed).

Assays

Samples were centrifuged at 4000r/min at 4 °C during 20 minutes, within 60 min of sampling. Subsequently, plasma was divided into separate aliquots and frozen at -80 °C until assays were performed. Samples of each subject were determined in the same assay run. Plasma TSH concentrations were measured with a time resolved immunofluorometric assay (Wallac, Turku, Finland) and its standard was calibrated against the WHO 2nd standard IRP (80/558) hTSH for immunoassays. The limit of detection is 0.05 mU/L and the inter-assay coefficient of variation was less then 5%. Plasma leptin concentrations were determined by RIA (Linco Research, St. Charles, MO) with a detection limit of 0.5 µg/L and the inter-assay coefficient ranged from 6-7%. Basal free thyroxine (fT_4) were estimated using an automated system (Elecsys 2010, Roche Diagnostics Nederland BV, Almere, Netherlands) and by dialysis as described before (23). Basal serum glucose, HbA1C, Apolipoprotein A-1 and triglyceride levels were measured using a fully automated system (P800, Integra 800 and Hitachi 747 respectively, Roche Diagnostics Nederland BV, Almere, Netherlands). Estradiol was determined by RIA (Diagnostic Systems Laboratory, Webster, TX).

Calculations and statistics

Cluster

The Cluster program describes various characteristics of pulsatile hormone concentration profiles (24). A concentration peak is defined as a significant increase in the test peak cluster vs. the test nadir cluster. We used a 2 x 1 cluster configuration

(2 samples in the test nadir and one in the test peak) and t-statistics of 2.0 for significant up- and downstrokes in TSH levels to constrain the false positive rate of peak identification to less than 5% of signal free noise. The locations and durations of all significant plasma hormone peaks were identified and the following parameters were determined: mean TSH concentration, peak frequency, mean peak width, mean peak height (maximum concentration attained within the peak), mean peak area (above the baseline), overall mean concentration of the inter-peak valley (nadir) and the total area under the curve.

Pulse

Deconvolution analysis estimates hormone secretion and clearance rates based on hormone concentration time-series. The Pulse algorithm is a waveform-independent deconvolution method, which can be used for calculation of hormonal secretion, without specifying shape, number and time of secretory events (25). The technique requires a priori specification of hormonal half-life in plasma. TSH disappearance from plasma is best described by a two compartment model, characterized by a fast component half-life of 18 ± 3 min and a slow component half-life of 90 ± 5 min where the fractional contribution of the slow component to the overall decay amounts to 32% (data kindly provided by J.D. Veldhuis, Mayo Clinic, Rochester, MN, USA). Pulse was used to quantify mean 24 h TSH secretion. Secretion rates were expressed per liter distribution volume (VD).

Approximate entropy

Approximate Entropy (ApEn) is a scale- and model- independent statistic that assigns a non- negative number to time series data, reflecting regularity of these data (26). Higher ApEn values denote greater relative randomness of hormone patterns. Normalized ApEn parameters of m = 1 (test range), r = 20% (threshold) and 1000 for the number of runs were used, as described previously (27). Hence, this member of the ApEn family is designated (1, 20%). The ApEn metric evaluates the consistency of recurrent subordinate (non pulsatile) patterns in a time series and thus yields information distinct from and complementary deconvolution (pulse) analyses (28). Data are presented as normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series. ApEn ratios close to 1.0 express high irregularity (maximum randomness) of pulsatile hormone patterns.

Diurnal Rhythmicity

Nyctohemeral characteristics of TSH concentration patterns were determined using a robust curve fitting algorithm described by Cleveland (LOWESS analysis, SYSTAT version 11 Systat Inc, Richmond, CA (29;30)). The acrophase (clock time during 24 h at which TSH concentration is maximal) was the maximal value of the fitted curve. The amplitude of the rhythm was defined as half the difference of the nocturnal zenith and the day-time nadir. The relative amplitude was the maximal percentage increase of the nadir value.

Statistics

Data are presented as mean ± SEM, unless otherwise specified. Means of TSH concentration and secretion parameters of both groups were compared using two-tailed independent Student's t-test. Significance level was set at 0.05. Regression analysis was used to determine the correlation of BMI and mean 24 h leptin concentrations vs. mean 24 h TSH secretion in the obese and normal weight premenopausal women together. Stepwise multiple regression analysis, with percentage total body fat, subcutaneous and visceral fat areas as independent variables, was used to determine correlations between specific measures of body fat distribution and diurnal TSH secretion rates in the obese subjects only.

Results

Subject Characteristics

Obese subjects had normal fasting glucose (mean 5.3 ± 0.4 range 4.8-6.0 mmol/L, ref. range lab 3.5-5.5 mmol/L), HbA1C (mean 4.7 ± 0.3 range 4.2-5.3 %, ref. range lab 4.3-6.3 %), triglyceride (mean 1.4 ± 0.7 range 0.7-2.9 mmol/L, ref. range

lab 0.80-1.94 mmol/L) cholesterol (mean 5.09 ± 0.81 range 3.94-6.17 mmol/L, ref. range lab 3.9-7.3 mmol/L) and apolipoprotein A-1 levels (mean 1.3 ± 0.1, range 1.0-1.7 g/L, ref. range lab 1.01-1.98 g/L). All subjects were clinically euthyroid and fasting free thyroxine (fT_4) concentrations as well as fT_4 fraction (fraction of total T_4) measured by dialysis were similar in obese and lean subjects (fT_4 obese 15.4 ± 1.5 vs. lean 16.4 ± 1.5 pmol/L, P = 0.147 obese vs. lean, ref. range lab 10-24 pmol/L and fT_4 fraction : obese 0.020 ± 0.001 vs. lean 0.022 ± 0.001 fraction of total T_4, P = 0.08 obese vs. lean). Relevant subject characteristics are presented in Table 1.

Plasma hormone concentration profiles and TSH secretion

Various characteristics of 24 h TSH hormone concentration profiles were determined using the Cluster program. Mean 24 h TSH concentration, mean peak height (maximum concentration attained within the peak), overall mean concentration of the inter-peak valley (nadir) and total area under the concentration curve were significantly higher, whereas peak frequency and peak width were unaltered in obese subjects compared to lean controls (Table 2, Figure 1). A graphical illustration of representative TSH concentration profiles of two obese and two lean woman of identical age are presented in Figure 2. Deconvolution analysis revealed that daily TSH secretion was significantly enhanced in the obese women compared to the lean controls (obese 43.4 ± 5.5 vs. lean 26.1 ± 2.2 mU/VD . 24 h, P = 0.011).

Diurnal variation 24 h TSH concentration profiles

The acrophase of the nyctohemeral TSH rhythm occurred at night at different clock-times in obese and lean subjects (obese 0100 h ± 01 h 26 min and lean 0009 h ± 50 min respectively, P = 0.033). The amplitude of the rhythm was not significantly increased in obese subjects (0.69 ± 0.11 mU/L vs. lean 0.51 ± 0.07 mU/L respectively, P = 0.206), whereas the relative increase in TSH concentration was significantly lower in the obese women (53.2 ± 5.0 % vs. lean 79.5 ± 5.4 % respectively, P = 0.019).

Regularity of plasma TSH concentration- time series

ApEn ratios as well as ApEn values, which reflect regularity of plasma TSH concentration time series, were similar in the obese and normal weight premenopausal women (ApEn ratios in obese 0.56 ± 0.03 vs. in lean 0.52 ± 0.04 respectively, P = 0.451 and ApEn values in obese 1.00 ± 0.06 vs. in lean 0.92 ± 0.08 respectively, P = 0.390).

Body composition and TSH secretion

Both obese and lean subjects (N = 23) were included in the regression analysis of BMI (range 18.3-39.4 kg/m^2) vs. daily TSH secretion. BMI was positively related to mean diurnal TSH release (R^2 = 0.29, P = 0.010). Correlations between specific measures of body fat distribution and diurnal TSH secretion rates were determined in the obese subjects only. The obese subjects had a mean percentage body fat of 40.7 ± 1.0 (36.9-46.3) %. Mean sizes of their visceral and subcutaneous fat areas were 392 ± 27 (274-539) cm^2 and 1326 ± 57 (1106-1709) cm^2 respectively. Multiple regression analysis, with percentage total body fat and visceral and subcutaneous fat areas as independent variables, revealed that there was no significant correlation between any of these specific body composition parameters and daily TSH secretion (% total body fat vs. 24 h TSH secretion R^2 = 0.12, P = 0.352; subcutaneous fat area vs. diurnal TSH release R^2 = 0.02, P = 0.692 and abdominal fat area vs. 24 h TSH secretion R^2 = 0.15, P = 0.306).

Leptin and 24 h TSH secretion

Both obese and lean subjects (N = 23) were included in regression analysis of mean 24 h leptin concentrations (range 4.9-50.3 µg/L) vs. 24 h TSH production. Regression analysis revealed that mean 24 h leptin concentrations were significantly positively related to mean diurnal TSH release (R^2 = 0.31, β = 0.577, P = 0.007 Fig 3). When both leptin and BMI were entered as independent variables into the model simultaneously, none of these parameters did significantly correlate with mean diurnal TSH release. Estrogen was not related to diurnal TSH secretion and did not influence the correlation between leptin and TSH, if added as independent variable to the model.

Discussion

This study demonstrates that spontaneous pulsatile TSH release over 24 hours is substantially enhanced in obese premenopausal women compared to lean controls of similar age and sex. The higher diurnal TSH secretion rate appears to be primarily attributable to an increase of TSH pulse amplitude, whereas the number of pulses is similar. The mean 24 h TSH secretion rate was positively related to circulating leptin concentrations.

As far as we are aware, this is the first study to directly compare spontaneous circadian TSH secretion in lean and obese humans. Previous investigators have primarily evaluated plasma hormone concentrations in a single blood sample or in response to TRH administration as a measure of HPT axis status in obese individuals (5-19;31). In this context, some authors report similar TSH concentrations in obese and normal weight humans (10;11), but others have shown that TSH concentrations are significantly elevated in proportion to BMI in obese women (6) which is in line with the findings of the present study. It seems important to emphasize, that we studied women of reproductive age in the early follicular phase of their menstrual cycle and that our data should therefore be judged within the framework of this physiological context. Indeed, it remains to be established if obese women in other stages of their cycle or obese men have similar elevations of circulating TSH. Also, one has to take into account that the waveform-dependent deconvolution technique we used requires a priori definition of TSH clearance. Therefore, we can not rule out the possibility that changes in plasma TSH clearance contribute to the elevated circulating TSH levels in obese women compared to normal controls.

TSH synthesis and secretion are primarily controlled by the stimulatory action of thyrotropin releasing hormone (TRH) and the negative feedback restraint by thyroid hormones (T_4 and T_3), whereas other factors, including leptin, dopamine, somatostatin and serotonin act to modulate release (for review see (32)). Several studies provide strong evidence that leptin stimulates TSH production in rodents and humans. Leptin counteracts the starvation-induced reduction of thyroid hormone and TSH release in rodents (33;34) by preventing the decline of TRH mRNA expression in paraventricular nucleus neurons that occurs during fasting (35). Furthermore, clinical studies have shown that leptin replacement significantly blunts the fasting-induced fall in TSH secretion in healthy lean men and in normal weight women of reproductive age in the early follicular phase of their menstrual cycle (36;37). Moreover, circadian plasma leptin and TSH concentration rhythms exhibit significant pattern synchrony of ultradian fluctuations in humans (38). Finally, indirect evidence for a stimulatory impact of leptin on TSH secretion has also been found in various human disease states characterized by low circulating leptin levels. For example, plasma TSH levels are reduced in proportion to circulating leptin in narcoleptic patients (39). Moreover, both circulating leptin and TSH concentrations appear to be low in patients with anorexia nervosa, while weight gain is accompanied by a significant increase of both hormones in these patients (40-43). The finding that 24 h TSH secretion was positively related to mean 24 h leptin concentrations in the present study is in line with the results of these studies. Although this simple correlation between leptin and TSH does not imply causality in a cross sectional study, this finding may be interpreted as circumstantial evidence of a stimulatory impact of hyperleptinemia on TSH release in obese individuals.

Additionally, reduced dopamine D2 receptor (D2R) mediated transmission in the brain may enhance TSH release in obese humans. Availability of D2R binding sites is considerably reduced in the brain of obese rodent models (44) and in striatal nuclei of obese humans (45). Moreover, we previously showed that spontaneous diurnal PRL release is enhanced in obese premenopausal women, which supports the concept of diminished D2R signalling in human obesity, as D2R activation is required for maintenance of low circulating PRL levels (46). Dopamine exerts its inhibitory influence on TSH synthesis and release through D2R activation in thyrotrophs of the pituitary gland, and it appears to specifically reduce the amplitude of pulsatile TSH release, whereas it does not affect TSH pulse frequency (32). The present study shows that the increase of TSH secretion rates in obese subjects is primarily attributable to enhanced TSH pulse amplitude, whereas the number of pulses was similar to that in controls. These findings are in keeping with a putative role of reduced D2R dopaminergic tone in the anomalous TSH release profile in obese humans. Furthermore, although dopamine has an inhibitory effect on TSH secretion at the pituitary level, dopamine and dopamine agonists stimulate TRH release by the hypothalamus in rats (47), acting through the dopamine 2 receptor (48). TRH plays an important role in the posttranslational processing of the oligosaccharide moieties of TSH and hence exerts an important influence on the biologic activity of TSH that is secreted (49). Thus, we speculate that reduced D2R signalling in hypothalamic nuclei may hamper the biological activity of TSH through diminution of TRH production in obese humans, which could explain why TSH levels are elevated in the face of normal free T_4 in our obese subjects. As D2R activity was not addressed directly in this study, it clearly requires further

investigation to establish if dopaminergic mechanisms indeed underlie enhanced TSH release in obese humans.
Alternatively, evidence has been provided that serotonin inhibits TSH secretion (50), although the literature on serotonergic control of TSH secretion is ambiguous. It has been suggested that a defect in hypothalamic serotonergic neurotransmission is involved in altered pituitary hormone release in obesity (51) and the elevated TSH response to TRH in obese subjects is normalized by serotonergic stimulation (16). Thus, reduced serotonergic signalling might be among the physiological cues explaining the elevated TSH levels in the obese women.
Finally, somatostatin inhibits TSH secretion (32). Somatostatin is known as the major inhibitor of GH release. Both spontaneous and stimulated GH secretion is profoundly impaired in obesity and a plethora of data implicates that obesity is associated with tonic somatostatin hypersecretion (52). Therefore, it seems not very likely that somatostatin is involved in the altered TSH secretion in obese women.
The fact that TSH plasma concentrations are elevated in obese humans in the face of normal free T_4 levels has not been reported before. Although this phenomenon might be explained by impaired biological activity of TSH through reduced dopaminergic signalling (see above), it has also been shown that human obesity is frequently associated with unresponsiveness to exogenous TSH (53). In this context, it is noteworthy that the sensitivity of the thyroid gland to TSH is regulated by the autonomic nervous system (54). Specifically, sympathetic activity appears to inhibit the thyroid hormone response to TSH stimulation (55;56). Thus, increased sympathetic activity associated with obesity (57-59), potentially contributes to the imbalance of the thyroid-pituitary axis observed here.
Finally, the acrophase of the TSH concentration patterns occurred significantly later during the night in the obese women than in the lean controls. The acrophase of TSH is believed to reflect the balance between the inhibitory effect of sleep and the increase of TSH release in the evening, regulated by neuronal signals emanating from the circadian master pacemaker, the suprachiasmatic nucleus (54). Thus, differential sleep patterns among obese and lean women may explain why the TSH phase shift occurs. Unfortunately, we did not perform EEG sleep monitoring to substantiate this thesis. Although phase shifts of other neuroendocrine systems have been described in viscerally obese premenopausal women (60), both cause and consequence of the epiphasia (delayed timing) of the 24 h TSH hormonal release remain elusive.
In conclusion, we here show that daily TSH secretion is enhanced in obese premenopausal women, while free thyroxine concentrations are similar to those in lean controls. The 24 h TSH secretion was positively correlated with mean circulating leptin concentrations and BMI, which suggests that hyperleptinemia is involved in this alteration of HPT axis setting in obese premenopausal women.

Tables and Figures

Table 1. Subject Characteristics Lean and Obese premenopausal women

Parameter	Obese (n = 12)	Controls (n = 11)	P-value [a)]
Age (years)	37.5 ± 2.0	36.0 ± 1.8	0.591
BMI (kg/m^2)	33.2 ± 0.9*	21.4 ± 0.5	<0.001
Body Fat (%)	40.7 ± 1.0	N.D.	
Visceral Fat Mass (cm^2)	392 ± 27	N.D	
Subcutaneous Fat Mass (cm^2)	1326 ± 57	N.D.	
Estradiol (E2) (pmol/L)	180 ± 30	162 ± 59	0.765
Free thyroxine (fT_4) (pmol/L) [b)]	15.4 ± 1.5	16.4 ± 1.5	0.147
Mean 24 h Leptin (µg/L)	29.5 ± 2.9*	14.5 ± 2.5	0.001

Data are presented as means ± SEM, range is given between brackets.

a) P- value independent Student's t-test obese vs. lean subjects

b) fT_4 values were measured by automatic system

* $P < 0.05$ obese vs. lean subjects

Percentage body fat was estimated by DEXA and was calculated as a fraction of total body weight. Visceral and subcutaneous fat areas were determined using MRI. N.D. Parameter was determined in obese controls only.

Table 2. TSH concentration and secretion parameters in lean and obese premenopausal women

Parameter	Obese (n = 12)	Controls (n = 11)	P-value [a)]
Mean 24 h plasma concentration (mU/L)	1.9 ± 0.2*	1.1 ± 0.1	0.009
Number of pulses (n/24 h)	19 ± 1	20 ± 1	0.376
Pulse width (min)	51 ± 3	49 ± 3	0.512
Pulse amplitude (mU/L)	2.1 ± 0.3*	1.3 ± 0.1	0.013
Pulse area (mU/Lxmin)	19.3 ± 4.0	10.5 ± 1.4	0.061
Nadir concentration (mU/L)	1.7 ± 0.2*	1.0 ± 0.1	0.012
Total area (mU/Lx24 h)	2750 ± 350*	1550 ± 190	0.008
Mean 24 h secretion (mU/V_{dl} x 24 h)	43.4 ± 5.5 *	26.1 ± 2.2	0.011

Concentration parameters were calculated from 24 h TSH concentration profiles using Cluster analysis. Mean TSH secretion was calculated from 24 h TSH concentration profiles using the Pulse algorithm, which is a waveform-independent deconvolution method. Secretion rates are calculated per liter distribution volume (V_{dl}).

Data are presented as means ± SEM.

a) P- value independent Student's t-test obese vs. lean subjects

* $P < 0.05$ obese vs. lean subjects

Figure 1.

Mean serum TSH concentration time series of the obese subjects (-●-) and control subjects (-○-). Data reflect sampling of blood every 10 min for 24 h. Blood sampling starts at 1800 h. Lights were switched off and subjects went to sleep at 2300 h until 0730 h next morning (grey horizontal bar). Sleep was not interrupted. Vertical arrows indicate the time point at which the acrophase occurred (obese 0100 h ± 01 h 26 min vs. lean 0009 h ± 50 min respectively, P = 0.033).

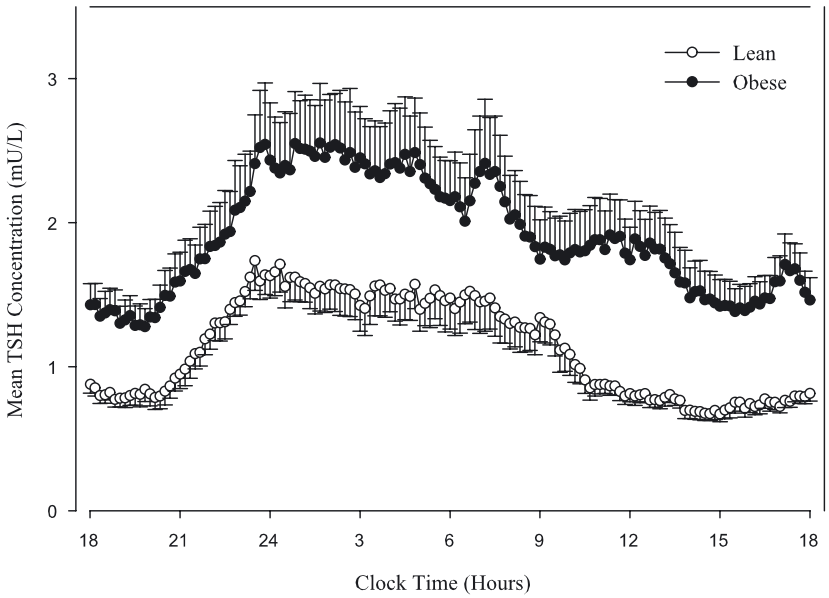
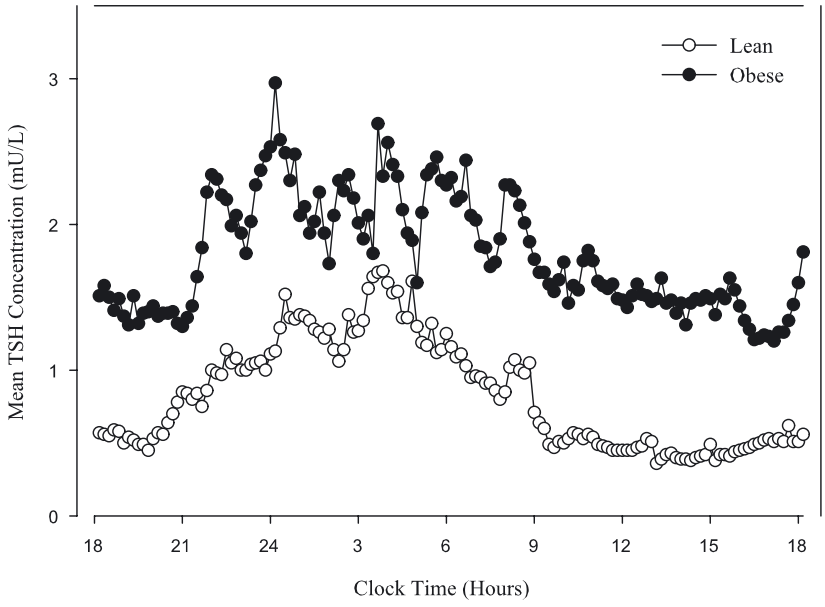


Figure 2.

Representative 24 h TSH concentration profiles of two lean (-•-) and two obese women (-○-). Data reflect sampling of blood every 10 min for 24 h. Blood sampling starts at 1800 h. Lights were switched off and subjects went to sleep at 2300 h until 0730 h next morning (grey horizontal bar). Sleep was not interrupted.

A) Lean woman Age = 39 yr, BMI = 21.0 (kg/m^2) and obese woman Age = 39 yr, BMI = 31.9 (kg/m^2)



B) Lean woman Age = 31 yr, BMI = 24.8 (kg/m^2) and obese woman Age = 31 yr, BMI = 39.4 (kg/m^2)

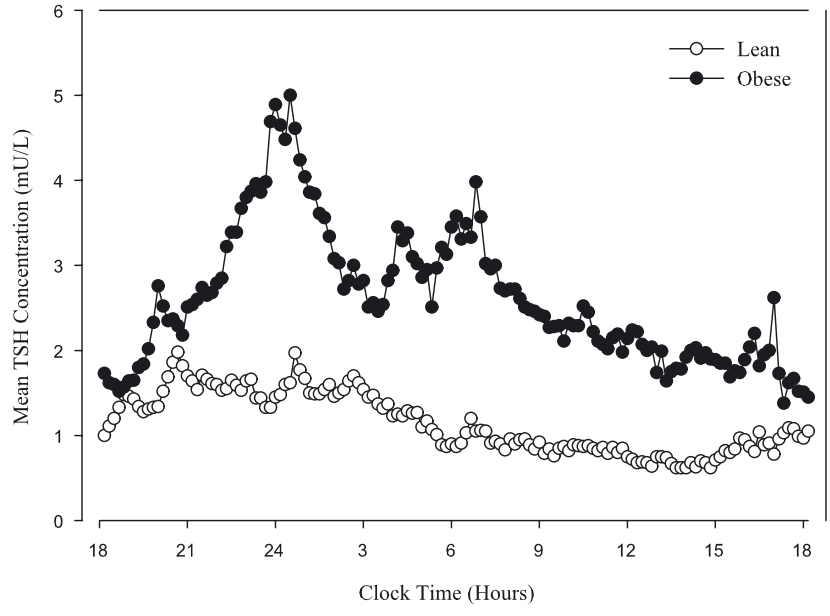
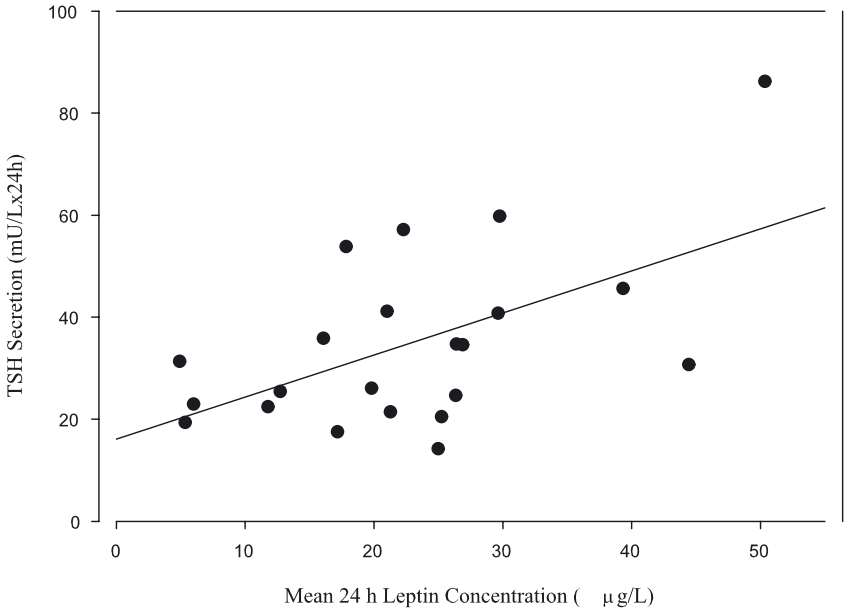


Figure 3.

Correlation between leptin and 24 h TSH secretion. Both obese and lean women (N = 22) were included in correlation analysis of 24 h mean leptin concentrations (range 4.9-50.3 µg/L) vs. daily TSH secretion.



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Chapter 5

High Circulating TSH Levels in Obese Women are Reduced after Body Weight Loss Induced by Caloric Restriction

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Abstract

1. Context. Previous clinical studies concerning the impact of body weight loss on single plasma TSH concentration measurements or the TSH response to TRH in obese humans have shown variable results.
2. Objective. To investigate the effect of weight loss induced by caloric restriction on diurnal TSH concentrations and secretion in obese humans.
3. Design. Clinical, prospective, cross over study (2004)
4. Setting. Clinical Research Center in the LUMC
5. Participants. Eleven obese premenopausal women (BMI 33.3 ± 0.7 kg/m^2).
6. Intervention. Weight loss (50% reduction overweight by caloric restriction).
7. Main Outcome Measure(s). 24 h plasma TSH concentrations (10 min intervals) and the 24 h TSH secretion rate, calculated by a waveform-independent deconvolution technique (Pulse).
8. Results. 24 h TSH secretion rate was significantly higher in obese women than in normal weight controls and weight loss was accompanied by diminished TSH release (before 43.4 ± 6.4 vs. after weight loss 34.4 ± 5.9 mU/Lx24 h, $P = 0.02$). Circulating free triiodothyronine levels dropped after weight loss from 4.3 ± 0.19 to 3.8 ± 0.14 pmol/L ($P = 0.04$). Differences in 24 h TSH release correlated positively with the decline of circulating leptin ($R^2 = 0.62$, $P < 0.01$).
9. Conclusions. Elevated TSH secretion in obese women is significantly reduced by the diet induced loss of overweight. Among various physiological cues, leptin may be involved in this phenomenon. The decrease of TSH and free triiodothyronine may blunt energy expenditure in response to long term calorie restriction, thereby frustrating weight loss attempts of obese individuals.

Introduction

The hypothalamic-pituitary-thyroid (HPT) axis regulates energy expenditure, oxygen consumption and fuel metabolism. HPT axis disorders impact metabolic rate, thermogenesis and body weight. Conversely, changes in body weight are accompanied by compensatory changes in energy expenditure (1), which may be brought about in part by adaptations of HPT axis activity.

All clinical studies evaluating the impact of body weight loss on the HPT axis have used single measurement of TSH and/or thyroid hormones and/or TSH release in response to TRH as a measure of activity. Most studies suggest that weight loss reduces TSH concentrations and the TSH response to TRH, whereas others report unchanged plasma TSH or TRH induced TSH responses in obese individuals after weight loss (2-6). As plasma TSH concentrations are characterized by circadian fluctuations, adequate appreciation of the impact of body weight loss on TSH release requires frequent measurement of TSH over time. Since circulating thyroid hormone levels are relatively stable, determination in a single sample suffices (7).

We recently showed that diurnal TSH secretion is significantly enhanced in obese women (Kok P et al. unpublished data), where TSH secretion rate appears to be positively correlated with circulating leptin levels and BMI. Other studies provide

strong evidence that leptin stimulates TSH production in rodents and humans (8;9). Here, we studied the impact of body weight loss induced by caloric restriction and associated decline of circulating leptin levels on spontaneous diurnal TSH release in obese humans.

Subjects and Methods

Subjects

Eleven healthy obese premenopausal women were enrolled after giving written informed consent for participation. A historical control group of 11 lean controls (BMI 21.4 ± 0.5 kg/m^2) of similar sex and age (obese 35.8 ± 2.3 vs. lean 36.0 ± 1.8 yr, P = 0.95) was included. All subjects underwent medical screening, including medical history, physical examination, standard laboratory haematology, blood chemistry and urine tests. Acute or chronic disease, smoking, weight change prior to the study (> 3 kg in 3 months), and use of medication (including oral contraceptives) or iodine supplements were exclusion criteria for participation. Subjects had not been exposed to radio contrast dyes and did not have personal or family history of thyroid dysfunction. All participants had regular menstrual cycles.

Weight Loss

Obese subjects started a weight loss program after the first study occasion to reduce their body weight by 50% of their overweight within a time period of 4 months (range 1.5-7 months) by dietary intervention, using a liquid very low calorie diet (2MJ/day; 43% proteins, 15% fat, 42% carbohydrates, iodine 159 µg/day; Modifast, Novartis, Netherlands), while physical activity level remained constant. Total body fat was quantified using bioelectrical impedance analysis (Bodystat, UK) and visceral/subcutaneous fat areas were assessed by MRI before and after weight loss.

Clinical Protocol

The protocol was approved by the Medical Ethics Committee of the LUMC. All subjects were studied in the early follicular stage of their menstrual cycle. Obese women were studied before and after weight loss. To limit the putative direct effects of feeding/VLCD on thyroid hormone release (which tend to occur almost immediately), all subjects used a standard eucaloric diet (1980 kCal/8.3 MJ per day) as of three days prior to each admission until the end of blood sampling. Subjects were admitted to the research unit at 0800 h. One hour after insertion of an iv cannula into an antecubital vein, blood sampling started using a constant withdrawal pump (Conflo, Carmeda AB, Sweden). Meals were served according to a fixed time schedule. No daytime naps were allowed. Lights were switched off at 2300 h. Vital signs were regularly recorded and great care was taken not to disturb and touch patients while sampling blood during their sleep (no EEG sleep recording was performed).

Assays

Samples of each subject were determined in the same assay run. Plasma TSH concentrations were measured with a time resolved immunofluorometric assay (Wallac, Finland), calibrated against the WHO 2nd standard IRP (80/558). Detection limit was 0.05 mU/L and inter-assay variation coefficient was < 5%. Leptin concentrations were determined by RIA (Linco Research,USA) with a detection limit of 0.5 µg/L and inter-assay coefficient of 6-7%. Total and free thyroxine was measured using an automated system (Elecsys 2010, Roche, Netherlands). Free triiodothyronine was measured with a micro particle enzyme immunoassay on an Imx (Abbott, USA). Total triiodothyronine and reverse T_3 was measured by an in-house RIA (Erasmus MC, Rotterdam, Netherlands). Estradiol was determined by RIA (Diagnostic Systems Laboratory, Webster, USA).

Calculations and statistics

Cluster delineates characteristics of pulsatile hormone concentration profiles. We used a 2 x 1 cluster configuration (10). The Pulse algorithm is a waveform-independent deconvolution method, which can be used for calculation of hormonal secretion based on plasma hormone concentration time-series, without specifying shape, number and time of secretory events, and was used to quantify mean 24 h TSH secretion (11). TSH disappearance from plasma is best described by a two

compartment model (fast component half-life 18 min, slow component 90 min, fractional contribution of the slow component 32%) (data kindly provided by J.D. Veldhuis, Mayo Clinic, USA). The Approximate Entropy (ApEn) statistic assigns a non- negative number to time series data, to quantify regularity of these data (7). Nyctohemeral characteristics of TSH concentration patterns were determined using Cleveland's' robust regression technique (7). Data were statistically analysed using parametric or non- parametric test when appropriate. Multiple regression analysis was performed to estimate the correlation between differences of these parameters vs. differences of 24 h TSH secretion before and after weight loss.

Results

Subjects

All subjects were clinically euthyroid. BMI, total body fat and visceral and subcutaneous fat areas were significantly reduced after weight loss (Table 1). Thyroxine (free T_4) levels in lean subjects were 16.4 ± 0.5 pmol/L (P = 0.09 vs. obese before VLCD). Free triiodothyronine levels were positively related to BMI in all subjects (R^2 = 0.51, P = 0.01), whereas BMI and total triiodothyronine were not related (R^2 = 0.19, P = 0.19). Total and free triiodothyronine was significantly reduced after weight loss. Differences in free or total triiodothyronine levels did not correlate with changes of body composition parameters before and after weight loss.

TSH concentration and secretion parameters

Mean 24 h TSH concentrations, mean peak height and peak area was significantly lower, whereas peak frequency was significantly increased after weight loss. Nadir concentration and peak width were not significantly altered by weight reduction (Table 2). Mean 24 h TSH secretion was significantly reduced in the obese women after weight loss (Table 2). Duration of the diet period did not correlate with differences in TSH secretion (R^2 = 0.12, P = 0.29). After weight loss, TSH concentration and secretion parameters did not differ significantly from those obtained in lean controls, except for pulse width, peak area and pulse frequency. Figure 1 shows the mean 24 h plasma TSH concentrations. ApEn ratios were similar before and after weight loss (0.51 ± 0.03 vs. 0.54 ± 0.04 respectively, P = 0.29) and were similar in obese and lean women (0.52 ± 0.04, P = 0.74). Clock-times of the acrophase were identical before and after weight loss (0020 h ± 30 min vs. 0010 h ± 30 min respectively, P = 0.82) and were similar in obese and lean subjects (0130 h ± 50 min, P = 0.16).

Leptin and 24 h TSH secretion

Mean 24 h leptin concentrations were significantly reduced after weight loss in obese women (P = 0.22 vs. leptin in lean subjects = 13.3 ± 2.5 µg/L). Multiple regression analysis, including body weight, BMI, percentage total body fat and mean 24 h leptin concentrations as independent variables revealed that the difference in 24 h TSH secretion was positively correlated with differences in mean 24 h leptin concentrations (P < 0.01, partial correlation R^2 = 0.62, Figure 2) and with differences in body weight (P = 0.01) and BMI (P < 0.01).

Discussion

The present study shows that weight loss blunts elevated circadian TSH secretion in obese women. Also, triiodothyronine levels were lower after weight loss, whereas the thyroxine concentration was not affected. The decline of TSH secretion was positively related with the decline of body weight, BMI and mean 24 h leptin concentrations in response to VLCD.

Previous studies have evaluated the impact of weight loss on the HPT axis using a single plasma TSH concentration and/or TSH release in response to TRH as a measure of activity. Most of these studies indicate that weight loss lowers TSH concentrations and blunts the TRH induced TSH response (2-6), which is in line with the present results.

Our subjects used a "eucaloric" diet for three days prior to each study to limit the putative impact of calorie restriction on TSH release. Although dietary intervention tends to impact circulating TSH rather quickly (i.e. within 3 days (12)), we can not exclude the possibility that the decline of TSH and triiodothyronine we observed was due to persistent effects of the VLCD rather than to the loss of body weight.

Thyroid hormones control pituitary TSH release by feed back inhibition at the pituitary and hypothalamic level (for review see (13)). In the present study, fT_4 and T_4 remained unchanged, whereas fT_3 and T_3 were lower after weight loss, which is in line with previous studies (14;15). Thus, other factors modulate TSH production so as to decrease in response to weight loss in obese women.

Several studies in rodents and humans provide strong evidence that leptin stimulates TSH production (8;9). In our study, the reduction of 24 h TSH secretion correlated with the decline of mean 24 h leptin concentration in response to weight loss (Fig 2). In concert, these data support the notion that leptin plays a role in the control of pituitary TSH release in (obese) humans.

Alternatively, activation of dopamine D2 receptors (D2R) may be involved. TSH release is inhibited by D2R activity (13) and D2R binding sites in the brain are reduced in obese humans (16). Calorie restriction and weight loss are accompanied by increased D2R signalling in animals (17) and probably also in humans (18). Thus, reduced central D2R neurotransmission may "unleash" TSH release in obese humans, and up-regulation of D2R tone in response to weight loss may then restore TSH secretion to normal.

Finally, it has been reported that exogenous estrogens raise TSH concentrations (19) and estradiol levels were significantly lower after weight loss in the present study, which has been documented previously (20). However, we did not find a significant relation between changes in TSH secretion and estradiol concentrations in response to weight loss, which argues against an important role of this hormone in the modulation of HPT axis activity.

Whatever the underlying mechanism, changes of HPT activity in response to body weight loss in obese humans may be of clinical and physiological relevance. Since thyroid hormones stimulate resting energy expenditure and basal metabolic rate (for review see (13)), a decline of TSH release and triiodothyronine concentrations may contribute to the compensatory reductions of energy expenditure and catabolism that typically accompany weight loss (21). Although such neuroendocrine adaptation surely protected us against the perils of famine in ancient times, it may hamper weight loss attempts in current times of plenty.

It seems important to emphasize, that the waveform-dependent deconvolution technique we employed requires a priori definition of TSH clearance. Therefore, we can not rule out the possibility that changes in plasma TSH clearance contribute to the decline of circulating TSH levels that we observed in response to weight loss.

In conclusion, the elevated diurnal TSH secretion rate in obese premenopausal women is blunted in response to body weight loss induced by long term caloric restriction, which is accompanied by a diminution of circulating triiodothyronine. The concomitant decline of circulating leptin levels or changes in central dopamine D2R neurotransmission may be among the regulatory cues involved in this neuroendocrine adaptation that potentially frustrates obese humans in their attempts to lose weight.

Tables and Figures

Table 1. Subject characteristics and fasting basal serum measurements before and after weight loss in obese premenopausal women

Parameter	Obese (N = 11)		P-value [a]
	Before Weight loss	After Weight loss	
Weight (kg)	92.7 ± 4.1	79.2 ± 3.2	< 0.01
BMI (kg/m^2)	33.1 ± 1.2	28.2 ± 0.8	< 0.01
WHR	0.85 ± 0.03	0.81 ± 0.02	0.02
Percentage Body Fat	41.2 ± 1.8	35.1 ± 1.3	< 0.01
Visceral Fat Mass (cm^2)	432 ± 8	258 ± 5	< 0.01
Subcutaneous Fat Mass (cm^2)	2659 ± 18	1961 ± 13	< 0.01
Estrogen (E2) (pmol/L)	203 ± 22	163 ± 25	< 0.01
Total Thyroxine (nmol/L)	110 ± 6	103 ± 5	0.10
Free Thyroxine (pmol/L)	15.1 ± 0.5	14.9 ± 0.5	0.50
Total Triiodothyronine (nmol/L)	1.83 ± 0.07	1.62 ± 0.09	0.01
Free Triiodothyronine (pmol/L)	4.3 ± 0.19	3.8 ± 0.14	0.04
Reverse Triiodothyronine (nmol/L)	0.33 ± 0.02	0.32 ± 0.02	0.40
Leptin (µg/L)	37.4 ± 6.7	19.7 ± 4.0	< 0.01

Data are presented as means ± SEM

a) P-values were determined by paired samples t-test, before vs. after weight loss obese women

Percentage body fat was estimated by bioelectrical impedance analysis and was calculated as a fraction of total body weight. Visceral and subcutaneous fat areas were determined using MRI.

Table 2. TSH concentration and secretion parameters

Parameter	Obese (N = 11)		P-value [a]	Controls (N = 11)
	Before Weight Loss	After Weight Loss		
Mean 24 h plasma concentration (mU/L)	1.9 ± 0.3 (1.7)	1.5 ± 0.3 (1.5)*	0.01	1.1 ± 0.1 (1.1)
Number of pulses (n/24 h)	8 ± 1 (7)	10 ± 1 (9)*	0.02	20 ± 1 (20)#
Pulse width (min)	116 ± 11 (114)	102 ± 12 (91)	0.16	49 ± 3 (49)#
Pulse amplitude (mU/V_{dl})	2.3 ± 0.3 (2.3)	1.8 ± 0.3 (1.7)*	0.05	1.3 ± 0.1 (1.2)
Pulse area (mU/V_{dl} x min)	69.5 ± 17.6 (54.8)	41.9 ± 8.2 (40.6)*	0.04	10.5 ± 1.4 (10.6)#
Nadir concentration (mU/V_{dl})	1.5 ± 0.2 (1.5)	1.3 ± 0.2 (1.2)	0.06	1.0 ± 0.1 (0.9)
Mean 24 h secretion (mU/V_{dl} x 24 h)	43.4 ± 6.4 (38.2)	34.4 ± 5.9 (33.8)*	0.02	26.1 ± 2.2 (24.7)

Data are presented as means ± SEM and between brackets the median is given

Concentration parameters were calculated from 24 h TSH concentration profiles using Cluster analysis. Mean TSH secretion was calculated from 24 h TSH concentration profiles using the Pulse algorithm, which is a waveform-independent deconvolution method. Secretion rates are calculated per liter distribution volume (V_{dl}).

a) P-values were determined by non-parametric Wilcoxon's signed-rank test

* $P < 0.05$ Before vs. after weight loss obese women

# $P < 0.05$ Obese women after weight loss vs. lean controls, determined by non-parametric Mann Whitney U- test

Figure 1.

Mean serum TSH concentration time series of the obese subjects before (-●-) and after weight loss (-⊙-) and mean serum TSH concentration time series of the historical control subjects (-○-). Data reflect sampling of blood every 10 min for 24 h. Sampling starts at 0900 h. Lights were switched off and subjects went to sleep at 2300 h until 0730 h next morning (vertical grey bar). Sleep was not interrupted.

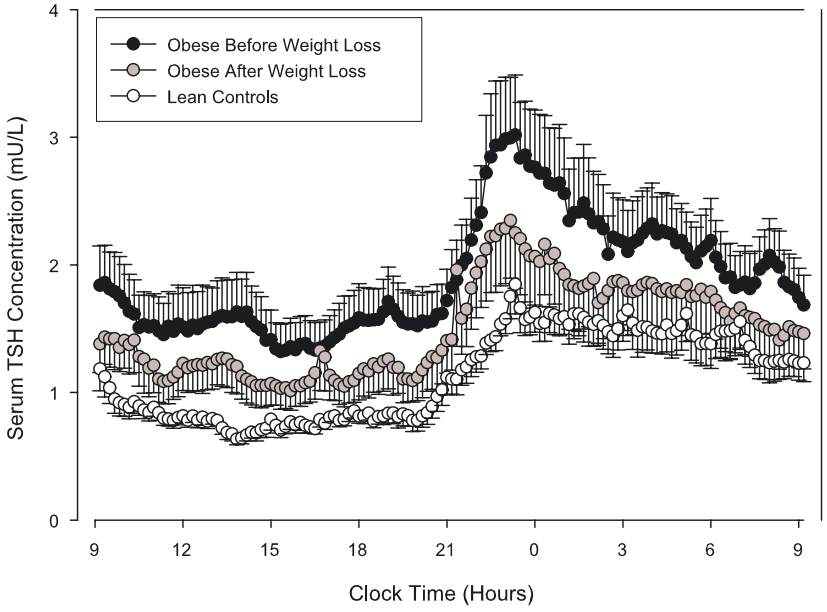
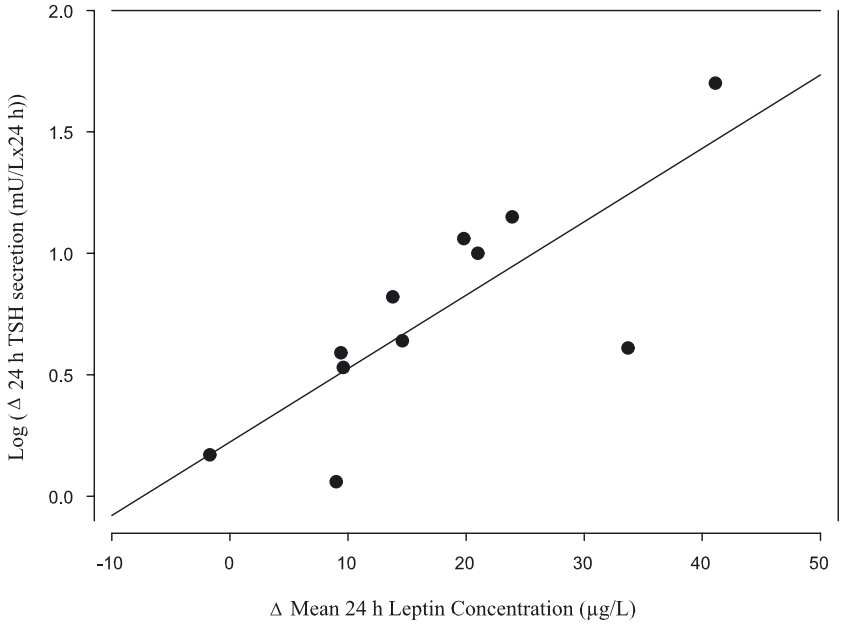


Figure 2.

Differences in TSH secretion were significantly positively related to differences in mean 24 h leptin concentrations ($R^2 = 0.62$, $P < 0.01$) before and after weight loss in obese women. Differences in TSH secretion were logarithmic transformed. The range of differences in TSH secretion was -4.0– 49.9 mU/Lx24 h. 24 h TSH secretion is calculated per litter distribution volume.



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Chapter 6

Acipimox Enhances Spontaneous Growth Hormone Secretion in Obese Women

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Abstract

We hypothesized that a high circulating FFA concentration is involved in the pathogenesis of hyposomatotropism associated with obesity. To evaluate this hypothesis, ten healthy premenopausal women (BMI 33.8 ± 1.0 kg/m^2) were studied in the follicular phase of their menstrual cycle at two occasions with a time interval of at least 8 weeks, where body weight remained stable. Subjects were randomly assigned to treatment with either Acipimox (an inhibitor of lipolysis, 250 mg orally four times daily) or placebo in a double blind cross-over design, starting one day prior to admission until the end of the blood sampling period. Blood samples were taken during 24 h with a sampling interval of 10 min for assessment of GH concentrations and GH secretion was estimated by deconvolution analysis. Identical methodology was used to study GH secretion in a historical control group of age-matched normal weight women. GH secretion, was clearly blunted in obese women (total daily release 66 ± 10 vs. lean controls: 201 ± 23 mU/V$_{dl}$/24 h, P = 0.005). Acipimox considerably enhanced total (113 ± 50 vs. 66 ± 10 mU/V$_{dl}$/24 h, P = 0.02) and pulsatile GH secretion (109 ± 49 vs. 62 ± 30 mU/V$_{dl}$/24 h, P = 0.02), but GH output remained lower compared to lean controls. Further analysis did not show any relationship between the effects of Acipimox on GH secretion and regional body fat distribution.

In conclusion, Acipimox unleashes spontaneous GH secretion in obese women. It specifically enhances GH secretory burst mass. This might mean that lowering of systemic FFA concentrations by Acipimox modulates neuroendocrine mechanisms that orchestrate the activity of the somatotropic ensemble.

Introduction

Spontaneous pulsatile GH release (36,49) and GH secretion in response to various provocative exogenous stimuli (1,7,14) are markedly blunted in obese patients. The mechanism underlying this neuroendocrine feature of obese humans remains elusive.

Obesity is associated with high circulating free fatty acid (FFA) concentrations (11,21) and FFA have been shown to suppress GH release in humans and animals (5,6,13,19,27,41). Thus, hyposomatotropism in obese individuals may be brought about by elevated plasma FFAs. Indeed, reduction of circulating FFA levels with Acipimox, a powerful anti-lipolytic drug, considerably enhances GH secretion in response to various secretagogues in obese humans (10,25,26,33,40). It remains to be established if Acipimox also unleashes spontaneous GH release in obese individuals.

Excess fat can be stored in various adipose depots. It appears that neuroendocrine alterations particularly occur in viscerally obese patients (28,36). Visceral fat is morphologically and functionally distinct from subcutaneous fat, in that cellularity and FFA turnover are higher per unit adipose tissue (22,29,42,47). Also, venous output of visceral fat drains directly into the portal system of the liver, while FFAs from subcutaneous fat enter the systemic circulation. FFA infusion into the portal vein enhances pituitary-adrenal axis and sympathetic nervous system activity, whereas systemic FFA infusion does not exert appreciable effects on these neuroendocrine ensembles (2,15). Thus, a high portal FFA flux, brought about by excess visceral fat, may particularly inhibit GH release.

We hypothesized that circulating FFAs are involved in the pathogenesis of hyposomatotropism in obese humans. Therefore, we measured 24 h spontaneous GH release in response to administration of Acipimox, a powerful inhibitor of lipolysis, in obese women. To further clarify the role of FFA released by visceral fat, we sought to determine the relationship between the effects of Acipimox and the size of various adipose depots.

Subjects and Methods

Subjects

Ten healthy, obese premenopausal women were enrolled in our study. Subjects were recruited taking body fat distribution into account. Conditions for participation were verified through medical screening, including medical history, physical examination, standard laboratory haematology, blood chemistry, urine and pregnancy tests and anthropometric measurements. A historical control group of lean women matched for age was included for comparison of GH secretion data with those in obese women. All obese subjects and the age-matched lean controls had an unremarkable medical history. Subjects were non-alcoholic, non-smoking and were not taking any medication, including hormonal contraception. All subjects gave written acknowledgement of informed consent.

Body composition

Total body fat mass (TBFM) was quantified on a separate day preceding the first study occasion using dual energy X-ray absorptiometry (DEXA)(3). Visceral and subcutaneous adipose tissue areas were assessed by MRI as described before (24), using a multi slice fast spin echo sequence (Gyroscan –T5 whole body scanner 0.5 Tesla, Philips Medical Systems, Best, The Netherlands). MRI images were analysed independently by two observers.

Drugs

Subjects were randomly assigned to 250 mg Acipimox or placebo in a double blind cross-over design by an independent investigator. Drug and placebo were taken four times daily (total 10 tablets) at 0700 h, 1300 h, 1900 h, 0100 h starting the day prior to admission until the end of the blood sampling period.

Diet

To limit confounding by nutritional factors, a dietician prescribed a eucaloric diet for each patient, taking basal energy requirement (calculated by the Harris-Benedict Formula) and physical activity into account. The macronutrient composition of the diet was exactly the same for each patient at both study occasions. The diet consisted of bread meals, prepared and supplied by the research center. Meals were served according to a fixed time schedule: breakfast at 0730 h, lunch at 1300 h and dinner at 1900 h and were consumed within limited time periods.

Clinical Protocol

The Medical Ethics Committee of the Leiden University Medical Center approved the protocol for both study groups. Apart from the fact that controls did not receive Acipimox treatment, the procedures and the clinical set-up of the experiments were exactly the same in obese subjects and controls. Subjects were admitted to the Clinical Research Unit of the Department of General Internal Medicine in the early follicular stage of their menstrual cycle at two separate occasions at 1600 h with an interval of at least eight weeks. A cannula for blood sampling was inserted into an ante cubital vein and blood samples for basal parameters were withdrawn. The cannula was attached to a 3-way stopcock and kept patent by a continuous saline infusion. Blood samples were taken with S-monovetten (Sarstedt, Etten-Leur, The Netherlands). Twenty four hour blood sampling started at 1800h and blood was collected at 10 minute intervals for determination of GH concentrations. Plasma FFA levels were measured every 6 hours. Plasma FFA concentrations were not measured in the historical control group. Subjects remained recumbent, except for bathroom visits. Lights were switched of at 2300 h. Vital signs were recorded at regular time intervals. The clinical set-up was the same during both occasions apart from the subject receiving the alternative treatment (Acipimox or placebo).

Assays

Blood sample handling and GH assays were performed using the same methodology in obese subjects and controls. Each tube, except the serum tubes, was immediately chilled on ice. Samples were centrifuged at 4000r/min at 4 °C during 20 minutes, within 60 min of sampling. Subsequently, plasma was divided into separate aliquots and frozen at -80 °C until assays were performed. GH concentrations were measured with a sensitive time-resolved fluoro immunoassay (Wallac, Turku, Finland) specific for the 22 kDa GH protein. The assay uses rhGH as standard (Genotropin, Pharmacia & Upjohn, Uppsala, Sweden), which is calibrated against WHO First International Reference Preparation (80-505). The limit of detection is 0.03 mU/L. Intra-assay coefficients of variation (CV) were 1.6-8.4% in the concentration range 0.26-47 mU/L, with corresponding inter-assay CV's of 2.0-9.9%.

The total serum IGF-I concentration was determined by RIA after extraction and purification on ODS-silica columns (Incstar Corp., Stillwater, MN). The inter assay CV was less than 11.8 %. The detection limit was 1.5 nmol/L. Age-related normative data were determined in the same laboratory. FFA levels were determined using a NEFA-C Free Fatty acid kit (Wako Chemicals GmbH, Neuss, Germany). The detection limit was 0.03 mMol/L and the inter- and intra-assay coefficients of variation were 1.1% and 2.6% respectively.

Calculations and statistics

Deconvolution Analysis

Multi parameter deconvolution analysis was used to determine kinetic and secretory parameters of 24 h spontaneous GH secretion, calculated from GH plasma concentrations. An initial guess of the secretion profile for waveform-independent estimates of GH secretion, was created with Pulse 2, an automated pulse detection program. Subsequent analysis with a waveform-dependent multi parameter deconvolution method was performed as described previously, using a first component half-life of 3.5 min, a second component half life of 20.8 min and a relative contribution of the slow component to the total elimination of 0.68 (50,51). This technique thus estimates the rate of basal release, the number and mass of randomly ordered secretory bursts and the subject-specific half-life. The daily pulsatile GH secretion is the product of secretory burst frequency and mean mass of GH released per event. Total GH secretion is the sum of basal and pulsatile secretion.

Approximate entropy

Approximate entropy (ApEn) is a scale and model independent statistic, applicable to a wide variety of physiological and clinical time-series data (16,38,39). ApEn quantities the orderliness or regularity of serial GH concentrations over 24 h. Normalized ApEn parameters of m = 1 (test range) and r = 20% (threshold) of the intra series SD were used, as described previously (37). Hence, this member of the ApEn family is designated ApEn (1, 20%). The ApEn metric evaluates the consistency of recurrent subordinate (non pulsatile) patterns in a time series, and thus yields information distinct from and complementary to cosinor and deconvolution (pulse) analyses (53). Higher absolute ApEn values denote greater relative randomness of hormone patterns. Data are presented as absolute ApEn values and normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series (54).

Statistics

TBFM is presented as a percentage of total body weight. Subcutaneous fat mass (SFM) and visceral fat mass (VFM) were expressed as a percentage of total fat mass. To determine the effect of Acipimox on daily GH secretion, numeric outcomes of deconvolution analysis and the ApEn metric were statistically analysed using one way ANOVA.

Differences between GH kinetic parameters between lean controls and obese women were analysed using Student's t-test for unpaired samples. Multiple regression analysis, using TBFM, VFM and SFM as independent variables, was done to determine specific correlations between measures of body fat distribution and GH secretory and kinetic parameters. All data are given as mean ± SEM and significance level was set at 0.05

Results

Subjects

Ten obese women (age 35.8 ± 2.0 yr, BMI 33.8 ± 1.0 kg/m^2) and 7 lean controls (age 35.1 ± 3.0 yr, BMI 21.5 ± 0.5 kg/m^2) were included. Body weight remained stable from 3 months before until the end of the study period.

Effect of Acipimox on spontaneous GH secretion

Mean 24 h plasma FFA levels were reduced during Acipimox treatment in all subjects (placebo 0.52 ± 0.04 vs. Acipimox 0.40 ± 0.03 mmol/L, P = 0.005). Under placebo conditions GH kinetic and secretory parameters were significantly lower in the obese subjects compared to the age-matched lean controls. Acipimox treatment significantly increased burst amplitude, burst mass, pulsatile and total daily GH production, while burst frequency, half-life, secretory half-duration and basal production were not significantly affected (Fig 1). However, Acipimox did not restore GH secretion to reference levels as determined in lean controls. Mean 24 h IGF-I levels were not affected by Acipimox (Table 1). An overview of GH secretory and kinetic parameters and reference values of GH secretory parameters in age matched premenopausal normal weight women, as determined in the control group, are given in Table 1. A graphical illustration of a representative 24 h GH concentration data set and corresponding secretory profile is shown in Fig 2.

Impact of body fat distribution

The obese subjects had a BMI of 33.8 ± 0.96 (range 31.0-39.4) kg/m^2, a WHR of 0.85 ± 0.01 (range 0.75-0.92) and their TBFM (% of total body weight) was 40.6 ± 1.1 (range 36.9-46.3) %. The seizes of their visceral and subcutaneous fat area were 392 ± 30 (range 274-539) cm^2 and 1348 ± 58 (range 1162-1709) cm^2 respectively.

Multiple regression analysis, including TBFM, VFM, and SFM as independent variables, showed no significant correlation (R^2 = 0.00, P = 0.48) between the size of visceral fat mass and the increase of total GH production during Acipimox treatment.

Approximate Entropy

ApEn ratios of plasma GH concentration time series were similar in obese and normal weight women. ApEn ratios were not affected by Acipimox (Table 1). Body fat distribution did not impact orderliness of the GH time series data either.

Discussion

Here we show that Acipimox unleashes spontaneous GH secretion in obese women. The drug particularly enhanced GH secretory burst mass, whereas burst frequency and basal GH secretion were largely unaffected. However, total daily GH production remained significantly lower than in normal weight controls. The distribution of excess fat over the various depots does not appear to impact the effect of Acipimox on GH secretion in obese individuals.

It has been repeatedly reported that the profound reduction of spontaneous GH secretion, that is invariably observed in obese humans, is primarily brought about by a diminution of secretory burst mass (20,52). The present data therefore suggest that Acipimox partially restores this primary neuroendocrine anomaly that underlies hyposomatotropism in obesity. GHRH input is a critical determinant of GH secretory burst mass, whereas other components of the somatotropic ensemble appear to control burst frequency and basal secretion (43). In vitro data show that incorporation of cis-unsaturated fatty acids into the plasma membrane of GH3 cells, disrupts signal transduction pathways that are pivotal for GHRH-induced GH release (34,35). Thus, Acipimox may enhance somatotroph sensitivity to GHRH feed forward inputs through lowering of circulating (cis-unsaturated) FFA and thereby specifically stimulate GH secretory burst mass, an inference that is in keeping with our observations.

A high portal FFA flux, released by excess visceral adipose tissue directly into the portal vein, could be responsible for the diminution of GH release in viscerally obese humans (3). In this scenario, one would expect Acipimox to exert its greatest effect on GH release in humans with large visceral fat stores. Our data are not in keeping with this postulate. However, the size of our study population was rather small, which considerably limits the statistical power to detect potential correlations in regression analyses.

Although Acipimox considerably enhanced GH secretion in obese subjects, it did not fully restore GH output to levels observed in normal weight controls. In this context, it seems prudent to emphasize that we used historical controls, which may limit the comparability of the data, although the clinical set-up, the applied assays and mathematical techniques for data analysis were identical in both groups.

Other physiological cues than FFA may also have affected GH release in our study. For example, insulin can blunt GH release (8,31). Acipimox can lower plasma insulin (48) and thereby enhance GH secretion. Also, high circulating insulin levels in our obese subjects might explain their persistently lower GH secretion rate with and without Acipimox treatment. Unfortunately, we did not measure insulin levels to further evaluate these possibilities. Another potential mechanistic explanation for the profound impact of Acipimox on GH secretion may relate to activation of dopaminergic neural circuits. Acipimox is a nicotinic acid derivative and nicotinic acid can activate dopaminergic neurons (9,17,18,23,30,46). Dopamine promotes (secretagogue-induced) GH release through activation of dopamine D2 receptors in rats and humans (4,12,32,44,45). Thus, Acipimox may also stimulate GH output through neural pathways.

In conclusion, the present data show that Acipimox acutely promotes spontaneous GH output in obese humans. It specifically enhances GH secretory burst mass, which might support the notion that Acipimox improves GHRH's ability to induce GH release by pituitary somatotrophs. The mechanism through which Acipimox exerts its effect on GH secretion in obese humans remains elusive.

Tables and Figures

Table 1. Effect of Acipimox on 24 h GH secretory Parameters in Obese Subjects

Treatment	Obese subjects (N = 10)			Controls (N = 7)
	Placebo	Acipimox	P-value [a)]	
Peak Frequency (number/24 h)	23 ± 2*	23 ± 2 *	0.97	16 ± 2
Half life (min)	16.5 ± 0.7	17.1 ± 0.6 *	0.42	14.7 ± 0.7
Secretory Half Duration (min)	19.7 ± 1.7*	22.1 ± 1.7	0.06	26.8 ± 1.9
Peak Amplitude (mU/V_{dl})	0.13 ± 0.02*	0.20 ± 0.02 *	0.03	0.46 ± 0.06
Burst Mass (mU/V_{dl}/peak)	2.7 ± 0.4*	4.7 ± 0.6 *	0.008	13.3 ± 2.1
Basal production (mU/V_{dl}/24 h)	3 ± 0.1*	5 ± 1.2 *	0.17	12 ± 2.3
Pulse Production (mU/V_{dl}/24 h)	62 ± 10*	109 ± 15 *	0.005	190 ± 23
Total Production (mU/V_{dl}/24 h)	66 ± 10*	113 ± 16 *	0.005	201 ± 23
ApEn 24 h GH concentration	0.43 ± 0.02	0.39 ± 0.03	0.41	0.39 ± 0.01
Mean 24 h IGF-1 (nmol/L)	17.9 ± 0.8	18.9 ± 1.2	0.27	ND

Data are presented as means ± SEM

ND = not determined

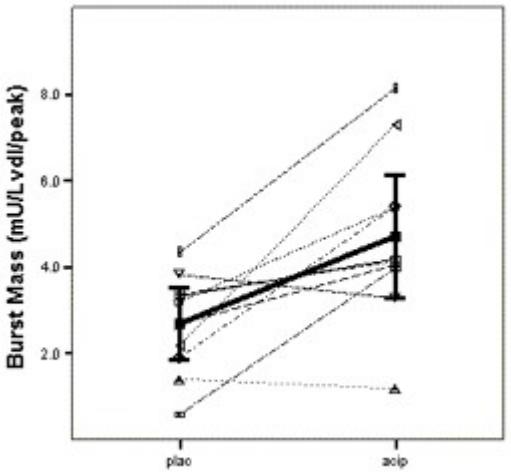
a) p-value placebo vs. Acipimox by one way ANOVA

* $p < 0.05$ vs. lean controls, using independent Student's t-test

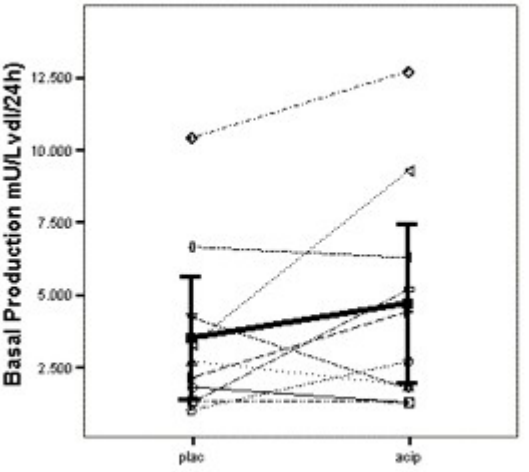
Figure 1.

Effect Acipimox on GH secretion parameters in obese subjects various parameters of spontaneous pulsatile GH release during placebo (plac) and Acipimox (acip) in obese women. P-values of the difference between means during Placebo vs. Acipimox treatment determined by one way ANOVA are presented.

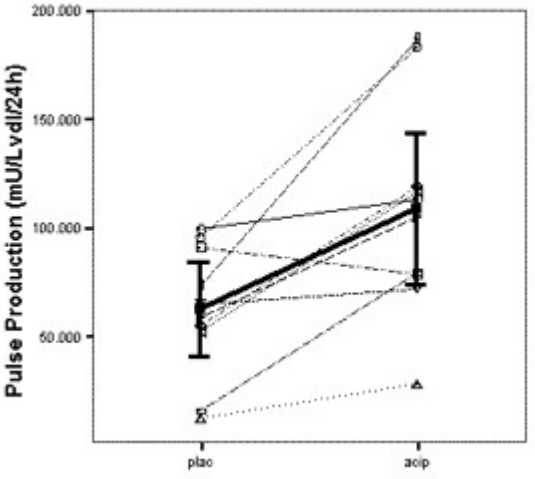
A) Burst Mass



B) Basal Production



C) Pulse Production



D) Total Production

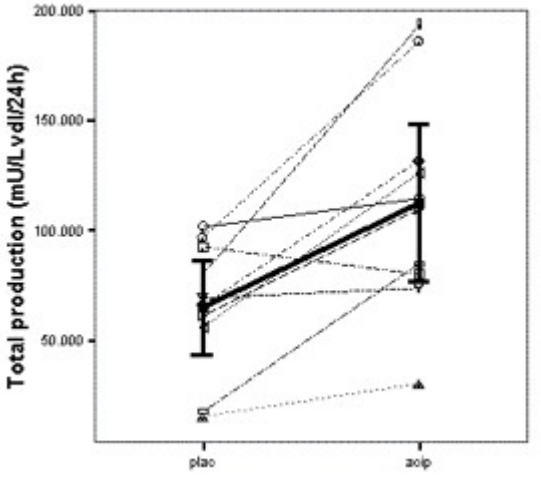
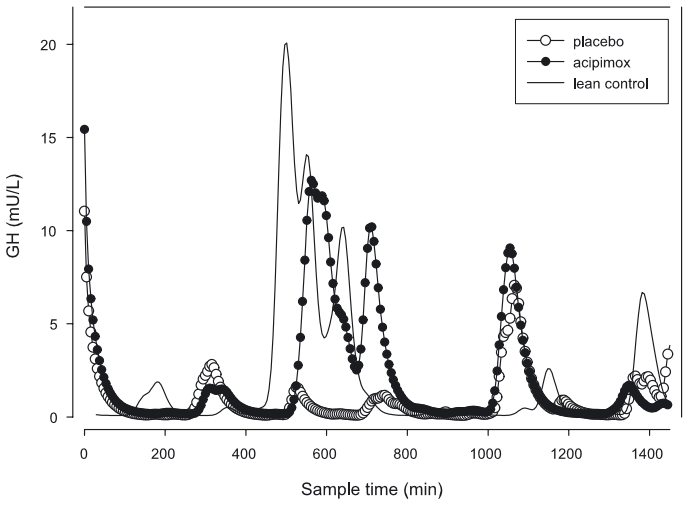


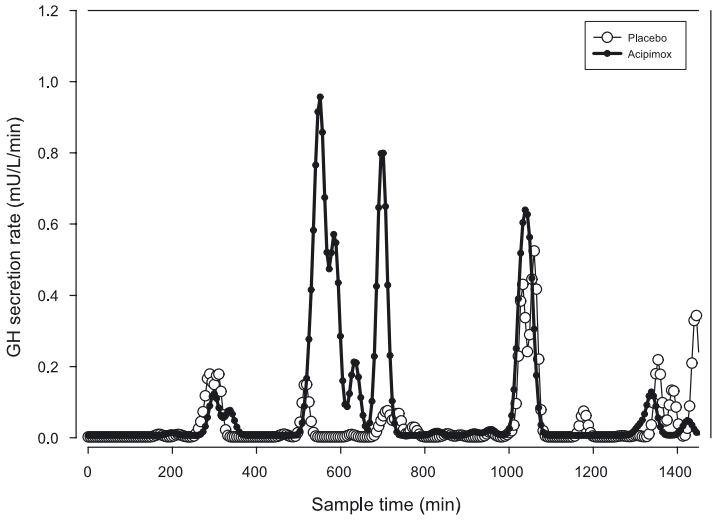
Figure 2.

24 h Plasma GH concentrations and corresponding secretion rates in 2 representative subjects.

A) Example of plasma GH concentration of one obese subject on placebo or Acipimox treatment and a lean control (age 36 resp. 34 yr)



B) Corresponding secretion rate profiles before and during Acipimox treatment in the obese woman



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Chapter 7

Enhanced Circadian ACTH Release in obese Premenopausal Women: Reversal by Short-term Acipimox Treatment

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Abstract

Several studies suggest that the hypothalamo-pituitary-adrenal (HPA) axis is exceedingly active in obese individuals. Experimental studies show that circulating free fatty acids (FFAs) promote the secretory activity of the HPA axis and human obesity is associated with high circulating FFAs. We hypothesized that HPA axis activity is enhanced and that lowering of circulating FFAs by Acipimox would reduce spontaneous secretion of the HPA hormonal ensemble in obese humans. To evaluate these hypotheses, diurnal ACTH and cortisol secretion was studied in 11 obese and 9 lean premenopausal women (BMI: obese 33.5 ± 0.9 vs. lean 21.2 ± 0.6 kg/m^2, P <0.001) in the early follicular stage of their menstrual cycle. Obese women were randomly assigned to treatment with either Acipimox (inhibitor of lipolysis, 250 mg orally four times daily) or placebo in a double blind cross-over design, starting one day prior to admission until the end of the blood-sampling period. Blood samples were taken during 24 h with a sampling interval of 10 min for assessment of plasma ACTH and cortisol concentrations. ACTH and cortisol secretion rates were estimated by multi parameter deconvolution analysis. Daily ACTH secretion was substantially higher in obese than in lean women (7950 ± 1212 vs. 2808 ± 329 ng/24 h, P = 0.002), whereas cortisol was not altered (obese 36 362 ± 5639 vs. lean 37 187 ± 4239 nmol/24 h, P = 0.912). Acipimox significantly reduced ACTH secretion in the obese subjects (Acipimox 5850 ± 769 ng/24 h, P = 0.039 vs. placebo), while cortisol release did not change (Acipimox 33 542 ± 3436 nmol/24 h, P = 0.484 vs. placebo). In conclusion, spontaneous ACTH secretion is enhanced in obese premenopausal women, whereas cortisol production is normal. Reduction of circulating FFA concentrations by Acipimox blunts ACTH release in obese women, which suggests that FFA's are involved in the pathophysiology of this neuroendocrine anomaly.

Introduction

The endocrine environment is a powerful regulator of body fat storage. For example, the hypothalamic-pituitary-adrenal (HPA) ensemble profoundly affects body composition in animals and humans. Glucocorticoid administration promotes body weight gain in rodents (19;26;77) and hyper cortisolism in patients with Cushing's syndrome leads to excess fat in visceral depots, which is readily reversed by lowering plasma cortisol levels (45;70).

Obese animal models are marked by an exceedingly active HPA ensemble. Genetically obese rodents have high levels of glucocorticoids (5;6), adrenalectomy reduces body weight in these animals (12;21) and subsequent corticosterone replacement restores the obese state (12;22;32;61;76). Adrenalectomy also attenuates diet-induced obesity. Removal of the adrenals reduces energy intake and adipose tissue weights in diet-induced obese rodents, which is reversed by glucocorticoid replacement (18;36;48;62).

Various clinical studies suggest that the HPA axis is also hyperactive in human obesity. Both plasma ACTH and cortisol concentrations rise to higher levels in response to Corticotropin Releasing Hormone (CRH) administration alone or in combination with arginine vasopressin (AVP) in obese humans compared to normal weight controls (51;54;69). Moreover, the cortisol response to ACTH is exaggerated in obese volunteers (29;49;53) and it has been reported that stress induced cortisol secretion is increased in abdominally obese women (20). Furthermore, urinary free cortisol excretion appears to be elevated in abdominally obese humans (49;53), while suppression of plasma cortisol levels by dexamethasone (43;59) or

hydrocortisone (35) is blunted. The cause of these endocrine perturbations remains elusive.
Considerable evidence obtained in experimental studies in rats shows that circulating free fatty acids (FFAs) are involved in the control of the HPA axis. Elevation of systemic or portal plasma FFA levels by intravenous lipid infusions enhances ACTH and cortisol secretion in rats (4;74). Moreover, prolonged high fat feeding raises circulating FFA levels and basal ACTH and cortisol concentrations in rodents (63). Circulating FFA concentrations are high in obese humans (15;34).
Acipimox is a powerful inhibitor of lipolysis. Its anti-lipolytic action is probably mediated through suppression of intracellular cyclic AMP levels, which inhibits cyclic AMP-dependent protein kinase activity. This precludes proper association of hormone-sensitive lipase with triacylglycerol substrate in the lipid droplet of adipocytes, thereby hampering lipolysis and lowering circulating free fatty acids (13).
We hypothesized that the spontaneous secretory activity of the HPA axis is elevated and that lowering of circulating FFAs by Acipimox would reduce HPA axis activity in obese humans.
To test these postulates, we measured 24 h spontaneous ACTH and cortisol release in lean and obese premenopausal women in the early follicular phase of their menstrual cycle. Obese women were studied twice, randomly assigned to short-term treatment with either Acipimox (250 mg orally four times daily) or placebo in a double blind crossover design.

Subjects and methods

Subjects

Eleven healthy obese premenopausal women (BMI > 30 kg/m^2) and 9 lean (BMI < 25 kg/m^2) controls with similar age and sex were recruited. All subjects enrolled in our study underwent medical screening, including medical history taking, physical examination, standard laboratory haematology, blood chemistry and urine tests. Acute or chronic disease, smoking, alcohol abuse and use of medication were exclusion criteria. All participants were required to have regular menstrual cycles and did not use oral contraceptives. All subjects gave written acknowledgement of informed consent for participation.

Body fat distribution

The obese subjects were recruited so as to vary widely with respect to girth, while their BMI was required to fall within a relatively narrow range to be able to specifically judge the effect of regional body fat distribution on hormone release. The total amount and location of excess body fat was determined in the obese women only. Percentage total body fat mass (fraction of total body weight) was quantified using dual energy X-ray absorptiometry (DEXA, Hologic QDR4500)(7). Visceral and subcutaneous adipose tissue areas were assessed by MRI as described before (41), using a multi slice fast spin echo sequence (Gyroscan –T5 whole body scanner 0.5 Tesla, Philips Medical Systems, Best, The Netherlands). MRI images were analysed by two observers independently.

Drugs

The obese subjects were randomly assigned to 250 mg Acipimox or placebo in a double blind crossover design by an independent investigator. Drug and placebo were taken four times daily (total 10 tablets) at 0700 h, 1300 h, 1900 h, and 0100 h starting the day prior to admission until the end of the blood-sampling period.

Diet

To limit nutritional confounding, a dietician prescribed a personal eucaloric diet for each obese woman, taking basal energy requirements (calculated by the Harris-Benedict Formula) and physical activity into account. The macronutrient composition of the diet was exactly the same for each obese woman at both study occasions. The diet consisted of bread meals, prepared and supplied by the research center. Meals were served according to a fixed time schedule (breakfast at 0730 h, lunch at 1300 h, and dinner at 1900 h) and were consumed within limited time periods. Lean women received a standardized eucaloric diet as well. No dietary restrictions were imposed on the obese women just before or between both study occasions.

Clinical Protocol

The protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center. All subjects were admitted at 1600 h to the Clinical Research Unit of the Department of General Internal Medicine in the early follicular stage of their menstrual cycle. Obese subjects were studied at two separate occasions with an interval of at least eight weeks and apart from the subject receiving Acipimox or placebo treatment, the clinical set-up was exactly the same during both study occasions. Identical methodology was used to study HPA hormonal secretion in obese and normal weight women. A cannula for blood sampling was inserted into an antecubital vein. The cannula was attached to a 3-way stopcock and kept patent by a continuous saline infusion. Blood samples were taken with S-monovetten (Sarstedt, Etten-Leur, The Netherlands). One hour after admission 24 h blood sampling started. 1.2 ml Blood was collected at 10-minute intervals for determination of plasma ACTH and cortisol concentrations. Blood samples (1.2 ml) for the measurement of plasma FFA levels were taken every 6 hours in the obese subjects only. The total amount of blood withdrawn for the measurement of ACTH, cortisol and FFA levels during each occasion was 187.5 ml. All subjects remained recumbent during the blood-sampling period, except for bathroom visits. Meals were served according to a fixed time schedule. Lights were switched off at 2300 h and subjects were not disturbed by withdrawal of blood samples during their sleep (sleep monitoring by EEG was not performed). Subjects were awakened at 0100 h for drug intake. Vital signs were recorded at regular time intervals.

Assays

Blood sample handling

Each tube, except the serum tubes (because of blood clotting), was immediately chilled on ice. All samples were centrifuged at 4000r/min at 4 °C during 20 minutes, within 60 min of sampling. Subsequently, plasma/serum was divided into separate aliquots and frozen at -80 °C until assays were performed. Plasma ACTH concentrations were measured by immunoradiometric assay with a detection limit of 3 ng/L (Nichols Institute Diagnostics, San Juan Capistrano, California, USA). The intra-assay coefficient of variation ranged from 2.8-7.5%. The ACTH IRMA was calibrated against the standard obtained from the National Pituitary Agency (University of Maryland School of Medicine) and the National Institute of Arthritis, Metabolism and Digestive Disease. Plasma cortisol concentrations were measured by Radioimmunoassay (RIA) with a detection limit of 25 nmol/L (DiaSorin, Stillwater, Minnesota, USA). The intra-assay coefficient of variation ranged from 2.0-4.0%. The cortisol RIA was calibrated against the U. S. P. Cortisol Reference standard.

FFA levels were determined using a NEFA-C Free Fatty acid kit (Wako Chemicals GmbH, Neuss, Germany). The detection limit was 30 µmol/L and the inter- and intra-assay coefficients of variation were 1.1% and 2.6% respectively. Basal estradiol concentrations were determined by RIA (Diagnostic Systems Laboratory, Webster, TX). The detection limit was 10 pmol/L and the inter- and intra-assay coefficients of variation were 6.8% and 15.8% respectively.

Calculations and statistics

Deconvolution Analysis

Multi parameter deconvolution analysis was used to estimate various kinetic and secretory parameters of spontaneous 24 h ACTH and cortisol plasma concentration time series data. Initial waveform- independent assessments of ACTH and cortisol secretion, were created with Pulse 2, an automated pulse detection program. Subsequent analysis with a waveform-dependent multi parameter deconvolution method was performed as described previously, using a first component half-life of 3.5 min, second component half life of 14 min and relative contribution of the slow component to the total elimination of 0.67 for ACTH and a first component half-life of 3.8 min, second component half life of 66 min and relative contribution of the slow component to the total elimination of 0.67 for cortisol (66). This technique thus estimates the rate of basal release, the number and mass of randomly ordered secretory bursts and the subject-specific half-life. The daily pulsatile secretion is the product of secretory burst frequency and mean secretory burst mass. Total secretion is the sum of basal and pulsatile secretion. Results were expressed per liter distribution volume. For the calculation of production rates per liter,

ACTH distribution volumes was estimated to amount to 40 ml/kg (65) and the distribution volume of cortisol was estimated to be 5.3 L/body surface area (m^2), which was calculated using the Dubois formula (37;71). The relationship between plasma ACTH and cortisol concentrations was determined by cross-correlation analysis.

Approximate Entropy

Approximate Entropy (ApEn) is a scale and model independent statistic that
assigns a non-negative number to time series data, reflecting regularity of these data (56). We used normalized ApEn parameters of m = 1, r = 20% and 1000 for the amount of runs, to test for regularity in 24 h plasma ACTH and cortisol concentration time series. Hence, this member of the ApEn family is designated ApEn (1, 20%). The ApEn metric evaluates the consistency of recurrent subordinate (non pulsatile) patterns in a time series, and thus yields information distinct from and complementary to deconvolution (pulse) analyses (67). Higher absolute ApEn values denote greater relative randomness of hormone patterns. Data are presented as normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series. Cross-ApEn was used to investigate joint regularity of the hormone pairs ACTH-cortisol (55).

Statistical analysis

Means of cortisol and ACTH secretion parameters of lean and obese volunteers were compared using independent Student's t-test. The means of ACTH and cortisol secretion parameters in obese subjects during Acipimox vs. placebo treatment were compared using Student's t-test for paired samples. Regression analysis was used to determine the correlation between BMI and daily ACTH and cortisol secretion in obese and normal weight women. Stepwise multiple regression analysis, including percentage body fat, subcutaneous fat area and visceral fat area as independent variables, was used to determine the relationship between the size of various fat depots and diurnal ACTH and cortisol production. The same technique was employed to determine the effect of Acipimox on ACTH and cortisol secretion in the obese subjects in relation to body fat distribution. Significance level was set at 0.05. Data are presented as mean ± SEM, unless otherwise specified.

Results

Subjects

Eleven obese and 9 lean subjects were enrolled in this study. Mean age of both groups was similar (obese 36.6 ± 1.9 vs. lean 36.4 ± 2.0 yr., P = 0.971) while BMI was significantly different (obese 33.5 ± 0.9 vs. lean 21.2 ± 0.6 kg/m^2, P < 0.001). All subjects were studied in the follicular phase of their menstrual cycle and basal estradiol (E2) levels in plasma were similar in both groups (obese 190 ± 31 vs. lean 208 ± 66 pmol/L, P = 0.795). Body weight of the obese subjects remained stable from 3 months before until the end of the study period.

Features of Spontaneous 24 h ACTH and cortisol secretion in lean and obese women

Total ACTH production was clearly higher in the obese subjects. In particular, pulsatile production, burst frequency and burst mass were enhanced, while basal secretion, half-life and secretory half-duration were not significantly different. Cortisol kinetic parameters were similar in the obese subjects compared to age-matched lean controls, except half-life, which was slightly prolonged in the obese women. A graphical illustration of representative ACTH and cortisol concentration profiles and corresponding secretion profiles of one obese and one lean woman of similar age are presented in Figure 1. Data of ACTH kinetic parameters of the obese and lean subjects are presented in Table 1 and Figure 2. An overview of cortisol kinetics, as estimated by deconvolution analysis, is given in Table 2.

Effect Acipimox on spontaneous 24 h ACTH and cortisol secretion parameters in obese women

Mean 24 h plasma FFA levels were reduced during Acipimox treatment in all subjects (Placebo 0.52 ± 0.04 vs. Acipimox 0.40 ± 0.03 mmol/L, P = 0.005). Total ACTH production was significantly lower in the obese subjects during Acipimox treatment (Figure 2). In particular, peak frequency and pulsatile production were reduced, while basal secretion, half-life

and secretory half-duration were not affected. Data of ACTH secretory and kinetic parameters during Acipimox and placebo treatment are presented in Table 1.
Acipimox did not affect cortisol kinetic and secretory parameters in the obese women. An overview of cortisol kinetics, as estimated by deconvolution analysis, during Acipimox and placebo treatment is shown in Table 2.

Regularity of plasma ACTH and cortisol concentration- time series
ApEn ratios of plasma ACTH concentration time series were significantly higher in obese women compared with controls (0.56 ± 0.03 vs. 0.45 ± 0.04 resp., P = 0.033), whereas the regularity of the 24 h cortisol concentration time series was similar in both groups (0.51 ± 0.03 vs. 0.52 ± 0.02 respectively, P = 0.738)(Figure 3). Cross-ApEn statistics showed that joint regularity of ACTH-cortisol hormone pairs was not significantly different between both groups (cross-ApEn ratio = 0.55 ± 0.03 vs. 0.49 ± 0.02 P = 0.143).
Acipimox did not impact the orderliness of plasma ACTH concentration time series of the obese women (ApEn ratios placebo 0.56 ± 0.03 vs. Acipimox 0.66 ± 0.05, P = 0.092), whereas the regularity of 24 h cortisol concentration time series was significantly less regular during Acipimox treatment (Placebo 0.51 ± 0.03 vs. Acipimox 0.56 ± 0.03, P = 0.009) (Figure 3). Cross-ApEn statistics showed that the joint regularity of ACTH-cortisol hormones pairs was lower during Acipimox treatment (Placebo vs. Acipimox: cross-ApEn ratio = 0.55 ± 0.03 vs. 0.63 ± 0.04, P = 0.029).

Correlation between ACTH and cortisol concentration- time series
Cross-correlation analysis revealed a high correlation between ACTH and cortisol concentration values, which was significantly higher in the obese women (Obese: R = 0.85 ± 0.02 vs. Lean: R = 0.77 ± 0.02, P = 0.016). ACTH was leading cortisol with a time lag of 10 minutes in both groups. Cross-correlation between ACTH and cortisol hormone pairs in the obese subjects was not significantly altered after Acipimox treatment (Placebo 0.85 ± 0.02 vs. Acipimox 0.74 ± 0.06, P = 0.065). ACTH was leading cortisol with a similar time lag during both treatments (Placebo 10 ± 2 vs. Acipimox 30 ± 24 min, P = 0.426).

BMI vs. daily ACTH and cortisol secretion in lean and obese women
Both obese and lean subjects (N = 21) were included in the correlation analysis of BMI (range 18.3-39.4 kg/m^2) vs. daily ACTH and cortisol production. A highly significant positive correlation was found for BMI vs. total ACTH production (R^2 = 0.39, P = 0.003, Figure 4). Also, BMI was positively related to peak frequency (R^2 = 0.39, P = 0.003) and peak burst mass (R^2 = 0.26, P = 0.022). Total cortisol secretion parameters were not related to BMI (Total cortisol vs. BMI: R^2 = 0.04, P = 0.413).

Body fat distribution vs. daily ACTH and cortisol secretion and the effect of Acipimox in the obese women
The obese subjects had a mean BMI of 33.5 (30.3-39.4) kg/m^2. The Mean of their percentage total body fat mass (% of total body weight) was 40.7 (36.9-46.3) %. Mean sizes of their visceral and subcutaneous fat area were 392 (274-539) cm^2 and 1326 (1106-1709) cm^2 respectively. Multiple regression analysis, with percentage total body fat mass, sizes of visceral and subcutaneous fat areas as independent variables, revealed that there was no significant correlation between any of these specific body composition parameters and the total daily ACTH production, 24 h cortisol production or the decrease of total daily ACTH production during Acipimox treatment. Additionally, regression analysis revealed that the reduction of FFA levels was not related tot the reduction of ACTH secretion after Acipimox treatment in the obese women (delta FFA (mmol/L) vs. delta ACTH (ng/24 h): R^2 = 0.05, P = 0.550).

Discussion
This study delineates differences of spontaneous diurnal ACTH and cortisol secretion in obese and lean premenopausal women and evaluates the effects of Acipimox, a powerful inhibitor of lipolysis, on the HPA hormonal ensemble in obese individuals.

The data show that daily ACTH secretion rates are substantially higher, while the ACTH release process is less regular (as evidenced by ApEn statistics) in obese than in lean women. Moreover, ACTH release rates correlate strongly with BMI, whereas the sizes of various fat areas (including visceral and subcutaneous fat depots) do not appear to be independently associated with ACTH production. The high ACTH secretion rate in obese subjects results from augmented peak frequency and secretory burst mass rather than enhanced basal secretion. Short-term treatment with Acipimox apparently restores these kinetic anomalies (except release process randomness) to a large extent, which suggests that circulating FFA concentrations may be involved in the pathophysiology. In contrast, cortisol production is not different in obese and lean premenopausal women and Acipimox does not significantly affect the secretory dynamics of this hormone (except for a slight increase in secretory process randomness).

To our knowledge, this is the first study to estimate the secretion rates of pituitary-adrenal hormones in obese vs. lean humans by deconvolution analysis. A few previous papers reported that diurnal plasma ACTH concentrations are higher in obese individuals, while circulating cortisol levels are similar to those in lean controls (44;52), which is in line with the results of the present study. Moreover, various other clinical studies showed that the incremental ACTH peak response to different exogenous stimuli is elevated in obese humans, which also corroborates our data (51;54;69).

The fact that ACTH release in obese women was blunted during Acipimox treatment is in keeping with data from experimental studies, showing that elevation of circulating FFA by intra lipid infusion raises plasma levels of ACTH (and corticosterone) (73;74). It has been suggested that the acute stimulatory effect of FFA infusion on blood pressure in rodents is mediated by afferent vagal inputs modulating central -adrenergic receptors (27). The hypothalamic paraventricular nucleus contains a high density of both 1 and 2 adrenoreceptors and there is considerable evidence that these receptors are involved in facilitating the secretion of CRH/AVP into the hypophyseal portal system, which ultimately leads to stimulation of ACTH secretion (2;3). Therefore, FFA-induced vagal inputs into PVN neurons may partake in the control of HPA activity. Alternatively, fatty acids are taken up by the brain (58) and exert direct effects on the electrical properties of neurons (46). Application of fatty acids into the ventromedial hypothalamus (VMH) inhibits neuronal firing in that area (63) and the VHM in its turn down regulates pituitary adrenal activity (16). Thus, FFA may directly reduce feedback restraint of the VMH on HPA activity at the hypothalamic level, ultimately leading to enhanced ACTH secretion by the pituitary gland. Collectively, these data suggest that FFA enhance HPA output through effects on neuronal control systems in brain centres at the supra pituitary level and that circulating FFA are involved in the pathophysiology of pituitary-adrenal hyperactivity in obese humans.

This inference is in apparent conflict with the results of a recent study, showing that elevation of circulating FFA through intravenous infusion of a lipid/heparin solution reduces plasma ACTH and cortisol levels in (normal weight) women (40). However, as the authors state in their discussion, the physiological relevance of their findings might be limited, because FFA plasma concentrations induced by intralipid infusion were 5-10 fold higher than those usually found in (obese) humans. Also, as various types of fatty acids (i.e. long-chain/short-chain, saturated/unsaturated) may have differential impact on neuronal membrane function (75), it seems unlikely that exogenous and endogenous lipids exert similar effects on the brain. Thus, although valuable in itself, the data reported by Lanfranco (40) do not necessarily argue against the position that elevation of circulating FFA is involved in the pathophysiology of ACTH hypersecretion in obese humans.

Interestingly, there was no correlation between the reduction of FFA levels and the decrease of ACTH production after Acipimox treatment in the obese subjects. Therefore, it is conceivable that Acipimox impacts ACTH release directly, through mechanistic pathways independent of its effect on plasma FFA levels. Acipimox is a nicotinic acid derivative, which can bind to nicotinic acid receptors. Activation of various subtypes of nicotinic acid receptors modulates neuronal activity in a variety of different regions in the central nervous system (17;25). To our knowledge, it is unknown if neural circuits involved in the control of pituitary-adrenal activity contain nicotinic acid receptors. Also, it is unclear if Acipimox can cross the blood-brain-barrier. However, we cannot exclude that Acipimox affects ACTH secretion directly at the level of the brain.

In the present study ApEn values of ACTH secretion data were significantly higher in the obese subjects compared to normal weight controls. Higher ApEn values denote greater irregularity (or higher process randomness). Regularity of hormonal secretion patterns mirrors the net result of feed forward signalling and feedback restraint (68). Since it seems unlikely that negative feedback restraint by cortisol per se can explain the enhanced ACTH secretion of the HPA axis in the present study, because daily cortisol secretion was not altered, the feed forward drive activating the HPA axis may be increased in obese

humans. CRH is one of the most potent hypothalamic secretagogues stimulating pituitary ACTH release. Thus, elevated CRH might lead to increased randomness of ACTH release. Indeed, it has been demonstrated that CRH levels are elevated in hypothalamic areas and neurons involved in the regulation of HPA axis activity in the brain of obese rodents compared to their wild-type counterparts (5;6). There is experimental evidence that leptin receptors are abundant in these CRH-containing neurons in the paraventricular nuclei of the rat brain (28) and intracerebroventricular (icv) leptin administration in animals enhances hypothalamic CRH content (23;33;60;64). Human obesity is marked by elevated plasma leptin concentrations (14;47), which may promote hypothalamic CRH release and thereby enhance ACTH release process irregularity. This might also explain the fact that circulating ACTH is only partially lowered by Acipimox in obese women.

As alluded to earlier, our finding that diurnal plasma cortisol levels are normal in obese women in the face of increased ACTH concentrations, corroborates other clinical studies (44), but remains unexplained. Although some papers report that urinary cortisol excretion is increased in (abdominal) obesity (implying that adrenal cortisol production is enhanced in the presence of normal circulating levels) (50;53) and others suggest that 5- reductase activity (which converts cortisol to inactive cortisone) is increased in obese humans (72), plasma half-life of cortisol was slightly (but significantly) longer in our obese subjects, which obviously does not support the notion that obesity is associated with enhanced cortisol clearance. Thus, the currently available clinical data suggest that the dynamics of the ACTH and cortisol ensemble are altered in obese humans, in the sense that cortisol release appears to be somewhat diminished in proportion to circulating ACTH. (In fact, even in absolute terms, cortisol production was slightly lower in our obese vs. normal weight women, although the difference was not significant. Unfortunately, due to the relatively small group-size, this study lacks the statistical power to significantly detect a 10% reduction of cortisol production, which could be physiologically relevant.) Various mechanistic explanations for this phenomenon were proposed, including insensitive adrenals (44) and reduced 21-hydroxylase activity (which would direct cortisol precursors towards androgen synthesis)(29). Alternatively, enhanced sympathetic neuronal inputs into the adrenocortical cells may be involved. Evidence from experimental animal studies suggests that the sensitivity of the adrenal cortex to ACTH is centrally regulated by the suprachiasmatic nuclei via the autonomic nervous system (11). Sympathetic inputs particularly desensitise adrenocortical cells to ACTH action. Since obesity appears to be associated with increased sympathetic activity (31), this might explain the occurrence of relatively low cortisol levels in face of elevated ACTH in the obese women enrolled in the present study. As a second alternative, it has also been described that leptin directly inhibits cortisol release directly at the adrenal gland (57). Leptin receptor expression was demonstrated in rat and human adrenal tissue and exposure of primary cultured rat and human adrenal cells to leptin led to a dose dependent decrease of ACTH stimulated adrenocortico-steroid secretion, whereas no effect was found in adrenal cells obtained from db/db mice, which lack a functional leptin receptor (9;24;57). It has been reported that there is a strict reciprocal diurnal relation between leptin and cortisol levels in both rats and humans (8;42). Also, reduced leptin levels are associated with enhanced cortisol secretion rates in narcoleptic humans (38;39). Thus, leptin mediated peripheral inhibition of adrenal glucocorticoid production appears to be another possible mechanistic explanation for the relatively low cortisol secretion in the obese women. Conclusive evidence to support either one of these postulates has not been reported to date.

The mere fact that plasma cortisol levels are normal in obese humans may limit the (patho) physiological meaning of the current findings, as cortisol is considered to be the main messenger conveying HPA signals to target tissues. In this context, it is important to keep in mind that adipocytes express ACTH receptors and that ACTH is a powerful lipolytic hormone, at least in some species (10). Therefore, a high circulating ACTH concentration in itself may promote lipolysis in obese subjects. Also, melanocortin receptors are distributed widely throughout the body, which suggest that these peptides partake in the control of a variety of (partly unknown) physiological functions (1). Thus, the exact implications of high plasma ACTH concentrations in the face of normal cortisol levels remain to be established. Furthermore, given the well known gender and age effects on HPA activity (30), one has to take into account that these results are not necessarily applicable to men or post-menopausal women.

In conclusion, this study documents enhanced circadian ACTH release in obese premenopausal women in the face of normal circulating cortisol concentrations. Reduction of plasma FFA levels by Acipimox blunts ACTH secretion in obese individuals, which suggests that circulating FFA are involved in the pathophysiology of this neuroendocrine perturbation associated with obesity.

Tables and Figures

Table 1. 24 h ACTH secretory parameters

	Controls (N = 9)	Obese subjects (N = 11)		P-value [a]	P-value [b]
		Placebo	Acipimox		
Peak Frequency (number/24 h)	23 ± 2	32 ± 1 *	28 ± 2	0.001	0.054
Half-life (min)	16 ± 1	14 ± 1	16 ± 1	0.116	0.229
Secretory Half Duration (min)	20 ± 2	23 ± 2	18 ± 3	0.267	0.258
Peak Amplitude (ng/V_{dl})	1.3 ± 0.2	1.7 ± 0.1	2.0 ± 0.4	0.103	0.456
Burst Mass (ng/V_{dl}/peak)	27.9 ± 4.2	40.8 ± 4.7	32.9 ± 4.5	0.059	0.191
Basal Production (ng/V_{dl}/24 h)	533 ± 62	679 ± 170	603 ± 108	0.467	0.307
Pulse Production (ng/V_{dl}/24 h)	606 ± 89	1320 ± 181 *	878 ± 103	0.004	0.051
Total Production (ng/V_{dl}/24 h)	1139 ± 105	2000 ± 289 *	1481 ± 193†	0.019	0.043
Total Production (ng/24 h)[c]	2808 ± 329	7950 ± 1212 *	5850 ± 769†	0.002	0.039
ApEn	0.45 ± 0.04	0.56 ± 0.03	0.66 ± 0.05	0.033	0.092

Multi parameter deconvolution analysis was used to estimate various kinetic and secretory parameters of spontaneous 24 h ACTH concentration time series data. Data are presented as means ± SEM.

a) P-value obese vs. lean, statistical analysis was performed by independent Student's t-test b) P-value placebo vs. Acipimox, statistical analysis was performed by paired samples t-test c) Distribution Volume = 40 ml/kg (Ref.(65)

* $P < 0.05$ Obese vs. lean subjects

† $P < 0.05$ Placebo vs. Acipimox obese subjects

Table 2. 24 h Cortisol secretory parameters

	Controls (N = 9)	Obese subjects (N = 11)		P-value [a]	P-value [b]
		Placebo	Acipimox		
Peak Frequency (number/24 h)	24 ± 1	21 ± 1	20 ± 1	0.126	0.148
Half-life (min)	61 ± 2	73 ± 5 *	73 ± 5	0.045	0.995
Secretory Half Duration (min)	12 ± 1	14 ± 3	11 ± 2	0.668	0.337
Peak Amplitude (nmol/V_{dl})	13.5 ± 0.9	13.0 ± 1.6	24.3 ± 8.5	0.811	0.191
Burst Mass (nmol/V_{dl}/peak)	172 ± 16	154 ± 22	152 ± 13.4	0.550	0.899
Total Production (nmol/V_{dl}/24 h)	4134 ± 369	3305 ± 579	3027 ± 351	0.267	0.453
Total Production (nmol/24 h) [c]	37 186 ± 4239	36 362 ± 5639	33 542 ± 3436	0.912	0.484
ApEn	0.52 ± 0.02	0.51 ± 0.03	0.56 ± 0.03†	0.738	0.009

Multi parameter deconvolution analysis was used to estimate various kinetic and secretory parameters of spontaneous 24 h cortisol plasma concentration time series data. Data are presented as means ± SEM.

a) P-value obese vs. lean, statistical analysis was performed by independent Student's t-test

b) P-value placebo vs. Acipimox, statistical analysis was performed by paired samples t-test

c) Distribution Volume = 5.3L/BSA (m^2), BSA was calculated by the Dubois formula (Ref. (37;71)

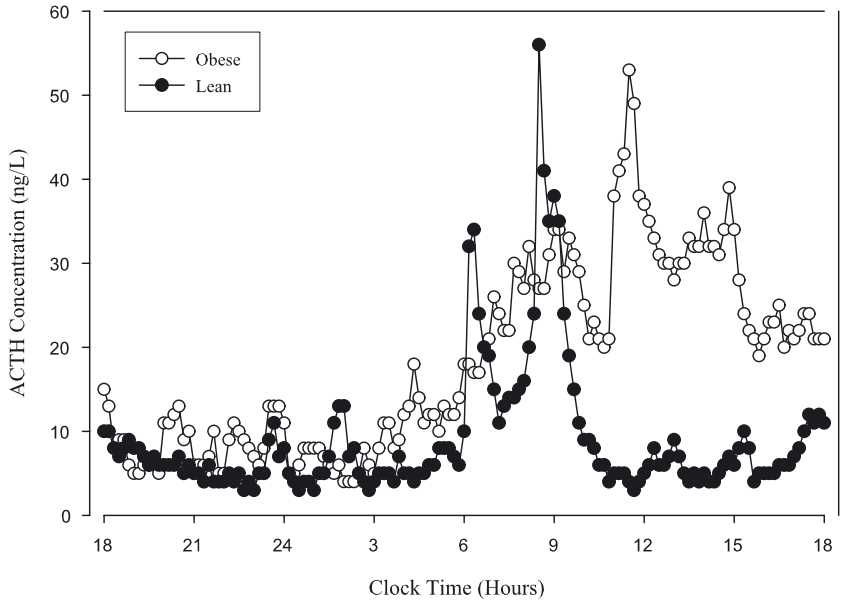
* $P < 0.05$ Obese vs. lean subjects

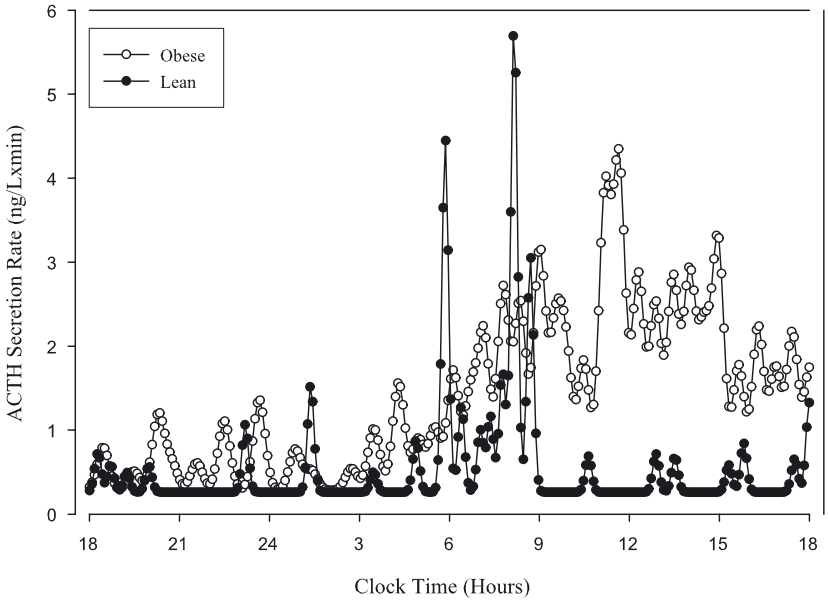
† $P < 0.05$ Placebo vs. Acipimox obese subjects

Figure 1.

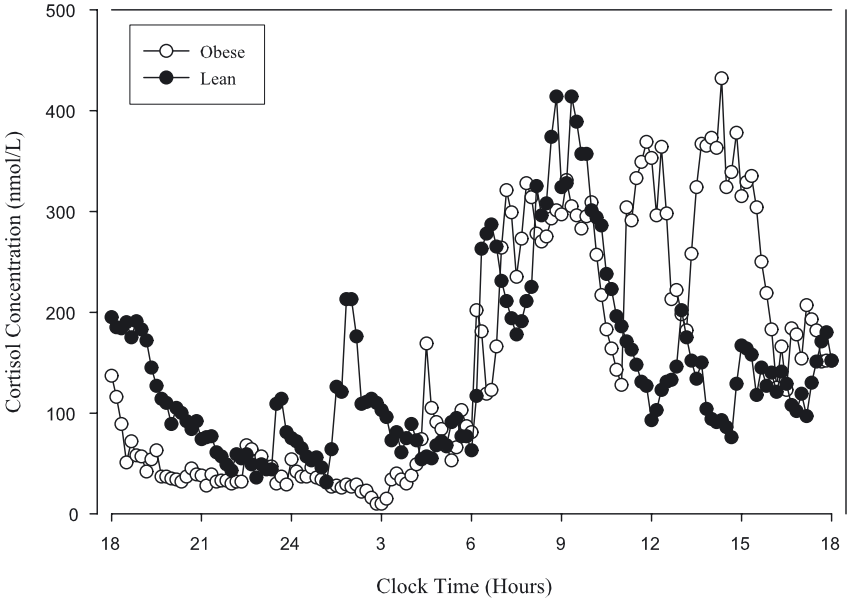
Representative 24 h ACTH (A) and cortisol (B) concentration profiles and corresponding diurnal secretion plots of one lean (-•-) and one obese woman (-o-). Lean woman Age = 33 yr, BMI = 18.3 (kg/m^2) and obese woman Age = 31 yr, BMI = 39.4 (kg/m^2)

A) 24 h ACTH concentration (ng/L) and corresponding secretion (ng/L x min) profiles





B) 24 h Cortisol concentration (nmol/L) and corresponding secretion (nmol/Lxmin) profiles



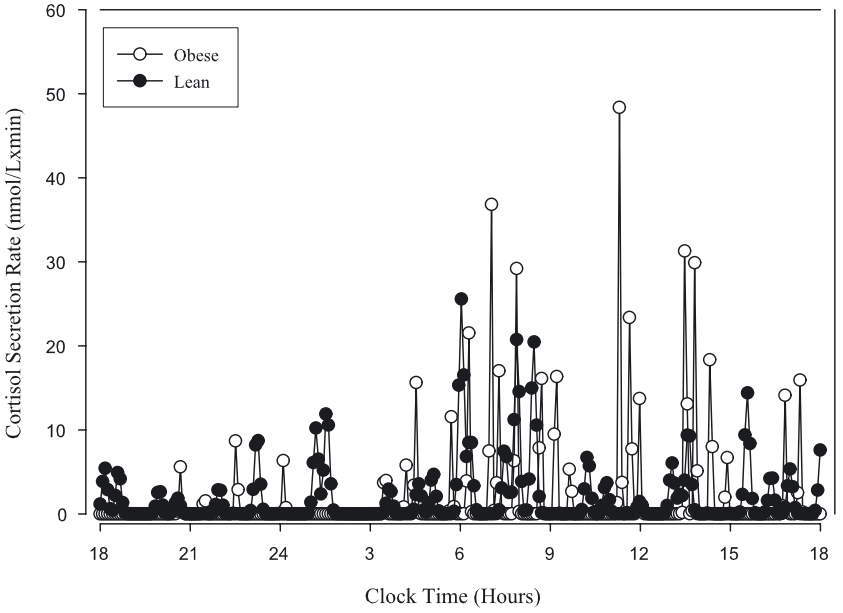


Figure 2.

Features of diurnal ACTH secretion in obese women during placebo (white bars) and Acipimox treatment (grey bars) and in lean controls (black bars). Error bars of the box plot represent SEM.

* P < 0.05 Obese vs. lean women, statistical analysis was performed using independent Student's t-test

** P < 0.05 Placebo vs. Acipimox obese women, statistical analysis was performed using paired samples t-test

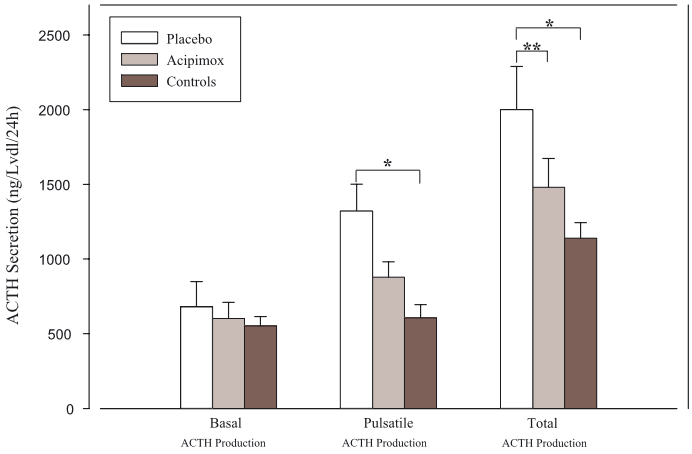


Figure 3.

Regularity of plasma ACTH and cortisol concentration- time series in obese women during placebo (open symbols) and Acipimox treatment (grey symbols) and in lean controls (closed symbols). Higher absolute ApEn values denote greater relative randomness of hormone patterns. Data are presented as normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series. Vertical marks indicate median ApEn ratio in each group. Mean ApEn ratios of plasma ACTH concentration time series were significantly higher in obese women compared with controls and the regularity of 24 h cortisol concentration time series was significantly less regular during Acipimox treatment.

* P < 0.05 Obese vs. lean women, statistical analysis was performed using independent Student's t-test

** P < 0.05 Placebo vs. Acipimox obese women, statistical analysis was performed using paired samples t-test

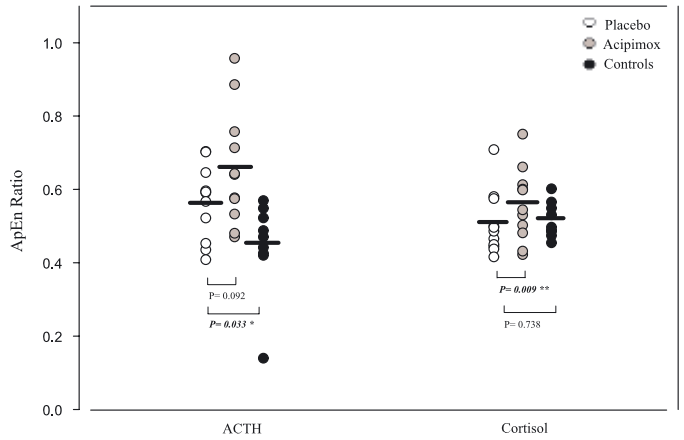
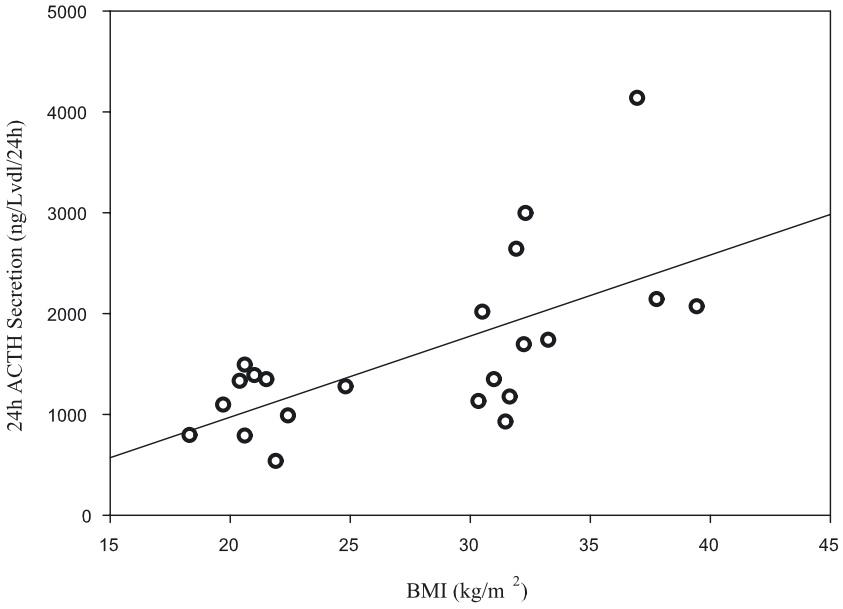


Figure 4.

Correlation BMI vs. diurnal ACTH secretion.

Both obese and lean women (N = 21) were included in correlation analysis of BMI (range 18.3-39.4 kg/m^2) vs. daily ACTH production. 24 h Total ACTH production is calculated per litter distribution volume.



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Chapter 8

Activation of Dopamine D2 Receptors Simultaneously Ameliorates Various Metabolic Features of Obese Women

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Abstract

The metabolic syndrome comprises a cluster of metabolic anomalies, including insulin resistance, abdominal obesity, dyslipidemia and hypertension. Previous studies suggest that impaired dopamine D2 receptor (D2R) signalling is involved in its pathogenesis. We studied the acute effects of bromocriptine (a D2R agonist) on energy metabolism in obese women, while body weight and caloric intake remained constant. 18 healthy obese women (BMI 33.2 ± 0.6 kg/m^2, mean age 37.5 ± 1.7 range 22-51 years) were studied twice in the follicular phase of their menstrual cycle in a prospective, single blind, cross-over design. Subject received either bromocriptine (B) or placebo (Pl) for eight days. At each occasion blood glucose and insulin were assessed every 10 minutes during 24 hours and circadian plasma free fatty acids (FFA) and triglyceride (TG) levels were measured hourly. Fuel oxidation was determined by indirect calorimetry. Body weight and -composition were not affected by the drug. Mean 24 h blood glucose ($P < 0.01$) and insulin ($P < 0.01$) were significantly reduced by bromocriptine, whereas mean 24 h FFA levels were increased ($P < 0.01$), suggesting that lipolysis was stimulated. Bromocriptine increased oxygen consumption ($P = 0.03$) and resting energy expenditure (by 50 kCal/day, $P = 0.03$). Systolic blood pressure was significantly reduced by bromocriptine. Thus, these results imply that short term bromocriptine treatment ameliorates various components of the metabolic syndrome, while it shifts energy balance away from lipogenesis in obese humans.

Introduction

The metabolic syndrome comprises a cluster of metabolic anomalies that are well-established risk factors for type 2 diabetes and cardiovascular disease, including insulin resistance, abdominal obesity, dyslipidemia and hypertension. Their concomitant occurrence suggests that a common pathophysiological denominator underlies these distinct metabolic features. Seasonally obese birds, fish and rodents spontaneously develop virtually all components of the metabolic syndrome in preparation for winter-time. A wealth of data indicates that fluctuations of dopaminergic neurotransmission in various brain nuclei are involved in these seasonal metabolic adaptations (1). In particular, reduction of dopaminergic neurotransmission in supra chiasmatic nuclei precedes the development of obesity and insulin resistance, and treatment with the dopamine D2 receptor (D2R) agonist bromocriptine effectively redirects the obese insulin resistant state towards the lean insulin sensitive state in these rodents (2-6). Compelling evidence suggests that D2R transmitted dopaminergic tone is also diminished in the brain of various models of non-seasonal obesity (7) and D2R agonist drugs ameliorate the metabolic profile of these animals as effectively as they do in seasonal obese models (8;9). D2R binding capacity in the brain of obese humans is reduced in proportion to body mass index (10), which thus may contribute to the metabolic anomalies associated with obesity. To investigate the potential impact of diminished dopaminergic D2 receptor mediated neurotransmission per se on the regulation of energy expenditure and fuel metabolism in humans, we studied the (sub) acute effects of short-term bromocriptine treatment on various metabolic parameters in obese humans.

Subjects and Methods

Subjects

Eighteen healthy obese premenopausal women (BMI 30.1-40.5 kg/m^2, mean age 37.5 ± 1.7, range 22-51 years) were recruited through advertisements in local news papers. All subjects had medical screening, including medical history taking, physical examination, standard haematology, and blood and urine chemistry. Acute or chronic disease, depression (present or in medical history), head trauma, smoking, alcohol abuse, recent trans-meridian flights, night-shift work, weight change prior to the study (> 5 kg in 3 months), recent blood donation, participation in another clinical trial (< 3 months) and use of medication (including oral anti-conceptives) were exclusion criteria for participation. All participants were required to have regular menstrual cycles. All studies were done in the early follicular phase of the menstrual cycle.

Body composition

Body mass index (weight (kg)/(length (m))^2) was calculated according to WHO recommendations. Percentage body fat (fraction of total body weight) was quantified using dual energy X-ray absorptiometry (DEXA, Hologic QDR4500) on a separate day between the two study occasions(11).

Drugs

Subjects were assigned to bromocriptine or placebo treatment for a period of 8 days in a single blind cross-over design, with a four week time interval between each study occasion. To avoid potential cross-over effects of bromocriptine treatment, all subjects received placebo during the first intervention period. A dose of 2.5 mg of bromocriptine or placebo was prescribed at the first day. Thereafter, drug or placebo were taken twice daily (totalling 5.0 mg daily) at 0800 h and 2000 h until the end of the blood sampling period, which took place at the 8th day of treatment. All subjects tolerated the drug well, although ten participants had gastro-intestinal complaints (nausea, vomiting) at the first day of bromocriptine treatment only.

Diet

To limit confounding by nutritional factors, all subjects were prescribed a standard eucaloric diet supplied by the research center and drinks other than water were prohibited as of one day prior to admission until the end of each study occasion. The macronutrient composition and caloric content of the diet was exactly the same for each individual at both study occasions. Meals were served according to a fixed time schedule (breakfast 0930 h, lunch 1300 h, diner 1830 h) and were consumed within limited time periods (30 minutes). No dietary restrictions were imposed on the obese women between both study occasions.

Indirect calorimetry

After resting for 45 minutes, subjects (fasting) were placed under a ventilated hood, while lying on a bed, awake, in a quiet room for 30 minutes. The volume of oxygen inspired (VO_2) and the expired volume of carbon dioxide (VCO_2) were measured every minute. Subsequently, resting energy expenditure (REE), glucose and lipid oxidation were calculated with the following equations:

Glucose oxidation = $4.57\ VCO_2\text{-}3.23VO_2\text{-}2.6N$

Lipid oxidation = $1.69\ VO_2\text{-}1.69VCO_2\text{-}2.03N$

REE = $3.91\ VO_2 + 1.10\ VCO_2\text{-}1.93\ N$

in which protein disappearance is ignored (N = Nitrogen) since the error thus introduced in the calculation of energy expenditure is negligible(12).

Clinical Protocol

The protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center and was performed according the Helsinki declaration. All subjects gave written acknowledgement of informed consent for participation and were admitted to the Clinical Research Unit of the Department of General Internal Medicine. Obese subjects were studied

twice with an interval of four weeks, wherein body weight remained stable and subjects were instructed to keep their physical activity level constant. The clinical set-up was the same during both occasions apart from the subject receiving the alternative treatment (bromocriptine or placebo). Subjects were admitted to the research center at 0700 h am, after an overnight fast. After resting for 45 minutes, indirect calorimetry was performed using a ventilated hood for 30 minutes. Thereafter a cannula for blood sampling was inserted into an antecubital vein, which was attached to a 3-way stopcock and kept patent by a continuous 0.9% NaCl and heparin (1 U/ml) infusion (500ml/24 h). Blood samples for basal parameters were withdrawn and twenty four hour blood sampling started. Blood was collected with S-monovetten (Sarstedt, Etten-Leur, The Netherlands) at 10-minute intervals for determination of plasma insulin and glucose concentrations. Blood samples for the measurements of plasma free fatty acid (FFA) and triglyceride (TG) levels were taken hourly. The total amount of blood withdrawn during each occasion was 246 ml. All subjects remained recumbent during the blood-sampling period except for bathroom visits (24 h urine was collected). No daytime naps were allowed. Well being and vital signs were recorded at regular time intervals (hourly). Meals were served according to a fixed time schedule (0930 h breakfast, 1300 h lunch, 1830 h diner) and consumed within limited time periods. Lights were switched off at 2300 h and great care was taken not to disturb and touch subjects during withdrawal of blood samples while they were sleeping. Periods of wakefulness and toilet visits during the night were recorded by the personnel performing nocturnal blood sampling. Polygraphic sleep monitoring by EEG was not performed. Lights were switched on and subjects were awakened at 0730 h am. All data were recorded on standard data collection forms and was entered after validation in a computer system for subsequent tabulation and statistical analysis.

Assays

Samples of each subject were determined in the same assay run. Serum insulin was measured with IRMA (Biosource Europe, Nivelles, Belgium) with a detection limit of 2 µU/L.The intra and inter-assay coefficients of variation were 4.4% and 5.9%, respectively. Plasma FFA levels were determined using a NEFA-C Free Fatty acid kit (Wako Chemicals GmbH, Neuss, Germany) with a detection limit of 30 µmol/L and the inter and intra-assay coefficients of variation of 2.6% and,1.1% respectively. Plasma TG concentrations were measured using an enzymatic colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) with a detection limit of 50 µmol/L and intra- and inter-assay coefficients of variation 1.5% and 1.8%, respectively. Progesterone concentrations were measured using a solid-phase RIA (Diagnostic Products, Los Angeles, CA, USA).

Estradiol concentrations were determined by RIA (Diagnostic Systems Laboratory, Webster, TX, USA). The detection limit was 10 pmol/L and inter and intra-assay coefficients of variation were 15.8% and 6.8% respectively. Plasma PRL concentrations were measured with a sensitive time- resolved fluoro immunoassay with a detection limit of 0.04 µg/L (Delfia, Wallac Oy, Turku, Finland). The PRL IFMA was calibrated against the 3rd WHO standard: 84/500, 1 ng/ml = 36 mU/L. The intra-assay coefficient of variation varies from 3.0-5.2% and inter-assay coefficient of variation is 3.4-6.2%. Serum glucose, cholesterol and triglyceride levels were measured using a fully automated Hitachi P800 system (Roche, Almere, The Netherlands). C-peptide concentrations were assessed by RIA (Adaltis Italia S.p.A., Casalecchio di Reno, Italy). Free thyroxine (T_4) concentrations were estimated using electro chemo luminescence immunoassay (Elecsys 2010, Roche Diagnostics Nederland BV, Almere, Netherlands).

Urine Analysis

From the moment the blood sampling period started 24 h urine was collected for the determination of catecholamine and urea nitrogen concentrations. Urinary urea concentrations were assessed by a fully automatic P 800 System (Roche, Almere, The Netherlands). Urinary epinephrine, nor-epinephrine and dopamine concentrations were assessed by high performance liquid chromatography with electron capture detection.

Calculations and statistics

Area under the Curves Metabolic Profiles
Area under the curves of insulin, glucose, FFA and TG concentration plots were calculated using the trapezoidal rule (Sigma Plot 2002 for Windows version 8.02).

HOMA model
Homeostatic model assessment (HOMA) was used to yield an estimate of longitudinal changes of insulin sensitivity before and after bromocriptine treatment in the obese subjects. The equation we used was: (fasting insulin (mU/L) x fasting glucose (mmol/l))/22.5), originating from the model firstly described by Matthews et al (13).

Statistics
Data are presented as means ± SEM, unless otherwise specified. Data was logarithmically transformed before statistical computations when appropriate and statistically analysed using a parametric test (paired samples t-test). Significance level was set at 0.05.

Results

Screening parameters obese subjects
Eighteen obese subjects were enrolled in the study. The mean age of all subjects was 37.5 ± 1.7 yrs (range 22-51 yrs). Subjects had a mean body weight of 93.9 ± 2.6 kg (range 81.2-124.1 kg), a BMI of 33.2 ± 0.6 kg/m^2 (range 30.1-40.5 kg/m^2) and total percentage body fat of 39.6 ± 0.8 % (range 32.1-44.8). Mean fasting glucose concentration was mmol/L 5.0 ± 0.1 (range 4.2-6.3 mmol/L), insulin mU/L 15.3 ± 1.7 (range 7-28 mU/L), HbA1C 4.7 ± 0.1 % (range 3.9-5.3 %), total cholesterol 4.7 ± 0.2 mmol/L (range 3.7-5.8 mmol/L), LDL cholesterol 2.99 ± 1.57 mmol/L (range 2.03-4.00 mmol/L) and HDL cholesterol 1.54 ± 0.08 mmol/L (range 1.03-2.32 mmol/L).

Baseline measurements at start study occasions
Body weight was similar at both study occasions. All subjects were studied in the early follicular phase of their menstrual cycle, as confirmed by plasma estradiol and progesterone. All subjects were clinically euthyroid. Bromocriptine significantly decreased systolic blood pressure and parameters of glucose metabolism in fasting conditions (glucose, insulin, C-peptide) at the beginning of each study occasion. Cholesterol concentrations were not affected by bromocriptine. PRL concentrations were significantly reduced by bromocriptine. An overview of body composition parameters and baseline serum measurements obtained at the start of both study occasions is given in Table 1.

Effect of bromocriptine on indirect calorimetry
Indirect calorimetry was performed in 12 subjects only (for technical reasons). Oxygen consumption (VO_2) was significantly increased by bromocriptine, whereas the drug did not affect VCO_2. Resting energy expenditure was significantly higher during bromocriptine treatment. Glucose oxidation was slightly decreased, while lipid oxidation was enhanced during bromocriptine treatment, although these differences were not statistically significant. An overview of the results is presented in table 2.

Effect of bromocriptine on circadian glucose profiles
Diurnal blood glucose concentrations as well as the AUC of the 24 h glucose concentrations were significantly reduced after bromocriptine treatment compared with placebo (Table 3 and Figure 1A). Both the maximum concentration and the AUC of the glucose peak in response to dinner was significantly decreased by bromocriptine (maximal concentration Pl 9.0 ± 0.4 vs. B 7.5 ± 0.4 mmol/L, $P < 0.01$ and AUC Pl 131 ± 5 vs. B 112 ± 4 mmol/L/3.5 h, $P < 0.01$, Figure 1A). Also, nocturnal glucose concentrations (0000 h-0700 h clock time) and the AUC of the nocturnal glucose curves were significantly lower

during bromocriptine treatment. Mean nocturnal glucose concentration Pl 4.8 ± 0.1 vs. B 4.5 ± 0.1 mmol/L, P < 0.01 and AUC P 198 ± 3 vs. B 184 ± 4 mmol/L/7h, P < 0.01, Figure 1A. Periods of wakefulness during the night were not different at both study occasions.

Effect of bromocriptine on circadian insulin profiles

Mean 24 h insulin concentrations and the AUC of 24 h insulin profiles were significantly reduced during bromocriptine treatment (Table 3 and Figure 1B). The maximum concentration of the insulin peak in response to dinner was significantly decreased (Pl 185 ± 19 vs. B 132 ± 19 mU/L, P < 0.01) and the AUC of the postprandial insulin peak was significantly lowered by bromocriptine (AUC Pl 1846 ± 209 vs. B 1216 ± 178 mU/L/3.5 h, P < 0.01). Both nocturnal insulin concentrations (0000 h-0700 h clock time) and the AUC of the nocturnal insulin curves were similar during bromocriptine and placebo treatment (Mean nocturnal insulin concentration Pl 14.0 ± 1.2 vs. B 13.1 ± 1.1 mU/L, P = 0.31 and AUC Pl 557 ± 47 vs. B 521 ± 42 mU/L/7h, P = 0.32)

Effect of bromocriptine on circadian lipid profiles

Circadian circulating plasma FFA concentrations as well as the AUC of the 24 h FFA concentration curves were significantly increased during bromocriptine treatment. TG concentrations and AUC of the 24 h TG curves showed the same (non significant) trend (Table 3 and Figure 2A and B).

Urine Analysis

Twenty four hour urea nitrogen excretion did not differ during placebo and bromocriptine treatment (Pl 357 ± 21 vs. B 362 ± 20 mmol/24 h, P = 0.64). Urine norepinephrine was significantly reduced during bromocriptine treatment (Pl 0.18 ± 0.02 vs. B 0.12 ± 0.01 umol/24 h, P < 0.01), whereas epinephrine (Pl 0.015 ± 0.005 vs. B 0.012 ± 0.004 umol/24 h, P = 0.42) and dopamine (Pl 1.58 ± 0.17 vs. B 1.55 ± 0.19 umol/24 h, P = 0.83) were not affected by bromocriptine.

Discussion

This study shows that short-term treatment with the dopamine D2 receptor agonist bromocriptine favourably affects energy metabolism and blood pressure in obese women. In particular, 8 days of bromocriptine treatment reduces diurnal glucose and insulin concentrations. In addition, bromocriptine enhances oxygen consumption and basal metabolic rate and lowers (systolic) blood pressure. Plasma free fatty acid and triglyceride concentrations were elevated during bromocriptine treatment. Notably, all of these effects come about without any change of body adiposity and independent of qualitative or quantitative changes in food intake.

As far as we are aware, this is the first study to show a beneficial effect of short-term bromocriptine treatment on energy expenditure and fuel metabolism in obese humans. The data indicate that activation of dopamine D2 receptors ameliorates various features of the metabolic syndrome in obese humans, even apart from its impact on food intake and body weight. Long-term bromocriptine treatment effectively reduces fasting insulin and glucose levels in rodents (4;9;14) and improves glucose tolerance in healthy and diabetic obese humans (15-18). However, chronic bromocriptine administration consistently reduces body fat, perhaps primarily via its inhibitory effect on food intake (19-21), which might explain the metabolic corollaries of treatment. We here show that activation of D2 receptors directly acts to reduce circadian plasma glucose and insulin concentrations, where both postprandial and nocturnal glucose levels are diminished, even without any measurable effect on body weight

Bromocriptine significantly enhanced resting energy expenditure and oxygen consumption in the current experimental context. This finding is corroborated by data documenting enhanced oxygen consumption in response to bromocriptine treatment in obese rodents (2;22). Conversely, loss of function mutations of the D2R gene are associated with reduced resting energy expenditure in humans (23). Our results further support the position that D2R signalling is involved in the control of basal metabolic rate in humans. Whether bromocriptine also affects the level of physical activity requires further investigation.

Bromocriptine significantly reduced systolic blood pressure. The autonomic nervous system plays a critical role in the

control of blood pressure (24) and sympathetic hyperactivity may indeed underlie hypertension in obese humans (25). Activation of dopamine D2R has sympatholytic effects (26;27), which thus may lower blood pressure. The fact that urinary catecholamine excretion was blunted by bromocriptine in the present study supports the notion that reduction of sympathetic tone may (in part) underlie the hypotensive effect of the drug. In addition, bromocriptine blocks 1-adrenergic receptors (28), which obviously may also contribute to the hypotensive effect of the drug (29).

The rise of circulating FFA levels induced by bromocriptine may reflect inhibition of net FFA uptake in adipocytes (4). In apparent contrast, long-term bromocriptine treatment either reduces or does not affect plasma FFA concentrations in rodents and humans (4;9;17;18). However, these effects on circulating FFA levels presumably result from loss of body fat induced by chronic bromocriptine administration, which did not occur in the present study.

The mechanisms through which dopaminergic neurons control energy balance and fuel metabolism remain to be established. Although D2R are expressed in various tissues (30), intracerebroventricular injections of bromocriptine at a very low dose completely reproduce the metabolic effects of high dose intravenous administration in rats, which suggests that the central nervous system D2R is a critical target of the drug. Activation of D2R reduces neuropeptide Y (NPY) mRNA expression in the arcuate nucleus of the hypothalamus (31-33). NPY is elevated in the arcuate nucleus of obese animal models (32-34)and icv administration of this neuropeptide directly induces (hepatic) insulin resistance and suppresses basal metabolic rate in rodents (35;36). Therefore, bromocriptine may facilitate glucose homeostasis through a reduction in hypothalamic NPY. Alternatively, bromocriptine may impact metabolism by virtue of its sympatholytic properties (37). High NE levels in the ventromedial hypothalamus are another neurochemical marker of obesity in rodents and NE infusion into this brain area produces all features of the metabolic syndrome (38). D2R activation inhibits NE gene expression and release in the arcuate nucleus and peripheral nerves (26;27) and bromocriptine's ability to act as such was supported by our data indicating that 24 h norepinephrine (NE) urine concentrations were significantly lower after bromocriptine treatment. Thus, the favourable effects of bromocriptine on glucose metabolism may also be due to reduced NE release in the ventromedial hypothalamus. Finally, activation of D2R inhibits the pituitary lactotroph axis and prolactin (PRL) has been reported to exert potent lipogenic and diabetogenic effects (for review see(39)). Thus, the ability of bromocriptine to favourably affect fuel flux in obese women could also be mediated by a decrease of circulating PRL levels.

Our data lend further support to the postulate that reduced dopaminergic D2R signalling in obese humans, as reported by Wang et al (10), has adverse metabolic consequences. In particular, deficient dopaminergic D2R transmission may be involved in the pathogenesis of various components of the metabolic syndrome in humans.

In conclusion, short-term bromocriptine treatment facilitates glucose metabolism, lowers systolic blood pressure and stimulates resting energy expenditure in obese humans. Notably, these effects occur through mechanistic routes distinct from reduction of food intake or loss of body fat. These data indicate that activation of D2R dopaminergic neurotransmission ameliorates various metabolic anomalies associated with obesity and lend further support to the thesis that reduced D2R availability in the brain of obese humans directly contributes to their adverse metabolic profile.

Tables and Figures

Table 1. Basal Measurements at the start of each study occasion

Parameter	Placebo	Bromocriptine	P-value [a]
Weight (kg)	94.1 ± 2.5	94.4 ± 2.5	0.33
BMI (kg/m^2)	33.2 ± 0.6	33.3 ± 0.6	0.35
Diastolic blood pressure (mmHg)	81 ± 3	79 ± 2	0.43
Systolic blood pressure (mmHg)	122 ± 4	112 ± 3	0.04
Fasting glucose (mmol/L)	5.3 ± 0.2	4.8 ± 0.1	<0.01
Fasting insulin (mU/L)	13.3 ± 1.4	10.9 ± 0.8	0.03
HOMA_IR [b]	3.25 ± 0.48	2.32 ± 0.19	0.01
C-peptide (nmol/L)	0.959 ± 0.108	0.724± 0.055	0.01
Cholesterol (mmol/L)	4.41 ± 0.16	4.45 ± 0.14	0.54
LDL cholesterol (mmol/L)	2.79 ± 0.16	2.83 ± 0.16	0.66
HDL cholesterol (mmol/L)	1.40 ± 0.07	1.43 ± 0.07	0.27
Ratio total cholesterol/HDL	3.26 ± 0.19	3.13 ± 0.17	0.15
Estrogen (E2) (pmol/L)	163 ± 21	209 ± 21	0.10
Progesteron (nmol/L)	2.13 ± 0.64	2.94 ± 1.13	0.56
Free thyroxine (pmol/L)	14.6 ± 0.4	14.4 ± 0.4	0.56
Prolactin (µg/L)	6.7 ± 1.1	2.3 ± 0.4	<0.01

Data are presented as mean ± SEM

a) P-values placebo vs. bromocriptine obese women, as determined by paired samples t-test

b) The homeostasis model was used to estimate insulin sensitivity from fasting insulin and glucose levels. HOMA_IR was calculated as (fasting insulin (mU/ml) x fasting glucose (mmol/l))/22.5)(13). Data was log-transformed before statistical analysis.

Table 2. Indirect Calorimetry in 12 obese women

Parameter	Placebo	Bromocriptine	P-value [a]
RQ	0.79 ± 0.01	0.78 ± 0.01	0.58
VO_2 (ml/min)	232.2 ± 5.7	243.6 ± 8.2	0.03
VCO_2 (ml/min)	182.8 ± 4.2	188.7 ± 6.3	0.20
Glucose oxidation (µmol/kg/min)	5.2 ± 0.9	4.6 ± 0.8	0.67
Lipid oxidation (µmol/kg/min)	3.4 ± 0.3	3.7 ± 0.3	0.38
REE (kcal/day)	1109 ± 26	1160 ± 39	0.03

Data are presented as mean ± SEM.

a) P-values placebo vs. bromocriptine obese women, as determined by paired samples t-test

Table 3. Metabolic Parameters in obese women

Parameter	Placebo	Bromocriptine	P-value [a]
Total Mean 24 h insulin (mU/L)	38 ± 4	29 ± 3	<0.01
Total AUC insulin (mU/Lx24 h)	55 130 ± 5 188	42 029 ± 4 704	<0.01
Total Mean 24 h glucose (mmol/L)	5.4 ± 0.1	4.9 ± 0.1	<0.01
Total AUC glucose (mmol/Lx24 h)	7497 ± 165	6987 ± 158	0.02
Mean 24 h FFA (mmol/L)	0.44 ± 0.03	0.57 ± 0.05	<0.01
AUC FFA (mmol/Lx24 h)	10.45 ± 0.72	13.59 ± 1.26	<0.01
Mean 24 h triglycerides (mmol/L)	1.24 ± 0.11	1.34 ± 0.10	0.15
AUC triglycerides (mmol/Lx24 h)	29.65 ± 2.69	32.04 ± 2.38	0.14

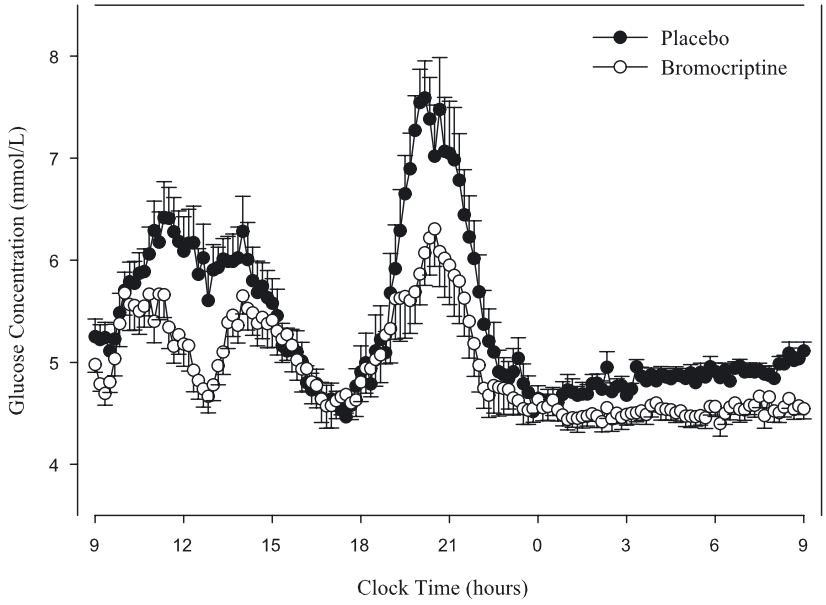
Data are presented as mean ± SEM.

a) P-values placebo vs. bromocriptine obese women, as determined by paired samples t-test

Figure 1.

Serum 24 h glucose concentration time series (A) and 24 h insulin concentrations (B) of the obese subjects (N = 18) during placebo (-•-) and bromocriptine treatment (-o-). Data reflect sampling of blood every 10 min. Error bars represent SEM. 24 h Blood sampling began at 0900 h. Lights were switched off and subjects went to sleep at 2300 h until 0730 h am, when lights were switched on (grey horizontal bar indicates dark period).

A)



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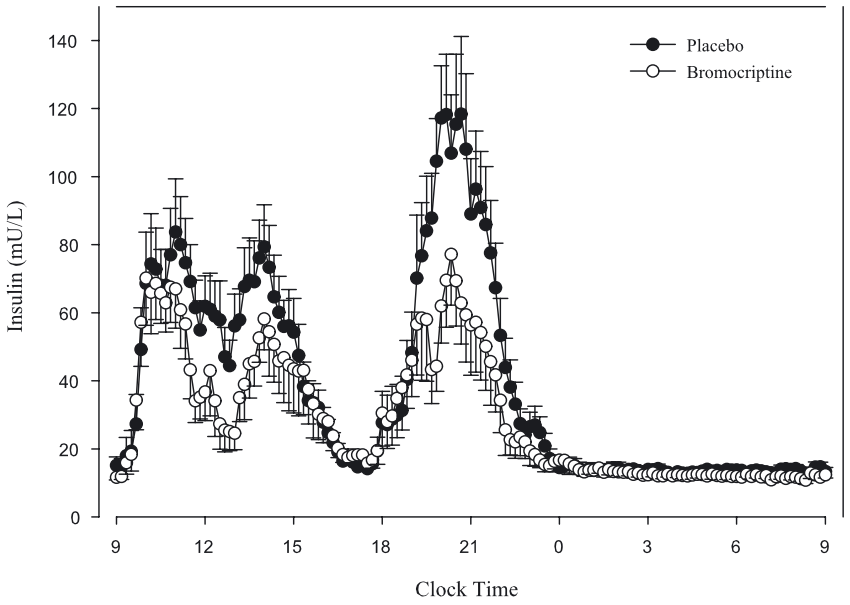
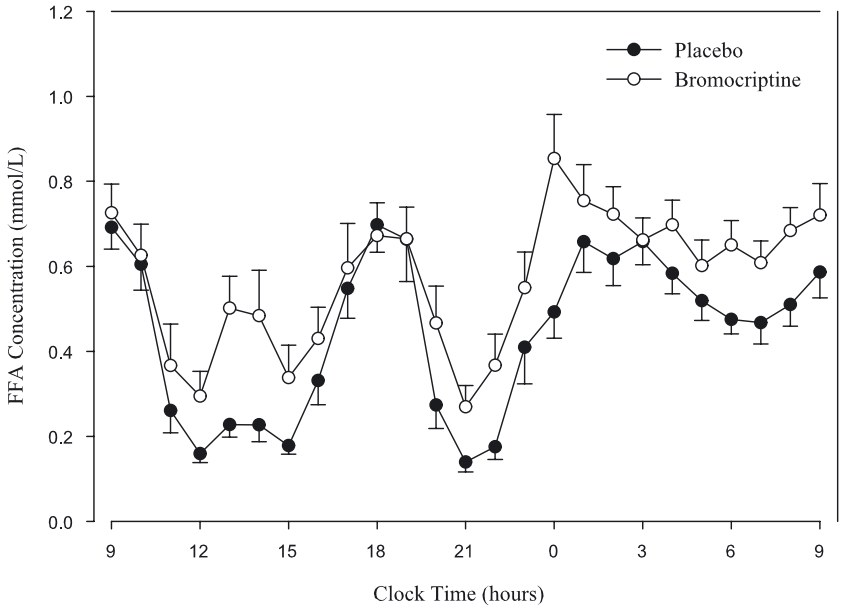


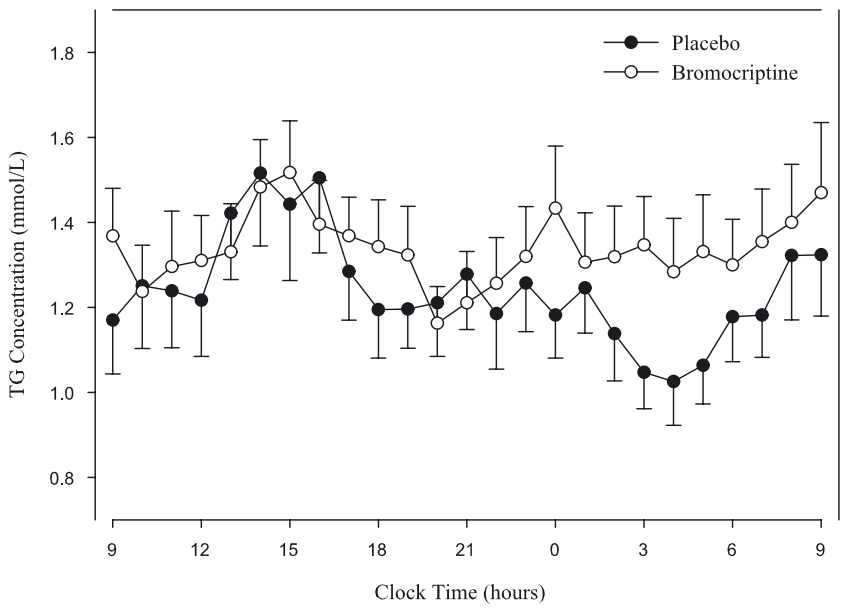
Figure 2.

Serum FFA (A) and TG (B) concentration time series of the obese subjects (N = 18) during placebo (-●-) and bromocriptine treatment (-○-). Data reflect sampling of blood every hour for 24 h. Error bars represent SEM. Blood sampling starts at 0900 h. Lights were switched off and subjects went to sleep at 2300 h until 0730 h next morning, when lights were switched on (grey horizontal bar indicates dark period).

A)



B)



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Chapter 9

Activation of Dopamine D2 Receptors Lowers Circadian Leptin Concentrations in Obese Women

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J Clin Endocrinol Metab. (Provisionally Accepted)

Abstract

1. Context. Leptin release is regulated by factors other than fat mass alone. Previous observations provide indirect evidence for an inhibitory effect of dopaminergic neurotransmission on leptin secretion. We hypothesized that short term bromocriptine treatment would lower circadian plasma leptin concentrations in obese humans.
2. Objective. To study the acute effects of bromocriptine (a D2R agonist) on circadian leptin levels in obese women, while body weight and caloric intake remained constant.
3. Design. Prospective, single blind, cross-over study (2004).
4. Setting. Clinical Research Center
5. Participants. Eighteen healthy obese women (BMI 33.2 ± 0.6 kg/m^2) were studied twice in the early follicular phase of their menstrual cycle.
6. Intervention(s). Treatment with bromocriptine (B) or placebo (Pl) for eight days
7. Main Outcome Measure(s). Blood was collected during 24 hours at 20-minute intervals for determination of leptin concentrations. Blood samples for the measurements of plasma insulin and glucose concentrations were taken at 10-minute intervals and hourly for the assessment of plasma free fatty acid and triglyceride levels.
8. Results. We here show that short-term treatment with bromocriptine significantly reduces circulating leptin levels in obese women (Pl 33.6 ± 2.5 vs. B 30.5 ± 2.5 µg/L, P = 0.03). FFA concentrations were increased by bromocriptine treatment and the increase of circulating FFA's during bromocriptine treatment was inversely related with the decline of leptin levels. The decline of glucose, insulin or prolactin concentration in response to bromocriptine was not correlated with the reduction of circulating leptin in the present study.
9. Conclusion. Activation of dopamine D2 receptors by bromocriptine lowers circulating leptin levels in obese women, which suggests that dopaminergic neurotransmission is involved in the control of leptin release in humans.

Introduction

Leptin is produced by adipocytes and serves as an endocrine signal to inform the brain about the size of body adipose tissue stores (1-3). Although circulating leptin levels are positively related to fat mass in groups of obese individuals (4), individual concentrations vary considerably for a given measure of adiposity (3). Circulating leptin levels are characterised by diurnal rhythm. The fact that plasma leptin concentrations acutely change in response to fasting (5;6), refeeding (6;7) and increased food intake (7), even without any measurable alteration of body fat content, also supports the contention that leptin release and/or clearance is regulated by factors other than fat mass alone. Indeed, corticosteroids, insulin, prolactin, various cytokines, nutrient flux through adipocytes and the sympathetic nervous system have all been shown to modulate leptin release by adipocytes (8).

In this context, previous observations provide indirect evidence for an inhibitory effect of dopaminergic neurotransmission on leptin secretion. In particular, it has been reported that treatment with bromocriptine, a dopamine D2 receptor agonist, significantly lowers the plasma leptin concentration in a single blood sample of humans with prolactinoma, even without affecting body weight (9). Furthermore, a single iv bolus injection of bromocriptine significantly reduced both basal and

lipopolysaccharide (LPS)-induced leptin release in rats (10). These data led us to hypothesize that short term bromocriptine treatment would lower circadian plasma leptin concentrations in obese humans. To test this postulate, we measured plasma leptin concentrations in obese women who were treated with bromocriptine or placebo for 8 days. Since plasma leptin levels clearly exhibit circadian fluctuation, concentrations were measured over 24 hours. As bromocriptine significantly affected various metabolic and endocrine parameters that may impact on leptin secretion, including circulating insulin, glucose and prolactin levels (Kok et al, to be published in a separate manuscript), we also report the statistical correlation between these parameters and leptin concentrations.

Subjects and Methods

Subjects

Eighteen healthy obese premenopausal women (BMI 30-35 kg/m^2, mean age 37.5 ± 1.7 yr, range 22-51 yr) were enrolled. Before participation, all subjects underwent medical screening, including medical history taking, physical examination, standard laboratory haematology, blood chemistry and urine tests. Acute or chronic disease, depression (present or in history), head trauma, smoking, alcohol abuse, recent trans meridian flights, night-shift work, weight change prior to the study (> 5 kg in 3 months), recent blood donation or participation in another clinical trial (< 3 months) and use of medication (including oral contraceptives) were exclusion criteria for participation. All participants were required to have regular menstrual cycles. All studies were performed in the early follicular phase of the menstrual cycle.

Drugs

Subjects were assigned to bromocriptine or placebo treatment for a period of 8 days in a single (patient) blind crossover design, with a four weeks time interval between each study occasion. To avoid potential crossover effects of bromocriptine treatment, all subjects received placebo during the first intervention period. A dose of 2.5 mg of bromocriptine was prescribed on the first day. Thereafter, drug or placebo was taken twice daily (totalling 5.0 mg daily) at 0800 h and 2000 h for 7 days. The drug was well tolerated, although ten participants had gastro-intestinal complaints (nausea, vomiting) on the first day of bromocriptine treatment only.

Diet

To limit confounding by nutritional factors, all subjects were prescribed a standard eucaloric diet, as from one day prior to admission until the end of each study occasion. The caloric content and macronutrient composition of the diet was exactly the same at both study occasions. Intake of alcohol and caffeine/theine containing beverages were not allowed. Meals were served according to a fixed time schedule (breakfast 0930 h, lunch 1300 h, diner 1830 h) and were consumed within limited time periods. No dietary restrictions were imposed between both study occasions.

Clinical Protocol

The protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center and was performed according the Helsinki declaration. All subjects gave written acknowledgement of informed consent for participation and were admitted to the Clinical Research Unit of the Department of General Internal Medicine in the early follicular stage of their menstrual cycle. Subjects were studied twice with an interval of four weeks, where body weight remained stable and subjects were instructed to keep their physical activity level constant. The clinical set-up was the same during both occasions apart from the subject receiving the alternative treatment (Bromocriptine or placebo). Subjects were admitted to the research center at 0700 h. A cannula for blood sampling was inserted into an antecubital vein, which was attached to a 3-way stopcock and kept patent by a continuous 0.9% NaCl and heparin (1 U/ml) infusion (500ml/24 h). Blood samples for basal parameters were withdrawn and twenty four hour blood sampling started. Blood was collected with S-monovetten (Sarstedt, Etten-Leur, The Netherlands) at 20-minute intervals for determination of leptin concentrations. Blood samples for the measurements of plasma insulin and glucose concentrations were taken at 10-minute intervals and hourly for the assessment

of plasma FFA and TG levels. Subjects remained recumbent during the blood-sampling period, except for bathroom visits (24 h urine was collected). No daytime naps were allowed. Well-being and vital signs were recorded at regular time intervals (hourly). Meals were served according to a fixed time schedule (0930 h breakfast, 1300 h lunch, 1830 h diner) and consumed within limited time periods. Lights were switched off at 2300 h and great care was taken not to disturb and touch subjects during withdrawal of blood samples while they were sleeping. Lights were switched on and subjects were awakened at 0730 h in the morning.

Assays

Samples of each subject were determined in the same assay run. Plasma leptin concentrations were determined by RIA (Linco Research, St. Charles, MO). The detection limit was 0.5 µg/L and the inter-assay coefficients of variation (CV) was 3.6-6.8 %. Estradiol concentrations were determined by RIA (Diagnostic Systems Laboratory, Webster, TX). The detection limit was 10 pmol/L and the inter-assay CV was 5.1-8.1 %. Serum insulin was measured with IRMA (Biosource Europe, Nivelles, Belgium) with a detection limit of 2 µU/L and inter-assay CV of 4.4 to 5.9 %. Plasma FFA levels were determined using a NEFA-C Free Fatty acid kit (Wako Chemicals GmbH, Neuss, Germany) with a detection limit of 30 µmol/L and inter-assay CV of 2.6%. Plasma triglyceride concentrations were measured using an enzymatic colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) with a detection limit of 50 µmol/L and inter-assay CV of 1.8%. Day long blood glucose concentrations were assessed using a blood glucose analyzer (Accutrend, Boehringer, Mannheim, Germany). Basal serum glucose was measured using a fully automated Modular P 800 (Hitachi, Tokyo, Japan) and Free thyroxine (fT_4) concentrations were estimated using electro chemo luminescence immunoassay (Elecsys 2010, Roche Diagnostics Nederland BV, Almere, Netherlands).

Urine Analysis

Urine was collected during the 24 h of blood sampling. Urinary epinephrine, nor-epinephrine and dopamine concentrations were assessed by high performance liquid chromatography with electron capture detection.

Calculations and statistics

Area under the Curves Leptin Profiles

Area under the curves of leptin concentration plots were calculated using the trapezoidal rule (Sigma Plot 2002 for Windows version 8.02).

Approximate Entropy

Approximate Entropy (ApEn) is a scale- and model- independent statistic that assigns a non- negative number to time series data, reflecting regularity of these data (11). Higher ApEn values denote greater relative randomness of hormone patterns. Data are presented as normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series. ApEn ratios close to 1.0 express high irregularity (maximum randomness) of pulsatile hormone patterns (12).

Circadian Rhythmicity

Circadian characteristics of leptin concentration patterns were determined using a robust curve fitting algorithm (LOWESS analysis, SYSTAT version 11 Systat Inc, Richmond, CA,(13;14)). The acrophase (clock time during 24 h at which leptin concentration is maximal) is the maximal value of the fitted curve. The mesor is the average value about which the diurnal rhythm oscillates. The amplitude of the rhythm was defined as half the difference of the nocturnal zenith and the day-time nadir. The relative amplitude is the maximal percentage increase of the mesor value.

Statistics

Data are presented as means ± SEM, unless otherwise specified. Data were logarithmically transformed before statistical computations when appropriate and statistically analysed using a parametric test (paired samples t-test). Significance level was set at 0.05. Multiple regression analysis was performed to estimate the correlation between changes in metabolic parameters (mean 24 h glucose, insulin, triglyceride and free fatty acid plasma concentrations) vs. changes of mean circadian leptin concentrations induced by bromocriptine treatment in the obese subjects. Differences were calculated subtracting values during bromocriptine treatment from values during placebo treatment. Negative differences reflect a decrease and positive differences reflect an increase induced by bromocriptine treatment of the parameter.

Results

Subjects

Eighteen obese subjects were enrolled in the present study. Body weight and BMI were similar after placebo and Bromocriptine treatment (Weight Pl 94.1 ± 2.5 vs. B 94.4 ± 2.5kg, P = 0.33 and BMI Pl 33.2 ± 0.6 vs. B 33.3 ± 0.6 kg/m^2, P = 0.35). All subjects were studied in the early follicular phase of their menstrual cycle (Estrogen Pl 163 ± 21 vs. B 209 ± 21 pmol/L, P = 0.10 and progesterone Pl 2.13 ± 0.64 vs. B 2.94 ± 1.13 nmol/L, P = 0.56). Subjects were clinically euthyroid (Thyroxine (free T_4) levels Pl 14.6 ± 0.4 vs. B 14.4 ± 0.4 pmol/L, P = 0.56).

Urine analysis

Urinary norepinephrine was significantly reduced after bromocriptine treatment (Pl 0.184 ± 0.020 vs. B 0.119 ± 0.015 µmol/24 h, P < 0.001). Urinary epinephrine (Pl 0.015 ± 0.005 vs. B 0.011 ± 0.004 µmol/24 h, P = 0.416) were not significantly different during placebo and bromocriptine treatment.

Leptin concentration parameters

Mean and AUC of 24 h leptin concentrations were significantly reduced by bromocriptine treatment (Table 1). A graphical illustration of mean 24 h plasma leptin concentrations during placebo and bromocriptine treatment vs. clock time is presented in Figure 1.

The Approximate Entropy (ApEn) ratio was not significantly affected by bromocriptine (Pl 0.88 ± 0.02 vs. B 0.87 ± 0.02, P = 0.81). Analysis of the circadian variation in plasma leptin concentrations revealed that the acrophase of the circadian leptin rhythm occurred at night at similar clock-times during placebo and bromocriptine treatment (Pl 0200 h ± 40 min and B 0100 h ± 40 min, P = 0.33). The mesor (Pl 33.1 ± 2.5 µg/L vs. B 30.0 ± 2.4 µg/L, P = 0.04) of the rhythm was significantly decreased by bromocriptine, whereas both the amplitude (Pl 8.0 ± 0.8 µg/L vs. B 7.0 ± 0.9 ng/L, P = 0.24) and the relative increase in leptin concentration (Pl 24.5 ± 1.7 % vs. B 22.5 ± 2.1 %, P = 0.39) were not significantly altered after bromocriptine treatment. An overview of the leptin concentration parameters is given in Table 1.

Correlations between leptin concentrations and metabolic parameters

The impact of bromocriptine treatment on metabolic parameters is reported in a separate paper (P. Kok et al unpublished). Multiple regression analysis, including differences (Δ) of mean 24 h glucose, insulin, free fatty acid and triglyceride concentrations as independent variables, revealed that differences in mean 24 h FFA concentrations (range Δ FFA-0.04 to 0.48 mmol/L) were significantly correlated with differences in mean 24 h leptin concentrations in response to bromocriptine treatment (partial correlation R^2 = 0.46, range Δ leptin-14.7 to 8.2 µg/L, P = 0.03, Figure 2). Changes in mean 24 h circulating glucose (range Δ glucose-1.22 to 0.72 mmol/L, P = 0.23), insulin (range Δ insulin-34.16 to 6.97 mU/L, P = 0.40) and triglyceride concentrations (range Δ triglyceride-0.41 to 0.63 mmol/L, P = 0.23) were not related to changes in leptin concentrations.

Discussion

We here show that short-term treatment with bromocriptine significantly reduces circulating leptin levels in obese women, while caloric intake was standardized and body weight remained stable. The increase of circulating FFA's during bromocriptine treatment was inversely related with the decline of leptin levels. Our finding is in keeping with a few previous reports documenting an inhibitory effect of bromocriptine on leptin release in rodents and humans (9;10).

Circulating leptin concentrations are the net result of concerted influences of prior and ongoing hormone secretion, distribution and elimination. As there is no evidence indicating that bromocriptine alters leptin clearance from the circulation, our observations suggest that activation of dopamine 2 receptors, directly or indirectly modulates leptin release by adipocytes. The brain is involved in the control of (circadian) leptin levels (15) perhaps via modulation of adipocyte metabolism by autonomic nerves (16), where sympathetic input inhibits leptin synthesis (8) Bromocriptine acts on presynaptic D2 receptors to inhibit (sympathetic) norepinephrine release (17;18). The fact that urinary norepinephrine excretion was reduced during bromocriptine treatment in our study corroborates this data. Thus, as sympathetic signals reduce leptin release by adipocytes (8), the reduction of circulating leptin levels we observe here, was not due to the inhibitory effects of bromocriptine on sympathetic activity. Alternatively, the decline of leptin during bromocriptine treatment was brought about via effects on other metabolic parameters that modulate leptin release. Glucose, insulin and prolactin all stimulate leptin synthesis (8;19-21). However, the decline of the concentration of either glucose or these hormones in response to bromocriptine was not correlated with the reduction of circulating leptin in the present study, which does not support the possibility that these factors are involved in bromocriptine's effect.

Interestingly, the decline of leptin in response to bromocriptine was correlated with the concomitant increase of circulating FFA's. Fuel flux through adipocytes is instrumental in the control of leptin synthesis, where net influx promotes, and net efflux inhibits leptin gene transcription (22). The rise of FFA levels is probably due to inhibition of the net influx of FFA in adipocytes by bromocriptine (23). Thus, the fact that changes of FFA and leptin in response to bromocriptine were inversely related supports the position that the drug reduces circulating leptin concentration via modulation of FFA flux in adipocytes.

Leptin levels are clearly increased in obese humans in proportion to fat mass, whereas dopamine D2 receptor availability in the brain is reduced in obese humans in proportion to body adiposity (24). The present findings allow for the postulate that these phenomena are related.

In conclusion, short-term bromocriptine treatment lowers circulating leptin levels in obese women, which suggests that dopaminergic neurotransmission is involved in the control of leptin release in humans.

Tables and Figures

Table 1. Features of 24 h plasma leptin concentration profiles

Parameter	Obese (N = 18)		P-value [a)]
	Placebo	Bromocriptine	
Mean 24 h plasma concentration (ng/L)	33.6 ± 2.5	30.5 ± 2.5	0.03
AUC Leptin (ng/Lx24 h)	48 423 ± 3615	44 046 ± 3604	0.03
ApEn	0.88 ± 0.02	0.87 ± 0.02	0.81
Acrophase (hours)	0200 ± 40	0100 ± 40	0.33
Mesor (ng/L)	33.1 ± 2.5	30.0 ± 2.4	0.04
Amplitude (ng/L)	8.0 ± 0.8	7.0 ± 0.9	0.24

Data are presented as means ± SEM.

a) P-values placebo vs. bromocriptine obese women, as determined by paired samples t-test

* P-value < 0.05 placebo vs. bromocriptine

Figure 1.

Mean diurnal serum leptin concentration time series the obese subjects (N = 18) during placebo (-•-) and bromocriptine treatment (-○-). Data reflect sampling of blood every 20 min for 24 h. Blood sampling starts at 0900 h. Lights were switched off and subjects went to sleep at 2300 h until 0730 h next morning, when lights were switched on (grey horizontal bar indicates sleeping period).

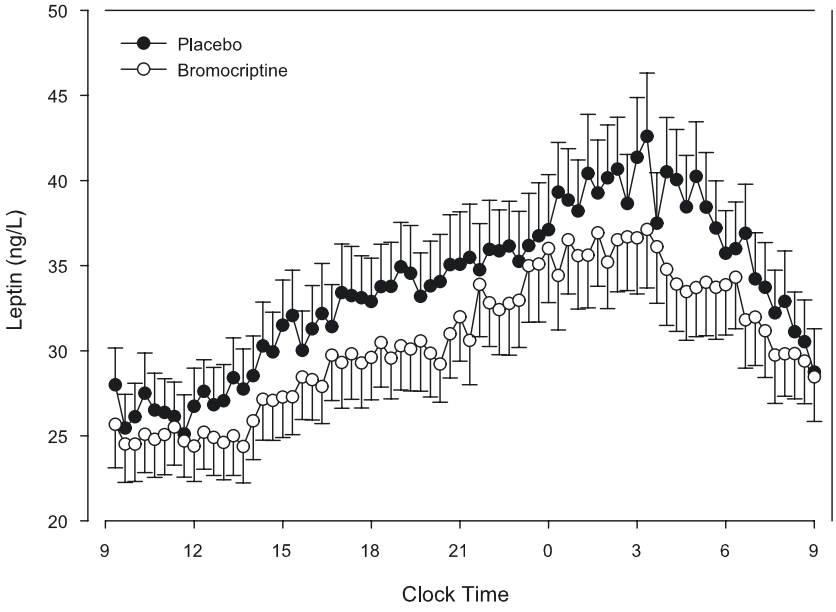
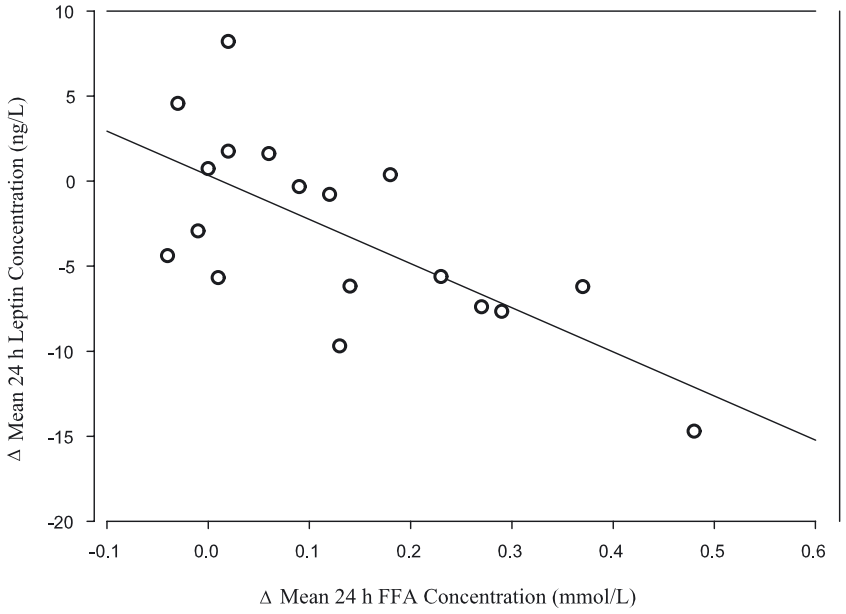


Figure 2.

Differences in mean FFA concentrations were significantly inversely related to differences in mean 24 h leptin concentrations ($R^2 = 0.46$, $P = 0.03$) during placebo and bromocriptine in obese women. The range of differences mean 24 h leptin concentrations was-14.7 to 8.2 µg/L.



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Chapter 10

Summary and Discussion

The evolutionary advantage of several animal species to conserve energy in the form of adipose issue in order to survive long periods of food shortage in the past, turned into a major health problem in current times of plenty. Excess accumulation of body fat, or "obesity", is associated with severely increased co-morbidity and mortality risks and is a global epidemical medical condition which is difficult to manage. The exact pathophysiologic mechanism of obesity remains elusive and various factors such as genetic, social, behavioural and physiological cues are involved in its development. From a biological point of view, obesity might be explained by differences in the regulation of energy intake, expenditure and storage (energy homeostasis) between obese and lean individuals. The neuroendocrine system provides a source of humoral messengers, which can modulate energy homeostasis. This thesis will focus on changes of the neuroendocrine environment of obese women. First of all, spontaneous diurnal plasma hormone concentrations and secretion of different hormonal systems were studied (the results of these studies will be discussed and summarized in **Paragraph 1**). Secondly, the effect of weight loss on neuroendocrine perturbations of some of these hormonal axes was evaluated (the results of these studies will be discussed and summarized in **Paragraph 2**). Finally, the impact of modulation of potential physiological cues which might be involved in the neuroendocrine changes and metabolic alterations (increased circulating FFAs and deficit dopaminergic signalling), was investigated (the results of these studies will be discussed and summarized in **Paragraph 3 and 4** respectively).

1. Changes of spontaneous diurnal plasma hormone concentration patterns and secretion in obese premenopausal women

The first aim of this thesis was to delineate differences of diurnal spontaneous hormonal concentrations and secretion of the lactotroph, thyrotroph and corticotroph axis in obese vs. lean premenopausal women. Therefore, blood samples were taken during 24 h with a sampling interval of 10 min for the assessment of plasma hormone concentrations in obese premenopausal women and lean premenopausal female controls of similar age. All subjects were studied in the early follicular stage of their menstrual cycle. 24 h Plasma hormone concentration rhythms were mathematically analysed as described in Appendix B. Hormonal secretion rates were estimated by (multi parameter or waveform-independent) deconvolution analysis. Sizes of regional body fat mass were measured using MRI, whereas total body fat mass was calculated using DEXA.

Lactotroph axis in obese vs. lean premenopausal women (Chapter 2)

The release of PRL by the pituitary is tonically inhibited by dopamine through activation of the dopamine D2 receptor (D2R) on lactotroph cells (1). Obese humans appear to have reduced D2R binding sites in their brain (2). Therefore, it is hypothesized that spontaneous PRL release is enhanced in obese humans. Results of this study showed that PRL secretion was significantly enhanced in obese women (total daily release 137 ± 8 vs. lean controls 92 ± 8 µg/L/24 h, $P = 0.001$) in proportion to their BMI ($R^2 = 0.55$, $P < 0.001$) and in particular the size of their visceral fat depot (total PRL secretion vs. visceral fat area $R^2 = 0.64$, $P = 0.006$). These findings are in conflict with previous studies reporting that basal (single measured) PRL levels were similar and exogenously stimulated PRL concentrations were blunted in obese individuals (3-11). These differences might either be explained by the methods used (spontaneous PRL secretion has not been estimated

in obese humans before) or subjects enrolled in the present and previous studies. The observation that PRL was enhanced in obese women in proportion to the size of their visceral fat mass is in line with previous studies showing that PRL has lipogenic effects (12-18) and knock-out of the PRL receptor gene in mice causes loss of body fat, primarily from the visceral depot(19). Since PRL is inhibited by D2R activation, the elevated PRL secretion may reflect reduced D2R availability in the brain in obese premenopausal women. Diminished dopaminergic neuronal activity promotes body fat accumulation in (seasonally) obese animal models and in humans. Furhtermore, anti psychotic drugs, blocking D2R, promote body weight gain (20-22). Thus, this study implicates that PRL may be one of the endocrine messengers that relay reduced D2R mediated dopaminergic neural signals to peripheral tissues to promote (visceral) fat storage.

Thyrotroph axis in obese vs. lean premenopausal women (Chapter 4)

The hypothalamic pituitary thyroid (HPT) hormonal ensemble regulates energy balance (23-25). Recent evidence implicates leptin as an important modulator of thyroid axis activity (23;26-30). As obesity might be considered as a phenotypic expression of energy imbalance (31) and obese humans are hyperleptinemic, it is hypothesized that obese individuals have altered HPT axis activity. Results of this study showed that mean TSH concentration (obese 1.9 ± 0.2 vs. lean 1.1 ± 0.1 mU/L, P = 0.009) and secretion rate (obese 43.4 ± 5.5 vs. in lean 26.1 ± 2.2 mU/V_{dl} x 24 h, P = 0.011) were significantly enhanced in obese women, whereas the fasting free thyroxine concentrations were similar compared to normal controls (free T_4 in obese 15.4 ± 1.5 vs. in lean 16.4 ± 1.5 pmol/L, P = 0.147). Furthermore, TSH secretion was positively related to 24 h leptin concentrations (R^2 = 0.31, P = 0.007). Previous studies documented that basal (single measured) serum TSH concentrations are normal in obese humans (32;33), whereas the stimulated TSH response to TRH is enhanced, normal or impaired in obese subjects compared to normal weight controls (7;9;33-39). However, spontaneous TSH concentration profiles over 24 hours have not been measured in obese humans before. Different physiological cues, such as the stage of the menstrual cycle in which the women were studied or sex differences, might explain the differences between results of this study and those of previous investigators. As several studies provide strong evidence that leptin stimulates TSH production in rodents and humans, the finding that 24 h TSH secretion was positively related to mean 24 h leptin concentrations in the present study and may be interpreted as circumstantial evidence of a stimulatory impact of hyperleptinemia on TSH release in obese individuals. Alternatively, dopamine inhibits TSH synthesis and release through D2R activation in thyrotrophs of the pituitary gland, whereby it appears to specifically reduce the amplitude of pulsatile TSH release (40). As the increased TSH secretion rates of the obese subjects were primarily attributable to enhanced TSH pulse amplitude and the availability of D2R binding sites is considerably reduced in human obesity (2), reduced dopamine D2 receptor (D2R) mediated neurotransmission may also be involved in the enhanced TSH release in the obese humans enrolled in the present study.

Although a few studies demonstrated that serum T_3 concentrations were elevated in obese subjects (32;44;45), the majority of data suggests that there is no change in basal thyroid hormone concentrations in obese humans (33;41-43), which is in line with the results of the present study. However, the finding that TSH levels are elevated in the face of normal free T_4 in our obese subjects has never been described before. This phenomenon might be explained by impaired biological activity of TSH (through reduced dopaminergic signalling (46-48)) or unresponsiveness to exogenous TSH through increased sympathetic activity, as autonomic nervous system regulates the sensitivity of the thyroid gland to TSH (49-51).

Corticotroph axis in obese vs. lean premenopausal women (Chapter 7)

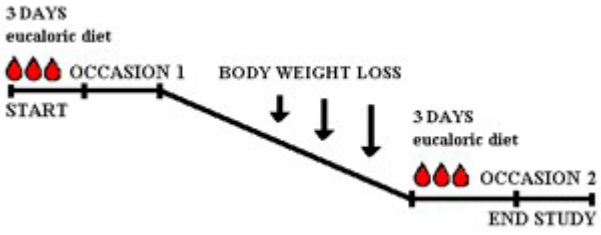
Based on several animal and clinical studies which document that obesity is associated with an exceedingly active hypothalamo-pituitary-adrenal (HPA) axis (52-63), it was hypothesized that the secretion rates of pituitary-adrenal hormones are enhanced in obesity. Daily ACTH secretion was substantially higher in obese than in lean women (7950 ± 1212 vs. 2808 ± 329 µg/24 h, P = 0.002), whereas cortisol was not altered (obese 36 362 ± 5639 vs. lean 37 187 ± 4239 nmol/24 h, P = 0.912). ACTH release rates correlate strongly with BMI, whereas the sizes of various fat areas (including visceral and subcutaneous fat depots) do not appear to be independently associated with ACTH production. Furthermore, the ACTH release process is less regular (as evidenced by ApEn statistics) in obese than in lean women. Regularity of hormonal secretion patterns mirrors the net result of feed forward signalling and feedback restraint. As cortisol secretion was not altered, it is stated that CRH, which is one of the strongest feed forward drives activating the HPA axis, may be increased

in obese humans. The occurrence of relatively low cortisol levels in face of elevated ACTH in the obese women enrolled in the present study, has been found in a few previous studies (64;65). Various mechanistic explanations for this neuroendocrine anomaly can be proposed, such as increased urinary cortisol excretion, insensitive adrenals (through increased sympathetic activity or leptin mediated peripheral inhibition of adrenal glucocorticoid production), reduced 21-hydroxylase activity (which would direct cortisol precursors towards androgen synthesis), increased 5- reductase activity (which converts cortisol to inactive cortisone) or increased 11βHSD type 1 (which catalyses the conversion of cortisone into active cortisol at tissue level). The exact pathophysiological implications of high plasma ACTH concentrations in the face of normal cortisol levels remain to be established.

2. Impact of weight loss on neuroendrocrine perturbations in obese premenopausal women

The second aim was to investigate the impact of body weight loss on the altered hormonal secretion of the lactotroph and thyrotroph axis in obese women. Therefore,
24 h plasma PRL and TSH concentrations were measured at 10 min intervals before and after weight loss (50% reduction of overweight, using a very low calorie diet) in eleven obese premenopausal women (BMI before weight loss 33.3 ± 0.7 kg/m^2) in the follicular phase of their menstrual cycle. Mathematical analysis of the hormone concentration patterns was performed (Appendix B). 24 h Hormone secretion rates were calculated using waveform-independent deconvolution technique (Pulse). Figure 1 is a schematic overview of the study.

Figure 1.



Lactotroph axis before and after weight loss in obese women (Chapter 3)

PRL release is inhibited by dopamine 2 receptor (D2R) and dietary restriction/weight loss are associated with increased dopaminergic signalling in animals(66). Therefore, it was hypothesized that enhanced PRL release in obese humans would be reversed by weight loss. Results of this study show indeed that elevated spontaneous 24 h PRL secretion was significantly reduced after weight loss in obese women (mean daily release before 128 ± 24 vs. after weight loss 110 ± 17 μg/V$_{dl}$ x 24 h, P = 0.05). Body weight loss particularly blunted PRL secretory burst mass (Pulse area before 230 ± 28 vs. after weight loss 221 ± 31 μg/V$_{dl}$ x min, P = 0.03), whereas burst frequency was unaffected (Number of pulses before 11 ± 1 vs. after weight loss 12 ± 1 n/24 h, P = 0.69). So far, variable results of studies evaluating the effects of caloric restriction and body weight loss on plasma PRL concentrations in humans have been described (67-71) and this is the first study to evaluate the effect of body weight loss on diurnal spontaneous PRL secretion rates in obese humans. Amelioration of deficit dopamine D2 receptor mediated neurotransmission can be involved in the physiology of this phenomenon, however dopaminergic neuronal activity was not directly assessed in the present study. The reduction of 24 h PRL secretion in response to weight loss in the present study was closely associated with the mean decrease of plasma leptin concentrations. Furthermore, findings of previous studies suggest that leptin plays a role in the control of PRL release (72-77). Thus, changes of leptin might be involved in the physiology of altered PRL secretion in response to body weight loss in the present study. In a variety of animal species PRL exerts potent lipogenic and diabetogenic effects and caloric restriction and weight loss tend to restore the metabolic profile to normal in obese individuals (78). Based on the data of this study it is postulated that the beneficial effect of long term caloric restriction on metabolic parameters in obese individuals may be brought about by amelioration of

deficit D2R mediated dopaminergic transmission in hypothalamic nuclei and that PRL serves as a messenger mediating the favourable effects of dopamine on glucose and lipid metabolism in peripheral tissues.

Thyrotroph axis before and after weight loss in obese women (Chapter 5)

Changes in body weight are accompanied by compensatory changes in energy expenditure (79), which may be brought about in part by adaptations of HPT axis activity (23-25). Studies in animals and humans show that leptin appears to be a regulator of the HPT axis. Therefore, it was hypothesized that weight loss induces adaptations of HPT axis activity in obese humans and that putative changes in leptin correlate with alterations of HPT axis activity. Results of this study show that weight loss significantly lowers TSH release (before 43.4 ± 6.4 vs. after weight loss 34.4 ± 5.9 mU/Lx24 h, P = 0.02) and circulating free triiodothyronine levels (from 4.3 ± 0.19 to 3.8 ± 0.14 pmol/L (P = 0.04). Differences in 24 h TSH release correlated positively with the decline of circulating leptin concentrations ($P < 0.01$, $R^2 = 0.62$). Most of the previous clinical studies evaluating the impact of body weight loss on the HPT axis showed that weight loss lowers single measurement of TSH and the TSH release in response to TRH, whereas others report unchanged thyroid hormones, plasma TSH or TRH induced TSH responses in obese individuals after weight loss (80-87). As the reduction of 24 h TSH secretion correlated with the decline of mean 24 h leptin concentrations in response to weight loss, this might implicate that leptin plays a possible role in the control of pituitary TSH release in (obese) humans.

Alternatively, other factors might modulate TSH production so as to decrease in response to weight loss in obese women. As TSH release is inhibited by D2R activity (40) and calorie restriction and weight loss are accompanied by increased D2R signalling in animals and probably also in humans (11;66), up-regulation of D2R tone in response to weight loss may reduce TSH secretion. As exogenous estrogens raise TSH concentrations (88) and estrogen levels significantly dropped after weight loss, estrogen might be involved in the modulation of HPT axis activity. Whatever the underlying mechanism, changes of HPT activity in response to body weight loss in obese humans may be of clinical and physiological relevance. Since thyroid hormones are among the regulatory cues involved in stimulating energy expenditure and basal metabolic rate (40), this neuroendocrine adaptation potentially frustrates obese humans in their attempts to lose weight.

3. Effect of Acipimox on neuroendocrine perturbations in obese premenopausal women

The **third aim** of this thesis was to study the impact of Acipimox, known as a lipid lowering drug which reduces circulating FFA levels, on the somatotroph and the corticotroph hormonal ensemble in obese premenopausal women. Therefore, plasma hormone concentrations of healthy obese premenopausal women were studied twice in the follicular phase of their menstrual cycle, with a time interval of at least 8 weeks where body weight remained stable. Obese women were randomly assigned to treatment with either Acipimox (an inhibitor of lipolysis, 250 mg orally four times daily) or placebo in a double blind cross-over design, starting one day prior to admission until the end of the blood sampling period. At each study occasion, blood samples were taken during 24 h with a sampling interval of 10 min for assessment of plasma hormone concentrations and hormone secretion was estimated by deconvolution analysis (**Appendix B**). Figure 2 is a schematic overview of the study.

Figure 2.

Somatotroph axis before and after Acipimox treatment in obese women (Chapter 6)

Both clinical as well as experimental animal studies have shown that Free Fatty Acids (FFAs) reduce hormonal secretion of the somatotroph axis (89-92). Obesity is associated with high circulating free fatty acid (FFA) concentrations (93;94) and hyposomatotropism (95). Therefore it hypothesized that reduction of circulating FFA levels with Acipimox, a powerful anti-lipolytic drug, enhances GH secretion in obese humans.

Results of this study showed that Acipimox unleashes spontaneous GH secretion in obese (Acipimox 113 ± 50 vs. Placebo 66 ± 10 mU/V_{dl}/24 h, P = 0.02). Diurnal GH secretion rates remained lower compared to lean controls (controls 201 ± 23 mU/V_{dl}/24 h, P = 0.005 vs. obese during Acipimox). Neuroendocrine alterations of the GH axis particularly occur in viscerally obese patients (96). Visceral fat is morphologically and functionally distinct from subcutaneous fat, in that cellularity and FFA turnover are higher per unit adipose tissue (97;98). Furthermore, venous output of visceral fat drains directly into the portal system of the liver, while FFAs from subcutaneous fat enter the systemic circulation. FFA infusion specifically into the portal vein enhances pituitary-adrenal axis and sympathetic nervous system activity, whereas systemic FFA infusion does not exert appreciable effects on these neuroendocrine systems (99-101). Thus, a high portal FFA flux, brought about by excess visceral fat, may particularly inhibit GH release. Therefore, we sought to determine the relationship between the effects of Acipimox and the size of various adipose depots. However, further analysis did not show any relationship between the effects of Acipimox on GH secretion and regional body fat distribution. This might be due to the limited size of our study population. The mechanism through which Acipimox stimulates GH secretion in obese individuals might be due to lowering circulating FFA, however, other potential mechanistic explanations for the profound impact of Acipimox on GH secretion may relate to its impact on plasma insulin levels or neural pathways such as dopamine. Finally, a direct effect of Acipimox on GH cannot be excluded. Findings of this study are in line with previous studies evaluating the effect of Acipimox on GH plasma levels in response to various exogenous secretagogues in obese humans (102-106). Thus, present and previous studies show that specifically enhances both exogenously as well as endogenously driven GH secretory burst mass. Therefore it is postulated that Acipimox may enhance somatotroph sensitivity to GHRH feed forward inputs, which appears to be a critical determinant involved in obesity related hyposomatotropism.

Corticotroph axis before and after Acipimox treatment in obese women (Chapter 7)

Experimental studies show that circulating free fatty acids (FFAs) promote the secretory activity of the HPA axis (99-101;107). Human obesity is associated with high circulating FFAs (93;94) and an exceedingly active hypothalamo-pituitary-adrenal (HPA) axis (52;54-60;62). Therefore, it is hypothesized that lowering of circulating FFAs by Acipimox would reduce HPA axis activity in obese humans. Results of this study showed that Acipimox significantly reduced ACTH secretion in the obese subjects (Acipimox 5850 ± 769 µg/24 h, P = 0.039 vs. placebo), while cortisol release did not change (Acipimox 33 542 ± 3436 nmol/24 h, P = 0.484 vs. placebo). This is the first study to evaluate the impact of Acipimox on secretion rates of pituitary-adrenal hormones in obese humans by deconvolution analysis. Findings of this study are in line with data from experimental studies, showing that elevation of circulating FFA by intralipid infusion raises plasma levels of ACTH (and corticosterone) (99-101;107). Although the exact mechanistic pathway through which Acipimox blunts ACTH secretion in obese individuals remains elusive, present results implicate that FFAs are indeed involved in the pathophysiology of this neuroendocrine anomaly.

4. Effect of Bromocriptine on neuroendocrine perturbations and food metabolism in obese premenopausal women

The **fourth aim** of this thesis was to study the impact of Bromocriptine, a dopamine D2 receptor (D2R) agonist which ameliorates dopaminergic neurotransmission, on food metabolism and leptin in obese premenopausal women. Therefore, eighteen healthy obese women were studied twice in the follicular phase of their menstrual cycle with a time interval of four weeks where body weight remained stable. Obese women were assigned to treatment with Bromocriptine or placebo in a single blind parallel design, starting eight days prior to admission until the end of the blood sampling period. At each study occasion, blood samples were taken during 24 h with a sampling interval of 10 min for the assessment of blood glucose and plasma insulin concentrations and with a 20 min sampling interval for the measurement of circadian plasma leptin concentrations. Plasma free fatty acids (FFA) and triglyceride (TG) plasma levels were measured hourly during 24 hours. Standardized eucaloric meals were served one day prior to admission until the end of the blood sampling period and caloric intake was identical at both study occasions. During each blood sampling period 24 h urine was collected. Fuel oxidation was determined by indirect calorimetry (ventilated hood) while subjects were fasting. Percentage total body fat was measured using DEXA. Figure 3 is a schematic overview of the study.

Figure 3.

Diurnal metabolic profiles and energy expenditure before and after Bromocriptine treatment in obese women (Chapter 8)

Diminished dopaminergic neuronal activity severely impairs insulin sensitivity and promotes body fat accumulation in (seasonally) obese animal models (108). In humans, anti psychotic drugs, blocking D2R, promote body weight gain and the development of type 2 Diabetes and hyperlipidemia (20-22). Obese humans appear to have reduced D2R binding sites in their brain (2). Therefore, it is hypothesized that short term amelioration of deficit D2R dopaminergic transmission by Bromocriptine would favourably affect diurnal metabolic profiles and energy balance in obese individuals.

Results of this study show that mean 24 h blood glucose (Bromocriptine 4.9 ± 0.1 vs. Placebo 5.4 ± 0.1 mmol/L, $P < 0.01$) and insulin (Bromocriptine 10.9 ± 0.8 vs. Placebo 13.3 ± 1.4 mU/L $P < 0.01$) were significantly reduced by Bromocriptine, whereas mean 24 h FFA and TG were increased (FFA Bromocriptine 0.57 ± 0.05 vs. Placebo 0.44 ± 0.03 mmol/L $P <0.01$ and TG Bromocriptine 1.34 ± 0.101 vs. Placebo 1.24 ± 0.1 mmol/L, $P = 0.14$). Bromocriptine increased oxygen consumption (Bromocriptine 243.6 ± 8.2 vs. Placebo 232.2 ± 5.7ml/min, $P = 0.03$) and resting energy expenditure by 50 kCal/day, $P = 0.03$). Finally, systolic blood pressure was significantly reduced by Bromocriptine (Bromocriptine 112 ± 3 vs. 122 ± 4 mmHg, $P = 0.04$). Previous studies have shown long-term Bromocriptine treatment effectively reduces fasting insulin and glucose levels in rodents and improves glucose tolerance in healthy and diabetic obese humans (109-115). However, chronic Bromocriptine administration consistently reduces body fat and food intake might have been altered in these studies, which could explain these metabolic corollaries of treatment. Data of this study strongly suggest that stimulation of D2R facilitates glucose metabolism in obese humans independent of body adiposity or food intake. The rise of circulating FFA levels induced by Bromocriptine may mirror the lipolytic properties of the drug, shifting energy balance away from lipogenesis in obesity. Although the exact mechanisms through which D2R dopaminergic neurotransmission impacts energy balance and fuel metabolism remain to be established, these findings support the notion that reduced D2R availability in the brain of obese humans directly contributes to their altered energy homeostasis and their metabolic anomalies.

Leptin before and after Bromocriptine treatment in obese women (Chapter 9)

Obese humans are hyperleptinemic and it has been postulated that obese individuals are leptin resistant (116). However, the mechanism involved with this neuroendocrine perturbation remains elusive and very little is know about the regulation of leptin secretion in vivo. Dopamine is among the neurotransmitters involved in the central adjustment of food intake, metabolism and hormonal secretion. A few previous studies provide evidence for an inhibitory effect of dopaminergic system activity on leptin secretion (117;118). As D2R binding capacity in the brain of obese humans is reduced, one might postulate that impaired dopaminergic signalling might be involved in the occurrence of hyperleptinemia in obese humans. Furthermore, short term treatment with the D2R agonist Bromocriptine profoundly alters metabolic profiles in obese women (P. Kok et al unpublished data) and previous studies have shown that changes of circulating metabolic parameters such as glucose, insulin and lipids are related to altered leptin secretion (119-130). Therefore, it is hypothesized that short term treatment with Bromocriptine reduces leptin concentrations in obese humans. Results of this study show that Bromocriptine significantly lowered diurnal leptin concentrations in obese premenopausal women (Mean 24 h concentration Bromocriptine 30.5 ± 2.5 vs. Placebo 33.6 ± 2.5 µg/L, $P = 0.03$). Furthermore, the decline of circadian leptin plasma levels is associated with the increase of FFA levels in response to Bromocriptine treatment in the obese subjects ($R^2 = 0.46$, $P = 0.03$). These results are in line with data obtained in these previous studies observing the effect of modulation of the dopaminergic activity on plasma leptin levels (117;118). Although the observed effect of Bromocriptine on leptin may also be mediated through other indirect mechanistic pathways, e.g. the effect of Bromocriptine on metabolic or hormonal

parameters, these findings implicate that leptin signalling/secretion is centrally regulated by neuronal dopaminergic systems in the brain. Thus, deficit dopaminergic signalling might be involved in the hyperleptinemic/leptin resistant state associated with obesity.

General Discussion and Future Perspectives

The studies of this thesis provide new insight of hormonal aberrations in obese women. In most of the previous studies investigating hormonal systems in obesity, single plasma hormone measurements were performed or exogenously stimulated hormone response peaks were studied. As the majority of plasma hormone concentrations fluctuate over the day and these circadian variations of serum hormone concentrations appear to be important for their biological function (17;131), proper appreciation of spontaneous hormonal concentrations requires analysis of circadian hormonal concentration patterns. Furthermore, circulating hormone concentrations result from combined influences of prior and ongoing hormone secretion, distribution and elimination. In the studies of this thesis different mathematical techniques were used to calculate these hormonal secretory and kinetic parameters from the hormone concentration time series data. It seems important to emphasize, that the design of the studies in this thesis also has some limitations. First of all, the studies were performed in a clinical setting under standardized physiological conditions which might be different from normal life outside the research center. For example, no changes of cortisol levels were found in obese and lean women when they remained recumbent in a clinical set-up. However, their cortisol levels in response to anticipatory stress or stressful experiences during daily activities could be different. Secondly, because of practical reasons, electro encephalogram (EEG) sleep recording was not performed during the studies. Therefore, great care was taken not to disturb and touch subjects during withdrawal of blood samples while they were sleeping. Lights were switched off/on and subjects went to sleep/were awakened at fixed time points. Periods of wakefulness and toilet visits during the night were recorded by the personnel performing nocturnal blood sampling. However, quantified data about sleep stages and sleep/wake cycles was not collected. In most of the studies standardized eucaloric meals were consumed at fixed time points at each study occasion, to limit nutritional confounding. It is important to note, that it is unclear from the literature how long a wash out period is needed exactly to “wash out” any potential confounding effect of calorie restriction per se on hormonal secretion. As, the secretion rate and/or plasma concentration of some hormones responds rather quickly (i.e. within hours to days) to changes in nutrient availability (132;133), we prescribed all obese subjects a standard liquid, eucalorie diet for 3 days prior to each study occasion to limit the putative impact of calorie restriction on hormonal release. However, we can not completely rule out the possibility of a persistent effect of the VLCD on the changes of hormonal secretion induced by weight loss. Finally, all subjects enrolled in the studies were premenopausal females. Whether the data of the studies in this thesis is extensible to obese men requires further investigation. Furthermore, all subjects were studied in the early follicular phase of their menstrual cycle. In this context, one might wonder whether the observed differences of hormonal secretion as measured in the follicular phase of lean and obese premenopausal women are a peculiarity of this phase of their menstrual cycle. Some clinical studies found altered basal hormone concentrations the peri-ovulatory and the luteal phase of the menstrual cycle (134-137), whereas others reported that no changes throughout different stages of the menstrual cycle (138-140). Thus, there is no conclusive evidence that the observed differences of hormonal secretion between obese and lean women are invariable during different stages of the menstrual cycle.

This thesis describes new observations and elucidated several facets of the altered hormonal milieu in obesity. Practising science is a never ending story; several fascinating questions remain to be answered. This paragraph will discuss some general conclusions and future perspectives. First of all, the changes of diurnal plasma PRL and TSH hormone levels and secretion in obese premenopausal women, provide indirect evidence for reduced dopaminergic signalling as a potential cue involved in the pathophysiology of hormonal alterations in obesity. Weight loss partly restores these neuroendocrine anomalies. Future studies are needed to directly assess the impact of weight loss on dopaminergic neuronal activity. For example, imaging studies assessing D2R availability in the brain of obese humans before and after weight loss could be performed. Alternatively, it is postulated that prolactin may be one of the endocrine messengers that relay reduced D2R mediated dopaminergic neural signals to peripheral tissues to promote (visceral) fat storage. Thus, PRL itself might have impact on peripheral glucose metabolism and adipogenesis in humans, which remains to be investigated.

Thirdly, TSH and ACTH were enhanced in face of normal thyroid hormones and cortisol levels in obese premenopausal women. Although we did not measure peripheral hormone metabolism and we therefore can not exclude the possibility that hormonal signalling at the level of the peripheral target organs was altered, these data implicate that peripheral sensitivity of the thyroid and adrenal gland towards the feed forward drive of the pituitary hormones (TSH and ACTH) is somehow hampered. Evidence from experimental animal studies suggests that the sensitivity of the adrenal cortex and thyroid gland to ACTH and TSH is centrally regulated by the suprachiasmatic nuclei via the autonomic nervous system. Since obesity appears to be associated with increased sympathetic activity, this might explain these endocrine phenomena. Present and previous studies show that Acipimox specifically enhances both exogenously as well as endogenously driven GH secretory burst mass. Furthermore, Acipimox specifically reduced the enhanced ACTH secretion by the pituitary gland. Collectively, these data suggest that FFA enhance HPA output and blunt GH secretion through effects on neuronal control systems in brain centres at the supra pituitary level. Although we cannot exclude that Acipimox itself directly impacts hormonal secretion, these findings implicate that circulating FFA are involved in the pathophysiology of pituitary-adrenal hyperactivity and hyposomatropism in obese humans.

Energy homeostasis is achieved by variable effects on energy intake, expenditure and storage, coordinated through the central nervous system (141;142). Signals related to either short term nutrient availability (e.g. nutrients and gastro intestinal peptides) or the amount of energy consumed over a more prolonged time period and proportion of body adiposity (the so called “long term” signals) emanate from adipose, endocrine, gastro-intestinal and neuronal systems. These efferent signals are received and integrated in the hypothalamus. On its turn, this specific brain area exerts homeostatic control over neuroendocrine secretion and energy homeostasis. Dopaminergic neurotransmission of this brain area is involved in the regulation of hormonal secretion and energy homeostasis. Hormonal changes described in chapter 2, 3, 4 and 5 provide indirect evidence that reduced dopaminergic signalling is involved in the pathophysiology of hormonal alterations in obesity and the studies in chapter 8 and 9 showed indeed that modulation of the dopaminergic system improves energy metabolism and blunts leptin levels in obese humans. Thus, next to its role regulating peripheral sensitivity of endocrine organs, the brain appears to be a central factor involved in the development and/or maintenance of the obese state and its associated metabolic perturbations.

Finally, one might wonder whether modulation of dopamine 2 Receptor signalling is a potential target for restoration altered neuroendocrine ensemble in human obesity. A few studies have shown long-term bromocriptine treatment consistently reduces body fat, fasting glucose and improves glucose tolerance in healthy and diabetic obese humans (109-115). However, chronic bromocriptine administration might have side effects and such corollaries of treatment have not been investigated in these studies. Long term follow up studies should be performed to evaluate long term effects and safety of chronic bromocriptine treatment.

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Samenvatting

Overgewicht en obesitas vormen een toenemend gezondheidsprobleem. Wereldwijd hebben nu meer dan 1 miljard volwassenen overgewicht en 300 miljoen mensen hiervan hebben obesitas. Obesitas wordt gedefinieerd als een toestand van excessieve vetophoping in het lichaam, waarbij gezondheid en welzijn negatief worden beïnvloed. De Body Mass Index (BMI) is heden ten dage de meest gebruikte index voor de classificatie en het in kaart brengen van overgewicht en obesitas. De BMI, ook wel Quetelet index genoemd, is lichaamsgewicht gecorrigeerd voor lengte en wordt berekend door het lichaamsgewicht (kg) te delen door lichaamslengte in het kwadraat (m^2). Tabel 1 geeft een overzicht van de classificatie van overgewicht en obesitas.

Tabel 1.

Classificatie	BMI (kg/m^2)	Risico op co-morbiditeit	Actie Niveau & Consequentie
Normaal	18.5-24.9	Gemiddeld	
Overgewicht	25-29.9	Licht verhoogd	1: Preventieve benadering: Voorkomen Gewichtstoename
Obesitas	≥ 30		2: Gewichtsreductie (10-15%) en
Niveau I	30-34.9	Matig verhoogd	stabilisatie van het lichaamsgewicht met professionele hulp
Niveau II	35-39.9	Ernstig verhoogd	
Niveau III	≥ 40	Zeer ernstig verhoogd	

Bron: Meinders AE, Fogteloo J. Ned Tijdschr Geneeskd. 2003 Sep 20;147(38):1847-51 en Richtlijnen WHO Tech Rep Ser 894, 2000

In Nederland heeft 28% van de volwassen vrouwen overgewicht en 10% obesitas, voor volwassen mannen zijn deze aantallen respectievelijk 43 en 10%. Ook de snelle toename van overgewicht bij kinderen en adolescenten is zorgwekkend. Wereldwijd zijn 30-45 miljoen kinderen obees.

Obesitas is niet goed voor de gezondheid. Het komt vaak voor in combinatie met insuline ongevoeligheid, hyperinsulinemie, dyslipidemie (hoog LDL cholesterol, hoge triglyceriden en laag HDL cholesterol) en hypertensie. Dit cluster aan symptomen heet Syndroom X of het metabool Syndroom en is gerelateerd aan hart- en vaatziekten. Obesitas op zich is een onafhankelijke risicofactor voor het krijgen van hart- en vaatziekten en suikerziekte en het speelt een rol bij het ontstaan van veel andere gezondheidsproblemen, zoals bijvoorbeeld ademhalingsproblemen, gewrichtsklachten, galsteenlijden, huidproblemen, infertiliteit en kanker.

Met name de aanwezigheid van overtollig vet in de buikholte verhoogt het risico op het ontstaan van de gezondheisproblemen geassocieerd met obesitas. Uit onderzoek blijkt, dat mensen met veel visceraal vet (vet in de buikholte) een hoge bloeddruk hebben en hogere spiegels van schadelijke vetten in het bloed hebben en hun lichaam minder gevoelig is voor het hormoon insuline, dat belangrijk is voor de suiker- en vetstofwisseling. Waarom visceraal vet de stofwisseling en de bloeddruk nadelig beïnvloedt, is nog niet bekend, maar de vetzuren in het bloed lijken hierbij een belangrijke rol te spelen. Vetzuren worden

opgeslagen in vetweefsel, maar kunnen ook weer aan het bloed worden afgegeven. Visceraal vet is anders dan vet elders in het lichaam, omdat het vetzuren afgeeft aan het grote bloedvat dat direct naar de lever stroomt (de vena portae), terwijl vet elders in het lichaam zijn vetzuren afgeeft verspeid over de bloedbaan in het hele lichaam. Zo veroorzaakt overmatig visceraal vet een toename van de stroom vetzuren naar de lever. Bij proefdieren bleek het toedienen van vetzuren in de vena portae te leiden tot verhoogde bloeddruk en verminderde gevoeligheid voor insuline. Na toediening van vrije vetzuren in de vena portae bleken ook delen van de hersenen geactiveerd te worden die hormoonspiegels regelen die weer invloed hebben op de bloeddruk en de insulinegevoeligheid. Het negatieve effect van visceraal vet bij de mens op de stofwisseling en de bloeddruk kan dus veroorzaakt worden door de grote stroom vetzuren uit visceraal vet naar de lever, en de daardoor veranderde hersenactiviteit.
De middelomvang weerspiegelt bij de mens goed de hoeveelheid visceraal vet en is een goede voorspeller voor ziekte en sterfte geassocieerd met excessieve toename van zowel het totale lichaamsvet als de hoeveelheid visceraal vet . Daarom zijn er op basis van populatiestudies actie-niveaus vastgesteld voor middelomvang met als doel groepen en individuen met verhoogd gezondheidrisico ten gevolge van stapeling van het visceraal vet te kunnen identificeren, hun lichaamsgewicht te optimaliseren en gezondsheidsrisico's te reduceren (tabel 2).

Tabel 2.

Middelomvang (cm)		Gezondheidsrisico	Actie-niveau
Vrouw	Man		
68-80	79-94	Normaal	
80-88	94-102	Verhoogd	1: Voorkomen gewichtstoename
> 88	> 102	Substantieel verhoogd	2: Gewichtsreductie met professionele hulp

Bron: Lean et al. Br Med J 1995;311:158 en Lean et al. Lancet 1998;351:853-856

Er zijn waarschijnlijk zowel genetische factoren, sociale factoren, gedragsfactoren als biologische factoren betrokken bij het ontstaan van obesitas. Dit proefschrift richt zich op één van de biologische factoren die mogelijk betrokken zijn bij de ontwikkeling of het in stand houden van obesitas en wel het hormonale milieu. Verschillende hormonen spelen een rol bij de energiehuishouding en de stofwisseling. In hoofdstuk 1 (**General Introduction**) wordt de invloed van diverse hormoonsystemen op de stofwisseling (het metabolisme) en de energiebalans nader toegelicht. Onderzoek naar hormoonveranderingen bij mensen met obesitas werd tot nu toe meestal verricht met slechts enkele metingen van hormoonconcentraties. Hormonen worden echter in pulsen uitgescheiden in het bloed en variatie van hormoonconcentraties gedurende de dag speelt een rol bij de biologische functie. Daarom zijn bij de studies in dit proefschrift 24 uurs hormoonritmes onderzocht. Circulerende hormoonconcentraties in de bloedbaan zijn het netto resultaat van continue uitscheiding, verdeling en klaring van het betreffende hormoon. Voor het berekenen van een aantal van deze parameters en het analyseren van hormoonritmes zijn speciale wiskundige computerprogramma's ontwikkeld. In **Appendix B** staat een overzicht van de methodes die gebruikt zijn bij de analyse van de hormoonritmes in dit proefschrift.
Het doel van dit proefschrift is om inzicht te krijgen in de veranderingen in de regeling van de hormoonhuishouding bij mensen met obesitas. Daartoe werden eerst de spontane 24 uurs ritmes van verschillende hormoonassen bestudeerd bij mensen met en zonder obesitas (zie paragraaf 1). Daarnaast werd het effect van gewichtsreductie op een aantal van deze hormoonsystemen onderzocht (zie paragraaf 2). Tenslotte werd bestudeerd wat er gebeurt als deze hormoonsystemen van buitenaf beïnvloed worden met medicijnen. (zie paragraaf 3 en 4).

§1. De hormoonhuishouding bij premenopausale vrouwen met en zonder obesitas.

Het eerste doel van dit onderzoek was om te zoeken naar verschillen in de spontane hormoonproductie bij mensen met en zonder overgewicht. Tijdens deze studies onderzocht de spontane 24 uurs hormoonproductie van obese (BMI>30-35 kg/m^2) en normaal gewichtige (BM I <25 kg/m^2) premenopausale vrouwen van gelijke leeftijd. De proefpersonen werden hiervoor opgenomen in het onderzoekscentrum van het LUMC. Na opname werd een infuus met een veneuze catheter in

de onderarm aangebracht. Hieruit werd gedurende 24 uur elke 10 minuten bloed afgenomen voor de bepaling van de hormonen. Tijdens deze meting lagen de vrijwilligers op bed. Overdag mochten de vrijwilligers niet slapen, maar konden ze lezen of video kijken. Er werd op vaste tijden gegeten. Om 23:00u gingen de vrijwilligers slapen, terwijl de bloedafnames doorgingen. Na de bepaling van de hormoonconcentraties werden de 24 uurs ritmes geanalyseerd met behulp van specifieke wiskundige technieken (Appendix B). De precieze hoeveelheid lichaamsvet werd door middel van een DEXA scan gemeten en de hoeveelheid visceraal vet met behulp van magnetische resonantie (MRI-scan).

Prolactine bij premenopausale vrouwen met en zonder obesitas (Hoofdstuk 2)

Prolactine wordt geseceneerd in de hypofyse voorkwab (pijnappelklier) en is een hormoon dat zeer veel verschillende functies heeft. Studies met dieren laten zien dat het de vorming van vetweefsel stimuleert. Bij genetisch gemanipuleerde muizen die geen functionele prolactine receptor hebben, is de hoeveelheid visceraal vet afgenomen. Verschillende resultaten zijn gevonden in studies naar prolactine secretie of basale prolactine concentraties in mensen met obesitas. Prolactine secretie wordt geremd door dopamine via de activatie van de dopamine 2 receptor op de celmembraan van de prolactine producerende cellen. De dopamine 2 receptor expressie is verlaagd bij mensen met obesitas. Daarom was de hypothese van het onderzoek beschreven in hoofdstuk 2, dat prolactine hoger is bij mensen met overgewicht. Resultaten van deze studie laten inderdaad zien dat de PRL secretie is verhoogd bij vrouwen met overgewicht vergeleken met controle personen met een normaal gewicht. Verder hangt de 24 uurs prolactine secretie samen met de hoeveelheid visceraal vet. Deze resultaten impliceren dat prolactine een van de endocriene signalen is die de verlaagde dopaminerge tonus in het brein bij obesitas weerspiegelt, wat leidt tot een stimulatie van vetstapeling in de buik.

Hypofyse schildklier as bij premenopausale vrouwen met en zonder obesitas (Hoofdstuk 4)

TSH wordt geproduceerd in de hypofyse voorkwab en stimuleert de schildklier tot productie van schildklierhormonen, waaronder thyroxine. De hypofyse schildklier as reguleert de stofwisseling. Een langzaam werkende schildklier (hypothyreoidie) gaat gepaard met gewichtstoename en verminderde eetlust, terwijl er bij een snel werkende schildklier (hyperthyreoidie) sprake is van gewichtsverlies en normale of verhoogde voedselinname. Een groot aantal studies heeft de hypofyse schildklier as onderzocht bij mensen met obesitas, maar er is geen eenduidige verandering beschreven van deze hormoonas bij obesitas. Resultaten van het onderzoek dat staat beschreven in hoofdstuk 4 van dit proefschrift, laten zien dat de productie van schildklier stimulerend hormoon (TSH) is verhoogd, terwijl het vrij thyroxine (fT_4) hetzelfde is in vrouwen met obesitas vergeleken met controle personen met een normaal gewicht. Verder wordt er in deze studie gevonden dat de TSH secretie postief correleert met het hormoon leptine. Leptine is een hormoon dat uitgescheiden wordt door de vetcel en een signaal doorgeeft aan de hersenen over de hoeveelheid vetreserve in het lichaam. Mensen met obesitas hebben zeer hoge leptine concentraties in het bloed. Studies in dieren laten zien dat leptine de TSH productie stimuleert. Dit zou kunnen betekenen dat leptine een van de factoren is die betrokken is bij de veranderde TSH productie bij obesitas. De bevinding van de verhoogde TSH productie bij onveranderde vrij thyroxine (fT_4) waarden zou verklaard kunnen worden door een verlaagde biologische activiteit van TSH of door ongevoeligheid van de schildklier voor TSH.

Hypofyse bijnier as bij premenopausale vrouwen met en zonder obesitas (Hoofdstuk 7)

In de hypofysevoorkwab wordt ook het hormoon ACTH geproduceerd, dat de afgifte van cortisol in de bijnierschors stimuleert. De hypofyse bijnier as heeft invloed op het vetmetabolisme en de lichaamsvetverdeling. Mensen met te veel circulerend cortisol (het syndroom van Cushing) hebben veel visceraal vet. Verder hebben verschillende studies in dieren en mensen laten zien dat de hypofyse bijnier as overactief is bij obesitas. De studie die staat beschreven in hoofdstuk 7 van dit proefschrift, laat zien dat de ACTH productie is verhoogd, terwijl de cortisol productie onveranderd is bij vrouwen met obesitas vergeleken met controle personen met een normaal gewicht. Verschillende verklaringen kunnen worden gevonden voor dit endocriene fenomeen, zoals bijvoorbeeld een verhoogde klaring in de urine, ongevoeligheid van de bijnieren voor ACTH of een verandering van het cortisol metabolisme op weefselniveau.

§2. De invloed van gewichtsverlies op de hormoonhuishouding bij premenopausale vrouwen met obesitas.

Gewichtsverlies verbetert de stofwisseling en de energiebalans bij obesitas. Verschillende studies laten zien dat gewichtsverlies gepaard gaat met hormonale veranderingen. De groeihormoonspiegels gaan bijvoorbeeld omhoog en leptine gaat naar beneden na gewichtsreductie bij mensen met obesitas. Studies waarin het effect van gewichtsreductie op de hypofyse schildklier as of prolactine wordt onderzocht laten echter wisselende resultaten zien. Het **tweede doel** van dit onderzoek was om te kijken wat het effect is van gewichtsreductie op de hormoonhuishouding van de hypofyse schildklier as en prolactine bij vrouwen met obesitas. Tijdens deze studies werd de spontane 24 uurs hormoonproduktie bij obese premenopausale vrouwen (BMI > 30-35 kg/m^2) tweemaal onderzocht. Na de eerste 24 uurs meting startten de vrijwilligers met een afvalprogramma met als doel de reductie van 50% van hun overgewicht. Het gewichtsreducerend programma bestond uit een zeer laag calorisch dieet (500kCal/2010 kJ per dag) en medische begeleiding in het onderzoekscentrum. Daarna werd er nogmaals een 24 uurs meting gedaan. Om invloeden van het zeer laag calorisch dieet zèlf op de hormoonmetingen te beperken kregen de deelnemers drie dagen voorafgaand aan de onderzoeksdag een eucalorisch dieet (tot het eind van de studie). Tijdens het tweede studiedeel ging alles precies hetzelfde als tijdens de eerste studiedag; het enige verschil was dat de proefpersonen de helft van hun overgewicht waren kwijt geraakt.

Prolactine voor en na gewichtsreductie bij obese premenopausale vrouwen (Hoofdstuk 3)
Prolactine secretie wordt geremd door dopamine en gewichtsverlies is geassocieerd met een verhoging van de dopaminergische activiteit. Resultaten van dit onderzoek laten zien dat de prolactine productie inderdaad lager is na gewichtsreductie in vrouwen met obesitas. De daling in prolactine was gecorroleerd met de daling in leptine wat zou kunnen betekenen dat leptine betrokken is bij de verandering van de prolactine secretie na gewichtsreductie. Gezien de effecten van prolactine op de suikerstofwisseling zou de daling van prolactine na gewichtsreductie een rol kunnen spelen bij de verbetering van het metaboolprofiel na gewichtsverlies bij obesitas.

Hypofyse schildklier as voor en na gewichtsreductie bij obese premenopausale vrouwen (Hoofdstuk 5)
Resultaten van deze studie laten zien dat gewichtsreductie leidt tot een daling van de TSH secretie bij vrouwen met overgewicht. De daling van de TSH productie correleerde met de daling van leptine. Dus leptine is mogelijk een van de betrokken factoren bij deze neuro-endocriene verandering bij afname van overgewicht. Omdat de schildklier as het energieverbruik en de basale stofwisseling reguleert, kan deze neuro-endocriene verandering mensen met obesitas hinderen bij het behoud van hun verloren lichaamsgewicht.

§3. De invloed van Acipimox (vetzuur verlagend geneesmiddel) op de hormoonhuishouding bij premenopausale vrouwen met obesitas.

Vrije vetzuren worden uitgescheiden door de vetcel in het bloed. Mensen met obesitas hebben een verhoogde concentratie circulerende vrije vetzuren in het bloed. Het **derde doel** van dit promotieonderzoek was om na te gaan wat het effect is van het verlagen van de afgifte van vetzuren door het geneesmiddel Acipimox op de hormoonhuishouding bij vrouwen met obesitas. Tijdens deze klinische studies met een prospectief, dubbel blind, gerandomiseerd, cross-over design, werd de 24 uurs hormoonproductie tijdens placebo en Acipimox behandeling (250 mg 4 d.d. gedurende 2.5 dagen) bij gezonde obese premenopausale vrouwen (BMI > 30 kg/m^2) onderzocht. Tussen de twee studiedagen zat een tijdsperiode van 8 weken. Tijdens het tweede studiedeel ging alles precies hetzelfde als tijdens de eerste opname; het enige verschil is dat de proefpersonen placebo i.p.v. Acipimox (of vice versa) gebruikten.

Het effect van Acipimox op Groeihormoon bij obese premenopausale vrouwen (Hoofdstuk 6)
Groeihormoon (GH) stimuleert de lipolyse (vrijkomen van vetzuren uit de vetcel) en remt de opslag van vet (lipogenese). De groeihormoonconcentraties in het bloed zijn lager dan normaal bij mensen met obesitas. Hoe dit komt, is niet bekend.

Vrije vetzuren verlagen de groeihormoonproductie. Daarom is de hypothese dat groeihormoonproductie omhoog gaat bij het verlagen van vrije vetzuren in het bloed bij mensen met obesitas. Resultaten van dit onderzoek laten zien dat de groeihormoonproductie inderdaad hoger is na vetzuurverlaging met Acipimox. Omdat dit effect vooral komt door een toegenomen pulsatiele productie van groeihormoon en andere studies laten zien dat de exogeen GHRH gestimuleerde groei hormoon response piek ook hoger is na toediening van acipimox, wordt er geconcludeerd dat er een verhoogde sensitiviteit is voor GHRH na Acipimox.

Het effect van Acipimox op de Hypofyse bijnier as bij obese premenopausale vrouwen (Hoofdstuk 7)
Studies bij dieren laten zien dat toediening van vrije vetzuren leidt tot een verhoogde activiteit van de hypofyse bijnier as. Mensen met obesitas hebben een verhoogde hoeveelheid circulererende vrije vetzuren en een hogere activiteit van de hypofyse bijnier as. Daarom is de hypothese dat verlaging van vrije vetzuren met Acipimox leidt tot een daling van de circulerende hormonen van de hypofyse bijnier as bij mensen met obesitas. Resultaten van dit onderzoek wijzen inderdaad uit dat de ACTH secretie daalt na Acipimox, terwijl de cortisol productie onveranderd is. Deze bevindingen impliceren dat de vrije vetzuren inderdaad betrokken zijn bij de veranderingen van de circulerende hormonen van de hypofyse voorkwab bij obesitas en dat aanpassing van de bijnierschorsfunctie plaats vindt.

§4. De invloed van Bromocriptine (geneesmiddel dat de dopamine receptor activeert) op de hormoonhuishouding bij premenopausale vrouwen met obesitas.

Dopamine is een neurotransmitter die betrokken is bij de regulatie van het voedselmetabolisme en de hormoonhuishouding. Het effect van dopamine op de doelwitcel vindt plaats door de activatie van de dopamine 2 receptor (D2R) op de celmembraan van de doelwitcel. Er zijn aanwijzingen in de literatuur dat de dopamine 2 receptor verminderd tot expressie komt in het brein van mensen met overgewicht. Verder laat een groot aantal studies bij dieren en mensen zien dat blokkade van de dopamine 2 receptor leidt tot de ontwikkeling van obesitas en het metabool syndroom. Het **vierde doel** van dit onderzoek was om na te gaan wat het effect is van dopamine 2 receptor activatie door het geneesmiddel Bromocriptine op de hormoonhuishouding en de energiebalans bij vrouwen met obesitas. Tijdens deze studies met een prospectief, single blind, parallel design, werden 24 uurs metingen van metabole parameters en leptine verricht tijdens placebo en Bromocriptine behandeling (5.0 mg d.d. gedurende 8 dagen) bij 18 gezonde obese premenopausale vrouwen (BMI>30-35 kg/m^2). Op de eerste dag van de menstruatie begonnen de vrijwilligsters met het nemen van Bromocriptine of placebo. Op dag zeven werd naast het medicament, een dieet bestaande uit drinkmaaltijden gebruikt waarbij alleen water werd gedronken. Dit dieet was een vervanging van het normale dieet en bestaat uit pakjes nutridrink. Het drinkmaaltijdendieet werd gegeven om invloeden van voedselinname op de hormoonmetingen en metabole parameters uit te sluiten en het duurde twee dagen (tot het eind van de studiedag). Bij opname in het onderzoekscentrum werd eerst het energieverbuik in rust gemeten door middel van indirecte caloriemetrie (ventilated hood). Daarna werd gedurende 24 uur elke 10 minuten bloed afgenomen voor de bepaling van de hormonen. Urine werd verzameld om onder andere de hormoonafgifte in de urine te meten. Na afloop van de eerste meting stopten de vrijwilligsters met de inname van de medicatie. Na ongeveer twee tot drie weken startten de vrijwilligsters met de tweede behandeling (Bromocriptine). Op dag 7 startte weer het drinkmaaltijdendieet en op dag 8 vond het tweede studiedeel plaats. Tijdens het tweede studiedeel was het onderzoeksprotocol hetzelfde als tijdens de eerste studiedag, het enige verschil is dat nu bromocriptine i.p.v. placebo werd gebruikt.

Het effect van Bromocriptine op het metabool profiel en het energiegebruik bij premenopausale vrouwen met obesitas (Hoofdstuk 8)
Verlaagde dopaminerge activiteit leidt tot obesitas en insulineongevoeligheid bij obese knaagdieren. Verder is behandeling van mensen met dopamine 2 receptor blockers geassocieerd met toename in lichaamsgewicht en insulineresistentie. Mensen met obesitas hebben een verlaagde dopamine 2 receptor expressie in het brein. Daarom is de hypothese dat activatie van de dopamine 2 receptor leidt tot een verbetering van het metabool profiel bij mensen met obesitas. Resultaten van deze studie laten zien dat de 24 uurs bloedglucose en insulineconcentraties daalden tijdens bromocriptine behandeling,

terwijl de vrije vetzuren stegen. Verder was er een stijging van het energiegebruik in rust en een daling van de systolische bloeddruk. Deze resultaten impliceren dat de suikerstofwisseling verbetert bij stimulatie van de dopamine 2 receptor, onafhankelijk van voedselinname of daling van lichaamsvet. Verder zou de stijging van de vetzuren de lipolytische ("vet afbrekende") effecten van Bromocriptine kunnen weerspiegelen, wat leidt tot een verschuiving van de energiebalans naar vetafbraak. Deze studie impliceert dus dat verlaagde dopamine 2 receptor expressie in het brein bijdraagt aan de minder gunstige energiehuishouding en het veranderde voedselmetabolisme bij obesitas.

Het effect van Bromocriptine op leptine bij premenopausale vrouwen met obesitas (Hoofdstuk 9)

Leptine stimuleert het energieverbruik en remt het de voedselinname. Leptine deficiente dieren en mensen zijn obees en hebben een toegenomen voedselinname. Mensen met obesitas hebben zeer hoge leptine concentraties in het bloed. Daarom wordt er verondersteld dat obese mensen leptine ongevoelig (resistent) zijn. Er is erg weinig bekend over de regulatie van leptine secretie in vivo. Dopamine is een van de neurotransmitters die betrokken is bij de regulatie van de energiebalans, het voedselmetabolisme en de hormoonhuishouding. Enkele studies leveren (indirect) bewijs voor een remmend effect van dopamine op plasma leptine concentraties. Verder zijn veranderingen van metabole parameters geassocieerd met veranderingen in leptine secretie en heeft modulatie van de dopaminerge tonus invloed op het metabole profiel. Daarom is de hypothese dat behandeling met de D2R agonist bromocritpine, de leptine concentraties reduceert. Resulaten van deze studie laten zien dat de leptine concentraties daalden na behandeling met bromocriptine. De daling van leptine concentraties was positief gecorroleerd met de stijging van de plasma vrije vetzuren bij behandeling met bromocriptine. De flux van vrije vetzuren uit de adipocyt leidt tot een daling van de leptine productie. Deze bevinding zou dus kunnen betekenen dat bromocriptine leptine inhibeert door een daling van de vrije vetzuur flux uit adipocyten.

§5. Algemene Conclusies

Tezamen leveren deze studies nieuwe inzichten in hormonale veranderingen bij obesitas. Een aantal van deze studies levert (indirect) bewijs dat veranderingen van het dopaminerge systeem een belangrijke rol spelen bij de hormonale en metabole veranderingen bij vrouwen met obesitas. Uit verder onderzoek zou moeten blijken wat de rol is van de hormonen zèlf (mn. Prolactine) op de suiker- en vetstofwisseling op weefselniveau bij obese mensen. Verder is het waarschijnlijk dat het dopaminerge systeem betrokken is bij het effect van gewichtsreductie op het hormonale milieu. De directe invloed van gewichtsreductie op het dopaminerge systeem zou nog verder kunnen worden uitgezocht.

Daarnaast wordt er in dit proefschrift gepostuleerd dat de gevoeligheid van de schildklier en bijnier voor hun stimulerende hormonen, lijkt te zijn afgenomen. Dit zou verklaard kunnen worden door een verhoogde activiteit van het sympatische zenuwstelsel bij obesitas.

Het verlagen van vrije vetzuren met behulp van Acipimox heeft een gunstige invloed op de onderzochte hormoonveranderingen bij vrouwen met obesitas. Waarschijnlijk komt dit effect van vertzuurverlaging of Acipimox zèlf door een veranderde aansturing op het niveau van de hypothalamus.

Het brein lijkt dus een majeure rol te spelen bij het veranderde endocriene milieu van obese individuen. De vraag of verandering van centrale dopaminerge neurotransmissie een potentieel effectief en veilig therapeutisch doel is bij de behandeling van obesitas is nog niet beantwoord en verdient nader onderzoek.

Curriculum Vitae

Petra Kok werd geboren op 8 september 1979 te Laren, Noord- Holland. Na het behalen van haar VWO diploma aan het Sondervick College te Veldhoven, studeerde ze een jaar Geneeskunde aan de Rijksunversiteit van Gent. In september 1998 begon ze met de studie Geneeskunde aan de Universiteit van Leiden. Tijdens haar studie deed ze onderzoek naar eiwitexpressie in goed- en kwaadaardige kraakbeentumoren bij de afdeling Pathologie van het LUMC onder begeleiding van Prof. Dr. P.C.W. Hoogendoorn. Daarnaast assisteerde ze 's nachts bij 24 uurs ritme studies in het onderzoekscentrum van de afdeling Algemene Interne Geneeskunde bij het promotieonderzoek van Dr. S.W. Kok en Dr. M.M. Buijs. Hierdoor ontstond haar interesse in het klinisch onderzoek en het vakgebied endocrinologie. Om ervaring op te doen met de diverse mathematische technieken voor de analyse van 24 uurs hormoonritmes volgde ze een extracurriculaire wetenschapsstage van 6 maanden in het General Clinical Research Center van de University of Virginia (Charlottesville, USA) bij de onderzoeksgroep van Prof. Dr. J.D. Veldhuis. Op 17 december 2002 behaalde ze haar doctoraal Geneeskunde. Daarna startte ze op de afdeling Algemene Interne Geneeskunde van het LUMC onder begeleiding van Prof. Dr. A.E. Meinders en Dr. H. Pijl met het promotieonderzoek dat staat beschreven in dit proefschrift.

Publicaties

Short term bromocriptine treatment lowers diurnal plasma leptin concentrations in obese premenopausal women

Petra Kok, Ferdinand Roelfsema, Marijke Frölich, Johannes van Pelt, A Edo Meinders, Hanno Pijl

J Clin Endocrinol Metab. (Accepted)

Bromocriptine facilitates glucose metabolism, enhances basal metabolic rate and lowers blood pressure in obese women: Mechanistic independence of food intake and loss of body fat

Petra Kok, Ferdinand Roelfsema, Marijke Frölich, Johannes van Pelt, Marcel P.M. Stokkel, A Edo Meinders, Hanno Pijl

Am J Physiol Endocrinol Metab. (Accepted)

Research Award- Oral presentation Internistendagen, April 2005, Maastricht, The Netherlands

Decrease of visceral fat following diet induced weight loss in upper body compared to lower body obese premenopausal women

Janneke G Langendonk, MD, PhD, **Petra Kok, MSc**, Marijke Frölich, MD, PhD, Hanno Pijl, MD, PhD, A. Edo Meinders, Prof, MD, PhD

Eur J Internal Medicine (Accepted for publication)

Spontaneous diurnal TSH secretion is enhanced in proportion to circulating leptin in obese premenopausal women

Petra Kok, Ferdinand Roelfsema, Marijke Frölich, A. Edo Meinders, Hanno Pijl

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Elevated diurnal TSH release is blunted after body weight loss in obese women

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De waarde en beperkingen van de Body Mass Index (BMI) voor het bepalen van het gezondheidsrisico van overgewicht en obesitas

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Ned Tijdschr Geneeskd 2004 27 November 148(48): 2379-2382

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Nawoord

Aan dit promotieonderzoek en de totstandkoming van het proefschrift leverden verschillende mensen een belangrijke bijdrage. Ik wil graag mijn paranimfen, collega's in het LUMC, student-assistenten, vrienden en familie bedanken voor hun steun en interesse tijdens dit promotieonderzoek.

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Appendix A

Abbreviations

ACTH	adreno corticotropin hormone
ApEn	approximate entropy
AVP	arginine vasopressin
AUC	area under the curve
BF	body fat
BMI	body mass index
CART	cocaine- and amphetamine-regulated transcript
CRH	corticotropin releasing hormone
CSF	cerebro spinal fluid
CV	inter-assay coefficients of variation
D2R	dopamine 2 receptor
DA	dopamine
DEXA	dual energy X-ray absorptiometry
E2	estrogen
EEG	electro encephalo gram
FFAs	free fatty acids
fT_4	free thyroxine
GH	growth hormone
GHRH	growth hormone releasing hormone
HOMA	homeostatic model assessment
HPA	hypothalamic-pituitary-adrenal
HPT	hypothalamic pituitary thyroid
ICV	intra cerebroventricular
IGF-1	insulin like growth factor type 1
IV	intra venous
LPS	lipopolysacharide
LUMC	leiden university medical centre
MET	metoclopramide
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
N	nitrogen
NE	norepinephrine
NPY	neuropeptide Y
POMC	pro-opiomelanocortin
PRL	prolactin
T_3	triiodothyronine
TG	triglyceride
TSH	thyroid stimulating hormone
TRH	thyrotropin releasing hormone
TBFM	total body fat mass
T_4	thyroxine
SEM	standard error of the mean
SFM	subcutaneous fat mass
REE	resting energy expenditure
VCO_2	volume of carbon dioxide
VD	distribution volume
VFM	visceral fat mass
VLCD	very low calorie diet
VMH	ventromedial hypothalamus
VO_2	volume of oxygen
WHO	world health organization

Appendix B:

Analysis 24 h hormone profiles

Endocrine glands secrete hormones in a temporal manner that is of major importance to achieve appropriate physiological functioning, e.g. the cellular response in target tissues. Quantifying secretion profiles rather then merely inspecting plasma hormone concentration patterns reveals additional information about pulse duration, pulse shape, pulse height, pulse timing and clearance rates (1). The prominent intermittency of hormonal release can be either rhythmic (regularly repeating secretion episodes over time) or episodic (apparently randomly scattered secretion events over time). Calculation of regularity and circadian rhythmicity of hormone concentration time series data provides additional insight of hormonal release (2). Various validated mathematical techniques have been developed to appraise these parameters from hormone concentration patterns (for review see (3)). Diurnal concentration patterns of different neuroendocrine systems in obese premenopausal women enrolled in the clinical studies described in this thesis were analyzed using Cluster, Deconvolution, Cosinor, Cleveland robust fitting and Approximate Entropy (ApEn) algorithms. The operating principles and a general introduction of these mathematical techniques will be discussed in this appendix.

Cluster Analysis

The first developed pulse detection method by Johnson and Veldhuis was the Cluster analysis method (4). This method uses a sliding pooled t-test to identify data points within the hormone time series that correspond to statistically significant increases and decreases in hormone concentrations (changes at the edges of times series are not identified). Thus, the Cluster program identifies locations and durations of significant plasma hormone peaks. In performing the analysis, one has to specify individual test cluster sizes for the nadir and the peak (i.e., number of points to be used in testing nadirs against peaks), a minimum and maximum of intra series coefficient of variation, a t-statistic to identify a significant increase and a t-statistic to identify a significant decrease. The following parameters are estimated: mean concentration, total area under the curve, peak frequency, mean peak height (maximum value attained within a peak), peak amplitude (mean incremental peak height), incremental peak height as a percentage of nadir, mean peak area (above the baseline) and mean inter peak valley concentration (nadir).

Deconvolution Analysis

As the Cluster program does not provide information about the secretion and elimination of hormones, the deconvolution analysis was developed (1). Deconvolution analysis is a statistically based algorithm that estimates hormone kinetics and secretion rates from hormone concentration time-series (5;6). The general approach of the deconvolution technique is to derive a mathematical model for the form of a hormone concentration pulse an then, using nonlinear least-square methods, fit the actual experimental data to a series of these mathematical forms (secretory bursts) occurring at various times. Each secretory burst has a specific waveform (shape), which is dependent on the appearance, distribution and the clearance of the hormone from plasma or serum. Disappearance of hormones from plasma is best described by a two compartment model, characterized by a fast component half-life, a slow component half-life and a fractional contribution of the slow component to the overall decay. The Pulse algorithm is a waveform-independent deconvolution method, which can be used for calculation of hormonal secretion, without specifying shape, number and time of secretory events (1). The technique

requires a priori specification of hormonal half-life in plasma, although the algorithm is relatively insensitive to the assumed values of elimination half-lives. Thus, before running the Pulse program, the fast and a slow component half-life and the fractional contribution of the slow component to the overall decay are entered for the hormone analyzed. Pulse can thus be used to quantify hormonal secretion. Secretion rates are expressed per liter distribution volume (VD). One limitation of this method is that the program does not identify small secretory events and a large number of tenuous assumptions must be imposed to propagate the uncertainties of the experimental observations into the uncertainties of the secretory profile. However, Pulse can be used to assess the initial parameters required for the waveform- dependent deconvolution method, as amplitudes and locations of large secretory events are easily defined. After the initial guess by waveform-independent estimates of hormonal secretion using Pulse, subsequent analysis with a waveform-dependent multi-parameter deconvolution method is performed. Mean best fit values and statistical confidence limits for each secretory and clearance parameter are taken into account by the program. Thus, the probability that a secretory burst has a significant amplitude is estimated. Furthermore, all underlying relevant secretory events are evaluated simultaneously, which enhances the statistical power of the deconvolution procedure. This technique also requires a priori specification of hormonal half-life in plasma. The fast and a slow component half-lifes and the fractional contribution of the slow component to the overall decay have to be specified for the specific hormone analyzed. This technique estimates the combined rates of basal release, number, duration, amplitude and mass of randomly ordered secretory bursts and the subject-specific half-life. The daily pulsatile secretion is the product of secretory burst frequency and mean mass released per event. Total secretion is the sum of basal and pulsatile secretion. Results are expressed per liter distribution volume. For the calculation of production rates per liter, the distribution volume of the hormone has to be calculated.

Cosinor and Cleveland robust fitting

Nyctohemeral characteristics of hormone concentration patterns can be determined using cosinor or Cleveland robust fitting analysis. The cosinor test is the oldest method proposed for and applied to 24 h rhythms. Cosinor analysis entails trigonometric regression of a cosine function on the full 24 h plasma hormone concentration profile vs. time: $y(t) = M + A \cos(\omega t + \phi) + e(t)$ with y(t) the value at time t of the periodic function of angular frequency ω (degrees per time unit, 360 degrees = complete circle), defined by parameters M (mesor), A (amplitude) and ϕ (acrophase). The cycle duration (τ) is fixed for each fit (e.g. 24 hours). This function is fitted to the data (using least squares method) to derive rhythm parameters estimates; the acrophase (clock time during 24 h at which hormone concentration is maximal), mesor (midline estimated statistic of rhythm, or the average value of the rhythmic cosine curve) and the amplitude (half of the total predictable change in the rhythm). The major disadvantage of this technique is that it assumes that the observed 24 h rhythm is best described by a symmetric sinusoidal curve. However, most 24 h concentration patterns have asymmetrical wave-shapes. Therefore, the robust curve (LOWESS) fitting algorithm described by Cleveland was used to determine the zenith, nadir and mesor of the day long hormone rhythms. The technique is described in more detail in ref (7). This program provides a more adequate description of asymmetrical wave shapes, based on periodogram calculations or non-linear regression procedures.

Approximate Entropy Analysis

Approximate entropy (ApEn) can be used to quantify the orderliness or regularity of serial hormone concentrations over 24 h (2;8). ApEn is a scale- and model-independent statistic developed and formulated by Pincus (9), which is applicable to a wide variety of physiological and clinical time-series data. ApEn measures the logarithmic likelihood that runs of patterns in a time series that are close for m consecutive observations remain close when considered as m + 1 consecutive observations. Greater regularity (higher probability to remain close) yields smaller ApEn values. Higher absolute ApEn values denote greater relative randomness or lower regularity of hormone patterns. Calculation of ApEn requires prior definition of two parameters: m (length of the run to be compared) and r (filter or the magnitude that will discern “close” and “not close”). For optimal statistical validity ApEn is typically implemented in hormone time series by using m values of 1 or 2 and r values of approximately 0.2 SD of the series being considered. The ApEn metric thus evaluates the consistency of recurrent subordinate (nonpulsatile) patterns in a time series. Regularity of hormonal secretion patterns mirrors the net result of feed forward signaling and feedback restraint (8). Thus, ApEn yields information about hormonal time series, distinct from and complementary to Cluster, deconvolution, cosinor and Cleveland analyses (2).

Some potential pitfalls of these mathematical techniques have been described (10). For example, the computer models require input of prolonged serial measurements of circulating hormone concentrations obtained by highly intensive sampling regimens. Furthermore, hormone concentration series can be noisy and experimental conditions may contribute to the uncertainty in the data (blood loss, subject manipulation, sample processing, assay methods). Another problem is that the waveform-independent deconvolution requires a priori knowledge of half-life. It is not possible to estimate secretion and clearance rates using waveform-dependent deconvolution analysis, without an initial assumption of the waveform. Finally, fluctuations of basal or tonic hormone secretion within the time series analyzed are not taken into account by the program, which in turn may yield different estimates of basal secretion rates. Secretion rates that are called basal (tonic secretion or inter-pulse secretion), cannot be interpreted as necessarily significant (i.e. distinct from zero or above assay noise and/or experimental uncertainty). Unfortunately, at present there is no useful and critical information regarding the analysis of low levels of tonic hormonal secretion.

Nevertheless, these techniques provide a way to obtain additional (physiologically relevant) information from hormone time series and these analyses are important non-invasive methods to calculate the temporal distribution of hormone pulses and secretion rates. In addition, the regularity of secretion (ApEn) can be measured, giving insight into the feedforward and feedback signaling of the particular neuroendocrine system. Finally, insight into the diurnal (circadian) properties of the neuroendocrine system can be obtained.

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