

Regulation of Brown Adipose Tissue by Stress and Sex



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Regulation of Brown Adipose Tissue by Stress and Sex

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CHAPTER 1

GENERAL INTRODUCTION

A ROLE FOR BROWN ADIPOSE TISSUE IN FIGHTING THE OBESITY EPIDEMIC

The incidence of obesity has increased over the last century in both Western and developing countries, making obesity a global health threat. We now have to deal with the consequences of obesity which include type 2 diabetes, dyslipidemia, coronary heart disease, hypertension, gall bladder disease and cancer (1) leading to a tremendous burden on healthcare budgets (2). Therefore, we need to look for potential targets to combat obesity. As a cause of this obesity epidemic, increased food intake and changed eating patterns in addition to decreased exercise are always mentioned. However, other hypotheses are also postulated. Obesity and its consequences might also be caused by sleep deprivation, endocrine disruptors, increasing maternal age, chronic inflammation possibly due to viral and parasitic infections, and even a changed gut microbiota is believed to play a role in the increased incidence of obesity (3,4). Although the etiology of the obesity epidemic is not totally clear and is likely multifactorial, obesity is eventually the result of a chronic positive energy balance. One of the tissues that is considered as a possible mediator of a changed energy balance is brown adipose tissue (BAT). This tissue has an enormous potential to utilize fatty acids through its ability to induce thermogenesis (5,6). By this use of fatty acids BAT has the potential to improve energy balance. Indeed, BAT has been found to be less active in humans with obesity and with increasing age (7). Thus, BAT is a promising target tissue in the search for methods to combat obesity. However, not much is known about the regulation of BAT activity and the influence of several hormones that can cause obesity, such as stress hormones and deregulation of sex hormones. The influence of these two groups of hormones on BAT activation will be studied in this thesis (Figure 1).

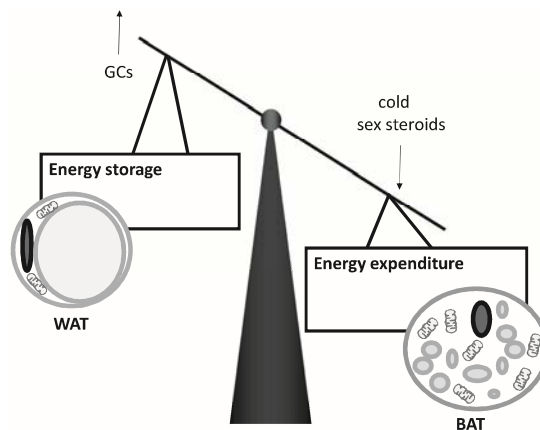


Figure 1: GCs increase energy storage and decrease BAT activity, cold exposure and sex steroid hormones increase BAT activity and subsequent energy expenditure thereby tipping the energy balance towards energy expenditure

BROWN ADIPOSE TISSUE

Historical view on BAT

BAT had already been designated as a distinct tissue with unique morphological features in 1551 by the Swiss naturalist Konrad Gessner. He described BAT as “neither fat, nor flesh, but something in between” (8,9). In the 1960s the thermogenic capacity of BAT was described for the first time (10,11). It was thought that BAT was only present in newborns and small children (12) but Heaton *et al.* (13) showed in 1972, based on histological criteria, that BAT was widely distributed in the adult human. BAT was found mainly around the larger vessels, neck region and surrounding the kidneys and adrenals (13,14) (Figure 2). Not long thereafter the function of uncoupling protein 1 (UCP1), the protein via which BAT can produce heat, was discovered (15) and new interest was aroused in BAT as a tissue that can influence the human energy balance and thereby possibly combat obesity (16).

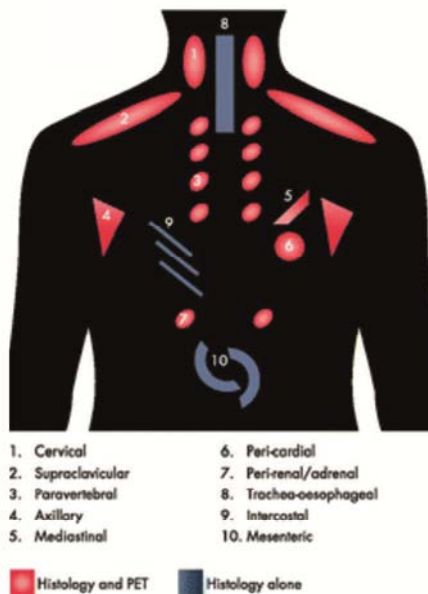


Figure 2. Presence of brown adipose tissue in humans, adapted from Lee et al. (14)

Function of BAT

BAT protects mammals against hypothermia by its ability to produce heat via non-shivering thermogenesis (17). BAT consists of smaller adipocytes compared to white adipose tissue (WAT), and has multiple small lipid droplets (Figure 3). BAT derives its brown color from the high concentration of mitochondria. In WAT, adipocytes are

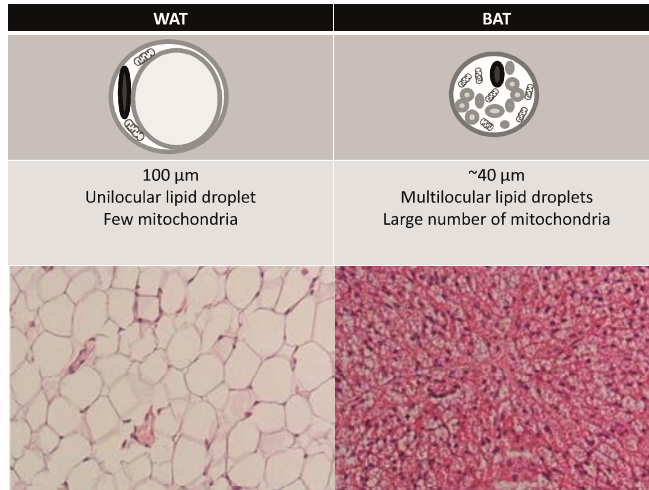


Figure 3. Characteristics of WAT and BAT.

optimized to store fatty acids and glucose, and other sources of energy, in the form of triglycerides (TGs) in large unilocular droplets. TGs in BAT are mainly stored as a source of energy for thermogenesis since BAT has the ability to generate heat by ‘futile’ use of fatty acids after lipolysis from TG. This futile use of fatty acids is due to the presence of UCP1 in the inner mitochondrial membrane (18). In every cell β -oxidation of fatty acids results in the production of succinate and fumarate (19) and these products can be used for oxidative phosphorylation resulting in a proton gradient over the inner mitochondrial membrane. This proton gradient is subsequently used by ATPsynthetase to convert ADP to ATP, allowing the cell to use the energy stored in ATP when needed. UCP1 is a proton pump and, when activated, transports protons back into the inner

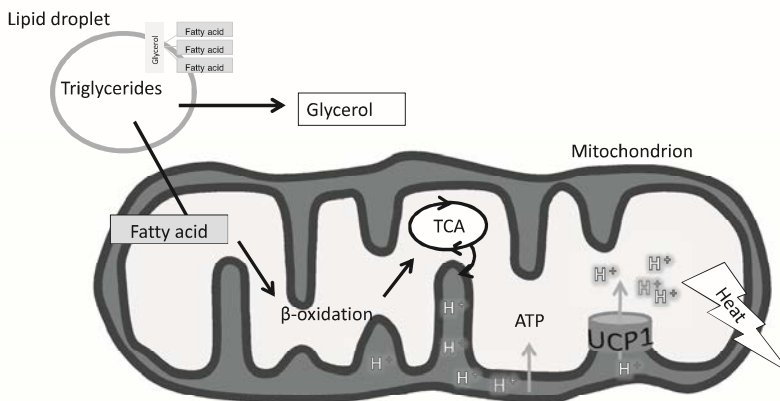


Figure 4. Mitochondrion with active UCP1 dissipates energy by creating a leak of protons into the inner mitochondrion resulting heat instead of ATP production.

mitochondrion lowering the proton gradient (20). As a result, energy is released as heat instead of ATP (Figure 4).

Beige adipocytes

Recently a third category of fat tissue next to WAT and BAT has been described; the beige adipose tissue (21). This fat tissue is a WAT depot wherein brown adipocytes can be found. Indeed, studies suggest that beige adipose tissue and not classical BAT might be the predominant tissue important for thermoregulation in human adults (22-24). Thus, if human beige adipocytes can be recruited, it may be possible to improve metabolic health by inducing 'browning' of WAT depots (22-24). The debate is still ongoing whether brown adipocytes in beige adipose tissue are derived from stem cells and thus are newly appearing in the adipose tissue, or whether they are adipocytes that have transdifferentiated from white adipocytes into brown adipocytes. Evidence has been presented for both possibilities (25,26). It might be that beige cells have the same precursor cells as classical brown adipocytes or that they originate from a different cell lineage (Figure 5). Seale *et al.* (27) have described two different precursor cell lineages which both give rise to brown adipocytes, depending on the absence or presence of Myf5. The Myf5+ precursor cells can give rise to the classical brown adipocyte or muscle (28), controlled by the transcription factor PRDM16. If PRDM16 is active, cells will differentiate into brown adipocytes instead of myocytes. Myf5- negative precursor cells can give rise to both the beige adipocytes and white adipocytes. This switch is controlled by transcription factor TLE3 that prevents PRDM16 from activating transcription of thermogenic gene programs (29,30). Although it is not clear whether brown adipocytes

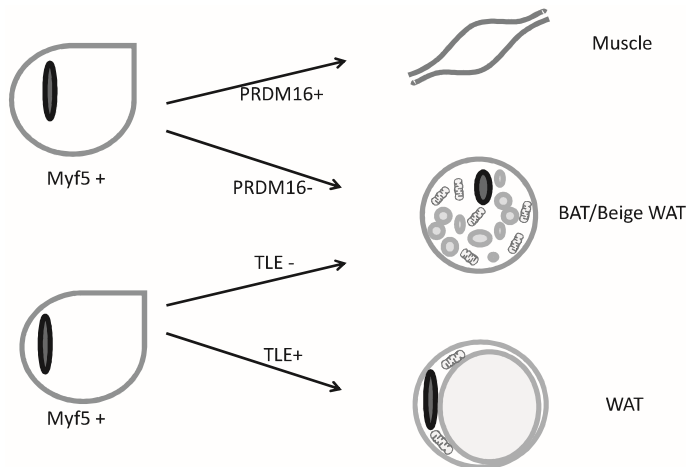


Figure 5. Schematic diagram of the two suggested lineages of precursor cells of classical BAT and beige/WAT adipocytes, TLE3 and PRDM16 are key transcription regulators which determine the switch to either muscle, beige/BAT or WAT.

found in adult humans are classical brown adipocytes or newly formed adipocytes derived from Myf5- precursors (31,32) it is of interest to study ways to increase the amount of beige adipocytes in addition to studying methods of activating classical BAT.

BAT AND METABOLISM

As described before, BAT might contribute to the prevention and treatment of obesity by increasing energy expenditure and decreasing storage of energy. Since carbohydrates and lipids, in the form of TGs, are the main energy substrates determining a positive or negative energy balance we will focus on the mechanism by which BAT activity can influence carbohydrate and lipid metabolism. Chronic excess of both carbohydrates and lipids is associated with dyslipidemia and type 2 diabetes, which are two major risk factors in the development of cardiovascular diseases (33). If we can increase the utilization of carbohydrates and lipids, this may provide a tool not only to treat obesity but also obesity's consequences and improve dyslipidemia and type 2 diabetes.

BAT and carbohydrate metabolism

Carbohydrates are an essential energy source. Energy obtained from glucose oxidation is temporarily stored in the form of ATP, which is used for every process in the cell. Carbohydrates are easy to metabolize in comparison to lipids or proteins. The human brain is solely dependent on glucose as an energy source, therefore it is of importance to keep glucose levels in a healthy range (34). The brain plays a key role in regulation of glucose homeostasis via the hypothalamus. Glucose sensing in the brain will result in autonomic nervous system mediated stimulation of the pancreas, which is the key organ of glucose homeostasis through the production of several hormones. These hormones include insulin and glucagon produced in the β -cells and α -cells of the pancreatic islets of Langerhans, respectively (35). When intake of glucose is low and glucose levels drop, glucagon is produced which stimulates gluconeogenesis and glycogenolysis by the liver, resulting in an increased hepatic glucose production (36). When intake of glucose is high and glucose levels rise, increased insulin stimulates the uptake of glucose by peripheral tissues such as adipose tissue, muscle and the liver. Insulin also inhibits hepatic glucose production and stimulates glucose storage in the liver as glycogen (36). In addition, a surplus of carbohydrates can be converted into fatty acids and TGs via de novo lipogenesis (DNL). These lipids are stored in liver, muscle and adipose tissue, but predominantly in WAT (37). The response to insulin and subsequent rate of uptake of circulating glucose by tissues such as the liver, muscle and adipose tissue determines the amount of insulin that is needed to keep the blood glucose levels in a healthy range, the so-called whole-body-insulin-sensitivity. In patients with type 2 diabetes the amount of insulin needed

is relatively high for the amount of glucose that is taken up by the peripheral tissues, meaning that their whole body insulin sensitivity is decreased (38). If we can increase whole body insulin sensitivity, we might be able to prevent type 2 diabetes (39).

In mice, BAT transplants improved glucose tolerance (40). In addition, several studies showed a higher metabolic rate and an improved glucose tolerance in mice when more beige fat was present (41-43). Interestingly enough, transplantation of BAT into *ob/ob* mice, mutant mice that lack the hormone leptin resulting in profound obesity, reversed obesity and improved insulin sensitivity and liver steatosis (44). In humans more and higher BAT activity on ^{18}F -fluorodeoxyglucose positron emission tomography (^{18}F -FDG PET) scans has been found to be associated with a lower body mass index and improved insulin sensitivity (7,45).

The role of BAT in the control of lipid homeostasis

After a lipid-rich meal, lipids are taken up via the intestine and transported via the lymph vessels into the circulation mainly as TGs in large lipoprotein particles, chylomicrons. Lipoproteins have a membrane of cholesterol and phospholipids. This membrane is hydrophilic at the outside but hydrophobic on the inside. Due to these characteristics lipoproteins can transport hydrophobic TG and cholesterol esters in serum. In peripheral tissues, such as WAT, BAT and muscle, fatty acids are taken up after breakdown of TGs from lipoproteins by lipoprotein lipase (LPL) and activation of CD36, a FA transporter. The chylomicron remnants are taken up by the liver by the low density lipoprotein receptor (LDLR). Fatty acids can then be stored as TG or metabolized by β -oxidation in the liver (46). The liver is the main regulator of lipid metabolism since stored hepatic TGs can be used to generate new lipoprotein particles, very low density lipoprotein (VLDL). These VLDL particles are formed to transport fatty acids from the liver to peripheral tissues to provide a continuous flux of lipids even when there has been no recent intake of lipids. Increased fluxes of lipids might lead to liver steatosis and increased levels of circulating VLDL and TGs (47). After LPL-mediated uptake of fatty acids by peripheral tissues, the remaining VLDL particle contains relatively more cholesterol and is now called Low Density Lipoproteins. LDL can also be taken up by the liver. Higher concentrations of LDL are associated with increased intravascular storage of cholesterol and subsequent plaque formation, leading to cardiovascular disease (48). We thus need to improve uptake of TG but also improve clearance of LDL by the liver to prevent cardiovascular disease. Using ^{18}F -FDG PET scanning to estimate BAT activity shows that activated BAT is utilizing more glucose than other tissues. However, lipids are the main energy source for thermogenesis where glucose uptake is presumably increased to supply energy for other processes than thermogenesis in the brown adipocyte (49). Since BAT is using fatty acids as the main source for thermogenesis, activated BAT is expected to diminish circulating

TG content in plasma resulting in a better balance between energy intake and energy expenditure. Decreased BAT activity will lead to increased VLDL and TG concentrations, as described recently (5,6,50). Uptake of TG from VLDL and FFA by BAT from the circulation is increased upon cold exposure (5,6,50). Although, we do not know if FA from WAT can be recruited towards active BAT, the burning of excess circulating FFAs will eventually lead to less storage of fatty acids in WAT. In addition, it was recently shown that β -adrenergic activation of BAT also leads to increased uptake of TG from VLDL particles and increased uptake of LDL by the liver, thereby preventing atherosclerotic disease (5). Activation of BAT is thus considered beneficial in improving metabolic disturbances such as dyslipidemia.

ACTIVATION OF BAT AND ITS REGULATION MECHANISM

BAT as a target for treatment of obesity

Since BAT can influence obesity and its metabolic consequences (51), BAT activity and its regulation mechanism has been a subject of study for the last ten years as a means to treat obesity. Although increased energy expenditure by increased BAT activity will eventually lead to less storage of energy in WAT and muscle, studies have not been performed to determine whether fatty acids from WAT are indeed recruited to BAT. Improvement of metabolic health by increased BAT activity might also be due to other mechanisms. Interestingly, transplantation of BAT from one mouse to another not only improved the metabolic health of the recipient mouse, now with double the amount of BAT, but also increased the activity of its existing BAT depot (52). Thus, BAT might play a role in changing metabolism by the production of several hormones, the so-called BATokines. For example, bone morphogenetic protein 8b (BMP8b) sensitizes BAT for β -adrenergic signaling, and stimulation of certain brain areas in the hypothalamus by BMP8b increases β -adrenergic signaling to BAT (53). Other factors belonging to the same family as BMP8b, i.e., the transforming growth factor β (TGF) family, are also produced in BAT and have been described to affect whole body metabolism. BMP4 and 7 have been shown to stimulate the differentiation of preadipocytes into brown adipocytes (54,55). But also other growth factors such as fibroblast growth factor 21 (FGF21) improve dyslipidemia and type 2 diabetes when administered to mice (56). Increased levels of FGF21 in humans are also associated with higher BAT activity (57). Thus, BAT is improving metabolic health not only by simply increasing energy expenditure, but also by autocrine, paracrine, or endocrine effects of BATokines that improve the metabolic function of BAT and other tissues (58,59).

Activation of BAT via the sympathetic nervous system

Cold exposure is the natural way to activate BAT and increase browning of WAT. Upon cold exposure, cold is sensed in peripheral tissues by primary afferent sensory neurons of the dorsal root and trigeminal ganglia. Temperature is sensed in the nerve endings via activation of the cold-sensing transient receptor potential (TRP) superfamily of cation channels. The most important cation channel to sense cold is TRPM8 that can sense temperatures below 28°C (60). This allows the entry of Na⁺ and Ca²⁺ into the cell, leading to the generation of an action potential and the activation of these primary afferent sensory neurons (61) that conduct the thermal information to the spinal cord and the brain, eventually leading to the sensation of cold (62). Subsequently, the sympathetic nervous system is activated and norepinephrine is released in BAT (63). Norepinephrine activates the β-adrenergic signaling pathway and thereby induces lipolysis (64). The fatty acids generated by lipolysis then activate UCP1 (65) and act as substrates for fatty acid oxidation, as described above. In addition, chronic stimulation with β-agonists results in expansion of the BAT depots and increases the number of beige adipocytes in mice (66,67). Thus, β-adrenergic stimulation effects a quick activation of thermogenesis and increases the amount of beige adipocytes (Figure 6).

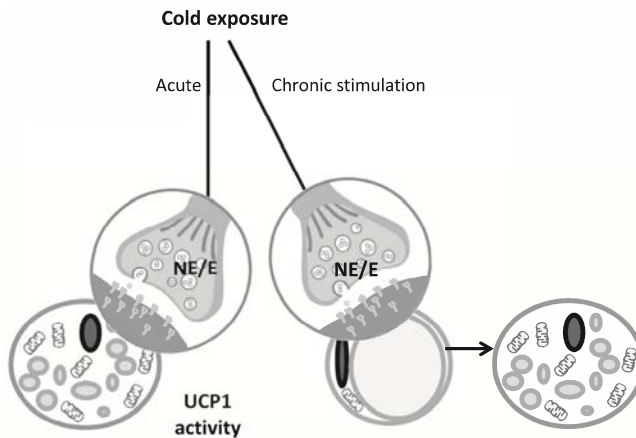


Figure 6. Cold exposure results in increased activity of β-adrenergic stimuli towards BAT and WAT. Acute stimulation results in increased UCP1 activity in BAT and long term increase in β-adrenergic stimulation of WAT results in browning of WAT.

Activation of BAT in adult humans

Earlier interest in BAT in adult humans was tempered by the difficulties involved in determining BAT activity in healthy live adult humans (68-70). In the last decade, however, the discovery of active BAT on ¹⁸F-FDG-PETscans has renewed interest in BAT in adult humans. In nuclear medicine, it was already known in 2002 that symmetrical metastases-like spots on ¹⁸F-FDG-PETscans were found in about 4% of the patients (71).

This phenomenon could be prevented by administration of the β -blocker propranolol and to take care that patients waited in a warm room before scanning (72,73). This knowledge did not reach the metabolic world until 2007 when Nedergaard *et al.* (12) described a few cases of detection of active BAT by ^{18}F -FDG-PET scanning. This new way of measuring BAT in humans resulted in a huge interest in BAT and its possible potential to treat obesity, even more so after 3 publications in the New England Journal of Medicine showing that presence of active BAT on ^{18}F -FDG-PET scans was inversely correlated with obesity and age (7,74,75). Interestingly, a higher percentage of people with active BAT was found during winter and when volunteers were cooled (7,41,74-76).

Individualized cooling in ^{18}F -FDG-PET studies

From the studies described above and later studies we learned that it is very important to cool patients in a standardized manner to measure the presence and activity of BAT accurately (77). Different techniques were used to determine active BAT on ^{18}F -FDG-PET scans. Individualized cooling, at a temperature a few degrees above shivering temperature, resulted in 96-100% detection of active BAT in lean subjects and 50% in obese subjects (77). BAT in adult humans can thus also be stimulated by cold exposure and subsequent β -adrenergic stimulation, resulting in increased energy expenditure and thereby reduced body weight (40,41,63,78). Indeed, exposure to cold in humans increased resting metabolism by 20% (79). In addition, several groups have reported activation of BAT in patients with pheochromocytomas, tumors localized in the medulla of the adrenal gland that produce large amounts of norepinephrine or epinephrine (80-85). These studies showed a high amount of active BAT or beige adipose tissue on ^{18}F -FDG-PET-CTs of these patients (80,81,85-88). Interestingly, browning of WAT in these patients is also increased, as was seen by histological analysis (82).

A better understanding of the mechanism of BAT activation in human adults might result in the development of an effective tool to treat or prevent obesity. β -adrenergic agonists unfortunately have many side-effects such as hypertension, palpitations and tremors (89,90). These side-effects possibly contributed to the fact that earlier research in the 1980s on β -agonist treatment of obesity has not resulted in therapeutic options.

REGULATION OF BAT BY STRESS HORMONES AND SEX STEROID HORMONES

Previous attempts to use catecholamines to reduce body weight led to severe side effects (91). We thus have to look for other regulatory mechanisms which can be used to develop alternative interventions to activate BAT and induce browning of WAT. In 2010 Nedergaard *et al.* had suggested that sex steroid hormones and glucocorticoids (GCs) could influence BAT activity, although good evidence was largely lacking (Figure 1) (92).

In this thesis we will discuss the role of the hypothalamic-pituitary-adrenal (HPA) axis hormones and sex steroid hormones in regulation of BAT activity.

Role of hypothalamic-pituitary-adrenal axis hormones on BAT activation

In times of stress, e.g. cold exposure, activity of the HPA axis is increased. Upon stress, corticotropin-releasing hormone (CRH) production by the hypothalamus is increased, which in turn stimulates the production of adrenocorticotropic hormone (ACTH) by the pituitary (93). In the adrenal cortex, ACTH activates the melanocortin 2 receptor (MC2R), inducing a signaling cascade that eventually results in increased production of GCs. The human GC is cortisol while the rodent adrenal gland mainly produces corticosterone. When GC levels are increased, ACTH and CRH levels are decreased due to the negative feedback of GC on the pituitary and hypothalamus (Figure 7).

Increased levels of GC increase circulating energy substrates exerting effects on different target tissues; GCs enhance muscle protein breakdown, increase lipolysis, increase hepatic glucose production and reduce glucose utilization by muscle and adipose tissue (94,95). In addition, high circulating levels of GC result in decreased whole body insulin sensitivity. Patients with GC excess due to a pituitary adenoma or adrenal adenoma, Cushing's disease or syndrome, or patients that are chronically treated with GCs suffer from metabolic derangements such as insulin resistance and hyperlipidemia (96). Since BAT is an important organ in maintaining lipid and glucose homeostasis, BAT might be one of the main target organs via which GCs exert their negative metabolic effects.

In the 1980s several studies were performed on BAT function in relation to GCs. Adrenalectomy in rodents increased BAT activity and administration of GCs in rodents decreased activity of BAT (97,98). Adrenalectomy induces production and secretion of

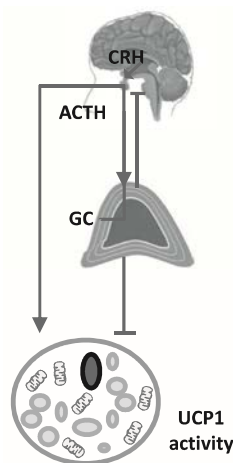


Figure 7. Schematic representation of the HPA axis. ACTH activates BAT and stimulates release of GCs. GCs inhibit BAT and inhibit the release of ACTH.

ACTH in the pituitary, and although these experiments were performed to study the effect of the absence of GCs, we might also hypothesize that ACTH can have an activating role in BAT metabolism. This possibility was investigated in the research described in chapter 2 of this thesis.

GCs also have a direct inhibitory effect *in vivo*. 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is an enzyme that converts cortisone to the active metabolite cortisol in humans and 11-dehydrocorticosterone to corticosterone in rodents. Pharmacological inhibition or genetic knockout of 11 β -HSD1 in adipose tissue led to increased expression and activity of UCP1 in BAT (99). In addition, locally produced GCs in transgenic mice that overexpress 11 β -HSD1 in adipose tissue resulted in decreased UCP1 expression in interscapular BAT and deteriorated lipid profile and insulin sensitivity (100), showing that GCs exert their effects locally at the tissue level. Moreover, GC administration to cell-lines resulted in decreased *Ucp1* mRNA expression and Ucp1 protein content (101,102).

Central stimuli can also be influenced by GCs. GCs are reported to inhibit the response to β -adrenergic stimulation by diminishing norepinephrine induced UCP1 transcription, for instance by reducing β 3 receptor mRNA content (101,102). Interestingly, human brown adipocytes that were stimulated by the synthetic GC dexamethasone were more active than the non-stimulated cells, but did show a reduced reaction on adrenergic stimuli. It was not known whether this interaction between adrenergic activation and GCs treatment on BAT activity is also present *in vivo*. In chapter 3 we describe our studies with corticosterone-treated mice that were kept at 4°C to induce adrenergic activation of BAT.

The data described above suggest that GCs inhibit BAT activity in murine models and in cultured human brown adipocytes. It is not known whether patients with GC excess indeed have decreased BAT activity and if this decreased BAT activity is causally related to the metabolic disturbances. We therefore studied BAT activity in patients with Cushing's disease before and after adrenalectomy. This is described in chapter 4. Reducing the BAT-inhibiting effect of GCs, e.g. via partial antagonists (103), can help to treat obesity and its subsequent complications (Figure 5).

Role of sex steroid hormones in regulation of BAT activity

Sex differences in BAT activity have been found in several retrospective ¹⁸F-FDG-PET-scan studies that report a higher prevalence and activity of BAT in women (7,42,104,105). It is not known why these differences exist, but this could be due to a difference in neuronal cold sensitivity between men and women (106,107) or a different set-point of the thermostat in the brain. Sex differences in BAT in humans have only been shown in retrospective PET scan studies in which temperature was not individually controlled. In an experimental setting with a warm environment no sex-differences are found in

^{18}F -FDG uptake (108). In our studies described in chapter 5 we studied whether sex differences in BAT activity would still be detectable in ^{18}F -FDG PET scans performed in subjects with the highest BAT prevalence, namely in a relatively young population, all premenopausal, that received ^{18}F -FDG in the winter. In addition, we studied the presence of brown adipocytes in the perirenal and subcutaneous adipose tissue in men and women at different temperatures, as described in chapter 6.

One of the most obvious differences between men and women are different levels of the sex steroids estrogen and testosterone. Estrogen might be one of the key players causing this gender difference in BAT activity. Silencing of estrogen signaling in the ventromedial nucleus in the hypothalamus, one of the brain centers regulating metabolism, results in decreased sympathetic nervous firing (109-111). This suggests that estrogens indeed modulate the set-point of the central thermostat, resulting in higher BAT activity. However, there is no evidence of central regulation of BAT activity by testosterone or progesterone.

In vitro studies showed effects of estrogens, testosterone and progesterone on UCP1 expression and mitochondrial biogenesis. Estrogens activated mRNA expression of the glucose pathway, thereby possibly stimulating glucose uptake; progesterone increased Ucp1 mRNA expression and expression of genes stimulating mitochondriogenesis; and testosterone inhibited the transcription of peroxisome proliferator-activated receptor- γ coactivator-1- α (Pgc1 α), an important factor in mitochondrial activity, as well as Ucp1 expression (112,113). Other non-steroid related factors that are differentially expressed between the sexes might be of even greater importance since sex steroids cannot be administered as treatments to increase BAT activity. Non-sex-steroid related factors that might be of interest given their role in differentiation and function of BAT might be bone morphogenic proteins (BMPs) and fibroblast growth factors (FGFs). For instance, as described above, BMP8b has been reported to activate BAT via peripheral and central action (53) and BMP4 and 7 have been shown to stimulate the differentiation of pre-adipocytes into beige adipocytes (54). Chapter 7 describes the differential expression of the BMP and FGF family members in the BAT depot of male and female mice and this shows that there are factors differentially expressed between the sexes and that *Bmp8b*, is increased by estrogens.

To summarize, we examine in this thesis the role of stress hormones and their role in the regulation of BAT activity and subsequently investigate sex-related factors that can influence BAT activity. Both topics are studied in cell-line models, in mice and in human adults.

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CHAPTER 2

DIRECT ACTIVATING EFFECTS OF ADRENOCORTICOTROPIC HORMONE (ACTH) ON BROWN ADIPOSE TISSUE ARE ATTENUATED BY CORTICOSTERONE

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ABSTRACT

Background: Brown adipose tissue (BAT) and brown-like cells in white adipose tissue (WAT) can dissipate energy through thermogenesis, a process mediated by uncoupling protein 1 (UCP1). We investigated whether stress-hormones ACTH and corticosterone contribute to BAT activation and browning of WAT.

Methods: ACTH and corticosterone were studied in male mice exposed to 4°C or 23°C for 24 hours. Direct effects were studied in T37i mouse brown adipocytes and primary cultured murine BAT and inguinal WAT cells. *In vivo* effects were studied using ¹⁸F-deoxyglucose positron emission tomography.

Results: Cold exposure doubled serum ACTH concentrations ($p=0.03$) and fecal corticosterone excretion ($p=0.008$). In T37i cells, ACTH dose-dependently increased *Ucp1* mRNA ($EC_{50}=1.8$ nM) but also induced *Ucp1* protein content 88% ($p=0.02$), glycerol release 32% ($p=0.03$) and uncoupled respiration 40% ($p=0.003$). In cultured BAT and inguinal WAT, ACTH elevated *Ucp1* mRNA by 3-fold ($p=0.03$) and 3.7-fold ($p=0.01$), respectively. In T37i cells, corticosterone prevented induction of *Ucp1* mRNA and *Ucp1* protein by both ACTH and norepinephrine in a glucocorticoid receptor (GR)-dependent fashion. ACTH and GR antagonist RU486 independently doubled BAT ¹⁸F-deoxyglucose uptake ($p=0.0003$ and $p=0.004$, respectively) *in vivo*.

Discussion: Our results show that ACTH activates BAT and browning of WAT while corticosterone counteracts this.

INTRODUCTION

In mammals, two different types of adipocytes are of major importance in the etiology of obesity and its metabolic consequences, namely brown adipose tissue (BAT) and white adipose tissue (WAT) (1). White adipocytes are involved in storage of energy as triglycerides (TGs) that can be utilized during periods of enhanced energy demand (2). Brown adipocytes, however, can oxidize fatty acids derived from TGs to generate heat due to the presence of the BAT-specific uncoupling protein 1 (UCP1). When UCP1 is activated, the energy generated by mitochondrial respiration is released as heat instead of being used by ATP synthetase to produce ATP (3). Because of this energy expenditure through futile use of TGs, activation of BAT and browning of WAT are considered attractive approaches to treat or prevent obesity (4).

Until a few years ago BAT was not considered to be of importance in adult humans. However, recent studies show that BAT is functionally active in adult humans and that its metabolic activity is inversely correlated with BMI, underscoring its potential role in regulating the energy balance (5-7). In humans, however, it appears that the metabolically most important BAT depots in humans are actually WAT that has gained BAT-like features such as UCP1 expression (8-14). In rodents, inguinal and subcutaneous WAT easily develops BAT-like properties whereas gonadal and visceral WAT are considered to be more resistant to browning (15,16).

Insight into mechanisms by which the body controls BAT activity and browning of WAT is of importance to develop new targets for the treatment or prevention of obesity. Cold exposure is the most robust way to increase BAT activity via centrally regulated catecholamine release into the BAT and WAT depots and subsequent β -adrenergic receptor activation in brown adipocytes. In BAT, catecholamines stimulate fatty acid oxidation as well as UCP1 activity via the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway (17,18). Prolonged stimulation with catecholamines induces browning of WAT (19,20,21). Nevertheless, catecholamines are not an obvious treatment candidate of obesity in humans, since high doses of sympathomimetics have severe cardiovascular side-effects (22-24).

Catecholamines are not the only hormones that are released during cold exposure. In behavioral studies, cold exposure is a commonly used method to increase the activity of the hypothalamic-pituitary-adrenal (HPA) axis (25). Upon stress, corticotrophin-releasing hormone (CRH) is released by the hypothalamus, stimulating the release of adrenocorticotrophic hormone (ACTH) by the pituitary. ACTH, in turn, stimulates synthesis and secretion of glucocorticoids (GC) by the adrenal cortex; cortisol in humans and corticosterone in rodents. In a negative feedback loop, GCs inhibit secretion of hypothalamic CRH and pituitary ACTH (26). *In vitro* studies performed in rodents showed that GCs have an inhibitory effect on BAT development and activity, most likely

mediated via the glucocorticoid receptor GR (27-29). In animal studies, increased BAT activity was seen after adrenalectomy (30,31), while GC replacement normalized BAT activity (30,32). It was thus implicated that long-term changes in plasma GCs affect BAT activity. However, these studies did not evaluate the direct effects of ACTH on BAT activity nor did they study a possible interaction between these two hormones in BAT function. Since GCs have a strong negative feedback on ACTH secretion, the increased BAT activity in the absence of GCs *in vivo* (30,31) might also have been the result of increased ACTH concentrations in the adrenalectomized mice. We therefore studied the interplay between the murine GC corticosterone and ACTH on BAT in a controlled *in vitro* system. In addition, we performed *in vivo* experiments to study the direct effects of ACTH on BAT activity using ^{18}F -deoxyglucose (^{18}F -FDG) positron emission tomography (PET) scans. Our data show that corticosterone and ACTH have opposite effects on BAT activity and browning of WAT: ACTH stimulates while corticosterone inhibits BAT activity and browning of WAT.

MATERIALS AND METHODS

Animals and cold exposure experiments

Male C57Bl/6J mice obtained from Charles River Laboratories (Maastricht, The Netherlands) at 8 weeks of age were acclimatized for one week under standard housing conditions before being individually housed and put in a temperature controlled climate chamber (Bronson, Nieuwkuijk, Netherlands) with normal light/dark cycle. Bodyweights and food intake were measured before and after exposure for 24 h to temperatures of either 23°C or 4°C. Feces were collected during the 24 h period. Mice were then terminated by cardiac puncture under isoflurane anesthesia. Plasma and various tissues were collected and either immediately frozen and stored at -80°C or fixed in 4% paraformaldehyde. All animal experiments were performed with the approval of the Animal Ethics Committee at Erasmus MC, Rotterdam, The Netherlands.

Histology and immunohistochemistry

For histological examination of adipose tissue, fixed interscapular BAT and inguinal WAT depots were embedded in paraffin. Eight μm sections were mounted on glass slides and stained with hematoxylin and eosin. For immunohistochemistry, sections were mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), deparaffinized, rinsed with water, dehydrated in alcohol and immersed in 3% H_2O_2 in methanol (Sigma) for 20 min to block endogenous peroxidase activity, then rinsed with water. After heat-mediated antigen retrieval for 3 x 5 min in a microwave oven in NaOH buffered 0.01 M citric acid, pH 6.0 (Merck, Darmstadt,

Germany), sections were cooled and rinsed in PBS and subsequently incubated with a UCP1 antibody raised in rabbit (ab10983, Abcam, Cambridge, United Kingdom) in a 1:500 dilution in PBS. After overnight incubation at 4°C sections were rinsed with PBS, then incubated for 30 min at room temperature with BrightVision-poly-HRP-anti mouse/rabbit/rat IgG (Immunologic, Duiven, The Netherlands) in a 1:2 dilution in PBS and rinsed with PBS. The peroxidase activity was developed with 0.07% 3,3-diaminobenzidine-tetrahydrochloride (Sigma). Finally, all sections were counterstained with hematoxylin. Lipid droplet content was assessed with ImageJ Version 1.45s (33).

Fecal corticosterone

Feces was air-dried for 2 days, weighed, crushed, homogenized and stored at -20°C. Corticosterone extraction was performed as previously described (34). Briefly, 50 mg feces homogenate was dissolved in 2.5 mL 100% ethanol (Sigma) and boiled for 20 min at 80°C followed by centrifugation for 15 min at 4500 rpm. The supernatant was transferred to a glass tube and the remaining pellet was dissolved in 1.5 mL 100% ethanol, boiled and centrifuged. The supernatants were then combined, dried under a stream of N₂ and redissolved in 250 µL methanol (Sigma). The corticosterone concentration was measured by ELISA (ENZO life sciences, Antwerp, Belgium).

Plasma corticosterone and ACTH measurements

Plasma corticosterone was measured by ELISA (ENZO life sciences) and plasma ACTH was measured by radioimmunoassay (MP biomedical, Orangeburg, NY) according to the manufacturer's protocols.

Primary cell culture

Interscapular BAT and inguinal WAT depots were collected from 9-week-old male C57Bl/6J mice, washed with PBS, minced and digested with 0.1% collagenase (Sigma), 0.1% dispase II (Roche Diagnostics, Mannheim, Germany) and 0.05% trypsin (Sigma) in serum-free culture medium (DMEM with 4.5 g/l glucose supplemented with 25 µg/mL Na-ascorbate, 10 mM HEPES, 4 mM glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (Gibco-Invitrogen, Breda, Netherlands)). Digestion was performed at 37°C for 30–60 min with gentle agitation. Enzymes were inactivated with an equal volume of culture medium containing 10% FCS. The samples were filtered through a 100-µm mesh filter to remove debris. The cells were spun down for 8 min at 300xg after which the pellets were resuspended in red blood cell lysis buffer (eBioscience, San Diego, USA) for 5 min to lyse red blood cells. After that, cells were centrifuged for 5 min at 500xg and resuspended in culture medium containing 10% FCS. Cells were cultured at a density of 125,000 cells/well in 24-well plates. After a 24 h attachment period, differentiation was induced by adding 10% FCS, 1 µM rosiglitazone

(ENZO life sciences) and 2.4 nM bovine insulin (Sigma) to the normal culture medium. Experiments were performed after 12 days of differentiation during which the medium was replaced every 2 or 3 days.

T37i preadipocyte cell culture

The murine brown preadipocyte cell line T37i (35,36) was cultured in HAM'S-F12 medium (Gibco-Invitrogen) containing glutamine that was supplemented with 10% FCS, 2 mM HEPES, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco-Invitrogen). For experiments, T37i cells were seeded in 6-wells plates for gene expression and protein analysis and in 24-wells plates for cAMP and glycerol release studies. Two days after reaching confluence, cells were differentiated using normal culture medium supplemented with 2 nM triiodothyronine (T3) (Sigma) and 112 ng/mL bovine insulin (Sigma). The differentiation medium was replaced every 2 or 3 days. Experiments were performed with 9-day differentiated cells. For the gene expression and protein studies, differentiated cells were treated for 4 or 8 h with different concentrations of ACTH (Polypeptide group, Strasbourg, France), NE (Sigma), corticosterone (Sigma) and RU486 (Sigma) or their respective vehicles.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA from mouse tissues and cultured cells was isolated using Tripure Isolation Reagent (Roche) according to the manufacturer's instructions. Genomic DNA was removed by DNase treatment (Promega Benelux BV, Leiden, The Netherlands) for 30

Table 1. Primer sequences used for Real Time PCR analysis

Gene		Sequence (5' to 3')	GenBank Acc.No.	Ref.
<i>Cyp11a1</i>	Forward	AGGTCCTTCAATGAGATCCCTT	NM_019779.3	
	Reverse	TCCTGTAAATGGGGCCATAC		
<i>Cyp11b1</i>	Forward	AAGAAACTTAGAGTCCTGGGATT	NM_001033229.3	(39)
	Reverse	GTGTCACTGCTCCAGCAATGAGT		
<i>Hprt</i>	Forward	GCAGTACAGCCCCAAAAT	NM_013556.2	
	Reverse	AACAAAGTCTGGCCTGTATCCAA		
<i>Hsd3b1</i>	Forward	GGATCATCAAGATGTTGGTGC	NM_008293.3	(40)
	Reverse	TGGTTTCTGGTCGGAATACTT		
<i>Ppargc1a</i>	Forward	CCCTGCCATTGTTAAGACC	NM_008904.2	(41)
	Reverse	TGCTGCTGTTCTGTTTTTC		
<i>Star</i>	Forward	TTCTCAACTGGAAGCAACACT	NM_011485.4	(40)
	Reverse	CTTCTGCATAGCCACCTCTC		
<i>Ucp1</i>	Forward	GGCCTCTACGACTCAGTCCA	NM_009463.3	(42)
	Reverse	TAAGCCGGCTGAGATCTTGT		

min at 37°C. Reverse transcription was performed using a cDNA synthesis kit (Roche) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SYBRgreen mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) with an ABI Prism 7900 Sequence Detection System. Sequences of the primers used are listed in Table 1. The expression of each gene was expressed in arbitrary units after normalization to the average expression level of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) using the $2^{-\Delta\Delta Ct}$ method (37).

Western blot analyses

Protein was extracted from differentiated treated and untreated T37i cells. Briefly, cells were lysed in cold PBS containing 0.05 M HEPES, 10mM EDTA, 20 mM NaF, 2 mM NaVO₃, 150 mM NaCl, 1% Triton, phosphatase and protein inhibitors. Protein extract was subsequently sonified for 10 s. Protein was diluted in Laemmli buffer and 30 µg of protein was electrophoresed on a 10% gel and blotted on a nitrocellulose membrane. Membranes were blocked in PBS containing 3% nonfat powdered skim milk before an overnight incubation at 4°C with a rabbit polyclonal anti-UCP1 antibody (1:500; Sigma) in PBS containing 0.1% Tween-20 and 5% nonfat powdered skim milk or a rabbit polyclonal anti- α -tubulin antibody (1:500; Santacruz, Heidelberg, Germany) in PBS containing 0.1% Tween-20 and 5% bovine serum albumin. Next, membranes were washed and incubated for 1 hour at room temperature with a goat-anti-rabbit IRDye 800 secondary antibody (1:15,000; Li-cor, Leusden, The Netherlands) in PBS containing 0.1% Tween-20 and 5% non-fat powdered skim milk. Ucp1 immunoreactivity was measured with an Odyssey fluorescence scanner (Li-cor, Leusden, The Netherlands) and was normalized for α -tubulin immunoreactivity in the same samples using Odyssey software.

cAMP measurements

For cAMP measurements, differentiated T37i cells were washed three times with PBS and treated with either 1 µM NE or different concentrations ACTH in differentiation medium containing 500 µM IBMX (Sigma). Media were collected after 15 min of incubation and cAMP was quantified by radioimmunoassay (Beckman Coulter, Woerden, The Netherlands) according to the manufacturer's protocol.

Glycerol measurements

Glycerol release in the culture medium was measured after 4 h incubation with 50 nM ACTH or 1 µM NE. We measured glycerol release using a glycerol colorimetric assay (Instruchemie, Delfzijl, The Netherlands) according to the manufacturer's protocol.

Oxygen consumption measurements

A Seahorse Bioscience XF24 extracellular flux analyzer was used (Seahorse Biosciences, North Billerica, MA, USA) to measure oxygen consumption rate (OCR) in differentiated T37i cells. T37i cells were seeded in poly-D-lysine (Sigma Aldrich) coated 24-well-Seahorse-assay plates, and differentiated as described above. Measurements were initiated after equilibration in freshly prepared seahorse medium comprising DMEM with 5 mM glucose and 1 mM pyruvate, pH adjusted with NaOH, for 1 hour in a 37°C incubator without supplemental CO₂.

Oligomycin and ACTH were preloaded in the reagent delivery chambers of the Seahorse sensor cartridge and then pneumatically injected into the wells to reach final working concentrations. Three baseline OCR measurements were performed, followed by injection with oligomycin to a final concentration of 0.5 μM to distinguish between ATP linked OCR and uncoupled oxygen consumption, subsequently ACTH (final concentration 50nM) was added to test whether uncoupled oxygen consumption could be increased by ACTH. Maximal respiration was determined by adding FCCP (1 μM). In each case, post exposure OCR was measured three times after 2 min of mixing. Experimental treatments were performed on 5 wells of each plate as technical replicates and each experiment had 2 biological replicates. OCR (picomoles per minute) was normalized for the amount of cellular protein in each well. The averages of three baseline measurements and three post-exposure OCRs were used for data analyses.

Small animal positron emission tomography (PET) studies

2-deoxy-2-[18F]fluoro-D-glucose (¹⁸F-FDG) small animal PET analyses were performed as previously described (38). 12-weeks old C57BL/6N mice were analyzed in 4 different imaging sessions. The different treatments were: vehicle; ACTH (Biofutura Pharma, Italy); co-treatment with RU486 (100 mg/kg, Sigma-Aldrich, Italy) followed by vehicle; and co-treatment with RU486 followed by ACTH. Mice were injected intraperitoneally with vehicle or 5 mg/kg ACTH in Acetic Acid/Sodium Acetate, 20 min later the tracer ¹⁸F-FDG was administered. One hour after tracer injection PET scanning was started. RU486 was given 30 min before vehicle or ACTH administration. The ¹⁸F-FDG PET studies were approved by the Central Veterinary Office of Bologna University in accordance with the European Community guidelines (2010/63/EU) for the use of laboratory animals.

Statistical analysis

Data were analyzed with Graphpad Prism 5 (San Diego, CA, USA) and expressed as mean ± SEM. Data were evaluated for statistical differences by unpaired Student's *t* test, one-way ANOVA or two-way ANOVA with Bonferroni post-hoc comparisons when appropriate. Differences were considered significant at *p*<0.05.

RESULTS

Twenty-four hours of cold exposure induces BAT activity and browning of WAT

To study the effects of cold exposure on both BAT activity and browning of inguinal WAT, mice were exposed to 4°C or 23°C for 24 h. Despite the relatively short period of exposure, clear morphological changes in BAT (Fig. S1) and inguinal WAT (Fig. S2) were found, suggestive for enhanced BAT activity and browning of inguinal WAT. Morphological analysis of inguinal WAT revealed areas that contained brown-like adipocytes in cold-exposed mice (Fig. S2). Immunohistochemistry confirmed the appearance of UCP1 protein in these areas (Fig. S2). BAT and iWAT expression of *Ucp1* and the gene encoding peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1α*) was increased after cold exposure (Fig. S1 and S2).

HPA axis activity is enhanced in cold exposed mice

Next, we determined whether cold exposure not only enhanced BAT activity and browning of inguinal WAT, but also induced the HPA axis. Plasma ACTH concentrations were 2-fold higher in cold-exposed than in control mice (Figure 1A). In line with this observa-

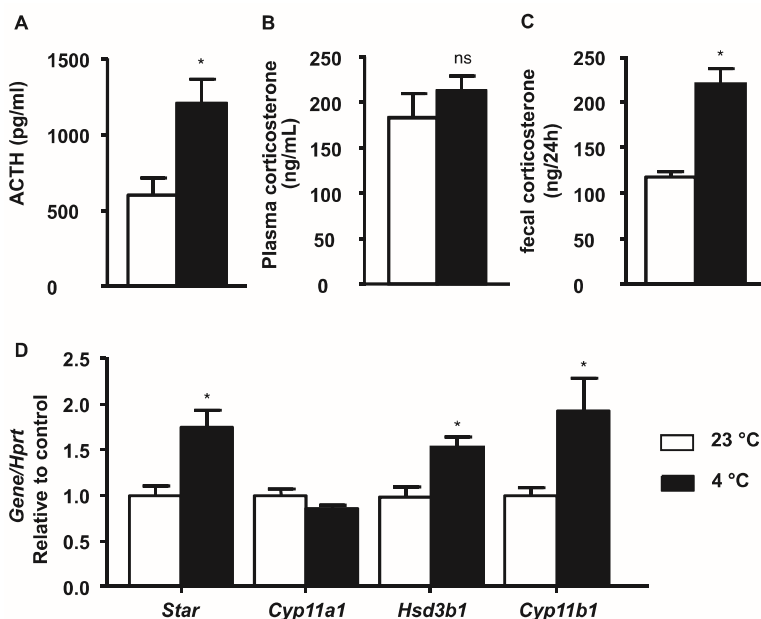


Figure 1. Effects of 24 h 23°C and 4°C on the HPA axis. A) Plasma ACTH concentrations after the 24 h; B) Plasma corticosterone concentrations after the 24 h; C) Fecal corticosterone excretion during the 24 h; D) Relative expression of genes encoding for steroidogenic enzymes in the adrenal gland, normalized to *Hprt* mRNA with data from mice kept at 23°C defined as '1'. Values are means \pm SEM; n=6; *, unpaired student's *t*-test $p < 0.05$.

tion, the 24 h fecal corticosterone excretion was 1.9-fold higher in cold-exposed mice than in control mice (Figure 1C), although plasma corticosterone was not significantly increased (Fig. 1B). The increase in fecal corticosterone excretion was accompanied by a corresponding upregulation of the expression of several genes encoding proteins involved in steroidogenesis in the adrenal gland (*Star*, *Hsd3b1* and *Cyp11b1*) (Figure 1C).

ACTH activates brown adipocytes and induces browning in white adipocytes in vitro

Previous publications report an inhibitory effect of GCs on BAT activity using adrenalectomy with or without GC supplementation (30,32,43). However, these studies overlook the effects of adrenalectomy or GC supplementation on ACTH *in vivo*. Since our cold exposure protocol resulted in markedly increased plasma ACTH, we questioned whether elevations in plasma ACTH might contribute to the induction of BAT activity

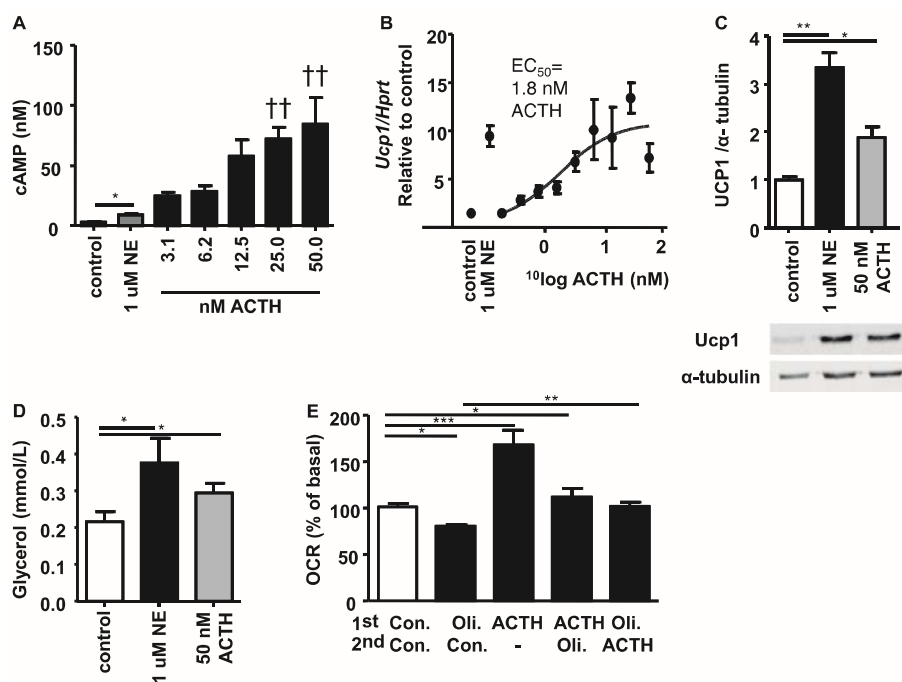


Figure 2. ACTH stimulates Ucp1 content and activity in T37i cells. A) cAMP concentration in medium of T37i cells treated with different concentrations ACTH or 1 μ M norepinephrine (NE) for 15 minutes, n=2; B) *Ucp1* mRNA expression normalized to *Hprt* mRNA expression in T37i cells treated with different concentrations of ACTH or 1 μ M NE for 4 h, n= 3; C) Relative Ucp1 protein concentrations normalized to α -tubulin protein concentrations in T37i cells treated with 50 nM ACTH or 1 μ M NE for 8 h, n=3; D) glycerol concentration in medium of T37i cells treated with 50 nM ACTH or 1 μ M NE for 4 h, n=3; E) Relative oxygen consumption rate of T37i cells compared to unstimulated situation, measured after two injections (1st and 2nd in the graph) adding control medium, ACTH, oligomycin or the combination of both, n=10. Values are means \pm SEM; *, unpaired student's t-test $p < 0.05$; †, one-way-ANOVA with post-hoc Bonferroni vs control †= $p < 0.05$, ††= $p < 0.01$

and browning of inguinal WAT. First, we tested the direct effects of ACTH on brown adipocytes *in vitro* using differentiated T37i cells. Since both the gene encoding the ACTH receptor melanocortin receptor type 2 (MC2R) and the MC2R accessory protein (MRAP) are expressed in T37i cells, BAT and iWAT (Fig. S3), a direct effect of ACTH on these cells is very likely. Indeed, treatment of differentiated T37i cells with ACTH caused a dose-dependent increase in cAMP to an even higher level than could be reached with a high concentration (1 μ M) of norepinephrine (NE) (Figure 2A). This increase in cAMP was accompanied by a strong dose-dependent increase in *Ucp1* mRNA expression with an EC₅₀ of 1.8 nM (Figure 2B). Of interest, the maximal induction of *Ucp1* mRNA comparable with the effect of 1 μ M NE. Based on these results, we decided to use a fixed 50 nM ACTH dose in all subsequent *in vitro* experiment.

Concomitant with the induced *Ucp1* mRNA expression, Ucp1 protein was also increased upon treatment with either 50 nM ACTH or 1 μ M NE (Figure 2C). Next, we determined whether the increase in Ucp1 by ACTH indeed resulted in greater metabolic activity. Lipolysis, as reflected by glycerol secreted into the medium, was found to be increased by both ACTH and NE in T37i cells (Figure 2D).

Oxygen consumption rate (OCR) of differentiated T37i cells was also measured. We first determined basal OCR, which includes both respiration driven by ATPsynthetase and uncoupling. ACTH increased basal OCR by 68%. Adding oligomycin, an ATPsynthetase inhibitor, to untreated cells resulted in a 21% decline in OCR (Figure 2E), thus 79% of total OCR of T37i cells was due to uncoupled respiration. This is in line with the abundance of Ucp1 protein in brown adipocytes. Subsequent ACTH administration to oligomycin-treated cells increased uncoupled OCR by 40% (Figure 2E), underscoring the stimulatory effect of this hormone on Ucp1 activity.

The effects of ACTH on T37i cells may be a peculiarity of this cell line. Therefore, we also treated primary cultured adipocytes from murine interscapular BAT and inguinal WAT with ACTH for 8h. In these cells, ACTH also increased *Ucp1* mRNA expression (Figure 3), further demonstrating the direct effect that ACTH has on both brown and white adipocytes.

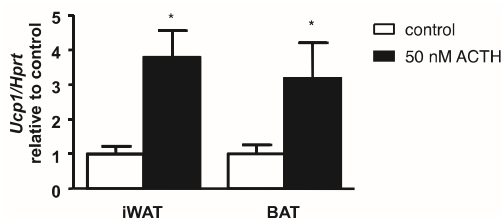


Figure 3. *Ucp1* mRNA expression normalized to *Hprt* mRNA expression of primary cultured murine white adipocytes from inguinal WAT (iWAT) and murine brown adipocytes from the interscapular BAT depot. Cells were treated with with 50 nM ACTH for 8 h. Values are means \pm SEM with data from the control situation defined as '1'; n=5-6; *, unpaired student's *t*-test $p < 0.05$.

Corticosterone inhibits ACTH and NE induced *Ucp1* mRNA expression and protein

Since both corticosterone and ACTH were increased in cold exposed mice, we next determined the interplay between corticosterone and ACTH on *Ucp1* mRNA expression in differentiated T37i cells. Corticosterone reduced basal *Ucp1* mRNA expression (Figure 4 A,C) but not *Ucp1* protein levels (Figure 4B,D). As reported before, corticosterone reduced the transcriptional response of *Ucp1* mRNA and *Ucp1* protein (Figure 4A,B) to adrenergic stimulation (27). Intriguingly, corticosterone also reduced *Ucp1* mRNA and *Ucp1* protein in ACTH treated T37i cells (Figure 4C,D). These inhibitory effects of corticosterone were mediated via the GR since addition of the GR antagonist RU486 prevented the corticosterone-mediated *Ucp1* mRNA (Figure 4A,C) and protein reduction (Figure 4 B,D). Addition of the mineralocorticoid receptor (MR) antagonist spironolactone could not prevent an inhibitory effect of corticosterone, showing that the MR is not involved in the corticosterone-mediated inhibition of *Ucp1* mRNA and *Ucp1* protein expression (Figure 4 A,B,C,D)

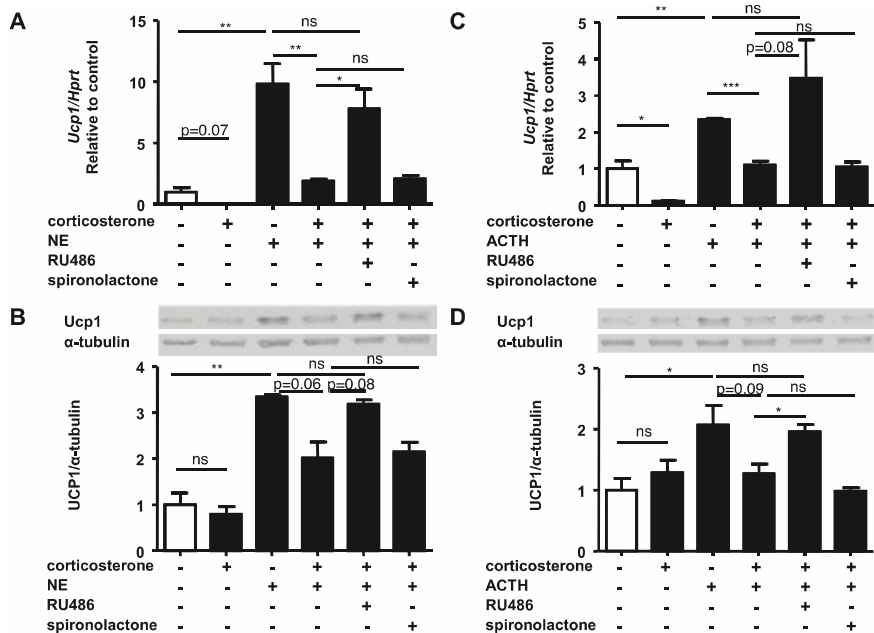


Figure 4. Effects of corticosterone on T37i cells. A) Relative *Ucp1* mRNA expression normalized to *Hprt* mRNA expression and B) relative *Ucp1* protein normalized to α -tubulin protein concentrations in T37i cells treated with 1 μ M corticosterone, 1 μ M NE, 10 μ M RU486 and/or 10 μ M spironolactone; C) Relative *Ucp1* mRNA expression normalized to *Hprt* mRNA expression and D) relative *Ucp1* protein normalized to α -tubulin protein concentrations in T37i cells treated with 1 μ M corticosterone, 50 nM ACTH, 10 μ M RU486 and/or 10 μ M spironolactone. Values are means \pm SEM with data from the control situation defined as '1'; n=3, unpaired students *t*-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

ACTH induces BAT activity and is counter regulated by corticosterone *in vivo* in mice.

Finally, we tested the effects of ACTH on BAT *in vivo*. For this, we injected mice with ACTH and determined uptake of ^{18}F -FDG by the interscapular BAT depot using PET, as described previously (38). A single ACTH bolus injection 20 minutes before ^{18}F -FDG administration resulted in a 2-fold induction of ^{18}F -FDG uptake in interscapular BAT (Figure 5), confirming the BAT activating role of ACTH. Pretreatment with the GR antagonist RU486 enhanced ^{18}F -FDG uptake in BAT. This is in line with the inhibitory effects of corticosterone on BAT activity via the GR, but could also be caused by the increase of ACTH upon RU486 treatment which could not be measured due to the experimental set-up. ACTH and RU486 showed no interaction, suggestive for independent effects of both ACTH and RU486. Unfortunately, due to the experimental set-up, ACTH levels could not be measured.

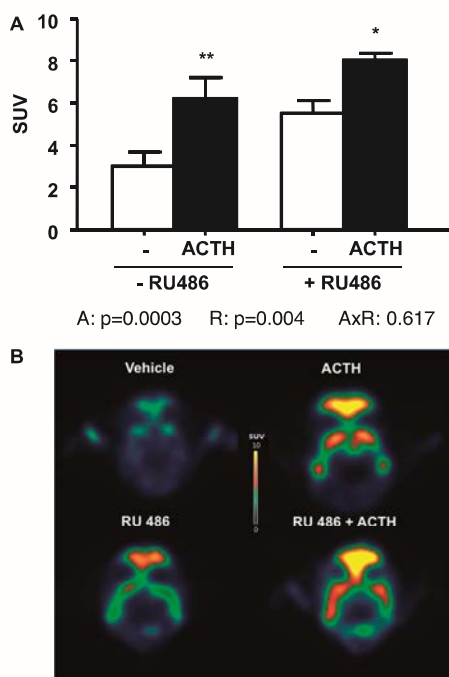


Figure 5. Effect of ACTH on BAT ^{18}F -FDG uptake. A) Standardized uptake value (SUV) in BAT of vehicle and ACTH injected mice expressed relative to the controls, with or without RU486 co-treatment. Values are means \pm SEM; n=6; *, 2-way-ANOVA with Bonferroni post-hoc comparisons, $p < 0.05$. Depicted below the graphs are the p-values of the 2-way ANOVA tests for either ACTH (A), RU486 (R) or the interaction between ACTH and RU486 (AxR). B) Representative PET image (transverse view) showing ^{18}F -FDG accumulation in the interscapular BAT of mice treated with either vehicle, ACTH, RU486 or both RU486 and ACTH.

DISCUSSION

The present study was designed to investigate the roles of the HPA-axis hormones corticosterone and ACTH on BAT activity and browning of WAT. Our results show that ACTH increases BAT activity and browning of WAT, while corticosterone largely inhibits ACTH-mediated BAT activating effects at a peripheral level. This thus indicates that both ACTH and corticosterone might play an important role in energy homeostasis through regulation of BAT activity, at least in mice. Via induction of the secretion of the BAT inhibitor corticosterone by the adrenal glands, ACTH restricts its stimulatory effects on BAT. The inhibitory effects of corticosterone are mediated via the GR and not the MR. Since these effects are seen *in vitro*, both ACTH and corticosterone have direct opposite effects on BAT and WAT.

Cold exposure is a generally accepted method to activate BAT and induce browning of WAT in animal models (44) and is also used as a stressor in behavioral studies (25). The remarkable flexibility of the adipose tissue depots, which has been reported previously (21,45), is illustrated by the fact that a relatively short period of 24 h causes morphological changes in BAT and WAT. Since cold exposure also enhances release of ACTH and subsequently corticosterone, it is very likely that these hormones also play a role in these cold-induced changes. Our results indicate that ACTH has physiological functions in addition to regulation of adrenal glucocorticoid release.

Previously, it has been shown that ACTH stimulated lipolysis in cultured 3T3-L1 cells via the adenylyl cyclase/cAMP/PKA pathway (46). It has also been shown that lipolysis can be induced with greater efficiency in WAT explants of rats after cold exposure of the animals (47). In the present paper we show that ACTH also has direct effects on BAT and on browning of WAT. We are the first to show that the increase in Ucp1 protein by ACTH, as has been shown previously with another BAT cell line (48), is functionally relevant since ACTH acutely induced uncoupled respiration of T37i cells and enhanced 18F-FDG uptake of mouse BAT *in vivo* within 20 minutes. The observed effects of ACTH are very similar to those of NE and are most likely mediated via the same signaling pathway, especially since the ACTH receptor MC2R and β -adrenergic receptors share the adenylyl cyclase/cAMP/PKA pathway (17,18). This shared pathway was indeed confirmed by our findings since ACTH elevated cAMP within 15 minutes.

The concentrations of ACTH used in our *in vitro* experiments are higher than the plasma concentration of 1.2 ng/ml (Figure 1A) that is equivalent to 0.26 nM. In our second set of *in vitro* experiments we chose to use a concentration of 50 nM ACTH to ensure a robust effect which is required for these proof-of-principle experiments. For our *in vivo* supplementation study, we chose a dose of 5 mg/kg ACTH to ensure that the intraperitoneally injection would result in a peak concentration of ACTH.

The finding that ACTH also stimulates WAT browning in the inguinal depot might be even more relevant in humans since Wu *et al.* (8) recently suggested that the areas considered to be BAT in humans are in fact WAT depots with large amounts of brown-like rather than brown adipocytes, although this is still under debate (10-12,49). In addition, since humans have more WAT than BAT, WAT depots are important targets in which to increase brown-like adipocyte density as a means of inducing energy expenditure.

Another important finding of our studies is the interaction between ACTH and corticosterone on BAT activity. *In vivo*, ACTH increases GC plasma concentrations and intriguingly corticosterone interacts with ACTH in regulating BAT activity. These effects are in line with earlier publications where GCs, administered simultaneously with NE, abolished the stimulatory effect of NE on *Ucp1* transcription but not mRNA translation (27). We showed that corticosterone has a negative feedback effect on ACTH not only at the level of the pituitary but also in BAT. This interaction of ACTH and corticosterone at a peripheral level has so far only been described for thymocyte development and peripheral blood mononuclear cell proliferation (50-53).

These inhibitory effect of GCs on *UCP1* transcription and function are possibly caused by GCs interfering with the adenylyl cyclase/cAMP/PKA signaling pathway (54) or might be mediated through reduction of β 3-adrenergic receptor number (54) although this requires more detailed investigation. Direct regulatory effects of the GR on transcription of *Ucp1* have been described (29) and could be the mechanism by which GCs regulate UCP1 activity. However, GR regulatory elements in the promoter region of *UCP1* have not been described. Since corticosterone is released after approximately 20-30 minutes (55-57) after ACTH is administered, it is most likely that ACTH is especially important in the very acute effects of stress on BAT activity. Thus far, only modest long-term effects of ACTH administration on BAT thermogenesis have been reported *in vivo* (58,59), but the thermogenic effects of ACTH could have been partially reduced by increased levels of circulating corticosterone. Our *in vivo* PET study shows that GCs inhibit the acute and direct stimulatory effects of ACTH since BAT activity after ACTH administration is higher when the GR antagonist RU486 is given shortly before the ACTH bolus. This interaction between GCs and ACTH on BAT have never been shown in an *in vivo* model before. In line with these *in vivo* data are the results from our T37i studies that also show that corticosterone attenuates the effect of ACTH on *Ucp1* gene and protein expression. We can thus conclude that corticosterone interferes with the activating signaling pathway induced by ACTH. However, the exact mechanism of interaction requires more research. Our observations show us to be careful in drawing conclusions from adrenalectomy studies, since the reported results might not only be due to reduced GC concentrations, but could also be due to elevated ACTH.

The ACTH receptor, MC2R and its co-receptor MRAP1 are both present in mouse brown and white adipocytes and T37i cells (Fig. S3). Of interest, a recent microarray study showed that human bone marrow-derived mesenchymal stem cells start to express MRAP1 during adipogenic development (60), suggesting a role for MRAP1 in adipocyte function and/or differentiation. MC2R, in contrast, has been reported to be absent in human WAT (61,62) although lipolytic effects of ACTH on human subcutaneous adipocytes have been described (63). Recent evidence indicates that MC4R, when co-expressed with MRAP2, can function as a sensitive ACTH receptor (64). Expression of MC2R or MC4R and MRAP in human brown or brown-like adipocytes has not yet been reported, but if these receptors are present in these cells, the question arises whether this is of importance in patients with an ACTH producing adenoma, i.e., those with Cushing's disease. It would be of interest to study whether these patients have more active brown and/or more brown-like adipocytes, although it might be possible that long term exposure to glucocorticoids overrules the stimulating effect of ACTH at fat tissue level. Indeed, Burt *et al.* (56) showed that energy expenditure in patients with Cushing's disease or syndrome (16 out of 18 patients studied suffered from ACTH overproduction) was not significantly decreased compared to matched healthy controls (65). Thus, dedicated human ^{18}F -FDG PET scans are required to study the effects of ACTH and cortisol in patients with Cushing's disease or syndrome.

In conclusion, we can add ACTH to the expanding list of compounds that directly activate BAT, such as catecholamines (66), thyroid hormone (67), natriuretic peptides (68,69), bone morphogenetic proteins 7 (70) and 8b (71), irisin (72), and sex steroid hormones (73). Interestingly, ACTH stimulates production of its own moderator, the glucocorticoids. It is important to take into account this finding, particularly when performing *in vivo* investigations into the long term role of GCs and ACTH on BAT activity. Thermogenesis in mammals is tightly controlled and it is therefore most likely that BAT activity is regulated by more than one hormone. More detailed studies of the role of glucocorticoids during stimulation of BAT activity might reveal new approaches to activate the lipid-burning properties of BAT and brown-like WAT as a way of combating obesity.

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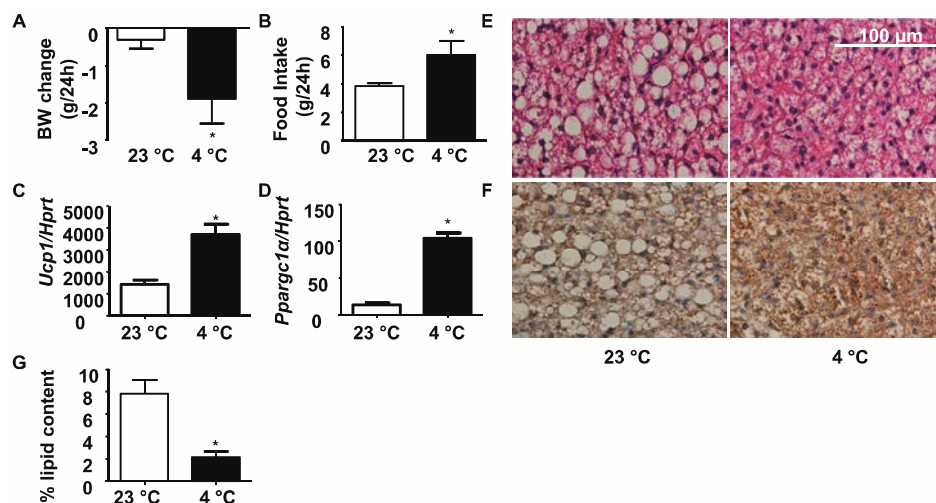
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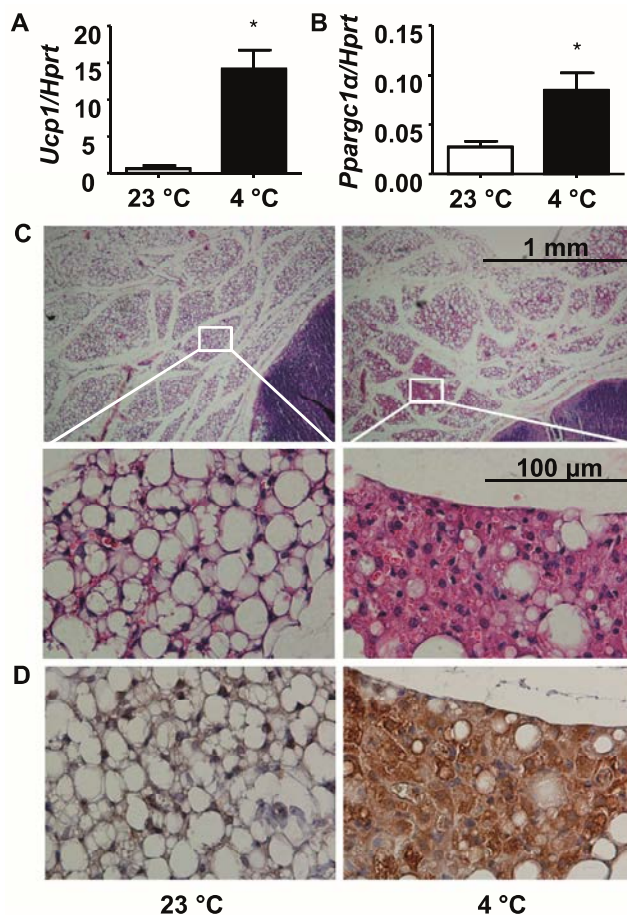
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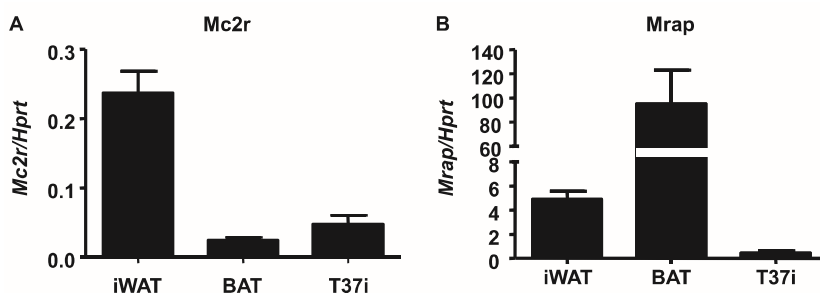
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Effects of 24 h 23°C and 4°C on bodyweight and BAT. A) Changes in bodyweight and B) food intake during the 24 h; C) Relative BAT *Ucp1* and D) *Ppargc1a* mRNA expression after the 24 h, normalized to *Hprt* mRNA expression; E) Representative H&E staining (400x original magnification) and F) UCP1 immunohistochemical staining (400x original magnification) of BAT after the 24 h; G) Percentage lipid content in BAT after the 24 h (3 slices were analyzed per animal, n=3) Values are means \pm SEM; n=6 unless otherwise indicated; *, unpaired students-*t*-test $p < 0.05$.



Supplemental Figure 2. Effects of 24 h 23°C and 4°C on inguinal WAT. A) Relative inguinal WAT *Ucp1* and B) *Ppargc1a* mRNA expression after the 24 h, normalized to *Hprt* mRNA expression; C) Representative H&E staining (40x and 400x original magnification) and D) UCP1 immunohistochemical staining (400x original magnification) of inguinal WAT after the 24 h. Values are means \pm SEM; n=6; *, unpaired students-*t*-test $p < 0.05$.



Supplemental Figure 3. Expression of A) *Mc2r* and B) *Mrap* mRNAs in interscapular BAT and inguinal WAT, n=6, and in T37i cells, n=3.

(re)

CHAPTER 3

COLD EXPOSURE PARTIALLY CORRECTS DISTURBANCES IN LIPID METABOLISM IN A MALE MOUSE MODEL OF GLUCOCORTICOID EXCESS

Johanna C. van den Beukel, Mariëtte R. Boon, Jacobie Steenbergen, Patrick C.N. Rensen, Onno C. Meijer, Axel P.N. Themmen, Aldo Grefhorst

ABSTRACT

Background: High glucocorticoid concentrations are accompanied by metabolic side effects such as high plasma triglyceride (TG) concentrations. Liver, brown adipose tissue (BAT) and white adipose tissue (WAT) are important regulators of plasma TG. Exposure to 4°C reduces plasma TG concentrations and we therefore aimed to study the interaction between glucocorticoid excess and 24 h exposure to 4°C on lipid metabolism.

Methods: Mice were implanted with 50 mg corticosterone or control pellets and housed for 24 h at 23°C or 4°C one week later, after which various aspects of TG metabolism in liver, BAT and WAT were studied.

Results: Corticosterone treatment resulted in a 3.8-fold increase of plasma TG concentrations. Increased TG was normalized by cold exposure, an effect still present 24 h after cold exposure. Corticosterone treatment increased hepatic TG content by 3.5-fold and provoked secretion of large, TG-rich very low density lipoprotein (VLDL) particles. Cold exposure reduced VLDL-TG secretion by approximately 50%. Corticosterone strongly decreased BAT activity: BAT weight increased by 3.5-fold while *Ucp1* mRNA expression and *Ucp1* protein content of BAT were reduced by 75% and 60%, respectively. Cold exposure partially normalized these parameters of BAT activity. The uptake of TG by BAT was not affected by corticosterone treatment, but was increased 4.5-fold upon cold exposure.

Discussion: cold exposure normalizes corticosterone-induced hypertriglyceridemia, at least partly via activating BAT.

INTRODUCTION

Glucocorticoid (GC) excess in patients with Cushing's disease or syndrome results in metabolic derangements such as insulin resistance and hyperlipidemia that are also part of the metabolic syndrome. Also patients undergoing chronic treatment with GCs have symptoms resembling the metabolic syndrome although interpretation of these observations is sometimes difficult due to possible confounding effects of the concomitant diseases that prompted the GC treatment (1).

The effects of GC excess on insulin resistance and glucose metabolism are well described (2,3), but GC-mediated disturbances in lipid metabolism are not completely understood. Since hyperlipidemia, including increased plasma triglyceride (TG) and free fatty acid (FA) concentrations, are probably associated with the increased incidence of cardiovascular disease (CVD) in Cushing patients (2), more insight in GC-induced hyperlipidemia is needed. Of note, even slight but prolonged elevations of plasma cortisol within the normal range are associated with an increased number of cardiovascular events (4). This might also have implications for patients in situations of chronic stress. Plasma TG concentrations are determined by three processes that are closely linked; secretion of TG-rich chylomicrons by the intestinal tract upon ingestion of fatty acids, secretion of TG-rich very low density lipoprotein (VLDL) particles by the liver, and hydrolysis of TGs from VLDL and chylomicrons by lipoprotein lipase (LPL) located on the capillary wall and subsequent uptake of released fatty acids by target tissues via fatty acid transporters such as CD36 (5). GCs have been reported to influence TG metabolism by increasing hepatic VLDL secretion but also by upregulation of *Lpl* gene expression and activity in various tissues, increasing the storage and availability of FA in adipose tissue (6,7).

The effects of GCs on plasma free fatty acid (FFA) concentrations are complex. The major source of circulating FFAs is the adipose tissue and GCs exert different effects on separate adipose tissue depots. Although it is thought that GCs increase white adipose tissue (WAT) lipolysis and hence increase plasma FFA concentrations (8,9), GCs at the same time induce expansion of specific WAT depots. In humans, central fat depots, such as visceral and abdominal fat, expand during GC excess, whereas peripheral fat depots, including subcutaneous fat, show increased lipolysis and diminished storage of TGs (6,10). Thus, the effects of GCs on WAT are depot specific.

Brown adipose tissue (BAT) plays an important role in the clearance of both VLDL-TG and FFA from the circulation (11). Activation of BAT by means of cold exposure results in increased uptake of VLDL-TG-derived FA (12) and FFA by this tissue. In addition, not only the 'classical' BAT appears to be pivotal for plasma TG and FFA clearance, but very likely also the recently characterized beige adipose tissue depots that contain white adipocytes with BAT-like characteristics are important (13,14). Appearance of the brown-like

adipocytes in these depots is induced by cold exposure and an increased amount of beige fat has been shown to contribute to a lean and healthy phenotype (15-17). We recently showed that a relative short, 24 h exposure to 4°C is enough to increase BAT activity and appearance of brown-like adipocytes in WAT (18). Thus, activation of BAT and browning of WAT by 24 h exposure to 4°C might be beneficial in improving metabolic disturbances such as dyslipidemia.

Since multiple tissues might be involved in GC-mediated hyperlipidemia, as we discussed above, we questioned *if, how* and *via* which tissues 24 h exposure to 4°C would be able to correct GC-mediated hyperlipidemia. Thus, we examined the effects of cold exposure and/or a high dose of corticosterone on plasma, liver, BAT and WAT lipid metabolism in a mouse model of GC excess.

MATERIALS AND METHODS

Animals

All animal experiments were performed with the approval of the Animal Ethics Committee at Erasmus MC, Rotterdam, the Netherlands. Eight weeks old male C57Bl/6J mice were obtained from Charles River Laboratories (Maastricht, The Netherlands).

Corticosterone supplementation and cold exposure

One week after arrival, mice at 9 weeks of age were implanted with a subcutaneous corticosterone pellet (50 mg corticosterone (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 50 mg cholesterol) or control pellet (100 mg cholesterol). Pellets were implanted under the skin of the neck under isoflurane anesthesia. Seven days after pellet implantation, the mice were exposed to 23°C or 4°C for 24 h, as previously described (18). Then, one of the experiments described below was performed.

Control experiment

After exposure for 24 h to either 23°C or 4°C, blood glucose was measured in tail blood obtained after tail incision by using a Freestyle mini glucometer and test strips (Abbot, Amersfoort, The Netherlands), and rectal body temperature was determined. Mice were then terminated by cardiac puncture under isoflurane anesthesia. Feces was collected during the 24 h period. Plasma and various tissues were collected and either immediately frozen and stored at -80°C or fixed in 4% paraformaldehyde.

Persistence study

For this study, blood was collected by orbital bleeding under light isoflurane sedation just before and immediately after 24 h of exposure to 4°C as well as 24 and 48 h after termination of cold exposure, and plasma TG was measured.

VLDL secretion experiment

The mice were deprived of food for the last 4 h after which they were sedated lightly with isoflurane and an orbital blood sample was taken. Next, the mice received an orbital injection of 100 µl 12.5% Triton WR-1339 (Sigma) in PBS. Orbital blood samples were taken under light isoflurane sedation 30, 60, 120 and 180 minutes after Triton WR-1339 injection. The mice were killed by cardiac puncture under isoflurane anesthesia directly after the last blood draw.

VLDL-TG derived FA uptake experiment

VLDL-like TG-rich emulsion particles (80 nm), labeled with glycerol tri³Holeate (triolein, TO), were prepared and characterized as described previously (19). The mice were deprived of food for the last 4 h while kept at either 23°C or 4°C after which they received an orbital injection of 200 µl VLDL-like TG-rich emulsion particles (1.0 mg TG per mouse) under light isoflurane sedation. Blood samples were obtained by tail bleeding at 2, 5, 10 and 15 minutes after injection of the VLDL-like TG emulsion particles. The mice were killed by cervical dislocation directly after the last blood draw after which they were perfused with ice-cold PBS via the heart to remove blood from all tissues. Various tissues were collected, weighed and dissolved in Soluene (Perkin Elmer, Groningen, the Netherlands). Plasma was separated from the tail blood samples. Retention of radioactivity in plasma and saponified tissue was determined.

Fatty acid uptake experiment

[³H]oleate (GE Healthcare, Hoevelaken, The Netherlands) was bound to FA-free bovine serum albumin (BSA) as described previously (20). In short, to remove FAs from BSA (Sigma-Aldrich, A3912), it was dissolved in aquadest with additional activated charcoal (0.5 g/g BSA) and pH adjusted to 3.0 with HCl. The suspension was stirred for 2 h on ice and centrifuged for 20 minutes at 13,000 rpm at 4°C. Supernatants were pooled and pH was normalized to 7.0 with NaOH and subsequently filtered (0.2 µm). For binding of [³H]oleate to this FA-free BSA, [³H]oleate was added in a 5 times higher molar concentration than FA-free BSA and incubated for 1 h at 37°C. Unbound [³H]oleate was removed using PD-10 columns (GE Healthcare). The FA-free BSA-[³H]oleate complex was rebuffered in PBS, filtered (0.2 µm) and stored at 4°C and used within 2 days.

The mice were deprived of food for the last 4 h while kept at either 23°C or 4°C after which they received an orbital injection of 100 µl of the FFA-free BSA-[³H]oleate

complex solution under light isoflurane sedation. Blood samples were obtained by tail bleeding at 2, 5 and 10 minutes after [^3H]oleate injection. The mice were killed by cervical dislocation directly after the last blood draw after which they were perfused with ice-cold PBS via the heart to remove blood from all tissues. Various tissues were collected, weighed and dissolved in Soluene (Perkin Elmer, Groningen, the Netherlands). Plasma was separated from the tail blood samples. Retention of radioactivity in plasma and saponified tissue was determined.

Measurements of plasma ACTH, corticosterone, cholesterol, TG and FFA

Plasma ACTH was measured by radioimmunoassay (MP Biomedicals, Orangeburg, NY). Plasma corticosterone and fecal corticosterone, which was extracted as previously described (18), were measured by ELISA (ENZO life sciences, Raamsdonksveer, The Netherlands). Plasma cholesterol and TG concentrations were measured with commercial available kits (ABX Pentra, Horiba, Irvine, CA) while plasma FFA was determined with a kit from Wako (Neuss, Germany). All measurements were performed according to the manufacturers' protocols.

Hepatic analysis

Hepatic concentrations of TGs were measured using a commercial available kit (ABX Pentra) after lipid extraction according to Bligh and Dyer (21).

VLDL isolation and particle size determination

Nascent VLDL particles were isolated from the plasma obtained from the cardiac puncture after the VLDL secretion experiment. For this isolation, 300 μl plasma was added to 3,700 μl PBS and centrifuged at 30,000 rpm for 17 h after which the top layer containing the VLDL was collected. VLDL size in this top fraction was determined using a Zetasizer Nano Z (Malvern Instruments, Malvern, United Kingdom).

Gene expression analysis

Total RNA isolation from mouse tissues and subsequent DNase treatment and reverse transcription was performed as previously described (18). Gene expression was measured using quantitative RT-PCR with SYBRgreen master mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems) and normalized to expression of housekeeping genes 18S and $\beta 2\gamma$ globulin (adipose tissue) or 18S and β actin (liver) using the $2^{-\Delta\Delta\text{Ct}}$ method (22). Expression of the housekeeping genes was not influenced by treatment and housing temperature. Primer sequences used for all measured genes are listed in Supplemental Table 1.

Histology and immunohistochemistry

For hematoxylin and eosin staining 8 μm sections of tissues preserved in paraformaldehyde and embedded in paraffin were used. Sections were mounted on microscope slides (Thermo Scientific, Breda, Nederland) and kept overnight at 37°C, deparaffinized in xylene and subsequently stained. For immunohistochemistry, sections were mounted on superfrost plus microscope slides (Thermo scientific), kept at 37°C for at least 10 h before staining for extra adherence. Sections were kept at 60°C for 1 h and subsequently deparaffinized in xylene for 6 minutes, rinsed twice in 100% EtOH, put in methanol containing 3% H_2O_2 to block endogenous peroxidase activity and rinsed in demineralized water. Heat antigen retrieval was achieved by cooking glasses in NaOH buffered citric acid (pH 6.0). After cooling down of the slides, sections were blocked with 5% normal goat serum (Dako, Heverlee, Belgium) in PBS for 5 minutes, rinsed in PBS and then incubated overnight with the first antibody at 4°C (UCP1 1:500, Sigma). BrightVision-poly-HRP-anti mouse/rabbit/rat IgG (Immunologic, Duiven, the Netherlands) in a 1:2 dilution in PBS was added for 30 minutes at room temperature as second antibody and peroxidase activity was developed with 0.07% 3,3-diaminobenzidine-tetrahydrochloride (Sigma) with subsequent counterstaining with hematoxylin.

Western blot analysis

Protein was extracted from tissues as previously described (18). Fifteen μg of protein was electrophoresed on a 10% gel and blotted onto a nitrocellulose membrane. Membranes were blocked in PBS containing 3% nonfat powdered skim milk before an overnight incubation at 4°C with a rabbit polyclonal anti-UCP1 antibody (1:1000; Sigma) in PBS containing 0.1% Tween-20 and 3% nonfat powdered skim milk or a rabbit polyclonal anti- α -tubulin antibody (1:200; Santa Cruz, Heidelberg, Germany) in PBS containing 0.1% Tween-20 and 5% BSA. Next, membranes were washed and incubated for 1 h at room temperature with a goat-anti-rabbit IRDye 800 secondary antibody (1:10,000; Li-cor, Leusden, The Netherlands) in PBS containing 0.1% Tween-20 and 3% non-fat powdered skim milk. Ucp1 immunoreactivity was measured with an Odyssey fluorescence scanner (Li-cor, Leusden, Belgium) and was normalized for α -tubulin immunoreactivity in the same samples using Odyssey software.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA). The effect of treatment and temperature was studied by two-way ANOVA. A post-hoc unpaired t-test was performed ($p < 0.025$ being considered significant) when a significance effect was found in the two-way ANOVA. For the persistence study, a paired t-test was performed with $p < 0.05$ being considered significant.

RESULTS

Corticosterone treatment creates a model of GC-excess

The primary aim of our experiments was to determine the interaction between GC excess and 24 h exposure to 4°C on lipid metabolism. We first characterized our mouse model of GC excess. On average, the mice treated with corticosterone showed a tendency towards lower increase in bodyweight over time than the placebo-treated mice, but this was not due to changes in food intake since the corticosterone-treated mice ate more than the control mice (Table 1). Plasma corticosterone concentrations and 24 h fecal corticosterone excretion were both increased ~6-fold by corticosterone (Table 1). As expected, due to its suppressive effects on the hypothalamic–pituitary–adrenal (HPA) axis, corticosterone treatment reduced plasma ACTH concentrations. In line with the high plasma corticosterone concentrations, thymus weights were reduced since GCs induce thymocyte apoptosis (23). Altogether, we created mice with a disturbed HPA axis, as is found in patients with Cushing and those receiving high doses of synthetic GCs.

Table 1. Effects of corticosterone treatment and cold exposure in mice

	23°C		4°C		Results 2WA		
	Control	Corticosterone	Control	Corticosterone	C	T	CxT
One week BW change (%)	2.6 ± 0.8 a	0.0 ± 1.0 a	1.9 ± 1.0 a	-1.5 ± 1.1 a	P<0.01	NS	NS
24 h BW change (%)	0.9 ± 0.9 a	0.7 ± 1.0 a	-0.9 ± 0.6 a	-1.7 ± 0.5 a	NS	P<0.05	NS
Food intake (g/24 h)	3.7 ± 0.1 a	4.5 ± 0.2 b	6.3 ± 0.2 c	5.1 ± 0.1 b	NS	P<0.001	P<0.001
Plasma corticosterone (ng/ml)	80 ± 9 a	469 ± 103 b	119 ± 12 a	688 ± 104 b	P<0.001	NS	NS
Fecal corticosterone (ng/24 h)	286 ± 54 a	1722 ± 331 b	433 ± 20 a	2139 ± 99 b	P<0.001	NS	NS
Plasma ACTH (pg/ml)	415 ± 81 a	65 ± 17 b	727 ± 129 a	85 ± 38 b	P<0.001	NS	NS
Thymus weight (g)	0.21 ± 0.01 a	0.10 ± 0.01 b	0.21 ± 0.02 a	0.10 ± 0.01 b	P<0.001	NS	NS

Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means ± SEM; n=6. BW, body weight. Statistical analysis was performed using 2-way-ANOVA analysis (2WA). C = effect of corticosterone treatment; T = effect of temperature, CxT = interaction between corticosterone and temperature. A post-hoc unpaired t-test was performed when a significant effect was found in the 2-way ANOVA and values that do not share a symbol are significantly different (p<0.025) in this post-hoc test.

Cold exposure is a stressor but does not change HPA axis activity in mice with GC excess.

To study the effects of cold exposure on corticosterone-induced changes, we kept corticosterone-treated and control mice at 23°C or 4°C for 24 h. Cold exposure reduced body weight independent of corticosterone treatment. Of interest, however, while the control mice almost doubled their food intake at 4°C, the corticosterone-treated mice continued their level of food intake (Table 1). Cold exposure itself tended to increase plasma corticosterone levels and fecal corticosterone excretion by 1.5-fold, but this was not significant (Table 1). Plasma ACTH concentrations were approximately 1.75-fold increased after cold exposure in control mice but cold did not change ACTH plasma concentrations in corticosterone treated mice. Thus, suppression of the HPA axis activity by corticosterone was not altered by cold exposure intervention.

Corticosterone treatment increases fasted plasma TG concentrations that are normalized by cold exposure

Next, we determined the effects of corticosterone and cold exposure on fasted blood and plasma parameters. Surprisingly, the corticosterone pellets did not affect fasted blood glucose concentrations (Figure 1A) but tended to increase fasted plasma FFA concentrations ($p=0.06$) (Figure 1B). The most striking effect of corticosterone treatment was seen for fasted plasma TG concentrations; plasma TG levels were increased 3.8-fold in corticosterone-treated mice (Figure 1C). Cold exposure increased circulating FFA independent of corticosterone treatment (Figure 1B). Although corticosterone increased plasma TG concentrations in mice kept at 23°C, this was not the case in mice kept at 4°C (Figure 1C). Next, we tested whether the TG-lowering effect of 24 h exposure to 4°C persisted even after the mice were placed back at normal housing temperature. Once again, cold exposure reduced plasma TG contents in both control and corticosterone treated mice, but interestingly, the plasma TG concentrations were still reduced in the corticosterone-treated mice 24 h after the mice were removed from the 4°C (Figure 1D). Since increased TG levels are an important negative side-effect of excess of GC, we decided to examine the mechanism by which corticosterone and cold exposure regulate plasma TG concentrations in mice in more detail. For this, we studied hepatic VLDL-TG production and tissue TG and FFA uptake.

Corticosterone treatment elevates while cold exposure lowers secretion of VLDL-TG

Corticosterone treatment markedly enhanced VLDL-TG secretion 1.5-fold ($p<0.0001$) (Figure 2A) and analysis of nascent VLDL particles revealed that corticosterone-treated mice secreted larger VLDL particles (Figure 2B). Analysis of the liver showed that corticosterone treatment resulted in heavier and fattier livers with lipids accumulation mainly localized in the perivenous (PV) compared to periportal (PP) area (Figures 2C-2E).

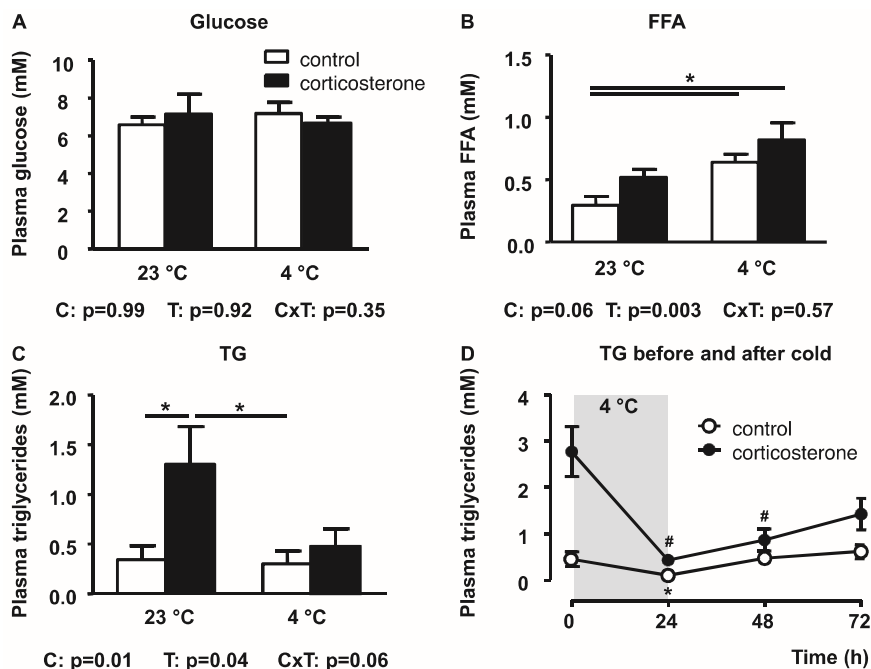


Figure 1. Plasma parameters of corticosterone-treated and/or cold-exposed mice

A) Plasma glucose concentrations; B) Plasma FFA concentrations; C) Plasma TG concentrations of control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either corticosterone treatment (C), temperature (T) or the interaction between corticosterone treatment and temperature (CxT). *, p<0.025 in the post-hoc unpaired t-test. D) Plasma TG concentrations before and after exposure to 4°C for 24 h. Data from control mice and mice treated with a corticosterone pellet for 1 week. Values are means \pm SEM; n=5; *, p<0.05 vs. value before exposure to 4°C for control mice in paired t-test; #, p<0.05 vs. value before exposure to 4°C for corticosterone-treated mice in paired t-test.

Cold exposure significantly reduced VLDL-TG secretion in control mice (Figure 2A). Although the exposure to 4°C also reduced VLDL-TG secretion in corticosterone-treated mice, this reduction was not statistically significant. In addition, cold exposure tended to slightly increase the VLDL size independent of corticosterone treatment (Figure 2B) and, unexpectedly, further increased the hepatic TG content in the liver (Figure 2D). Since FFA are the main substrates of TG esterification in the liver and, hence, VLDL-TG secretion (24), we next determined hepatic uptake of VLDL-TG derived FA and FFA, using injections of glycerol tri[³H]oleate-labeled VLDL-like TG-rich particles and [³H]oleate respectively. While the hepatic uptakes of FFA and VLDL-TG derived FA were not affected by corticosterone treatment, cold exposure lowered FFA uptake but did not influence VLDL-TG derived FA uptake (Figure 3). Thus, these data suggest that the increase in plasma TG levels upon corticosterone treatment might be due to enhanced hepatic

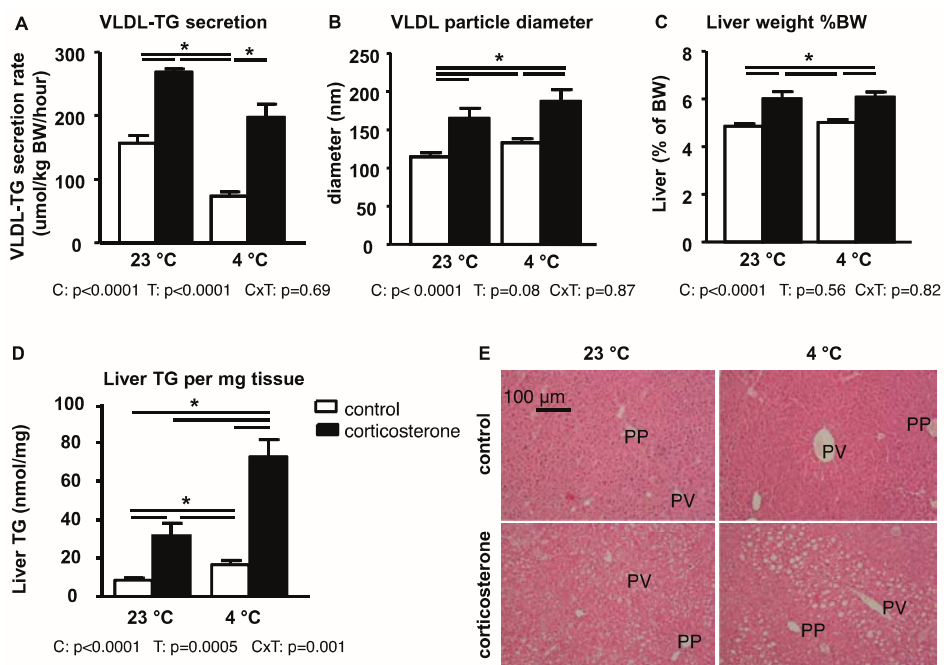


Figure 2. Liver lipid metabolism in corticosterone-treated and/or cold-exposed mice

A) VLDL-TG secretion rate; B) Nascent VLDL particle diameter; C) Relative liver weight as percent of body weight (BW); D) Liver TG content per mg tissue. Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either corticosterone treatment (C), temperature (T) or the interaction between corticosterone treatment and temperature (CxT). *, p<0.025 in the post-hoc unpaired t-test. E) Representative H&E staining of livers from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. PP = periportal vein, periportal area; PV = portal vein, perivenous area.

VLDL-TG secretion but the normalization of hypertriglyceridemia upon cold exposure cannot be explained by changes in VLDL secretion.

Cold exposure normalizes the corticosterone-induced hepatic de novo lipogenic gene expression

Since hepatic lipid accumulation upon corticosterone treatment was apparently not due to changes in FA uptake and predominantly found in the perivenous area where both *de novo* lipogenesis and VLDL secretion are thought to be localized (25-29), we determined whether genes encoding enzymes involved in *de novo* lipogenesis were changed by corticosterone. In addition, we determined hepatic expression of genes encoding other enzymes involved in lipid metabolism. Expression of *de novo* lipogenesis gene *Fasn* was markedly increased upon corticosterone treatment (Figure 4A) but, interestingly, gene

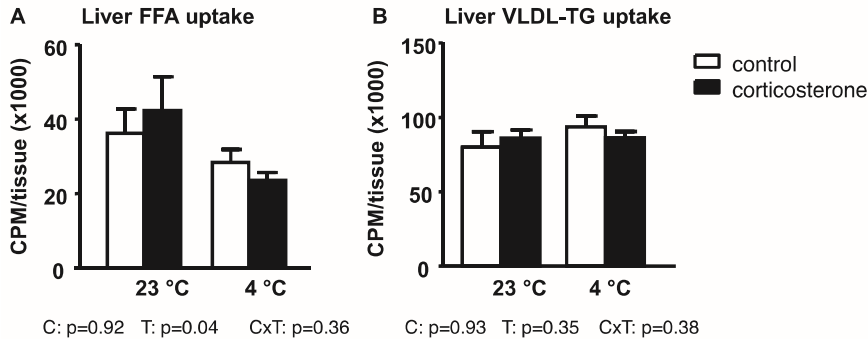


Figure 3. Hepatic FA and TG uptake

A) Hepatic [^3H]oleate uptake; B) Hepatic uptake of fatty acids derived from VLDL-like TG-rich emulsion particles (80 nm) enriched with glycerol tri[^3H]oleate in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either corticosterone treatment (C), temperature (T) or the interaction between corticosterone treatment and temperature (CxT). *, p<0.025 in the post-hoc unpaired t-test.

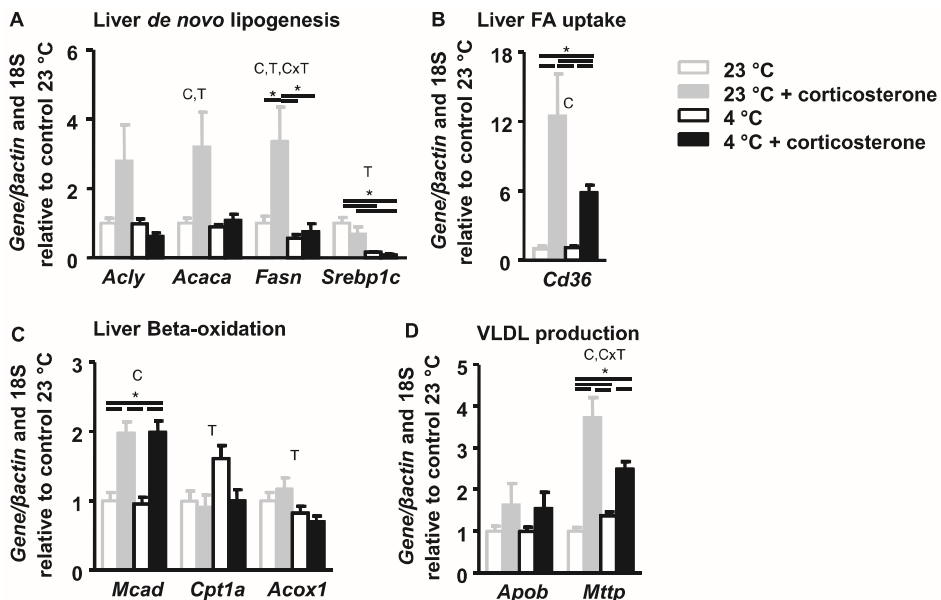


Figure 4. Hepatic gene expression

A) Hepatic expression of lipogenic genes; B) Hepatic *Cd36* expression of *Cd36*; C) Hepatic expression of genes encoding proteins involved in beta-oxidation; D) Hepatic *Apob* and *Mttp* expression in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. C, p<0.05 for corticosterone treatment in 2-way ANOVA test. T, p<0.05 for temperature in 2-way ANOVA test. CxT, p<0.05 for the interaction between corticosterone treatment and temperature. *, p<0.025 in the post-hoc unpaired t-test.

expression of the major regulator of *de novo* lipogenesis, sterol-regulatory element-binding protein-1c (*Srebp1c*), was not affected by corticosterone treatment. With respect to hepatic fatty acid uptake, expression of the gene encoding the fatty acid transporter CD36 (*Cd36*) was 12.5-fold upregulated by corticosterone treatment (Figure 4B). For the genes encoding enzymes involved in fatty acid oxidation, the effects are less obvious. Only *Mcad* expression was increased by corticosterone (Figure 4C). The expression of *Apob* and *Mttp*, two genes important in VLDL secretion, was increased by corticosterone treatment albeit that this was only statistically significant for *Mttp* (Figure 4D).

Cold exposure reduced the increased *Fasn* expression and reduced *Srebp1c* expression by 5-fold (Figure 4A). Likewise, the increase of *Cd36* by corticosterone was reduced by a factor 2 upon cold exposure (Figure 4B). Cold exposure partially normalized the corticosterone-induced *Mttp* expression.

BAT activity is reduced by corticosterone and increased by cold exposure

The increase of hepatic VLDL-TG secretion provides an explanation for increased plasma TG concentrations in corticosterone-treated mice, but cannot explain the correction of plasma TG in cold-exposed corticosterone-treated mice since livers of these mice still secrete more TGs than those of non-treated mice (Figure 2A). Since BAT is known to be a marked contributor to VLDL-TG clearance from the circulation, especially when activated (11,12), we next studied lipid metabolism in BAT.

BAT weight was 3-fold higher ($p < 0.025$) in corticosterone-treated mice than in control mice (Figure 5A) and histological examination showed much more lipid content per adipocyte (Figure 5B). *Ucp1* staining (Figure 5C), *Ucp1* mRNA expression ($p = 0.006$ in unpaired t-test for control mice kept at 23°C and corticosterone-treated mice kept at 23°C; Figure 5D) and *Ucp1* protein content (Figure 5E) in BAT were all lower in corticosterone-treated animals compared to non-treated animals, indicative for reduced BAT activity. However, both the uptake of FFA and VLDL-TG derived FA by BAT were not affected by corticosterone treatment (Figures 5F and 5G). Thus, the increased lipid content in BAT of corticosterone-treated mice is likely due to reduced lipid turnover in BAT, which is indeed supported by the reduced *Ucp1* expression and *Ucp1* content.

As expected, cold exposure strongly upregulated *Ucp1* mRNA expression in BAT of control mice and in corticosterone-treated mice (Figure 5D). The weight and lipid content of BAT were also partially corrected by cold in corticosterone-treated mice (Figures 5A and 5B). Effects of cold exposure on the uptake of FFA and VLDL-TG derived FA by BAT did not differ between control and corticosterone-treated mice (Figures 5F and 5G). Next, we studied the expression of various genes that encode proteins involved in BAT differentiation/activity, e.g., *Ppargc1a*, *Prdm16* and *Cidea*, and genes involved in stimulation of BAT-specific genes: *Cebpa* and *Pparg*. We found that corticosterone treatment had no effect on these markers while cold exposure enhanced BAT activity,

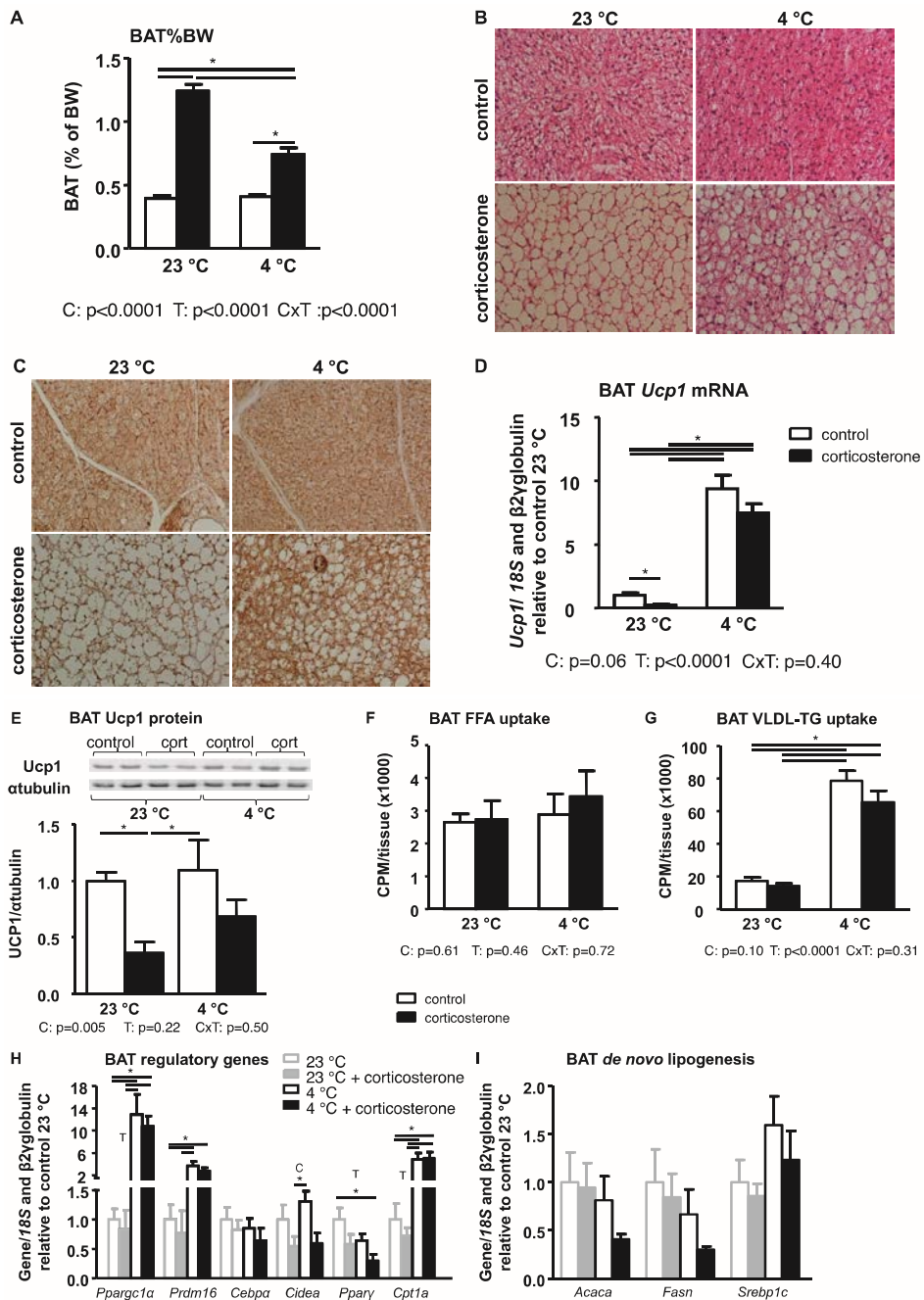


Figure 5. Effects of corticosterone treatment and/or cold-exposure on BAT

A) Relative intrascapular BAT weight as percent of body weight (BW); B) Representative H&E staining of intrascapular BAT; C) Representative *Ucp1* immunohistological staining of intrascapular BAT; D) Intrascapular BAT *Ucp1* expression; E) Representative intrascapular BAT *Ucp1* and α -tubulin

Western blot and quantification of 3 Ucp1 vs. α -tubulin Western blots; F) Intrascapular BAT [^3H] oleate uptake; G) Intrascapular BAT uptake of fatty acids derived from VLDL-like TG-rich emulsion particles (80 nm) enriched with glycerol tri[^3H]oleate. Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either corticosterone treatment (C), temperature (T) or the interaction between corticosterone treatment and temperature (CxT). *, p<0.025 in the post-hoc unpaired t-test. H) Intrascapular BAT expression of various regulatory genes and genes encoding for proteins involved in fatty acid oxidation; I) Intrascapular BAT expression of lipogenic genes in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. C, p<0.05 for corticosterone treatment in 2-way ANOVA test. T, p<0.05 for temperature in 2-way ANOVA test. CxT, p<0.05 for the interaction between corticosterone treatment and temperature. *, p<0.025 in the post-hoc unpaired t-test.

as expected. *Cpt1a* expression can be considered a readout of fatty acid oxidation and thus likely BAT activity and it is clear that cold exposure increased expression of *Cpt1a* in BAT of both control and corticosterone-treated mice (Figure 5H). Neither corticosterone treatment nor cold exposure affected the expression of the lipogenic genes *Acaca*, *Fasn* and *Srebp1c* in BAT (Figure 5I).

Browning of inguinal WAT is reduced by corticosterone but partially corrected by cold

Since the WAT depots deliver their fatty acids to the liver and BAT, we questioned whether WAT is also affected by corticosterone and cold exposure. We therefore studied browning and lipogenic and lipolytic activity of two different WAT depots: inguinal and gonadal WAT. Inguinal WAT has been shown to brown easily and gonadal WAT depot is considered a classical WAT depot that is more resistant to ‘browning’.

Weight and adipocyte cell size of inguinal WAT were increased upon corticosterone treatment (Figures 6A and 6B). Corticosterone also diminished Ucp1 protein staining (Figure 6C) and *Ucp1* mRNA expression (Figure 6D), indicative for reduced ‘browning’. Uptake of FFA and TG by inguinal WAT was increased by \sim 2-fold and \sim 4-fold, respectively (Figures 6E and 6F) in corticosterone-treated mice. In line with this, corticosterone treatment increased *Cd36* mRNA expression (Figure 6G).

Cold-induced browning of inguinal WAT in both control mice and corticosterone-treated mice is evident from the decreased cell size, increased Ucp1 staining and 10-fold increased *Ucp1* mRNA expression (Figures 6B-D). While the effect of cold on inguinal WAT FFA uptake did not reach statistical significance (Figure 6E), the 5-fold increased VLDL-TG derived FA uptake induced by corticosterone treatment was completely and statistically significant normalized by cold exposure (Figures 6F). *Cd36* expression was increased by cold exposure in both control and corticosterone-treated mice (Figure 6G). Altogether, these data indicate that corticosterone diminishes browning but stimulates

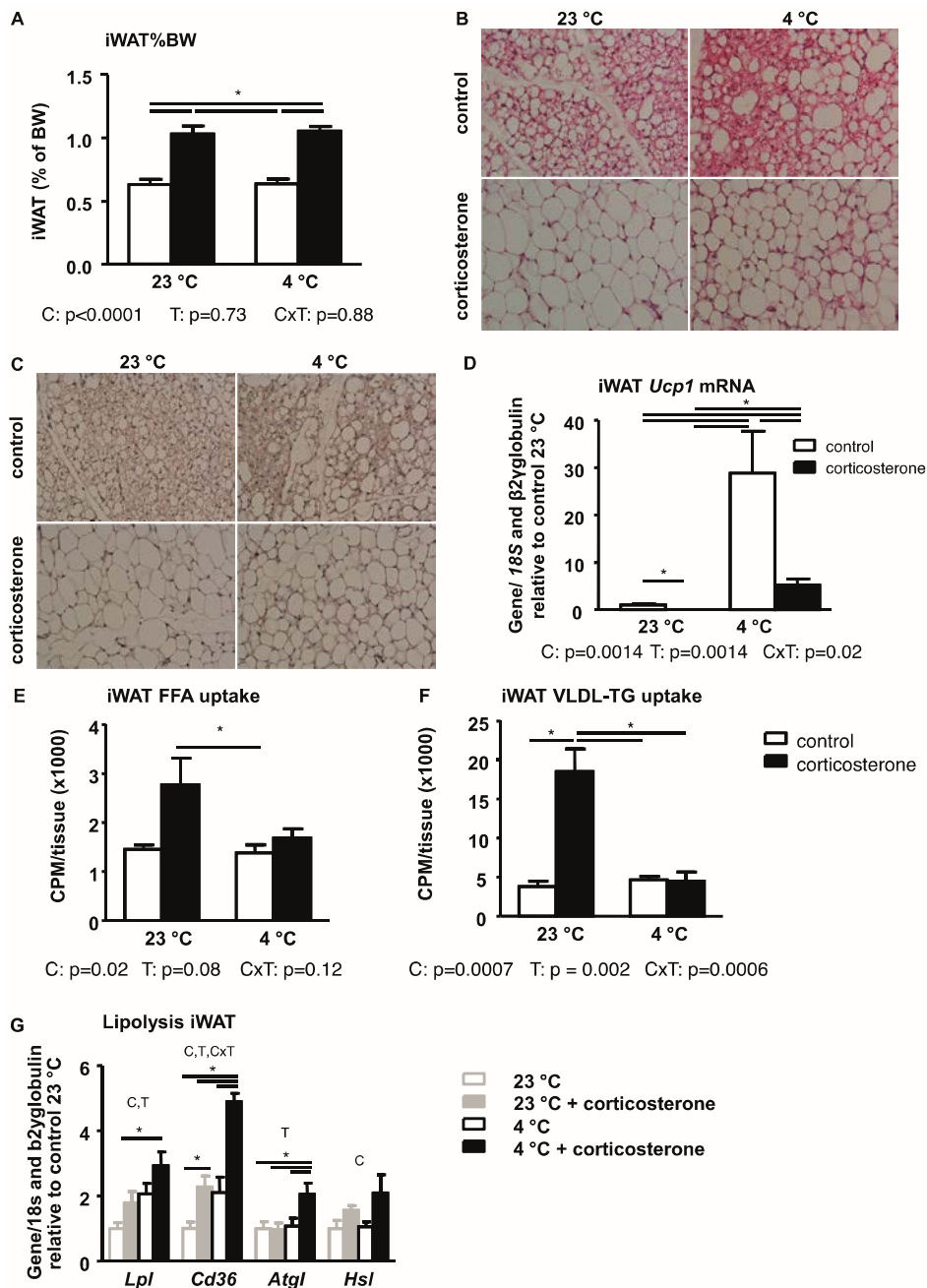


Figure 6. Effects of corticosterone treatment and/or cold-exposure on inguinal WAT

A) Relative inguinal WAT weight as percent of body weight (BW); B) Representative H&E staining of inguinal WAT; C) Representative Ucp1 immunohistological staining of inguinal WAT; D) Inguinal WAT *Ucp1* expression; E) Inguinal WAT [3 H]oleate uptake; F) Inguinal WAT uptake of fatty acids derived from VLDL-like TG-rich emulsion particles (80 nm) enriched with glycerol tri[3 H]oleate.

Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either corticosterone treatment (C), temperature (T) or the interaction between corticosterone treatment and temperature (CxT). *, p<0.025 in the post-hoc unpaired t-test. G) Inguinal WAT expression of lipolytic genes in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. C, p<0.05 for corticosterone treatment in 2-way ANOVA test. T, p<0.05 for temperature in 2-way ANOVA test. CxT, p<0.05 for the interaction between corticosterone treatment and temperature. *, p<0.025 in the post-hoc unpaired t-test.

storage of lipids in the inguinal WAT depot. Importantly, cold exposure partially corrects these corticosterone-induced disturbances.

Corticosterone increases lipid uptake in the gonadal WAT and this is normalized by cold exposure

Finally, we investigated the effects of corticosterone and cold exposure on gonadal WAT. As for inguinal WAT, corticosterone treatment increased weight and adipocyte cell size of gonadal WAT, increased uptake of FFA and TG-derived FA, and resulted into a tendency towards increased *Lpl* and *Cd36* mRNA expression (Figure 7). In contrast to inguinal WAT, however, corticosterone increased mRNA expression of the lipolytic gene *Atgl*, suggesting that gonadal WAT is a source for plasma FFA. Both cell size and weight of gonadal WAT were not affected by cold exposure but cold exposure increased *Ucp1* and *Ppargc1a* expression in gonadal WAT (Figure 7F). The uptake experiments revealed that cold exposure enhanced the uptake of FFA but did not affect the uptake of VLDL-TG derived FA by gonadal WAT of control mice. Of interest, 24 h exposure to 4°C resulted in a normalization of VLDL-TG derived FA uptake by gonadal WAT in corticosterone-treated animals. Finally, the combination of corticosterone-treatment and cold exposure resulted in enhanced mRNA expression of *Lpl*, *Cd36*, *Atgl*, and *Hsl* in gonadal WAT compared to control mice kept at 23°C (Figure 7E).

DISCUSSION

Our mouse model of GC excess shows that chronic corticosterone treatment of C57Bl/6J mice elevates plasma TG concentration and that this is very likely the result of enhanced hepatic *de novo* lipogenesis and, hence, elevated VLDL-TG secretion. The relative short, 24 h exposure to 4°C reduced the corticosterone-mediated elevation in plasma TG concentration, an effect still present 24 h after termination of cold exposure. Although cold exposure reduced hepatic VLDL-TG secretion, the VLDL-TG secretion rate was still significantly higher in cold-exposed corticosterone-treated mice compared to control mice. In addition, we found that 24 h exposure to 4°C was able to partially correct the severe inhibition of

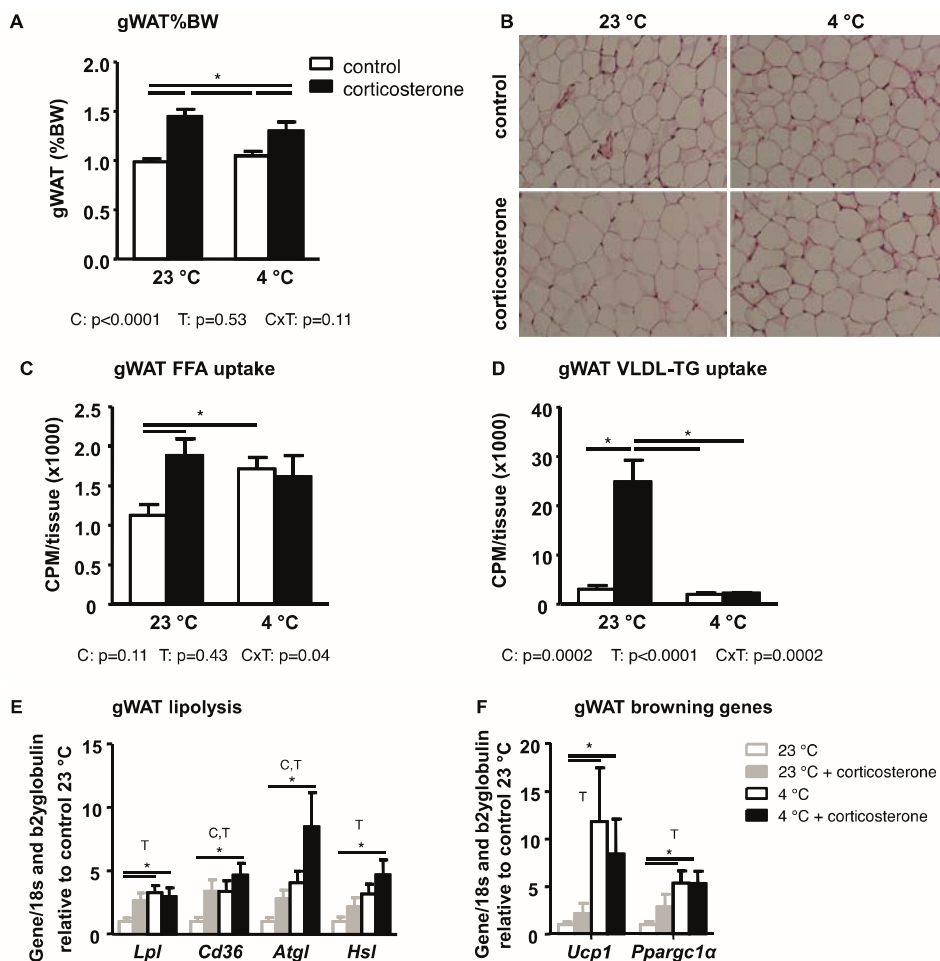


Figure 7. Effects of corticosterone treatment and/or cold-exposure on gonadal WAT

A) Relative gonadal WAT weight as percent of body weight (BW); B) Representative H&E staining of gonadal WAT; C) Gonadal WAT [^3H]oleate uptake; D) Gonadal WAT uptake of fatty acids derived from VLDL-like TG-rich emulsion particles (80 nm) enriched with glycerol tri[^3H]oleate. Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either corticosterone treatment (C), temperature (T) or the interaction between corticosterone treatment and temperature (CxT). *, p<0.025 in the post-hoc unpaired t-test. E) Gonadal WAT expression of lipolytic genes; F) Gonadal WAT *Ucp1* and *Ppargc1 α* expression in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 h. Values are means \pm SEM; n=6. C, p<0.05 for corticosterone treatment in 2-way ANOVA test. T, p<0.05 for temperature in 2-way ANOVA test. CxT, p<0.05 for the interaction between corticosterone treatment and temperature. *, p<0.025 in the post-hoc unpaired t-test.

BAT activity upon GC excess. Finally, in our model of GC excess, the adipocyte size of WAT was increased, likely due to enhanced uptake of both FFA and VLDL-TG-derived FA. Cold exposure normalized both features of WAT. Altogether, in our murine model of GC excess, multiple features of disturbed lipid metabolism located in different tissues are corrected by a relative short, 24 h exposure to 4°C, albeit not fully on all accounts.

Cold exposure is a well-known stressor for rodents (30) and we also found that in our experiments since exposure to 4°C for 24 h resulted in elevated plasma ACTH concentrations and fecal corticosterone excretion in control mice by approximately 50% and 75%, respectively. These effects of cold exposure are comparable to what was found before (18) but failed to reach statistical significance upon multiple testing due to inclusion of the corticosterone-treated mice.

In our studies, we found that both corticosterone treatment and 24 h exposure to 4°C elevated the hepatic TG concentration. In general, GC excess results in insulin resistance of glucose metabolism with elevated plasma insulin concentrations. Since hepatic *de novo* lipogenesis remains insulin sensitive under these conditions (31,32), the high plasma insulin concentrations upon GC excess might stimulate hepatic *de novo* lipogenesis. Insulin is known to enhance nuclear translocation increase of SREBP-1, resulting in increased expression of lipogenic SREBP-1c target genes such as *Acaca* and *Fasn* (33). Indeed, we found enhanced hepatic *Fasn* and *Acaca* expression in corticosterone-treated mice. Interestingly, mRNA expression of *Srebp1c* was reduced upon corticosterone treatment as is in line with the findings of Laskewitz *et al.* (34) who found a similar effect with prednisolone in livers of *ad lib* fed mice and Erhuma *et al.* (35) who found downregulation of hepatic *Srebp1c* mRNA by GCs in rats *in vivo* but upregulation *in vitro*. As discussed by the authors of the latter study, these data suggest that the effects of GCs on *Srebp1c* mRNA *in vivo* may be through indirect mechanisms rather than a direct effect on the SREBP-1c promoter level. In addition, also other nuclear transcription factors such as the liver X receptor (LXR) and the carbohydrate response element binding protein (ChREBP) facilitate lipogenic gene expression in the liver (36,37), thus maybe activation of these factors might have enhanced *Acaca* and *Fasn* expression.

Of interest, cold exposure resulted in a massive hepatic TG accumulation in the corticosterone-treated mice, even significantly higher than in cold exposed control mice. This corticosterone-induced hepatic TG content is most probably not the result of a changed balance in lipid secretion and uptake since VLDL-TG secretion and hepatic uptake of VLDL-TG-derived FA and FFA did not differ between control and corticosterone-treated mice. Therefore, the increase in hepatic TG concentration is very likely a result of reduced hepatic fatty acid oxidation in cold exposed corticosterone-treated mice compared to cold exposed control mice. Indeed, the gene encoding one of the rate controlling enzymes in fatty acid oxidation, *Cpt1a*, is upregulated upon cold exposure in control mice but not in corticosterone-treated mice, providing an explanation of the disbalance between fatty acid and TG synthesis and oxidation.

Although previous studies by others have shown that GC excess results in elevated VLDL-TG secretion, the mechanisms remained largely unknown (6,7). Since TG availability is an important driver of VLDL-TG secretion, it is very likely that enhanced hepatic *de novo* lipogenesis upon chronic corticosterone treatment was the driving force for elevated VLDL-TG secretion.

The effects of cold exposure on VLDL-TG secretion have not been studied in great detail. Cold exposure increases BAT activity and hence the uptake of VLDL-TG-derived FA by this tissue (11,12) thus one might expect the body to compensate for this by enhancing the hepatic VLDL-TG secretion. In rats, 3 h exposure to 10°C increased VLDL secretion and this effect of cold could be prevented by administration of the non-specific beta-adrenergic antagonist propranolol (38). The latter finding is in sharp contrast to what we find. Very likely, the timing of the experiments is of crucial importance. We determined VLDL-TG secretion after 24 h cold exposure, *i.e.*, much later than the previous study, and found a decreased VLDL-TG secretion. Thus, duration of cold exposure likely differentially affects VLDL-TG secretion.

Another important regulator of VLDL-TG secretion in cold-exposed mice might be sympathetic nervous activity, *i.e.*, elevated catecholamines. In line with our data, Rasouli and colleagues (39) found that hepatic denervation, resulting in a 99% reduction of hepatic catecholamine content, enhanced VLDL-TG secretion, while treatment of primary hepatocytes with both alpha- and beta-adrenoreceptor agonists suppressed VLDL secretion (40). In contrast, others found that selective denervation of sympathetic input towards the liver resulted in decreased VLDL secretion in 19 h-fasted but not in 4 h-fasted rats (41). Interestingly, cold-exposure interacts with corticosterone treatment at the level of lipogenic gene expression, diminishing some deteriorating effects of corticosterone. However, more research is required to understand via which mechanisms cold-exposure and corticosterone affect hepatic lipid metabolism and VLDL-TG secretion.

A number of *in vivo* and *in vitro* studies show that GCs inhibit BAT activity, most likely via a glucocorticoid receptor (GR) mediated pathway (42-46). In addition, adrenalectomy of rodents resulted in increased BAT activity which was normalized by GC replacement (47,48). Our results are in line with these findings, but we are the first to show that activation of BAT by means such as cold exposure partially reverse this GC-mediated inhibition. This is confirmed by our previous *in vitro* observations that norepinephrine was still able to increase *Ucp1* mRNA expression in cultured brown adipocytes treated with corticosterone (18). Since we found profound inhibition of BAT activity in our mouse model of GC excess and BAT can take up large amounts of VLDL-TG derived FA, we initially hypothesized that reduced BAT activity in corticosterone-treated mice would result in reduced uptake of TG-derived FA by BAT and thus contribute to increased plasma VLDL-TG levels. However, we did not find a decrease in uptake of TG-derived FA by BAT upon GC treatment. Thus, the accumulation of lipids in the BAT depot of GC-treated

mice is very likely due to reduced lipid catabolism in BAT. Indeed, the severely reduced *Ucp1* mRNA expression and *Ucp1* protein content in BAT of corticosterone-treated mice kept at 23°C points towards reduced lipid catabolism.

Cold exposure is not the sole method to activate BAT *in vivo*. For instance, treatment with the beta3-adrenoreceptor agonist CL316,243 is also a commonly used method to activate BAT in laboratory animal models (49). In rats, Suárez et al. (50) did not find an effect of CL316,243 on percentage of total liver fat, suggesting that it is not activation of BAT *per se* that induces hepatic TG concentrations upon cold exposure.

The effects of GCs and the interaction with the 24 h exposure to 4°C seem to differ between the studied WAT depots. In gonadal WAT, GC treatment resulted in a tendency towards increased expression of both *Atgl* and *Hsl* that both encode important enzymes involved in lipolysis of intracellular TGs into glycerol and fatty acids. In inguinal WAT, corticosterone treatment did not affect *Atgl* expression. Interestingly, in inguinal WAT, corticosterone treatment induced expression of the gene encoding the fatty acid transporter CD36 independent of cold exposure. In gonadal WAT, in contrast, corticosterone treatment resulted in a tendency towards increased *Cd36* expression in mice kept at 23°C, not in those exposed to 4°C. However, both the gonadal and the inguinal WAT depot have an increased uptake of VLDL-TG derived FA and FFA upon corticosterone excess, but the differences in uptake between the control and corticosterone-treated mice disappeared when mice were kept at 4°C for 24 h. This shows, once again, that 24 h exposure to 4°C is beneficial in controlling disturbed lipid metabolism in our mouse model of GC excess.

In summary, we show that treatment of mice with corticosterone resulted in hypertriglyceridemia which was likely the result of enhanced VLDL-TG secretion due to increased hepatic *de novo* lipogenesis. Twenty-four h exposure to 4°C normalized the corticosterone-induced hypertriglyceridemia, an effect that was still present 24 h after the cold exposure was ended. Although cold exposure reduced VLDL-TG secretion, corticosterone-treated mice kept at 4°C still had higher VLDL-TG secretion rates than control mice at this temperature. Since exposure to 4°C corrected the corticosterone-mediated reduction of BAT activity, activation of BAT was at least partly responsible for normalization of corticosterone-induced hypertriglyceridemia. Alternative methods to activate BAT such as treatment with a beta3-adrenoreceptor agonist might be useful to study whether BAT activation *per se* is indeed sufficient to correct GC-induced hypertriglyceridemia.

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Supplemental Table 1. Primer sequences

Gene	Accession No.	Forward primer	Reverse primer
<i>Rn18s</i>	NR_003278	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
<i>B2m</i>	NM_009735	ATCCAAATGCTGAAGAACGG	CAGTCTCAGTGGGGTGAAT
<i>βactin</i>	NM_007393	AAGGCCAACCGTAAAAGAT	GTGGTACGACCAGAGGCATAC
<i>Ucp1</i>	NM_009463	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT
<i>Fkbp5</i>	NM_010220	TCTGCCATTTATTATGTAAACCAGAA	TTTGAATCAAGGAGCTCAATCT
<i>Pck1</i>	NM_011044	ATGTGTGGGCGATGACATT	AACCCGTTTTCTGGGTGAT
<i>Acly</i>	NM_001199296	GAGCCACACTCAGGCTTTCT	CTGCTCTGAAATGCCTTGG
<i>Acaca</i>	XM_006531958	GGATGTGGATGATGGTCTGA	AGGCCTTGATCATCACTGGA
<i>Fasn</i>	NM_007988	GCTGCTGTTGGAAGTCAGC	AGTGTTCGTTCTCGGAGTG
<i>Srebf1</i>	XM_006532716	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCAGCATA
<i>Cd36</i>	XM_006535625	CCTCCAGAATCCAGACAACC	CACAGGCTTTCCTTCTTTGC
<i>Mcad</i>	NM_007382	GATCGCAATGGGTGCTTTTGATAGAA	AGCTGATTGGCAATGTCTCCAGCAA
<i>Cpt1a</i>	NM_009948	TGCCTTTACATCGTCTCAA	GGCTCCAGGGTTCAGAAAGT
<i>Acox1</i>	NM_001271898	TATGGGATCAGCCAGAAAGG	ACAGAGCCAAGGGTCACATC
<i>Apob</i>	NM_009693	AAACATGCAGAGCTACTTTGGAG	TTTAGGATCACTTCTGGTCAAA
<i>Mttp</i>	NM_008642	CGAGTAAAAATCGGGTGCC	GGCTTCAGCCTTGCCATCT
<i>Pgc1α</i>	NM_008904	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCTGTTTTTC
<i>Prmd16</i>	NM_181325	GACATTCCAATCCCACCAGA	CACCTCTGTATCCGTCAGCA
<i>Cebpa</i>	NM_001287514	GACATCAGCGCTACATCGA	TCGGCTGTGCTGGAAGAG
<i>Pparγ</i>	NM_001127330	CAAGAATACCAAAGTGCATCAA	GAGCTGGGTCTTTTCAATAATAAG
<i>Lpl</i>	NM_008509	GCTGGTGGGAAATGATGTG	TGGACGTTGTCTAGGGGGTA
<i>Atgl</i>	NM_025802	GAGCCCCGGGTGGAACAAGAT	AAAAGGTGGTGGGCAGGAGTAAGG
<i>Hsl</i>	NM_001039507	CGCTACACAAAGGCTGCTT	GGATGGCAGGTGTGAACTG

(re)

CHAPTER 4

BAT ACTIVITY AND METABOLISM IN PATIENTS WITH CUSHING'S SYNDROME

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ABSTRACT

Background: Brown adipose tissue (BAT) can burn fatty acids due to the presence of active uncoupling protein 1 (UCP1). Due to this ability, active BAT is an important target tissue to combat obesity and its metabolic consequences. Glucocorticoids diminish and inactivate Ucp1 in rodents but it is not known whether this is also true in humans.

Methods: ^{18}F -fluorodeoxyglucose Positron Emission Tomography (^{18}F -FDG PET) scans, oral glucose tolerance tests and lipid profiles of patients with a glucocorticoid producing adrenal adenoma were studied before and 6 months after adrenalectomy.

Results: Adrenalectomy resulted in a doubling of ^{18}F -FDG uptake in the intrascapular BAT and improved glucose tolerance. In addition, plasma triglyceride (TG) concentrations were decreased while plasma HDL cholesterol concentrations were decreased after adrenalectomy.

Discussion: BAT activity was negatively correlated to plasma cortisol concentrations. In line with increased BAT activity, plasma TG concentrations and glucose metabolism were also improved by adrenalectomy.

INTRODUCTION

Brown adipose tissue (BAT) in adult humans was thought to be irrelevant for temperature control until several studies with ^{18}F -fluorodeoxyglucose Positron Emission Tomography (^{18}F -FDG PET) showed that BAT is present and active in human adults (1,2). The clear negative correlation between the presence of active BAT and body mass index (BMI) suggests an important role of BAT in regulating energy balance in humans (3). Uncoupling protein 1 (UCP1) is a protein only present in brown adipocyte mitochondria that uncouples oxidative phosphorylation from ATP synthesis by enabling a leak of protons over the inner mitochondrial membrane and is thereby essential in generating of heat. Due to this process of thermogenesis, energy is not stored but released as heat by brown adipocytes. In theory, this futile use of energy by BAT will eventually lead to a reduction of bodyweight and hence improve metabolic health. In adult humans not only the classical BAT depot contributes to energy expenditure, but also white adipose tissue (WAT) depots that gained brown-like characteristics such as the presence of UCP1-positive cells (4). However, UCP1 protein is not innately active thus we should not only increase the amount of UCP1 but also activate UCP1 (5). Cold-exposure is the most physiological method to activate BAT and increasing browning of WAT (6,7) and consequently maximizes uptake of ^{18}F -FDG (8).

Glucocorticoids are reported to diminish not only *Ucp1* mRNA expression but also *Ucp1* protein content and activity in rodents (9-11) but the role of glucocorticoids on BAT and/or WAT with brown-like characteristics in humans has not been studied. Humans with high plasma glucocorticoids such as those with Cushing's disease or syndrome or those receiving high doses of glucocorticoids for therapeutic reasons, show several symptoms related to the metabolic syndrome, e.g., abdominal obesity, high plasma triglyceride (TG) concentrations, insulin insensitivity and glucose intolerance (12-14). All of these symptoms strongly suggest that BAT activity is reduced in these patients and thus prompted us to look into BAT activity in patients diagnosed with Cushing's syndrome before and after removal of the glucocorticoid producing adrenal adenoma by adrenalectomy (ADX). For this, we studied BAT activity on ^{18}F -FDG-PET scans after cold exposure and metabolic health by oral glucose tolerance tests (OGTTs) and lipid profiles in these patients before and 6 months after ADX.

METHODS AND MATERIALS

Patients

The study was approved by the Medical Ethics Committee of the Erasmus MC, University Medical Center Rotterdam (protocol no. MEC-2012-238) and written informed consent

was obtained from the patients. Two patients with Cushing's syndrome were enrolled in the study. Patients' clinical data were obtained from medical records and anonymized before analyses were performed. Patients underwent a ^{18}F -FDG PET computed tomography (CT) scan, a Dual X-ray Absorptiometry (DXA) scan and an oral glucose tolerance test (OGTT).

^{18}F –FDG PET-CT scanning

BAT activity was measured by ^{18}F -FDG PET-CT scans that were performed twice: the first scan before ADX and the second scan 6 months after ADX. Patients were fasted for 5 hours before administration of ^{18}F -FDG. Patients were cooled 1 hour before administration of ^{18}F –FDG using a cooling device (BLANKET III Model 233 Hyper - Hypothermia Units, Cincinnati Sub-Zero). Torso, thighs, neck and head were covered with cooling vests (MAXI - LITE THERM, Patient Vest, Cincinnati Sub-Zero). Initially, the device was set to a temperature of 24°C and the temperature was lowered until shivering temperature was reached and cooling procedure was then continued with 2°C above shivering temperature until the ^{18}F -FDG PET-CT scan was performed. ^{18}F -FDG PET-CT was performed following a standard procedure. Since lean body mass (LBM) and hence fat percentage can influence distribution of ^{18}F -FDG, BAT activity was measured by mean Standardized uptake value (mean SUV) and corrected by LBM resulting in mean SUL.

DXA-scanning

LBM, and total fat percentage were determined with a DXA scan (Lunar Prodigy, GE Healthcare, Madison, WI, USA). DXAs were performed before and 6 months after ADX.

OGTT

OGTT was performed before and 6 months after ADX and was started at approximately 9 am after an overnight fast. Blood glucose levels were measured at 5 min prior to administration of glucose, 15 and 30 minutes after glucose intake (75 mg in 200 mL) and then every 30 minutes until 210 minutes.

Laboratory measurements

Laboratory evaluations for cortisol, insulin and glucose were performed following standard care protocol. Plasma lipid profiling was performed on fasted plasma samples using fast protein liquid chromatography (FPLC) on a Superose 6 column (Amersham Biosciences, Roosendaal, The Netherlands) coupled to a Superdex 200 column (Amersham Biosciences). Cholesterol and TG content were measured in the separated fractions using commercial available kits (ABX Pentra, Horiba, Irvine, CA).

Statistical analysis

Data were analyzed using Graphpad Prism.

RESULTS AND DISCUSSION

Effective treatment of Cushing's syndrome by ADX

Before ADX, urinary 24-hour urinary free cortisol was above the normal upper limit of 133 nmol/24 hour in both patients. Six months after ADX, plasma cortisol concentrations were low and an intramuscular injection of 250 µgram ACTH₁₋₂₄ could not increase cortisol above the reference level 540 nmol/L 1h after injection. Thus, in both patients, the function of contralateral adrenal was not normalized yet 6 months after ADX (Table 1). Of interest, ADX improved bodyweight, LBM and BMI in both subjects.

Table 1. Patient characteristics

	Patient 1		Patient 2	
	Before ADX	After ADX	Before ADX	After ADX
Body weight (kg)	80.3	77.6	76.2	64.6
BMI (kg/m ²)	22.0	21.3	29.4	24.9
LBM (%)	61.0	74.8	57.0	64.9
24h-urinary cortisol (nmol/24h) Ref: < 133 nmol/24h	5855	-	582	-
Maximum cortisol after ACTH stimulation (baseline-1h after (nmol/L)) Ref: > 540 nM	-	212→255	-	91→276

Uptake in intrascapular BAT is doubled after ADX

We measured ¹⁸F-FDG uptake in the supraclavicular region, the area where human BAT is localized (1-3,15-18). Uptake in this region was doubled after ADX, even as soon as 6 months of the surgical procedure (Figure 1). This strongly suggests that BAT activity is inhibited in patients with glucocorticoid excess and that this activity can be restored by lowering exposure to cortisol by ADX.

OGTT is improved after ADX

In both patients, AUC for insulin and glucose were improved after ADX (Figure 2). These data confirm an improved glucose metabolism, which is previously reported after successful treatment of patients with Cushing's syndrome or disease (19). We cannot correlate increased BAT activity with improved glucose metabolism due to the number of patients. Further research with more patients will be necessary to address this question.

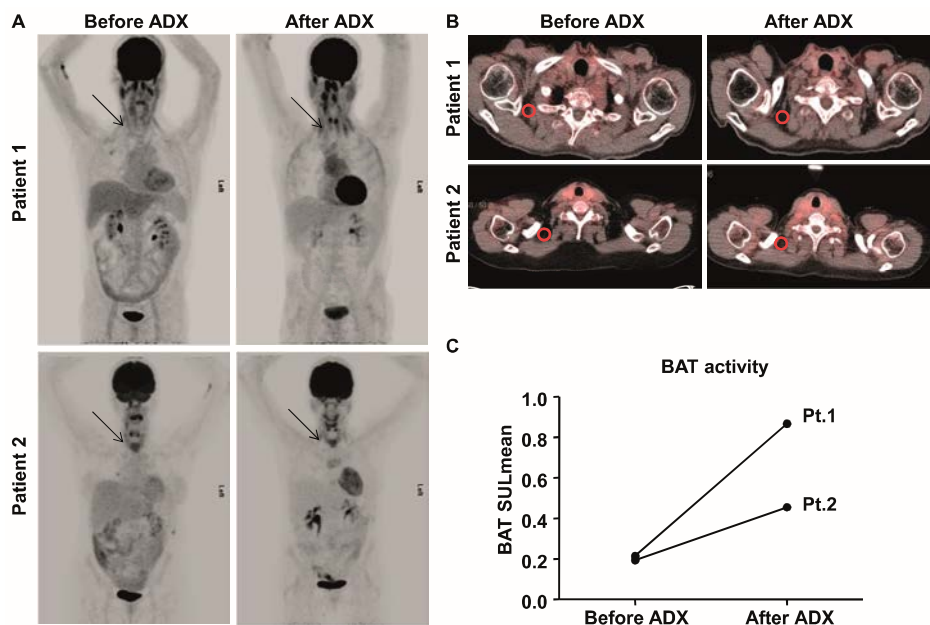


Figure 1. ^{18}F -FDG PET scans of patients with Cushing's syndrome.

5) Total body PET scan of 2 patients before and after ADX. Arrows indicate place of measurement. B) Transverse section of the neck region circles indicate place of measurement. C) Quantification of ^{18}F -FDG uptake in the supraclavicular fat depot before and after ADX of patient 1 (pt.1) and patient 2 (pt. 2). SUV is corrected for LBM resulting in SUL.

Plasma TG concentrations are decreased and HDL cholesterol is lowered after ADX

In line with our previous work in rodents (van den Beukel et al, submitted), we found that plasma TG concentrations were much higher before compared to 6 months after ADX (Figure 3). It will be very interesting to correlate TG levels to BAT activity but we need more patients to be able to draw any conclusions. However, previous mouse studies have revealed that high plasma TG concentrations upon glucocorticoid excess in mice were mainly the result of elevated hepatic VLDL secretion (van den Beukel et al, submitted). In line with this, Taskinen *et al.* (23) showed that also Cushing patients have a significantly higher VLDL-TG synthetic rate than healthy controls.

To our surprise we found a decreased level of HDL cholesterol after ADX (Figure 3). Increased HDL is associated with decreased risk for cardiovascular disease and patients with Cushing's syndrome or disease have clearly increased risk for cardiovascular events (20). Thus the reduced HDL upon adrenalectomy is unexpected. Some reports show that glucocorticoids inhibit cholesteryl ester transfer protein (CETP), which trades TGs from VLDL particles for cholesterol esters from HDL and vice versa (21). Inhibition of CETP results in elevated HDL (22). However, increased HDL might also be due to direct effects

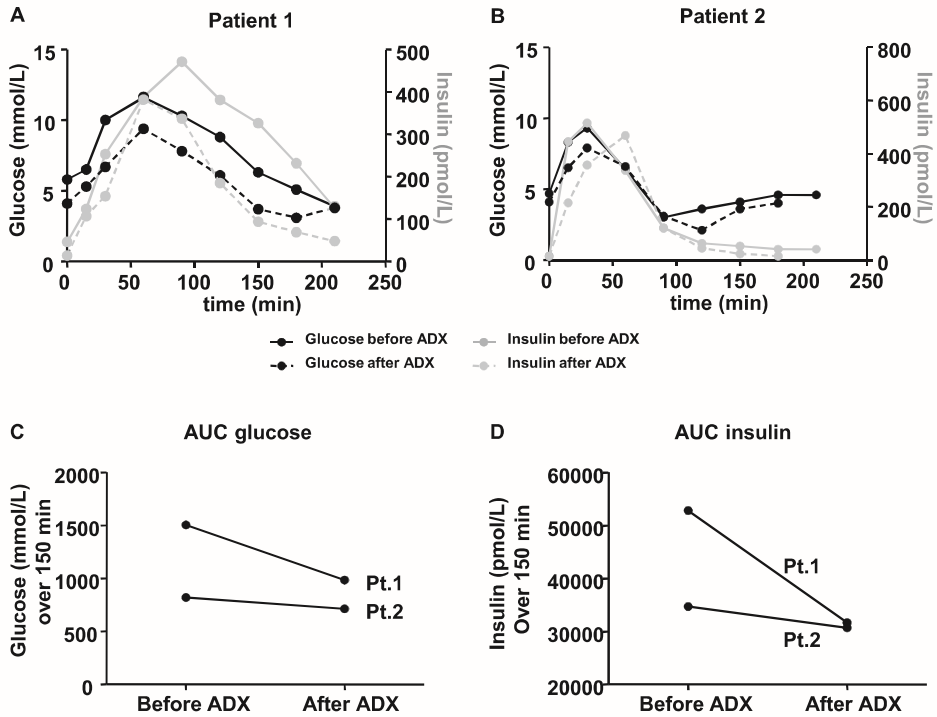


Figure 2. OGTT in patients with Cushing's syndrome before and after ADX. A, B) Glucose and insulin levels after oral glucose intake. C) Area under the curve for glucose of both patients and D) area under the curve for insulin of both patients.

of cortisol on the liver cholesterol metabolism (23,24). Also in patients with Cushing's syndrome, a trend in increased plasma HDL has been reported previously albeit that the differences were not significant (25).

In conclusion, cortisol very likely inhibits BAT activity but this can be corrected by normalization of plasma cortisol concentrations by ADX. In addition, plasma TG concentrations and glucose tolerance also improved after ADX. Increasing the number of patients will give us the opportunity to study possible interactions between increased BAT activity and improved plasma TG levels and increased glucose tolerance. Interestingly, also changes in HDL cholesterol were observed. We recommend further studies on the possible role of glucocorticoids on CETP activity.

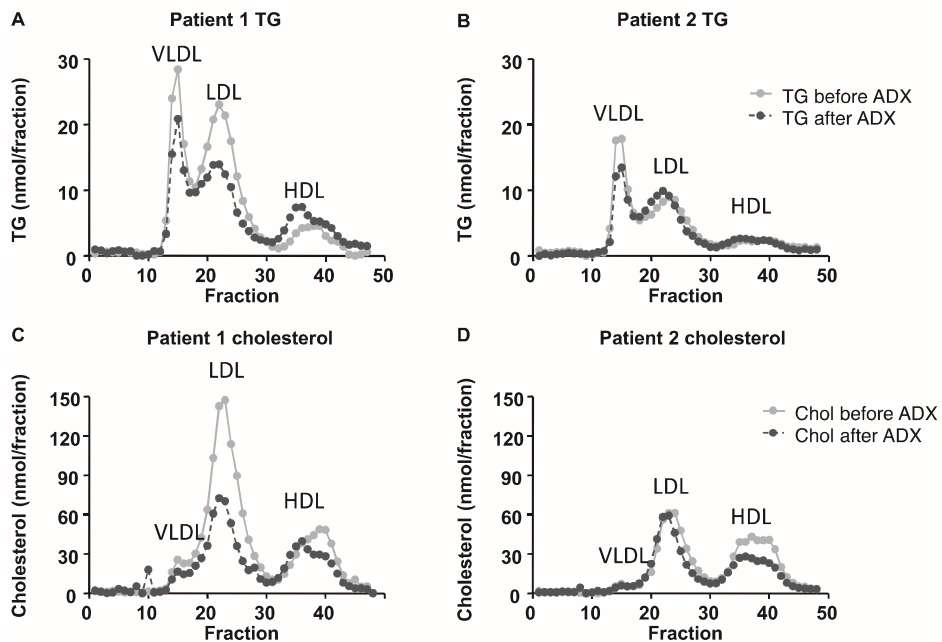
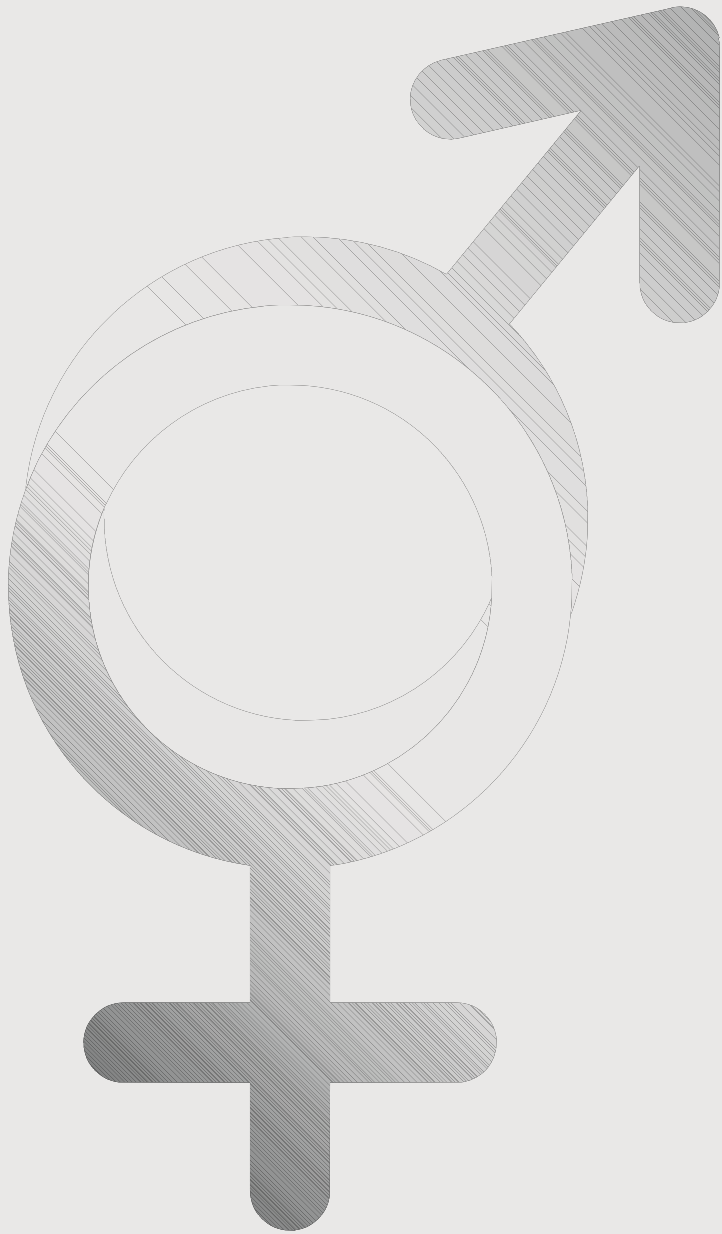


Figure 3. FPLC lipid profiles of patients with Cushing's syndrome before and after ADX. A,B) TG levels per fraction. C,D) Cholesterol levels per fraction.

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CHAPTER 5

ESTROGEN INCREASES EXPRESSION OF BONE MORPHOGENETIC PROTEIN 8B IN BROWN ADIPOSE TISSUE OF MICE

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ABSTRACT

Background: In mammals, white adipose tissue (WAT) stores fat and brown adipose tissue (BAT) dissipates fat to produce heat. Several studies showed that females have more active BAT. Members of the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) families are expressed in BAT and are involved in BAT activity. We hypothesized that differential expression of BMPs and FGFs might contribute to sex differences in BAT activity.

Methods: We investigated the expression of BMPs and FGFs in BAT of male and female C57BL/6J mice upon gonadectomy, cold exposure and exposure to sex steroids.

Results: Of the FGF family, BAT *Fgf1*, *Fgf9*, *Fgf18* and *Fgf21* expression was induced upon cold exposure, but only *Fgf1* expression was obviously different between the sexes: females had 2.5-fold lower BAT *Fgf1* than males. Cold exposure induced BAT *Bmp4* and *Bmp8b* expression, but only *Bmp8b* differed between the sexes: females had 35-fold higher BAT *Bmp8b* than males. Ovariectomy almost completely blunted BAT *Bmp8b* expression, while orchidectomy had no effect. Male mice and ovariectomized female mice treated with diethylstilbestrol (DES) had ~350-fold and ~36-fold higher BAT *Bmp8b* expression, respectively. Ninety-day and 7-day treatment of female mice with dihydrotestosterone (DHT) decreased BAT *Bmp8b* expression by ~5-fold and ~4-fold, respectively. Finally, treatment of primary murine brown adipocytes with DES did not result in changes in *Bmp8b* expression.

Discussion: BAT *Bmp8b* expression in mice is positively regulated by presence of ovaries and estrogens such as DES.

INTRODUCTION

Adipose tissue is an important mediator of energy balance in mammals. White adipose tissue (WAT) stores energy in the form of energy-dense triglycerides (TGs) while brown adipose tissue (BAT) has the unique ability to oxidize fatty acids released from TGs to generate heat, a process termed thermogenesis (1). The mitochondrial uncoupling protein 1 (UCP1) is predominantly expressed in BAT and controls the thermogenetic properties of this tissue. UCP1 uncouples ATP synthesis from oxidative phosphorylation in the mitochondria; a process that generates heat. Cold exposure of adult humans causes enhanced ^{18}F -deoxyglucose (^{18}F -FDG) uptake in the upper chest and neck regions (2-4), showing that BAT is present and active in adult humans.

Animal experiments have shown that females have more active BAT than males (5-7). For instance, BAT from female rodents contains more and bigger mitochondria and has a higher lipolytic activity upon caloric restriction. More importantly, human studies also show that, compared to men, women have more often ^{18}F -DFG uptake in areas considered to contain BAT as determined by positron emission tomography (PET) scans (2,8-10). A recent study confirmed that the relative contribution of fat mass to resting metabolic rate and the metabolic rate per kilogram adipose tissue were both higher in women than in men (11). Furthermore, tissue gene expression of genes involved in mitochondrial function suggested that women have an increased number of brown adipocytes (11). It is not entirely clear how this sex difference is regulated but certain aspects have been elucidated. The sex steroid hormone estradiol (E2) may be one of the regulators. Pedersen *et al.* (12), for instance, showed that treatment of ovariectomized rats with 17- β -estradiol pellets prevented ovariectomy-mediated reduction of BAT *Ucp1* mRNA expression. In cultured primary mouse brown adipocytes, E2 suppressed transcription of the α_2 -adrenergic receptor that inhibits rather than elevates cAMP upon norepinephrine (NE) activation (13) while testosterone suppressed *Ucp1* expression (14). Presumably the most important activator of UCP1 and BAT is sympathetic innervation (1) and Martínez de Morentin *et al.* (15) recently showed that E2 acts in the central nervous system to regulate BAT thermogenesis, specifically in the ventromedial nuclei of the hypothalamus.

A number of additional BAT activators such as thyroid hormone (1) have been discovered in recent years, but the hunt for new physiological relevant BAT activators continues, since autocrine and/or paracrine hormones or growth factors that activate the BAT depot may be considered novel candidates that can be used in treatment modalities to combat obesity. Two classes of paracrine/autocrine hormones or growth factors might be of particular interest given their role in brown adipocyte differentiation and function: bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs). BAT expresses several BMP family members (16), among which BMP7 (17,18) and BMP8b

(19) have been shown to activate BAT directly. Compared to their wild-type littermates, *Bmp7*^{-/-} mice have reduced amounts of BAT (18) and *Bmp8b*^{-/-} mice have impaired thermogenesis and reduced metabolic rate (19). In addition, BMP7 induces brown preadipocyte differentiation (18). Likewise, BAT expresses several FGF family members (20). FGF21 is secreted by activated BAT (21,22) and both FGF1 and FGF2 have been shown to upregulate UCP1 expression in cultured rat brown adipocytes (23).

In the present studies we investigated the differential expression of BMP and FGF family members in BAT of male and female mice. We therefore determined the mRNA expression of the most abundant BMP and FGF family members in male and female BAT upon cold exposure, gonadectomy and sex steroid treatment. In short, we found that BMP8b might be one of the factors involved in sex-specific differences in BAT activity since BAT *Bmp8b* expression was higher in female mice than in male mice. Moreover, BAT *Bmp8b* expression in female mice was reduced by ovariectomy and induced by treatment of male mice and ovariectomized female mice with diethylstilbestrol (DES).

METHODS

Animals

Male and female C57Bl/6J mice were obtained from Charles River Laboratories (Maastricht, The Netherlands) at the indicated age and were kept one week under standard housing conditions before they were enrolled in an experimental set-up.

In the first experiment, 9-week-old male mice were individually housed in a temperature controlled climate chamber (Bronson, Nieuwkuijk, The Netherlands) with normal light/dark cycle at 23°C or 4°C for 24 hours (n=6 mice per group). After these 24 hours, the mice were terminated by cardiac puncture under isoflurane anesthesia.

In the second and third experiment, 9-week-old male and female mice underwent gonadectomy or a sham operation under isoflurane anesthesia (n=10 mice per group). For the female mice, gonadectomy involved a small incision in both flanks after which the ovaries were removed. In the male mice, small incisions were made in the lower abdomen through which the testis were removed. Sham-operated animals underwent the same procedures without removal of ovaries or testis. After the surgery, the mice were allowed to recover for 45 days. For the second experiment, mice were fasted for 4 hours and terminated by cardiac puncture under isoflurane anesthesia. For the third experiment, the mice were put in the climate chamber for 24 hours at 23°C or 4°C after which they were terminated by cardiac puncture under isoflurane anesthesia (n=4-6 mice per group). Uterus weight of female mice in both experiments was measured to determine whether ovariectomy was successful.

For the fourth experiment, 9-week-old male mice received daily subcutaneous injections with 100 µg/kg diethylstilbestrol (DES) (Steraloids Inc., Newport, RI) dissolved in olive oil or the olive oil vehicle alone for one week before they were terminated by cardiac puncture under isoflurane anesthesia (n=6 mice per group).

For the fifth experiment, 9-week-old female mice underwent gonadectomy or a sham operation as described above (n=6 mice per group). After one week of recovery, these mice received daily subcutaneous injections with 100 µg/kg DES dissolved in olive oil or the olive oil vehicle alone for one week before they were terminated by cardiac puncture under isoflurane anesthesia.

For the sixth experiment, 9-week old female mice received daily subcutaneous injections with 100 µg/animal dihydrotestosterone (DHT) (Steraloids Inc.) dissolved in olive oil or the olive oil vehicle alone for one week before they were terminated by cardiac puncture under isoflurane anesthesia after which the uterus was weighed (n=6 mice per group).

For the seventh experiment, 19-day-old female mice received a DHT or placebo pellet (Innovative Research of America, Sarasota, FL) as described previously (24) (n=6 mice per group). After 90 days, the mice were terminated by decapitation under isoflurane anesthesia.

For all experiments, the intrascapular BAT depot was collected and stored at -80°C until analysis. All animal experiments were performed with the Approval of the Animal Ethics Committee at Erasmus MC, Rotterdam, The Netherlands.

Primary cell cultures

The intrascapular BAT depot was harvested from 18 male mice that were terminated by cardiac puncture under isoflurane anesthesia. The depots were kept in ice cold PBS until they were minced into small pieces. The pieces were digested with 0.1% m/v collagenase (Sigma-Aldrich, Zwijndrecht, The Netherlands), 0.1% m/v dispase II (Roche Diagnostics, Mannheim, Germany) and 0.05% m/v trypsin (Sigma-Aldrich) in serum-free culture medium (DMEM with 4.5 g/l D-glucose supplemented with antibiotic-antimycotic (Gibco, Bleiswijk, The Netherlands)) for 45 minutes at 37°C with gentle agitation. The enzymes were inactivated with an equal volume of culture medium with 10% FCS (Gibco) after which the samples were filtered through a 100-µm mesh filter to remove debris and spun down for 8 minutes at 1200 rpm. The pellets were resuspended in RBC Lysis buffer (eBioscience, San Diego, CA) and lysed for 5 minutes after which the samples were spun down for 5 minutes at 1500 rpm. The pellet was resuspended in culture medium with 10% FCS, the amount of viable cells counted and plated with 300,000 alive cells in conventional 24-well plates and cultured at 37°C with 5% CO₂. Twenty-four hours later, the medium was replaced with differentiation medium: DMEM with 4.5 g/l D-glucose supplemented with 10% FCS, antibiotic-antimycotic, 4 nM bovine insulin (Sigma-Aldrich),

10 mM HEPES (Gibco), 4 mM glutamine (Gibco), 25 µg/ml ascorbate (Sigma-Aldrich), and 1 µM rosiglitazone (ENZO Lifesciences, Raamsdonkveer, The Netherlands). This differentiation medium was replaced every 2 or 3 days. After 12 days of differentiation, the medium was replaced with culture medium with 10% charcoal stripped FCS (Gibco). After 8 hours, this medium was replaced with culture medium with charcoal stripped FCS supplemented with or without 10 µM DES. The cells were harvested and stored at -80°C until RNA isolation 24 hours later.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA from mouse tissues and cultured cells was isolated using Tripure Isolation Reagent (Roche) according to the manufacturer's instructions. Genomic DNA was removed by DNase treatment (Promega Benelux BV, Leiden, The Netherlands) for 30 minutes at 37°C. Reverse transcription was performed using a cDNA synthesis kit (Roche) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SYBRgreen mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands)

Table 1: Primer sequences

Gene	Accession No.	Forward primer	Reverse primer	Reference
Rn18s	NR_003278	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG	
B2m	NM_009735	ATCCAAATGCTGAAGAACGG	CAGTCTCAGTGGGGGTGAAT	
Hprt	NM_013556	GCAGTACAGCCCCAAAAT	AACAAAGTCTGGCCTGTATCCAA	
Ucp1	NM_009463	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT	(47)
Bmp2	NM_007553	TGCTTCTTAGACGGACTGCG	CTGGGGAAGCAGCAACACTA	
Bmp4	NM_007554	GAGCCATTCCGTAGTGCAT	AACGACCATCAGCATTTCGGT	
Bmp5	NM_007555	ATGCCACCAACCATGCCATA	GCCACACGAACGTACTACCA	
Bmp6	NM_007556	AACGCACACATGAATGCCAC	CCACAAGTCTCACGACCAT	
Bmp7	NM_007557	CGGGAGTTCCGGTTTGATCT	GCAAGAAGAGGTCCGACTCC	
Bmp8b	NM_007559	CAACCACGCCACTATGCAG	CACTCAGCTCAGTAGGCACA	
Fgf1	NM_010197	GAAGCATGCGGAGAAGAAGCTG	CGAGGACCGCGCTTACA	(48)
Fgf2	NM_008006	CAACCGGTACCTTGCTATGA	TCCGTGACCGGTAAGTATTG	(20)
Fgf6	NM_010204	ACACACGAGGAGAACCCTA	TGGAAGCTGGGCGTTGTGTA	
Fgf9	NM_013518	TATCCAGGGAACCAAGAAAGAC	CAGGCCACTGTACTACTGATAAA	(48)
Fgf10	NM_008002	GCGGGACCAAGAATGAAGA	AGTTGCTGTTGATGGCTTGA	(20)
Fgf11	NM_010198	TTGTACAGCTCGCCACATTC	GTAATTCTCAAAGACGCACTCCTT	(20)
Fgf13	NM_010200	AATGAACAGCGAGGGATACTTG	ACTGATTCTTTGAATTTGCACTCA	(20)
Fgf14	NM_207667	ACCCATCAGAACTTTTTACCCCT	ACATGGCAACTTCCAATGGCT	
Fgf16	NM_030614	GGCCTGTACCTAGGAATGAATGA	TTCCCGAAAACACATTACAC	(20)
Fgf18	NM_008005	CATTCAAGTCCTGGGCCGTA	ATGAACACGCACTCCTTGCT	
Fgf21	NM_020013	CTGGGGGTCTACCAAGCATA	CACCCAGGATTTGAATGACC	(49)

with an ABI Prism 7900 Sequence Detection System. Sequences of the primers used are listed in Table 1. The expression of each gene was expressed in arbitrary units after normalization to the average expression level of the housekeeping genes 18S and beta-2 microglobulin using the $2^{-\Delta\Delta Ct}$ method (25).

Immunoblot analysis

The murine intrascapular BAT depot was lysed in cold PBS supplemented with Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich) and cComplete Protease Inhibitor Cocktail Tablets (Roche) followed by sonification for 10 seconds. The protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Individual samples were mixed with Laemmli loading buffer (Pierce), heated for 5 min at 96 °C and subjected to SDS-PAGE on a 10% gel. UCP1 was determined using a polyclonal anti-UCP1 antibody raised in rabbit (Sigma-Aldrich). As loading controls, the concentrations of α -tubulin was determined using a polyclonal anti- α -tubulin antibody raised in rabbit (Santacruz, Heidelberg, Germany). Finally, goat-anti-rabbit IRDye 800 secondary antibody (Li-cor, Leusden, The Netherlands) and an Odyssey fluorescence scanner (Li-cor) were used. The immunoblots were analyzed with Odyssey software.

Statistical analysis

Statistics were performed with GraphPad Prism (GraphPad Software, Inc.). When 4°C was compared with 23°C, a Mann-Whitney U test was performed ($p < 0.05$ was considered significant). When the effect of sex and gonadectomy or sex and treatment was studied, a 2-way ANOVA was performed. Post-hoc Mann-Whitney U tests were performed ($p < 0.025$ being considered significant) when a significance ($P < 0.05$) was found in the 2-way ANOVA.

RESULTS

Multiple BMPs and FGFs are induced in BAT upon cold exposure

BMPs and FGFs are proteins with autocrine and/or paracrine actions of which many are expressed in BAT, as previously described (18,20,26). We determined which of these BMP and FGF family members are associated with activated BAT. For this, male mice were exposed to 4°C for 24 h. Exposure to the 4°C resulted in a significant more loss of bodyweight (1.87 ± 0.24 vs. 0.30 ± 0.09 gram lost during 24 hours, 4°C vs. 23°C, $p < 0.05$). Cold exposure indeed activated BAT since *Ucp1* expression was almost 10-fold upregulated (Figure 1A). Of the BMP family members, only expression of *Bmp4*, *Bmp6* and *Bmp8b* was significantly upregulated in BAT upon cold exposure by 3.08 ± 0.64 , 1.89 ± 0.11 , and 110.06 ± 16.67 fold, respectively ($p < 0.05$) (Figure 1B). For the FGFs, cold

exposure significantly increased *Fgf1*, *Fgf9*, *Fgf10*, *Fgf11*, *Fgf18*, and *Fgf21* expression by 3.02 ± 0.41 , 11.60 ± 2.21 , 2.12 ± 0.20 , 2.63 ± 0.34 , 2.97 ± 0.71 , and 12.78 ± 2.05 fold, respectively ($p < 0.05$) (Figure 1C). Since we aimed to study the link between BAT activity and BMPs and FGFs in relation to sex differences, the expression of these three

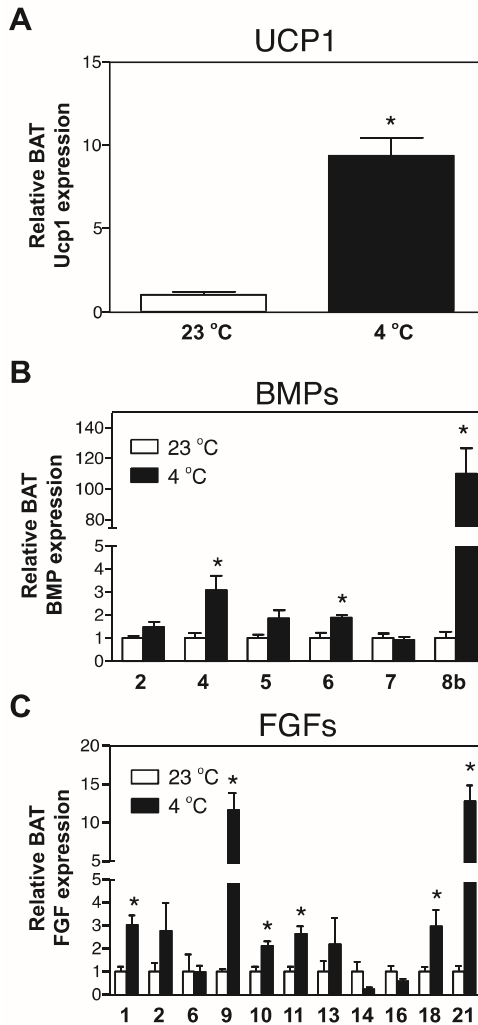


Figure 1. Induction of three BMP family members and seven FGF family members upon cold exposure.

A. *Ucp1* mRNA expression in BAT of male mice kept at 23°C or 4°C for 24 hours. **B.** Expression of genes encoding for BMP family members in BAT of male mice kept at 23°C or 4°C for 24 hours. **C.** Expression of genes encoding for FGF family members in BAT of male mice kept at 23°C or 4°C for 24 hours. Results were normalized to 18S ribosomal RNA (*Rn18s*) and beta-2 microglobulin (*B2m*) with data from mice kept at 23°C defined as '1'. Values are averages \pm SEM; $n = 6$; *, $p < 0.05$ vs. 23°C (Mann-Whitney U test).

BMP family members and six FGF family members was further analyzed in subsequent experiments.

Female mice have higher BAT *Bmp8b* and lower BAT *Fgf1* expression

Next, to determine whether BMP and FGF family members associated with activated BAT are differentially expressed in male and female BAT, we compared the expression of the three BMPs and six FGFs in the BAT depot of male and female mice. In addition, we determined whether gonadectomy (GDX) had an effect on their expression levels, in order to investigate the contribution of gonadal function to the regulation of BAT activity. In male mice, GDX resulted in a reduced body weight while female mice gained weight when GDX'd (Figure 2). Remarkably, BAT *Ucp1* mRNA expression did not differ between male and female mice (Figure 2). Of the three BMP family members tested, only BAT *Bmp8b* expression showed a striking difference between the sexes (Figure 2). Sham-operated female mice had a 35.4 ± 8.3 fold higher BAT *Bmp8b* expression compared to sham-operated male mice ($p < 0.025$) which was significantly reduced to levels observed in male mice upon ovariectomy. In contrast, orchidectomy had no effect on BAT *Bmp8b* expression. Of the six FGF family members, BAT *Fgf1* expression showed the largest significant difference between male and female mice. Sham-operated female mice had lower BAT *Fgf1* expression than sham-operated male mice and ovariectomy tended to increase BAT *Fgf1* expression, however, this failed to reach significance. BAT *Fgf9*, *Fgf11*, *Fgf18* and *Fgf21* mRNA expression was not significantly different between the four experimental groups. Finally, BAT *Fgf10* expression was affected by GDX, independent of sex

Cold induces BAT *Bmp8b* and *Fgf1* expression, independent of sex or presence of gonads

The data so far show that of the genes studied, expression of *Bmp8b* and *Fgf1* in BAT differs between the sexes. Since both genes are also upregulated upon cold exposure, we next investigated what the effect of cold exposure on these two genes was in mice of both sexes combined with GDX. The effect of GDX on body weight of the male and female mice was comparable to what was seen in the previous experiment (data not shown). Cold exposure enhanced metabolism as is evident from the effects of the 24-h exposure to 4°C on body weight and food intake (Figure 3A). The 4°C sham-operated mice ate ~50% more than those kept at 23°C and this resulted in marginal effects on body weight. However, GDX'd mice did not eat more at 4°C compared to 23°C and this resulted in a marked body weight loss of 1 gram/24 hour. Cold exposure induced BAT *Ucp1* mRNA expression up to 6-fold (Figure 3B) but UCP1 protein content was only marginally induced (Figure 3C). In general, female mice kept at 23°C have more UCP1 protein than male mice housed at the same temperature.

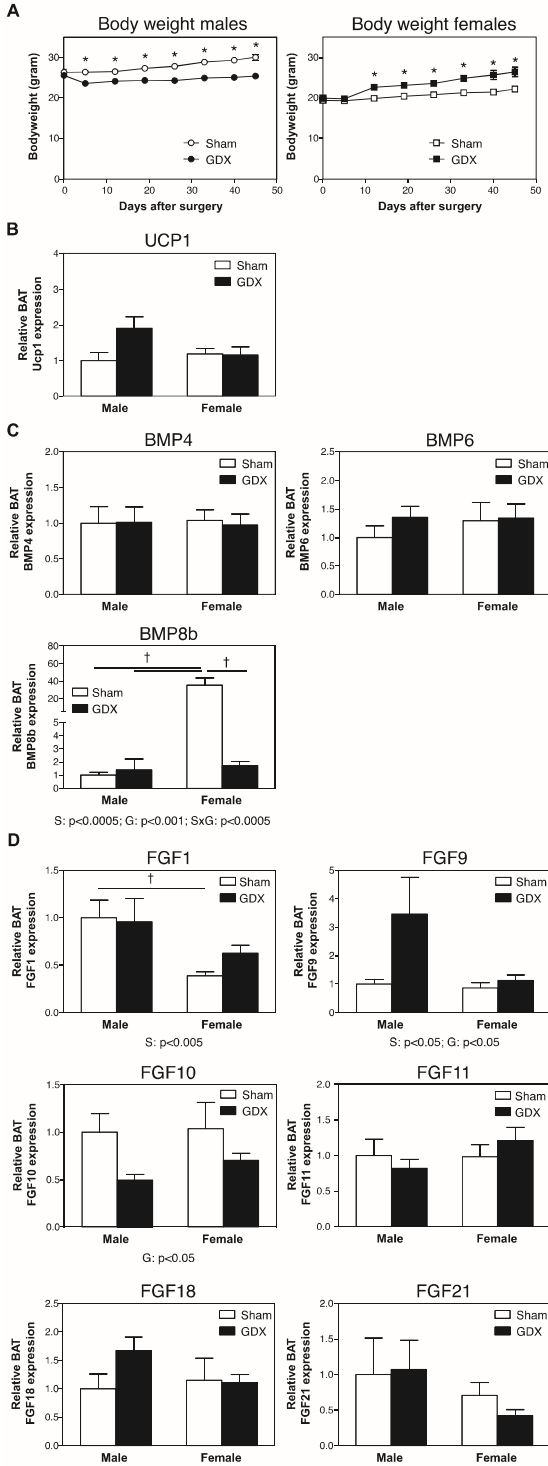


Figure 2. Female mice have higher BAT *Bmp8b* expression and lower BAT *Fgf1* expression.

A. Body weight of male and female mice after they received gonadectomy (GDX) or a sham operation. **B.** *Ucp1* mRNA expression in BAT of male and female mice that received GDX or a sham operation. **C.** *Bmp4*, *Bmp6* and *Bmp8b* mRNA expression in BAT of male and female mice that received GDX or a sham operation. **D.** *Fgf1*, *Fgf9*, *Fgf10*, *Fgf11*, *Fgf18* and *Fgf21* mRNA expression in BAT of male and female mice that received GDX or a sham operation. mRNA expression results were normalized to 18S ribosomal RNA (*Rn18s*) and beta-2 microglobulin (*B2m*) with data from sham-operated male mice defined as '1'. Values are averages \pm SEM; n = 10; †, p < 0.025 (Mann-Whitney U post hoc test when a significance was detected by 2-way ANOVA). Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either sex (S), GDX (G) or the interaction between sex and GDX (SxG).

Female mice had much higher BAT *Bmp8b* expression than male mice and ovariectomy almost completely ablated BAT *Bmp8b* expression (Figure 3D). The effects of the cold challenge on BAT *Bmp8b* expression in sham-operated male mice was comparable to what we found with intact males: a ~44-fold induction ($p < 0.025$). Cold exposure also elevated BAT *Bmp8b* expression in sham-operated female mice, but with a ~17-fold induction, the cold effect was less strong than in male mice. This different relative effect of cold on BAT *Bmp8b* expression between the sexes can be explained by the already higher BAT *Bmp8b* expression in female mice compared to male mice. As in the previous experiment, BAT *Fgf1* expression was lower in female mice than in male mice ($p < 0.025$) while ovariectomy resulted in elevated BAT *Fgf1* expression (Figure 3D). Cold exposure resulted in a similar and in general higher BAT *Fgf1* expression in all four groups of mice. Thus, cold exposure induces BAT *Bmp8b* and *Fgf1* expression independent of sex and presence of gonads.

DES induces BAT *Bmp8b* expression in vivo but not in vitro

Since estrogens are mainly produced by the ovaries and are only present at lower concentrations in male, the data so far suggest that they are obvious candidate hormones controlling *Bmp8b* and *Fgf1* expression in BAT. To test whether estrogens indeed regulate BAT *Bmp8b* and *Fgf1* expression *in vivo*, male mice, sham-operated female mice and ovariectomized female mice were injected with the stable E2 analogue DES. In male mice, those injected with DES significantly gained more weight than the control mice ($10.2 \pm 1.5\%$ vs. $1.2 \pm 0.5\%$, DES vs. control, $p < 0.05$, Mann-Whitney U test) and showed a strongly induced BAT *Bmp8b* expression (Figure 4). However, DES did not affect BAT *Fgf1* expression. In both sham-operated and ovariectomized female mice, 1 week DES treatment also significantly increased body weight (Table 2). Interestingly, two weeks after

Table 2. Effect of GDX and DES on bodyweight of female mice.

	Sham		GDX		Results 2-way ANOVA		
	Vehicle	DES	Vehicle	DES	Surgery	Treatment	Interaction
BW before surgery	19.8 ± 0.4	19.6 ± 0.4	19.9 ± 0.6	19.8 ± 0.4	NS	NS	NS
BW 1 week after surgery	20.2 ± 0.5	20.1 ± 0.4	21.6 ± 0.3	21.4 ± 0.5	$P < 0.005$	NS	NS
Change in BW (%) 1 week after surgery	2.1 ± 0.5	3.0 ± 1.3	8.9 ± 2.5	8.3 ± 2.7	$P < 0.01$	NS	NS
BW after 1 week treatment	21.3 ± 0.5	23.0 ± 0.4	23.2 ± 0.2	24.1 ± 0.4	$P < 0.005$	$P < 0.005$	NS
Change in BW (%) After 1 week treatment	5.8 ± 1.0	14.3 ± 1.6	7.5 ± 1.3	12.7 ± 1.2	NS	$P < 0.0001$	NS

Female mice received gonadectomy (GDX) or a sham operation. After 1 week, the mice were injected daily with diethylstilbestrol (DES) or olive oil vehicle for 1 week. Values are averages ± SEM; n = 6; NS, not significant.

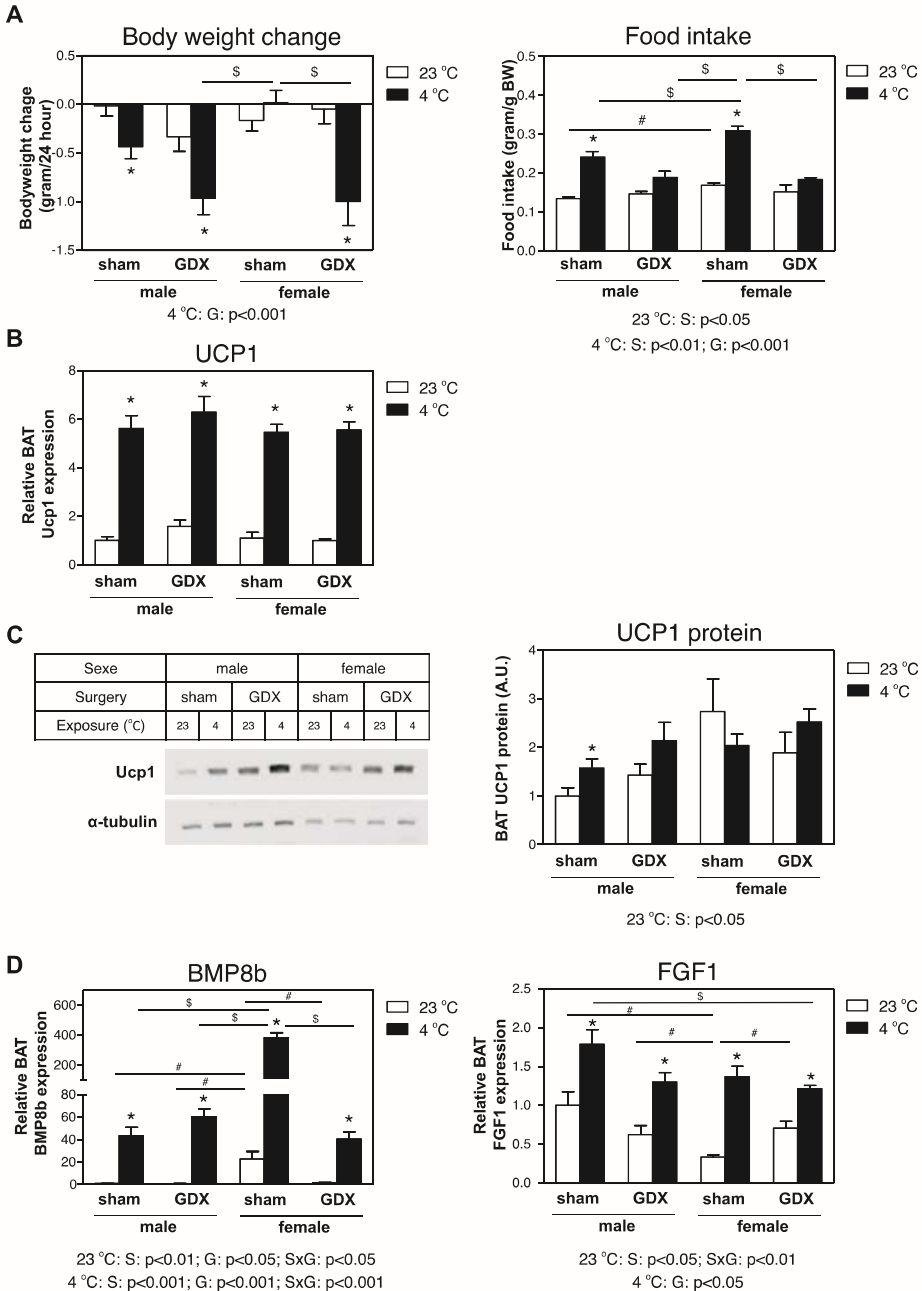


Figure 3. Independent of sex and presence of gonads, cold exposure induces BAT *Fgf1* and *Bmp8b* expression.

A. Change in body weight and food intake of sham operated and GDX'd male and female mice during exposure to 4°C or 23°C for 24 hours. **B.** *Ucp1* mRNA expression in BAT of male and female mice that received GDX or a sham operation, exposed to 4°C or 23°C for 24 hours. **C.** Immunoblot

of UCP1 and α -tubulin and quantification of UCP1 protein vs. α -tubulin protein content in BAT of male and female mice that received GDX or a sham operation, exposed to 4°C or 23°C for 24 hours. UCP1 protein results were normalized with data from sham-operated male mice defined as '1', n=4. **D.** *Bmp8b* and *Fgf1* mRNA expression in BAT of male and female mice that received GDX or a sham operation, exposed to 4°C or 23°C for 24 hours. mRNA expression results were normalized to 18S ribosomal RNA (*Rn18s*) and beta-2 microglobulin (*B2m*) with data from sham-operated male mice kept at 23°C defined as '1'. Values are averages \pm SEM; n = 4-6; *, p < 0.05 4°C vs. 23°C for same animal model (Mann-Whitney U test); #, p < 0.025 (Mann-Whitney U post hoc test when significance was detected by 2-way ANOVA for mice kept at 23°C); \$, p < 0.025 (Mann-Whitney U post hoc test when significance was detected by 2-way ANOVA for mice kept at 4°C). Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either (S), GDX (G) or the interaction between sex and GDX (SxG) for mice kept at either 23°C or 4°C.

ovariectomy, BAT *Ucp1* expression was severely reduced but this was not corrected by DES (Figure 5). Ovariectomy also reduced BAT *Bmp8b* and *Fgf1* mRNA concentrations. DES treatment enhanced *Bmp8b* expression in sham-operated and ovariectomized female mice by $504 \pm 261\%$ and $3472 \pm 970\%$, respectively. In addition, DES reduced BAT *Fgf1* expression by 74% in sham-operated females.

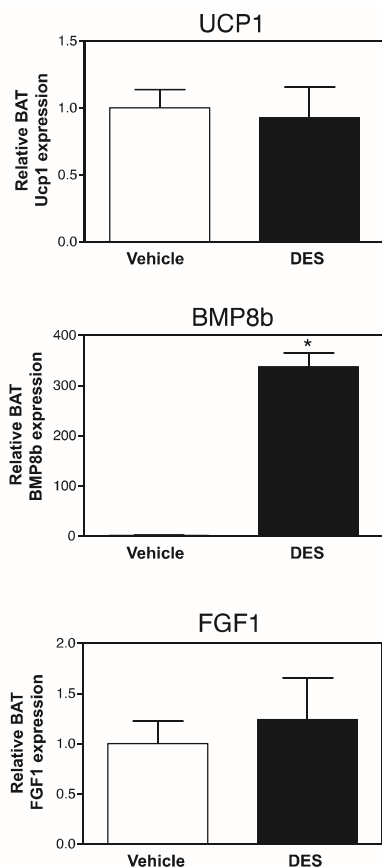


Figure 4. DES induces BAT *Bmp8b* expression in male mice.

A. *Ucp1*, *Bmp8b* and *Fgf1* mRNA expression in BAT of male mice injected with diethylstilbestrol (DES) or olive oil vehicle for 1 week. Results were normalized to 18S ribosomal RNA (*Rn18s*) and beta-2 microglobulin (*B2m*) with data from placebo or vehicle mice defined as '1'. Values are averages \pm SEM; n = 6; *, p < 0.05 vs. vehicle (Mann-Whitney U test)

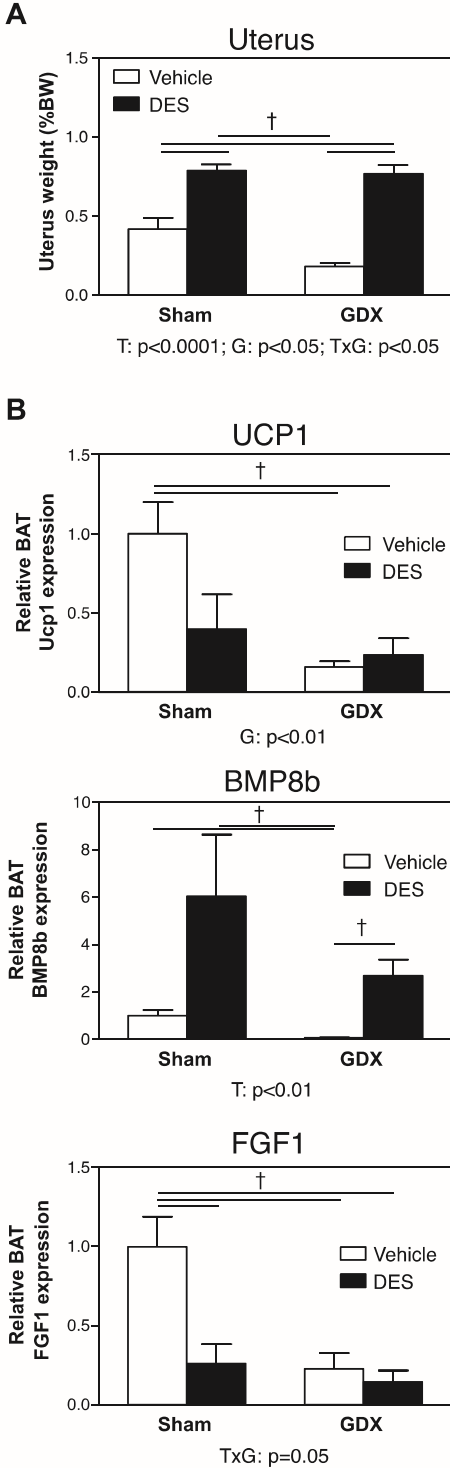


Figure 5. DES increases BAT *Bmp8b* expression in female mice.

Ucp1, *Bmp8b* and *Fgf1* mRNA expression in BAT of female mice two weeks after they received gonadectomy (GDX) or a sham operation and who were injected daily with diethylstilbestrol (DES) or olive oil vehicle for the last week. Results were normalized to 18S ribosomal RNA (*Rn18s*) and beta-2 microglobulin (*B2m*) with data from placebo or vehicle mice defined as '1'. Values are averages \pm SEM; $n = 6$; †, $p < 0.025$ (Mann-Whitney U post hoc test when significance was detected by 2-way ANOVA). Depicted below the graphs are the significant p-values of the 2-way ANOVA tests for either treatment (T), GDX (G) or the interaction between treatment and GDX (TxG).

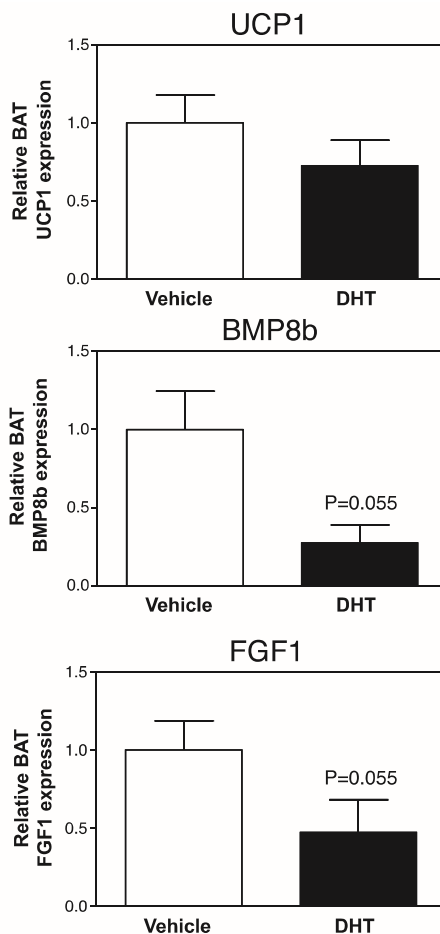


Figure 6. One week DHT reduces BAT *Bmp8b* expression in female mice.

Ucp1, *Bmp8b* and *Fgf1* mRNA expression in BAT of female mice who were injected daily with dihydrotestosterone (DHT) for 1 week. Results were normalized to 18S ribosomal RNA (*Rn18s*) and beta-2 microglobulin (*B2m*) with data from vehicle mice defined as '1'. Values are averages \pm SEM; n = 6; *, p < 0.05 vs. vehicle (Mann-Whitney U test).

To decrease estrogen concentrations in female mice via another mechanism than removal of the ovaries, we injected intact female mice with dihydrotestosterone (DHT) for 1 week, which resulted in a tendency towards higher body weights (induction of $8.4 \pm 1.0\%$ vs. $5.0 \pm 1.0\%$, DHT vs control, p=0.065, Mann Whitney U test). One week DHT treatment resulted in a tendency towards reduced BAT *Bmp8b* and *Fgf1* expression, albeit not significant (Figure 6). However, the once daily DHT injections were not sufficient to constantly suppresses release of gonadotropin-releasing hormone (GnRH) by the hypothalamus since uterus weights were only marginally affected by DHT (data not shown). When female mice were treated with DHT for a much longer period of 90 days

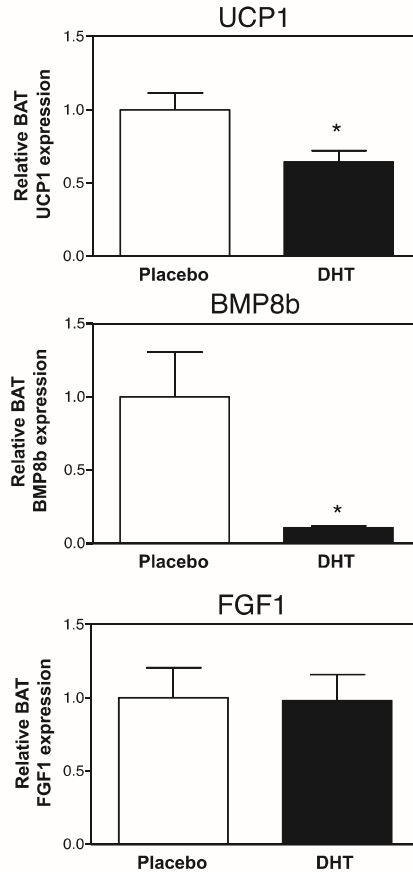


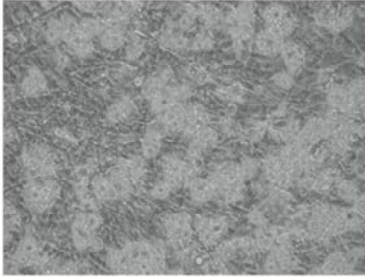
Figure 7. Long term effects of DHT treatment on BAT gene expression in female mice.

Ucp1, *Bmp8b* and *Fgf1* mRNA expression in BAT of female mice who were treated with a DHT or placebo pellet for 90 days. Results were normalized to 18S ribosomal RNA (*Rn18s*) and beta-2 microglobulin (*B2m*) with data from placebo mice defined as '1'. Values are averages \pm SEM; n = 6; *, p < 0.05 vs. placebo (Mann-Whitney U test).

they were approximately 21% heavier, as described before [24], but also had a severe reduction of BAT *Bmp8b* expression while BAT *Fgf1* expression was not affected (Figure 7). Altogether, these data clearly show that estrogens regulate BAT *Bmp8b* expression *in vivo*.

Next, to investigate whether the effects *in vivo* on BAT *Bmp8b* expression are due to direct effects of DES on the brown adipocytes, we treated cultured primary brown adipocytes with DES (Figure 8). As reported before for the 3T3-L1 pre-adipocyte cell line (27), DES induced expression of the gene encoding the lipogenic transcription factor proliferator-activated receptor gamma (PPAR γ) by 45%, albeit this did not reach statistical significance. Since DES did not affect *Bmp8b* expression in brown adipocytes *in vitro*,

A



B

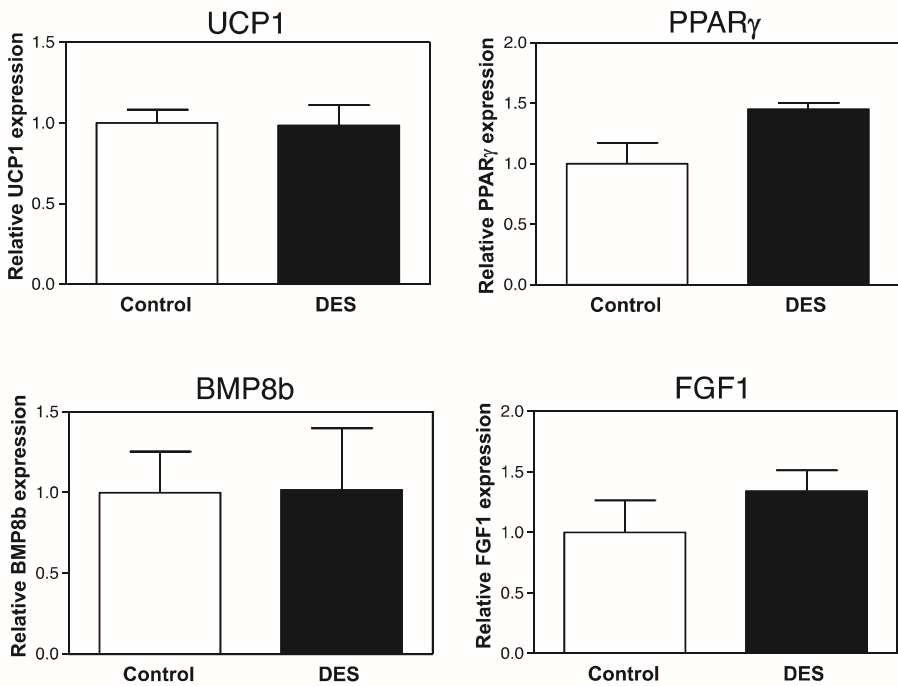


Figure 8. Effect of DES on brown adipocyte gene expression *in vitro*.

Primary cultured murine brown adipocytes were treated with or without 10 μ M diethylstilbestrol (DES) for 24 hours. A. Representative picture of the primary murine brown adipocyte culture after differentiation. B. *Ppar γ* , *Ucp1*, *Bmp8b* and *Fgf1* mRNA expression in the brown adipocytes. Expression is normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) with data from control adipocytes defined as '1'. Values are averages \pm SEM; n = 4; *, p < 0.05 (Mann-Whiney U test).

the effect of DES on BAT *Bmp8b* expression *in vivo* are likely mediated via an indirect mechanism, presumably via the hypothalamus.

DISCUSSION

The presence of UCP1 in the mitochondrial membranes allows BAT to produce heat instead of ATP upon oxidation of fatty acids. Since BAT has very high potential to oxidize large amounts of lipids from the system, BAT is an attractive target tissue to combat obesity. However, despite renewed interest in this tissue after the discovery of BAT in adult humans (2-4), no specific therapeutics to activate BAT have been identified yet. Catecholamines and thyroid hormone T_4 are known to induce BAT activity (1) but systemic administration of these compounds results in severe side effects such as hypertension and tachycardia. Detailed investigation of paracrine and autocrine factors, such as BMPs and FGFs, that mediate BAT activity might tell us how to pursue the hunt for novel BAT activators. Studies have shown that female mice have more active BAT than male mice and that estrogens might be key hormones in these differences (6,7,12,14,15). Therefore, we also compared the sex differences in BAT in mice with or without gonads, thus with or without their sex steroid hormones, using the physiologically most relevant method to activate BAT *in vivo*: cold exposure (28). In summary, we found that expression of the gene encoding BMP8b in the BAT depot is upregulated by both cold exposure and the presence of ovaries. Additional experiments in which mice had received DES and female mice that had a changed estrogen/androgen ratio to a male-like nature showed that increased estrogen/androgen ratios result in a higher BAT *Bmp8b* mRNA expression *in vivo*.

It has been proposed that females have more active BAT than males (6,7,12,14,15) and one would expect that female mice also have higher BAT *Ucp1* mRNA expression and protein content. In our hands, however, BAT *Ucp1* mRNA expression did not differ between male and female mice (Figure 2B and Figure 3C). In contrast, we found that, compared to male mice, female mice have more BAT UCP1 protein than male mice (Figure 3D), which is the protein involved in thermogenesis (29). Of interest, the 45-day period after ovariectomy did not affect BAT *Ucp1* mRNA expression (Figure 2 and Figure 3C), but when mice were analysed only two weeks after ovariectomy, BAT *Ucp1* mRNA expression was significantly reduced (Figure 5). Thus, a direct effect of ovariectomy is indeed reduced BAT *Ucp1* mRNA expression, but a longer period of ovariectomy results in a compensatory effect on *Ucp1* mRNA expression. How this latter mechanism is regulated is, to our knowledge, not known.

Since recent and older literature show that E2 induces BAT *Ucp1* mRNA expression (7,12,13,15), our findings that one week daily injections with DES did not significantly affect the expression of *Ucp1* in BAT (Figures 4 and 5) is unexpected. DES has a similar affinity for the estrogen receptor α (ER α) as E2 albeit five-fold more potent (30), thus similar effects of DES and E2 on mRNA expression profiles are to be expected. A plausible explanation for the differences between our DES study and previous studies might

be the method of delivery. While most research groups implanted estrogen containing pellets, we give daily DES injections and collected tissue samples 24 hours after the last injection. Since the clearance of DES in pregnant mice is very rapid during the first 30 minutes, although slowed down after 1 hour (31), it is possible that analysis of *Ucp1* mRNA expression 24 hours after the last injection is too late to find any effects. Unfortunately, we are unaware of studies on DES kinetics in murine BAT.

That DES failed to induce *Ucp1* mRNA expression despite its effects on BAT metabolism via ER α might also be the result of reduced estrogen-receptor-related receptor (ERR) α , ERR β , and/or ERR γ transcriptional activity. Tremblay *et al.* (32) found that DES inhibits transcriptional activity of all three ERR subtypes in trophoblast stem cells. Other studies showed that both ERR α and ERR β mediate *Ucp1* expression in brown adipocytes (33,34). However, whether DES also inhibits transcriptional activity of ERR subtypes and hence reduces *Ucp1* mRNA expression in (brown) adipocytes *in vivo* needs to be confirmed by additional experiments.

Our results suggest that the observed higher expression of *Bmp8b* in female mice may be the result of regulation by estrogens, since ovariectomy abolished BAT *Bmp8b* expression (Figure 2) while DES administration to intact male mice, sham-operated female mice and ovariectomized female mice induced BAT *Bmp8b* expression (Figures 4 and 5). In addition, we show that changing the estrogen/androgen ratio to a male-like nature by treating female mice with DHT for either one week or 90 days also negatively affected BAT *Bmp8b* expression (Figures 6 and 7). As such, *Bmp8b* may be a direct target of estrogen action via ER α and/or ER β in brown adipocytes. However, additional *in vitro* experiments with cultured brown adipocytes showed that DES had no direct effect on *Bmp8b* mRNA expression in these cells (Figure 8). Thus, it is more likely that DES indirectly mediates BAT *Bmp8b* expression. For instance, DES may act on ER α located in the ventromedial nucleus of the hypothalamus that will result in an inhibition of AMP-activated protein kinase (AMPK), subsequently leading to induction of BAT activation, as recently shown for E2 (15), and cause *Bmp8b* expression to rise.

Of interest, BAT *Bmp8b* expression did not correlate with changes in body weight in our experiments. Both DHT and DES treatment resulted in elevated body weights, but DHT reduced whereas DES induced BAT *Bmp8b* expression. The increase of bodyweight upon DES treatment has been reported before and has been attributed to enhanced expansion of the WAT depot (27). Altogether, our findings underscore that changing the estrogen/androgen ratio *per se* affects BAT *Bmp8b* expression, irrespective of effects on body weights.

Our studies also showed an induction of the gene encoding for FGF1 in cold-exposed mice (Figure 1C). FGF1 has recently been shown to be crucial for the hyperplastic effects of peroxisome proliferator-activated receptor gamma (PPAR γ) agonists in adipose tissue depots which most importantly also involves angiogenesis (35). Of interest, PPAR γ is a

nuclear receptor that is also required for brown adipocyte differentiation (36). Effects of FGF1 on brown adipocytes themselves are conflicting. On the one hand, FGF1 has been reported to upregulate *Ucp1* mRNA expression (23) while others found that FGF1 downregulated expression of the gene encoding lipoprotein lipase (LPL) (37). This latter observation is counterintuitive since cold exposure increases BAT *Lpl* mRNA expression (38) and LPL is thought to be crucial in the uptake of the fatty acid substrates by activated BAT. Hence, the precise roles of FGF1 in BAT remain to be elucidated.

The finding that a member of the BMP family and a member of the FGF family are expressed in a sex-dependent manner in BAT is intriguing. The interaction between the signalling pathways of various BMP and FGF family members is well known; a strong balance between both pathways is important in several developmental processes (39). For instance, BMP and FGF ligands have opposing effects in cardiomyocyte differentiation (40), apical ectodermal ridge and hindbrain development (41,42), and oligodendrocyte precursors generation (43). However, it remains to be determined whether such a crosstalk exist between FGF1 and BMP8b signalling in BAT.

The data collected in this article are generated from mouse experiments leaving the translational question open. Mejhert *et al.* (44) mapped the presence of FGFs in human WAT and found that only *FGF1*, *FGF2*, *FGF7*, *FGF9* and *FGF18* were present in human WAT. They almost exclusively used female subjects, making comparisons between the sexes not possible. The same study showed that only FGF1 was released by the white adipocytes but this secretion did not contribute to FGF1 in the circulation, suggesting again that FGF1 very likely has a paracrine and/or autocrine function. Another study found that human WAT from obese men expresses *FGF1*, *FGF2*, *FGF7*, *FGF9*, *FGF10*, and *FGF18* (45). *FGF1* and *FGF9* were more abundantly expressed in omental WAT. Since this WAT depot is considered less likely to gain BAT-like properties, the presence of *FGF1* and *FGF9* in omental WAT is rather unexpected since expression of *Fgf1* and *Fgf9* was induced in activated murine BAT (Figure 1C). In mice, *Bmp8b* expression responded strongly to cold exposure and also showed a large sex-differential expression pattern. However, it is unknown whether human BAT expresses *BMP8b*. One of the BMP type I receptors (BMPRI1A) is expressed in human WAT (46), but no sex-differences have been reported yet.

CONCLUSIONS

In conclusion, we discovered that BAT *Bmp8b* expression is regulated by presence of ovaries and ER α ligands such as DES in mice.

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CHAPTER 6

WOMEN HAVE MORE POTENTIAL TO INDUCE BROWNING OF PERIRENAL ADIPOSE TISSUE THAN MEN

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ABSTRACT

Background: Brown adipose tissue (BAT) can generate heat by burning fatty acids, a process mediated by uncoupling protein 1 (UCP1). White adipose tissue (WAT) depots can gain BAT-like properties and various studies suggest that females have more active BAT or BAT-like WAT. We studied sex differences in BAT-like properties of human perirenal adipose tissue.

Methods: Perirenal and subcutaneous adipose tissue was obtained from 20 male and 24 female healthy live kidney donors. Mesenchymal stem cells (MSCs), adipocyte precursor cells, were isolated from these depots to study whether intrinsic factors control BAT-like properties of the adipose tissue depots.

Results: When average outside temperature a week before harvesting was below 11°C, brown-like adipocytes expressing UCP1 were present in perirenal adipose tissue of women, but not of men. MSCs derived from perirenal adipose tissue expressed significantly more UCP1 when from female origin compared to male origin ($p=0.009$). However, UCP1 protein content and oxygen consumption rate did not differ between adipocytes derived from male and female perirenal MSCs.

Discussion: Female perirenal adipose tissue has a higher potency to gain BAT-like properties than male perirenal adipose tissue. The degree of gaining BAT-like properties depends on sex-specific intrinsic factors and environmental triggers such as temperature.

INTRODUCTION

Adipose tissue plays an important role in the imbalance between energy intake and expenditure that causes obesity. Mammals have two types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). Whereas WAT stores energy in the form of triglycerides, which can be mobilized as FFA and glycerol during fasting, BAT uses fatty acids to produce heat, thus increasing energy expenditure. This process, also called non-shivering thermogenesis, depends on the presence of uncoupling protein 1 (UCP1) which is almost exclusively expressed in BAT (1). We can distinguish two distinct types of brown adipocytes that originate from two different precursor cells: 'classical' brown adipocytes originating from myf5⁺ cells on one hand and white adipocytes from myf5⁻ precursor cells that gained BAT-like properties such as multiple lipid droplets and high expression of UCP1 on the other hand (2). Cold exposure activates BAT through stimulation of the sympathetic nervous system, which has been extensively studied in rodent models. In BAT of rodents, sympathetic nerve firing stimulates fatty acid oxidation as well as *Ucp1* mRNA expression and *Ucp1* protein activity (3). In addition, prolonged *in vivo* exposure to catecholamines induces 'browning' of WAT depots (4). Positron emission tomography (PET) scans after administration of ¹⁸F-deoxyglucose (¹⁸F-FDG) to cold exposed subjects made clear that active BAT or WAT with BAT-like properties is present in adult humans (5,6). Increased uptake of ¹⁸F-FDG was predominantly found in the neck and paravertebral regions that also had UCP1 gene and protein expression (5-7). However, the debate is still ongoing whether the UCP1-positive cells in adult humans are 'classical' brown adipocytes and/or white adipocytes that gained BAT-like properties (2,7,8).

Several studies have suggested that the amount and activity of BAT or WAT with BAT-like properties is higher in women than in men since women have more often and higher ¹⁸F-FDG uptake in areas considered to contain BAT or WAT with BAT-like properties compared to men (5,9). In addition, female adipose tissue has a higher expression of genes involved in mitochondrial function and the relative contribution of fat mass to resting metabolic rate is higher in women than in men (10,11).

Thus, it appears that clear differences between the sexes exist. Nevertheless, the exact physiological mechanism causing this difference between men and women remains unclear, although certain aspects have been studied. The focus has been predominantly on the differences in sex steroid hormone levels between men and women. The female hormone estradiol (E2) for instance has been reported to increase resting metabolic rate, suggesting that E2 induces BAT thermogenesis (12,13), either via direct effects on BAT or indirectly via the central nervous system (CNS). The existence of the latter, indirect route of activation of BAT by E2 has recently been shown by Martínez de Morentin *et al.* (14). Direct effects of E2 have also been demonstrated in cultured primary mouse

brown adipocytes; E2 suppresses transcription of the α 2-adrenergic receptor. Stimulation of the α 2-adrenergic receptor results in an inhibition rather than an elevation of adrenergic signaling upon catecholamine activation (15), thus E2 facilitate activation of BAT. In contrast to the well-studied effects of E2, less is known about the role of the male hormone testosterone on BAT activity, but some studies showed that testosterone suppresses *Ucp1* expression in cultured primary mouse brown adipocytes (15,16) and thus probably inhibits BAT activity.

A largely neglected area of research is the regulation of tissue function by sex-specific intrinsic mechanisms, such as sex chromosome-mediated effects or sex-specific epigenetic alterations. It has been found that adipocytes isolated from female WAT depots are more insulin sensitive than those isolated from males (17) indicating the existence of such mechanisms in WAT, but we are unaware of studies on the role of sex-specific intrinsic factors in regulation of BAT activity and 'browning' of WAT.

Since the human perirenal adipose tissue depot gains BAT-like properties with reduced environmental temperatures (18), this depot may provide a useful tool to determine whether 'browning' of the perirenal adipose tissue depot differs between men and women. We therefore investigated the perirenal adipose tissue from healthy male and female live kidney donors and compared these to the subcutaneous adipose tissue depot of the same subjects. To study whether sex-intrinsic factors might mediate differences between the sexes and adipose tissue depots, mesenchymal stem cells (MSCs) were isolated from male and female perirenal and subcutaneous adipose tissue depots and differentiated into mature adipocytes *in vitro* in the absence of sex steroid hormones.

METHODS AND PROCEDURES

Patients and adipose tissue collection

Perirenal and subcutaneous adipose tissues were collected from healthy live kidney donors during donor nephrectomy. The study was approved by the Medical Ethics Committee of the Erasmus MC, University Medical Center Rotterdam (protocol no. MEC-2006-190,3) and written informed consent was obtained from the donors. Donors' clinical data were obtained from medical records and anonymized before analyses were performed. Data on menopausal status was retrospectively obtained by asking the women if they were postmenopausal and if they were using estrogens or estrogen-like substances when donating their kidney. Twenty-four women and 20 men participated in this study, their age ranged from 29 - 81 years and their BMI from 19.0-38.2 kg/m². The study was initially performed with adipose tissue from 8 women and 8 men which was used for histology, mRNA analysis and primary culture. Since adipose tissue of men was collected during winter, adipose tissue from an additional 7 women and 6 men

Table 1. Healthy live kidney donor characteristics.

RNA fresh tissue	Women (n=8)	Men (n=8)	P-value
Age (years)	48.7 ± 3.9	56.5 ± 5.6	0.276
BMI (kg/m ²)	28.9 ± 2.2	29.2 ± 1.6	0.918
OutsideTemperature (°C)	12.1 ± 1.4	7.2 ± 1.9	0.058
Histology	Women (n=15)	Men (n=14)	P-value
Age (years)	48.7 ± 2.8	56.2 ± 3.3	0.097
BMI (kg/m ²)	28.3 ± 1.4	27.5 ± 1.3	0.665
Outside Temperature (°C)	14.8 ± 1.1	12.1 ± 1.9	0.222
Primary culture	Women (n=17)	Men (n=13)	P-value
Age (years)	49.8 ± 2.6	51.2 ± 3.9	0.770
BMI (kg/m ²)	27.4 ± 1.3	27.3 ± 1.3	0.951

Analysis performed with unpaired t-tests. Values are mean ± SEM. Outside temperature is the average outside temperature in Rotterdam the week before surgery.

was only used for histological examinations to ensure that in warmer environmental circumstances, perirenal adipose tissue did indeed not possess brown-like structures. To verify the differences between *UCP1* expression between men and women in cultured adipocytes, adipose tissue of an additional 9 women and 5 men was collected and only used for primary culture (Table 1).

Meteorological data

All meteorological data of Rotterdam during inclusion were obtained from the Royal Dutch Meteorological Institute (KNMI) via a publicly accessible database (<https://data.knmi.nl/portal/KNMI-DataCentre.html>).

Histology and immunohistochemistry

For histological examination, adipose tissue was directly fixed in 4% formaldehyde and after 24 h stored in 70% ethanol until further investigation. Ten µm sections of formaldehyde fixed and paraffin embedded perirenal and subcutaneous adipose tissue were mounted on glass slides and stained with hematoxylin and eosin. For immunohistochemistry, sections were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, Zwijndrecht, The Netherlands). Endogenous peroxidase activity was blocked by 3% H₂O₂ in methanol (Sigma-Aldrich) for 20 min. After heat mediated antigen retrieval, sections were incubated over night at 4°C with an UCP1 antibody raised in rabbit (Sigma-Aldrich) in a 1:2000 dilution in PBS and subsequently incubated for 30 min at room temperature with BrightVision poly-HRP-anti mouse/rabbit/rat IgG (Immunologic, Duiven, The Netherlands) in a 1:2 dilution in PBS. Next, peroxidase activity

was developed with 0.07% 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Finally, all sections were counterstained with hematoxylin.

Primary cultures

For isolation of mesenchymal stem cells (MSCs), samples of perirenal and subcutaneous adipose tissue were collected in PBS (Gibco-Invitrogen, Breda, Netherlands). MSCs were isolated as previously described (19). In short, fat tissues were minced, washed with PBS and digested with 0.5 mg/mL collagenase (Sigma) in minimum essential medium- α (MEM- α) (Sigma) enriched with 4 mM glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin and 0.25 μ g/mL amphotericin B (Gibco) at 37°C. After centrifugation at 1200 rpm for 10 minutes, cell pellet was dissolved and filtrated (70 μ m) and cultured in minimum essential medium- α (MEM- α) (Sigma) with 15% heat-inactivated FCS, 4 mM glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin and 0.25 μ g/mL amphotericin B (Gibco) to select for MSCs. The cells were cultured at 37°C with 5% CO₂ and 95% humidity and medium was refreshed every 2 or 3 days. When cells had reached 95-100% confluence, differentiation was started by supplementation of the culture medium with 500 μ M IBMX (Sigma), 60 μ M indomethacine (Sigma) and 10 nM dexamethasone (Sigma). This differentiation medium was replaced every 2 or 3 days. Experiments were performed 15 days after start of differentiation, passage used for experiments were 2-5.

RNA isolation, cDNA synthesis and real-time PCR

For RNA isolation, adipose tissue samples and differentiated MSCs were directly frozen and stored at -80°C until further processing. Total RNA was isolated as previously described (20). In short, RNA was isolated using Tripure Isolations Reagent (Roche Applied Sciences, Almere, The Netherlands), DNase treated for 30 minutes at 37°C and reverse transcribed using a cDNA synthesis kit (Roche Applied Sciences). Quantitative RT-PCR was determined with an ABI Prism 7900 Sequence Detection System using SYBRgreen mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and FAM-TAMRA duo-label probe (Roche, Woerden, The Netherlands) was used for the detection of hypoxanthine-guanine phosphoribosyltransferase-1 (HPRT1). Sequences of the primers used are listed in Table 2. The expression of each gene is given in arbitrary units after normalization to the average expression level of HPRT1 in case of cultured adipocytes or RNA18S5 in case of fresh tissue using the $2^{-\Delta\Delta Ct}$ method (21).

Western blot analyses

Western blot analysis was performed as previously described (20) on differentiated MSCs of 4 men and 4 women that had the highest *UCP1* expression mRNA. In short, protein was extracted from differentiated MSCs with lysis buffer containing 0.05 M

Table 2. Characteristics of BAT positive and BAT negative women

Women	BAT positive (n=5)	BAT negative (n=10)	P-value
Age (years)	45.6 (38-55)	50.3 (36-70)	0.429
BMI (kg/m ²)	29.2 (±3.1)	27.9 (±1.6)	0.693
Plasma glucose (mM)	4.9 (±0.3)	5.3 (±0.4)	0.416
Plasma TG (mM)	0.9 (±0.2)	1.3 (±0.2)	0.234
Plasma cholesterol (mM)	5.1 (±0.3)	5.8 (±0.3)	0.212
MAP (mmHg)	88 (± 5)	93 (±2)	0.335

P-values are obtained with unpaired t-test. Values are means and range for age and \pm SEM for other parameters. MAP = mean arterial pressure and is calculated as 2 times diastolic pressure plus systolic pressure, divided by 3.

HEPES, 10mM EDTA, 20 mM NaF, 2 mM NaVO₃, 150 mM NaCl, 1% Triton, phosphatase inhibitors and protein inhibitors and subsequent sonification. Next, 30 μ g protein was diluted in Laemmli buffer (Bio-rad, Veenendaal, The Netherlands) and subsequently electrophoresed on a 10% gel and blotted on a nitrocellulose membrane. Membranes were blocked in PBS with 3% nonfat powdered skimmed milk, followed by overnight incubation at 4°C with a rabbit polyclonal anti-UCP1 antibody (1:500; Sigma) or a rabbit polyclonal anti- α -tubulin antibody (1:1000; Santacruz, Heidelberg, Germany). Goat-anti-rabbit IRDye 800 was used as secondary antibody (1:10,000 for UCP1 and 1:20,000 for α -tubulin; Li-cor, Leusden, The Netherlands). UCP1 and α -tubulin immunoreactivity was measured with Odyssey fluorescence scanner (Li-cor, Leusden, The Netherlands) and was normalized to α -tubulin immunoreactivity using Odyssey software.

Oxygen consumption measurements

Oxygen consumption rate in differentiated MSCs was measured using a Seahorse Bioscience XF24 extracellular flux analyzer (Seahorse Biosciences, North Billerica, MA, USA). Oxygen consumption was determined on differentiated MSCs of 4 men and 4 women that had the highest *UCP1* mRNA expression. For this, MSCs were seeded in poly-D-lysine (Sigma Aldrich) coated 24-well-Seahorse-assay plates, and differentiated as described above. Measurements were performed as described before (20). In short, measurements were initiated after equilibration in freshly prepared seahorse medium. Three baseline OCR measurements were performed, followed by injection of oligomycin (Sigma) to a final concentration of 0.5 μ M, subsequently OCR was measured six times after 2 min of mixing. Measurements were performed on 5 wells of each plate as technical replicates. The averages of three baseline OCR measurements and three OCR measurements after oligomycin addition were used for data analyses.

Statistical analysis

Data were analyzed with Graphpad Prism 5 (San Diego, CA, USA) and expressed as mean \pm SEM. Data were evaluated for statistical differences by Mann–Whitney U test. Differences were considered significant at $P < 0.05$. Correlations between *UCP1* mRNA and metabolic variables and temperature were analyzed using linear regression models.

RESULTS

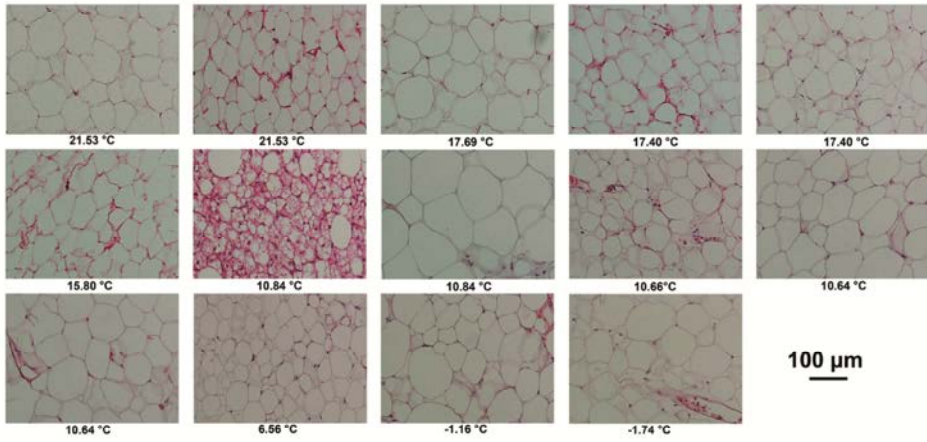
More ‘browning’ of perirenal adipose in women than in men

To determine the degree of ‘browning’ in the perirenal and subcutaneous adipose tissue depots, we histologically examined these two depots and found that small, multilocular adipocytes were present in perirenal adipose tissue (Figure 1A) but not in subcutaneous adipose tissue (Figure S1). As expected, these small, multilocular cells could be identified as brown-like adipocytes since they stained positive for UCP1 (Figure 1B). Of interest, the brown-like adipocytes were present in 33% of the female perirenal adipose tissue, while only 1 out of the 14 men (7%) had brown-like cells in his perirenal adipose tissue depot. Moreover, *UCP1* mRNA was predominantly detectable in the perirenal fat tissue of women but not in the subcutaneous adipose tissue of both men and women (Figure 1C). This was only significantly different when we excluded the only male with high *UCP1* mRNA expression, since this value could be considered as an outlier with a value more than 2SD above average *UCP1* expression in males. A similar pattern as the *UCP1* mRNA expression was found for *PPARGC1A* mRNA expression, albeit not statistically significant (Figure 1D).

Cold induces ‘browning’ of the perirenal adipose tissue in women but not in men

Since cold exposure is a well-known trigger for WAT depots to gain brown-like features (22), we analysed whether outside temperatures shortly before collection of the samples might have affected the degree of ‘browning’ of the perirenal adipose tissue. For this, we calculated the average outside temperature of the week before surgery and the change in temperature during that week. Surprisingly, female perirenal adipose tissue depots contained brown-like adipocytes when the average outside temperature was below 11°C or when temperature dropped with more than 5°C in the week before surgery. An effect of outside temperature or temperature change on the presence of brown-like adipocytes was not detectable in the male perirenal adipose tissue. The sole man that had brown-like adipocytes in his perirenal adipose tissue was exposed to an average outside temperature of 10.8°C with a delta change in temperature of 3°C during that week. We did not find a significant correlation between *UCP1* mRNA expression and outside temperature, although a trend could be observed for women (Figure 2).

Figure 1A
Men



Women

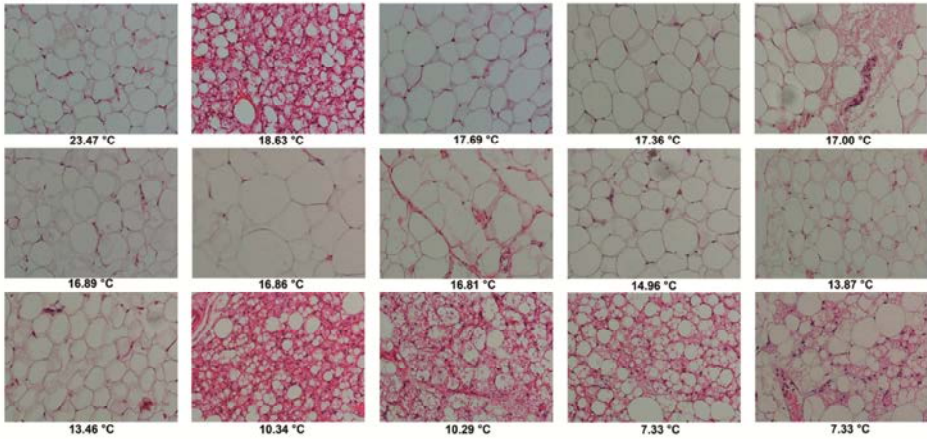
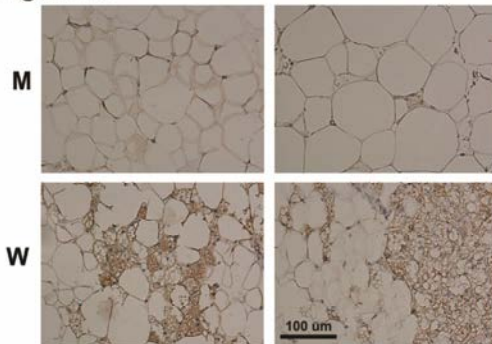


Figure 1B



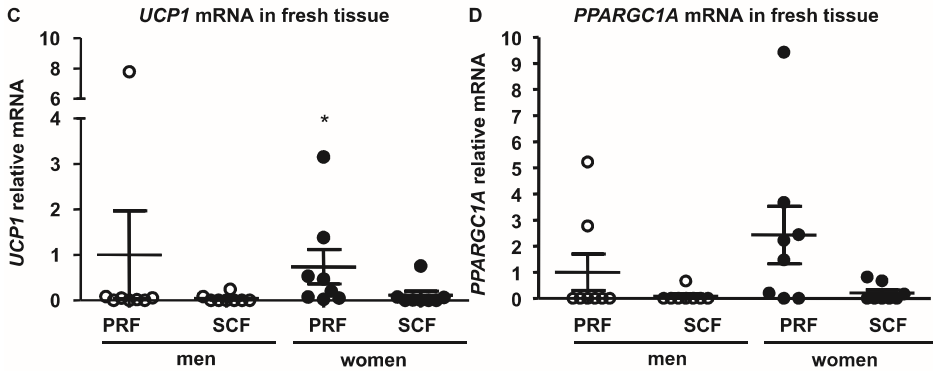


Figure 1. Analysis of perirenal fat of men and women.

A) Histological staining of fresh perirenal fat. H&E staining revealed that women (lower panel) have more BAT in perirenal fat when the average maximum outside temperature a week before surgery drops below 11°C, this pattern is not observed in men (upper panel). B) Representative images of immunohistochemical UCP1 staining of fresh perirenal fat obtained from male (upper panel) and female (lower panel) healthy live kidney donors. C) *UCP1* mRNA expression in Perirenal Fat (PRF) is significantly different between men and women in fresh tissue but not significantly different in Subcutaneous Fat (SCF). D) *PPARGC1A* mRNA expression in PRF and SCF is not significantly different between men and women in fresh tissue. A Mann–Whitney U test was used to test for significant differences in all cases.

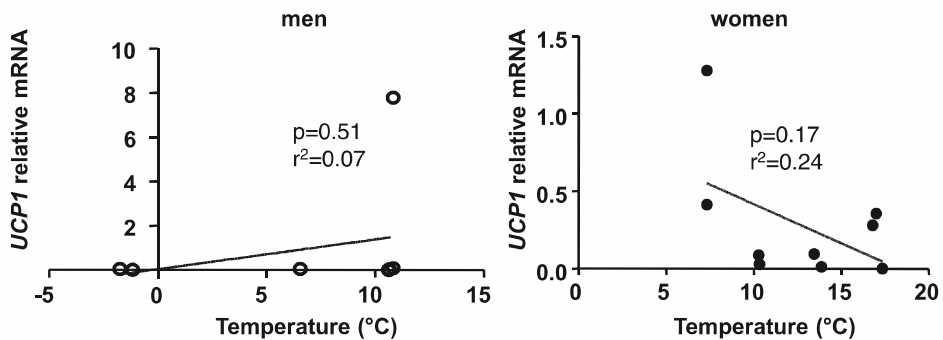


Figure 2. Correlation between average outside temperature during one week before surgery and *UCP1* mRNA expression in fresh perirenal fat tissue.

Presence of brown-like adipocytes and *UCP1* mRNA expression do not correlate with metabolic plasma markers.

Estrogens are thought to induce BAT activity and browning of WAT, thus one would expect that postmenopausal women have less browning of their perirenal adipose tissue. There was no difference in age between the females that had multilocular cells and *UCP1* protein present (BAT positive subjects) and those without multilocular cells and *UCP1* protein (BAT negative group) (Table 2). Age also did not correlate with *UCP1* mRNA expression (Figure S2). In addition, menopausal status did not predict the pres-

Table 3. Menopausal status in women with BAT positive histology and BAT negative histology.

Menopause:	yes	no	Unknown	Total
BAT +	2	3		5
BAT -	4	5	1	10

Table 4. Primer sequences

Gene		Primer sequences 5'-3'	Probe 5'FAM-3'TAMRA
HPRT	Forward	TGCTTTCCTTGGTCAGGCAGTAT	TGACTTCAGCCCAGCTGTGGTTCCA
	Reverse	TCAAATCCAACAAAGTCTGGCTTATATC	
UCP1	Forward	CTGGAATAGCGGCTGCTT	No probe (SYBR Green)
	Reverse	AATAAACTGGACGTCGGGC	
RNA18S5	Forward	ATGGCCGTTCTTAGTTGGTG	No probe (SYBR Green)
	Reverse	ATGCCAGAGTCTCGTTCGTT	
PPARGC1A	Forward	TCTGAGTCTGTATGGAGTGACAT	No probe (SYBR Green)
	Reverse	CCAAGTCGTTACATCTAGTTCA	

ence of brown-like cells (Table 3). Increased activity of brown adipocytes and brown-like adipocytes increases the futile use of triglycerides and glucose and may have a metabolically beneficial effect. Therefore, we examined whether presence of multilocular cells and perirenal adipose tissue *UCP1* mRNA expression in perirenal WAT in women were associated with markers of the metabolic syndrome: BMI, fasted blood glucose, plasma triglycerides, plasma total cholesterol and blood pressure. No statistically significant correlations could be found (Table 2 and Figure S2).

Adipocytes generated from stem cells of female perirenal adipose tissue have the highest *UCP1* mRNA expression but not *UCP1* protein and function

In the next experiment we examined whether intrinsic factors might determine 'browning' of the perirenal adipose tissue. For this, we isolated mesenchymal stem cells (MSCs) from the subcutaneous and the perirenal adipose tissue depot of both women and men and studied their potency to develop brown-like characteristics in exactly the same environment in the absence of sex steroid hormones. Differentiation of the MSCs towards adipocytes (Figure 3A) resulted in the highest *UCP1* mRNA expression in those originating from female perirenal adipose tissue, which was not correlated to outside temperature. No *UCP1* mRNA was detected in MSCs originating from subcutaneous adipose tissue from either sex (Figure 3B). Notwithstanding the clear difference in *UCP1* mRNA expression, adipocytes originating from female and male perirenal adipose tissue had the same amount of *UCP1* protein (Figure 3C). In line with this, both total oxygen consumption and the uncoupled oxygen consumption did not differ between adipocytes generated from MSCs of male and female perirenal adipose tissue (Figure

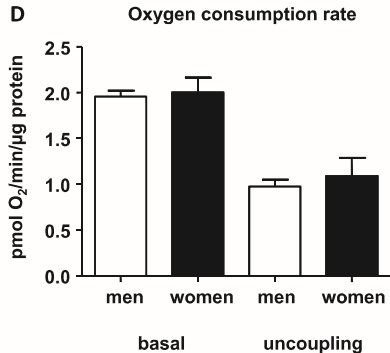
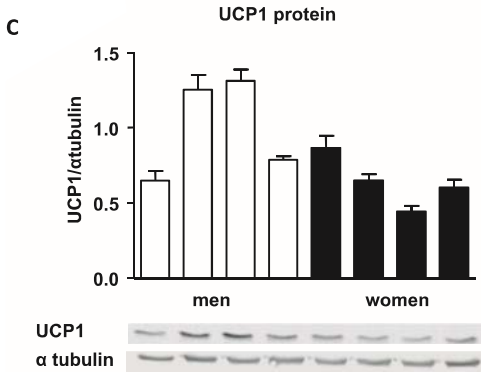
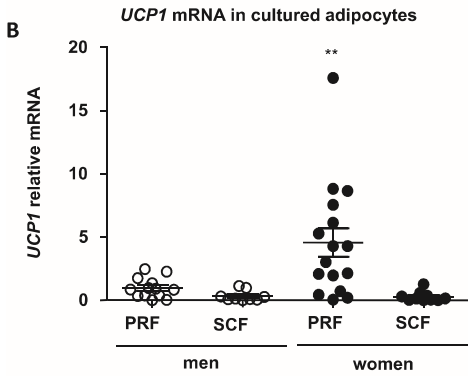
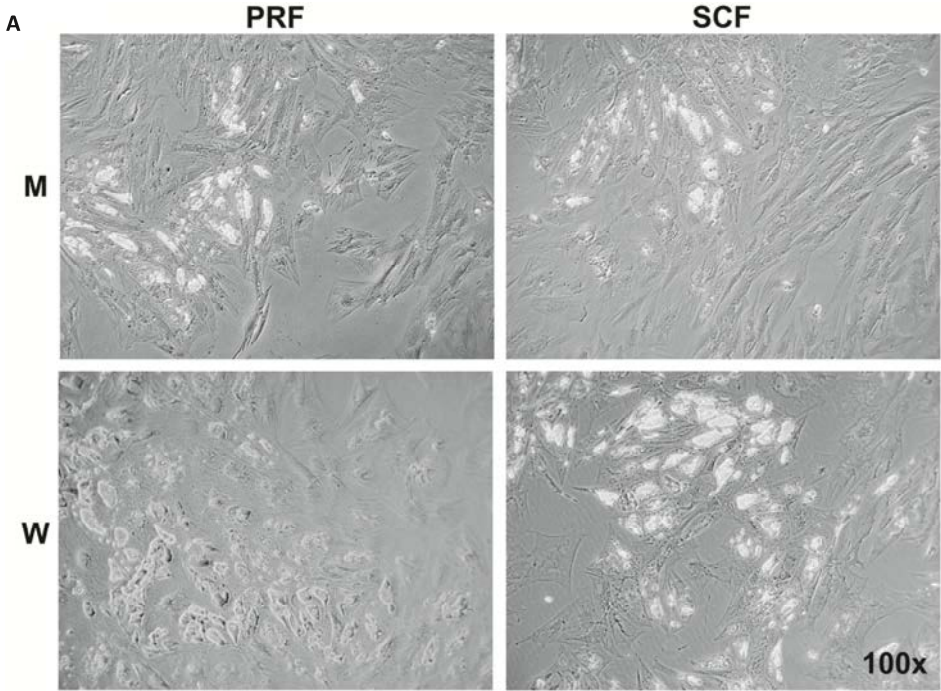


Figure 3. Analysis of MSCs derived from subcutaneous and perirenal fat.

A) Representative pictures of differentiated adipocytes derived from perirenal and subcutaneous fat, both from a man and a woman. B) *UCP1* mRNA expression is increased in adipocytes derived from stem cells of perirenal fat of women. C) *UCP1* protein is not higher in cultured adipocytes of females derived from perirenal fat. Values are average values of *UCP1* protein over α -tubulin ($n=3$) per subject, 4 males and 4 females. Below a representative blot of *UCP1* and α -tubulin. D) Oxygen consumption is not different between males and females. Oxygen consumption driven by uncoupling (measured after addition of oligomycin, a ATPsynthetase inhibitor) is not different in cultured adipocytes from males and females ($n=4$ per group). A Mann–Whitney U test was used to test for significant differences in all cases.

3D). In cultured adipocytes from female perirenal origin 54% of the oxygen consumption was due to uncoupling while this value was 50% in cells from male perirenal origin.

DISCUSSION

In this study, we found that sex and environmental temperature are strong determinants of ‘browning’ of human perirenal adipose tissue. At colder temperatures, women are more likely to have brown-like adipocytes in their perirenal adipose tissue than men. Analysis of differentiated MSCs isolated from the perirenal adipose tissue depot showed that female perirenal adipose tissue has the highest potency *per se* to gain brown-like features, at least with respect to *UCP1* mRNA expression. We were able to find these differences despite the fact that our population is relatively heterogeneous for age and BMI. This indicates that our findings are applicable for people of all ages with a broad range of BMI.

Perirenal fat tissue was easy to obtain due to the large number of live donor nephrectomies in our hospital. This created the unique opportunity to study the perirenal fat depot of healthy individuals. This fat depot is an ideal model to study browning, since it has been reported that brown adipocytes are found in perirenal fat tissue (23-25), a depot that ‘browns’ easily upon cold exposure (18). Interestingly, *UCP1* mRNA expression was not significantly correlated with outside temperature. This lack of correlation might be due to the heterogeneous character of the perirenal adipose tissue depot, but also underlines the importance of *UCP1* protein measurements, since this reflects the actual activity of brown adipocytes. It is not clear why the perirenal depot has this potential to brown easily but one can elaborate that heat produced by the perirenal fat depot is easily transported through the body, since the kidney receives approximately 20% of the cardiac output. In addition, this depot is close to the adrenal gland which produces steroids and catecholamines. Glucocorticoids have been reported to inhibit development and activity of BAT in rodents (26,27) whereas high levels of catecholamines increase browning of the perirenal depot (28,29). The anatomical proximity to

the adrenal gland might be one of the reasons why browning of this depot is easily regulated.

Our data show that regulation of browning is different between men and women. A number of studies have found higher prevalence of active BAT in women compared to men (5,30,31) while others have not (22). We have to be aware, however, that active BAT was detected by ^{18}F -FDG PET scanning, a method that only detects uptake of the radioactive glucose as marker of BAT activity. In our experiments, in contrast, we did not determine BAT *activity*, but *presence* of BAT-like cells in perirenal adipose tissue.

We will discuss three factors where men and women can differ; gonadal sex hormone differences, chromosomal differences (XX and XY) and possible epigenetic differences. Hormonal status was, in our study, not a determinant for 'browning' of the perirenal adipose tissue. This suggests that sex steroids are at least not the sole determinants of browning the perirenal adipose tissue. Direct effects of estrogens and other sex steroid hormones can be studied *in vitro*, but such studies have rarely been performed and are mainly focused on transcriptional responses (15,16). Direct effects of sex steroids on more physiologically relevant markers of BAT activity and browning of WAT such as uptake of glucose, fatty acids and triglycerides and respiration have not been investigated. The same cell culture conditions were used for all perirenal MSCs, but *UCP1* mRNA expression was predominantly detected in adipocytes derived from female origin. This points to a sex-specific intrinsic factor which determines transcription of *UCP1*. Thus, it is of importance whether cells were generated from male or female origin. Pollitzer also recently stressed the importance of the sex of cells, even when cultured (32). Cells derived from male and female mice might respond differently to stress (33) and muscle stem cells from female mice can regenerate muscle faster than their male equivalents (34). Also within the field of endocrinology male-female differences are currently under investigation, since the prevalence of diseases such as autoimmune thyroid disorders and thyroid nodules is significantly higher in women than in men (35).

The biggest genetic difference between men and women is the presence of two X-chromosomes in women and one X and one Y chromosome in men. Recently Chen *et al.* (36) showed that the presence of the Y-chromosome suppresses BAT *Ucp1* expression in mice. In addition, due to inactivation of one X-chromosome, women have a mosaic expression of both the maternally and paternally derived X-chromosomes, where men only express genes of the maternally derived X-chromosome. Gene expression profiling studies are needed to find out whether the presence of different sex chromosomes, X-inactivation and the apparent potency of some genes to escape this inactivation are important in controlling BAT activity and 'browning' of WAT.

Although *UCP1* mRNA was higher in adipocytes derived from female perirenal adipose tissue compared to these from male perirenal adipose tissue, no differences could be detected in *UCP1* protein content and *UCP1* function, i.e., uncoupled oxygen consump-

tion. Thus, the presence of *UCP1* mRNA does not simply determine the uncoupling status, a matter already discussed by Nedergaard *et al.* (37). Translation of *UCP1* mRNA into UCP1 protein and UCP1 function is regulated by various factors such as epigenetic control (38) and availability of substrates required for uncoupled respiration such as fatty acids, glucose and ADP (1,39). Thus, the differentiated MSCs apparently need an additional factor which might be present in BAT and WAT with BAT-like properties upon cold exposure *in vivo*.

In conclusion, women have more potency to induce browning of their perirenal adipose tissue, very likely due to sex-specific intrinsic characteristics of the MSCs in the perirenal adipose tissue. For activation of the brown adipocytes, other factors that might be sex-specific are required.

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JvdB and AG designed the study, carried out experiments and analyzed data and generated figures, JS carried out experiments and analyzed data, MH, PM, and FD carried out experiments and FD, MH and AT contributed to study design, data analysis and interpretation. All authors were involved in writing the paper and had final approval of the submitted and published versions.

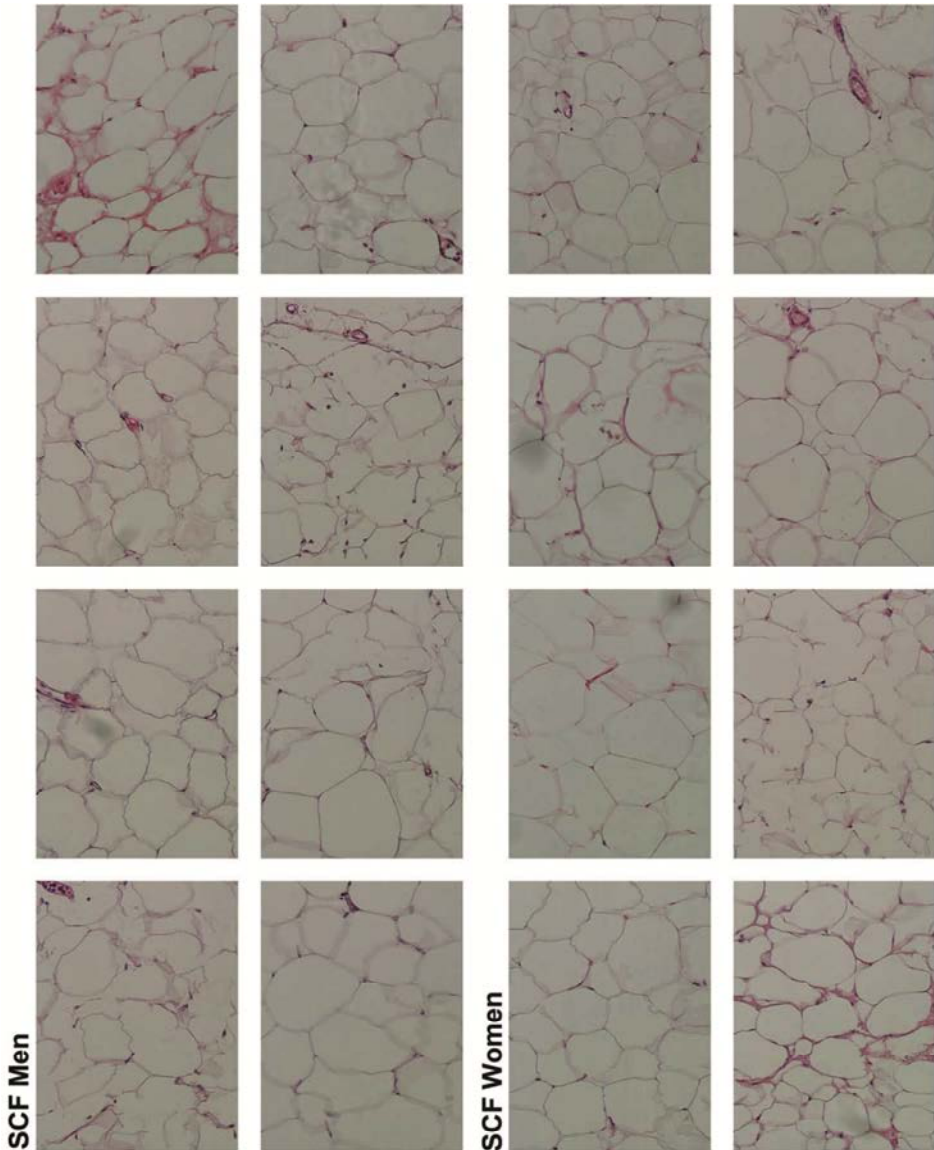
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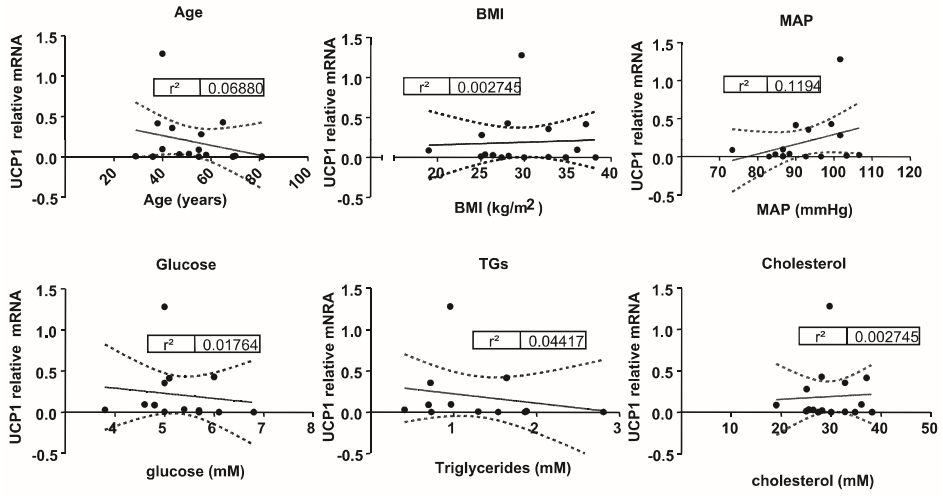
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SUPPLEMENTAL FIGURES

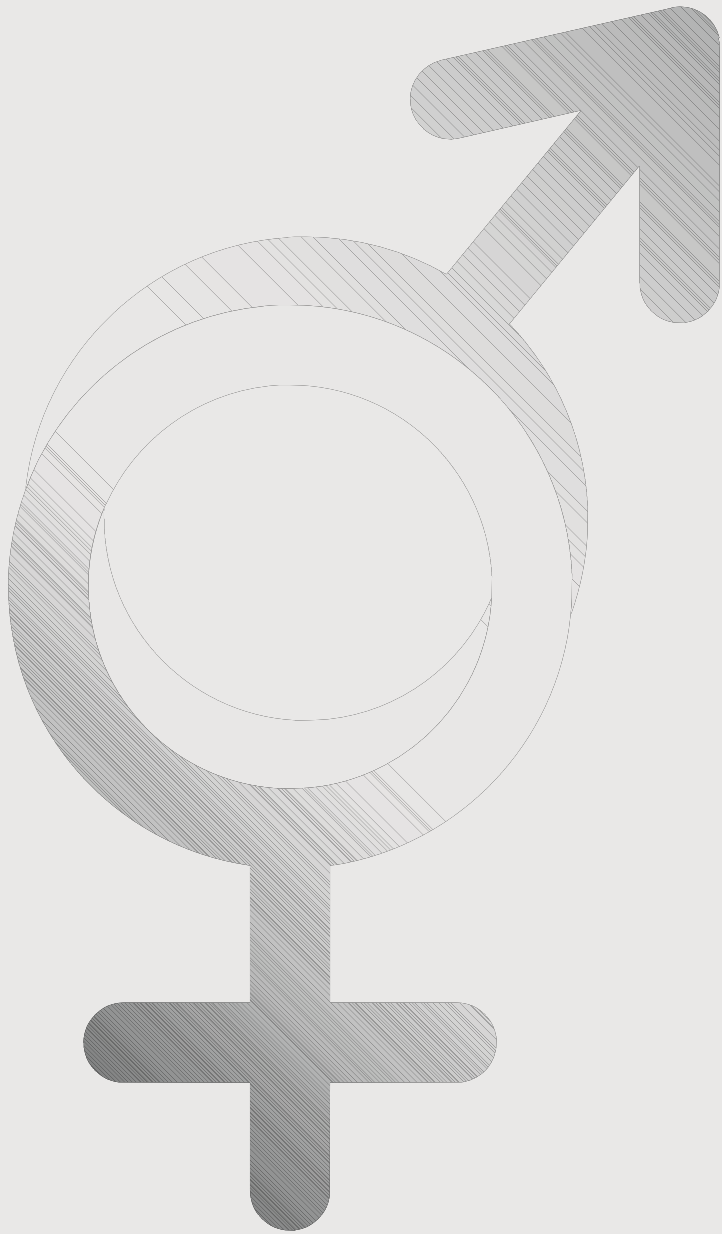


Supplemental figure 1. Subcutaneous fat of males and females.

H&E staining of subcutaneous fat. No brown-like adipocytes in subcutaneous fat of men (upper panel) and women (lower panel) could be detected



Supplemental figure 2. Correlations between UCP1 expression in fresh perirenal fat tissue and metabolic parameters



CHAPTER 7

EVEN IN WINTER, YOUNG FEMALES
TEND TO HAVE MORE ACTIVE
BROWN ADIPOSE TISSUE THAN
YOUNG MALES

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ABSTRACT

Background Since brown adipose tissue (BAT) has been detected on ^{18}F -fluoro-deoxy-glucose positron emission tomography (^{18}F -FDG PET) scans in adult humans, BAT has gained interest as potential target for obesity treatment. BAT activity is higher at young age and is induced upon cold exposure. In addition, studies have reported higher prevalence of activated BAT in women compared to men. We questioned whether this sex difference is also detectable in a young population during the winter.

Methods Retrospective analysis of supraclavicular BAT on ^{18}F -FDG PET/CT whole-body scans of 53 men and 46 women (20-35 years of age) collected between October 1 and April 30 of 2011/2012, 2012/2013 and 2013/2014. Tissue standard uptake value (SUV) of ^{18}F -FDG was calculated as the ratio of radioactivity concentration (MBq/kg) and injected activity (MBq) divided by body weight (BW) (kg). Correction for lean body mass (LBM) instead of BW results in SUV per LBM (SUL). Liver SUV and SUL were determined to study which value was BW independent. BAT was considered active when ^{18}F -FDG uptake was $\geq 3\text{SD}$ above the mean of maximal ^{18}F -FDG uptake in subcutaneous WAT of all subjects.

Results Liver SUL but not SUV was independent of BW. Using BAT SUL, no difference prevalence of active BAT between men and women was found. However, BAT was most frequently activated in patients in the lowest BMI tertile ($<21.7 \text{ kg/m}^2$) ($p < 0.05$). In all BMI tertiles, women had a tendency of higher prevalence of activated BAT.

Discussion In a young population studied in the winter, the prevalence of active BAT was inversely correlated with BMI, but did not differ between males and females, although there was a tendency towards more active BAT in females across BMI tertiles.

INTRODUCTION

Previously it was thought that only small mammals and young children possess brown adipose tissue (BAT). In 2009, using ^{18}F -fluoro-deoxy-glucose positron emission tomography (^{18}F -FDG PET), three independent studies reported that most adult humans also have metabolically active BAT (1-3). While white adipose tissue (WAT) stores energy in the form of triglycerides, BAT can dissipate energy by production of heat (thermogenesis) and is thereby partly responsible for the maintenance of our body temperature (4). In most tissues, fatty acids and glucose are metabolized in the mitochondria to produce ATP via oxidative phosphorylation. BAT, however, is capable to produce heat due to the presence of uncoupling protein 1 (UCP1) in its numerous mitochondria. UCP1 uncouples oxidative phosphorylation from ATP production, resulting in the release of energy as heat (5).

In adult humans, BAT can be stimulated by cold exposure (1-3,6-12) and the chance to detect activated BAT is highest in subjects of younger age (1,3,7,8,12-16). In theory, activation of BAT has the potential of increasing energy expenditure and thereby reducing body weight. Stimulation of BAT thermogenesis is thus considered an important potential target to reduce stored energy in WAT and circulating glucose in the treatment of obesity and its metabolic consequences. Indeed, several observational studies in humans showed an association between increased BAT activity and improved insulin sensitivity (10,15,17).

Various studies have reported that, in general, the prevalence of active BAT is higher in women than in men (3,15,16,18). It is however not known whether this sex difference remains under physiological conditions that likely result in very active BAT, *i.e.*, in a young population in wintertime. We therefore studied the level of BAT activity and the prevalence of active BAT in the supraclavicular BAT depot using ^{18}F -FDG PET scans from young (20-35 years old) subjects obtained for diagnostic purposes during 3 winters.

METHODS

PET/CT scan analyses

^{18}F -FDG PET/CT whole-body scans from 53 young men and 46 young women aged between 20-35 years that were performed at the Erasmus Medical Center from October 1-April 30 in the seasons 2011/2012, 2012/2013 and 2013/2014 were retrospectively analyzed. Scans were performed mainly for oncological diagnostic reasons. Data about age, sex, height, weight, medication use and diagnosis were obtained for all patients. PET/CT scans were acquired with the use of a mCT-128 or a mCT-40 PET/CT scanner (Siemens Healthcare, Erlangen, Germany). Scanners were calibrated according to the

recommendations of the European Association of Nuclear Medicine (EANM) procedure guideline for tumor PET imaging (19). Both scanners had gained EANM Research Ltd. (EARL) accreditation. Briefly, the scanning procedure was as follows: after a 6-hour fasting period the injected activity was 2.3 MBq/kg ^{18}F -FDG, with PET acquisition starting 60 ± 5 minutes after the injection. All parameters were according to the EANM procedure guidelines. For standard uptake value (SUV) measurements, PET was reconstructed with standard iterative reconstruction, without inclusion of time-of-flight or resolution enhancement parameters to adhere with the EARL calibration.

Analysis of ^{18}F -FDG uptake was performed using Hybrid Viewer™ version 2 software (Hermes, Medical Solutions AB, Stockholm, Sweden). Tissue activity was measured by maximal and mean SUV's of ^{18}F -FDG, defined as the ratio of the tissue radioactivity concentration (in MBq/kg) and the injected activity (in MBq), divided by body weight (in kg). BAT SUV was determined in the supraclavicular area while subcutaneous WAT measurements were taken lateral to the musculus gluteus maximus at the height of os pubis or at the distal point of os ischium.

SUVs of all regions of interests were also corrected for lean body mass (LBM) instead of body weight to determine the SUV per LBM (SUL). LBM was calculated according to Hume *et al.* (20):

$$LBM(\text{women}) = (0.29569 \times BW) + ((0.41813 \times \text{height}) - 43.2933)$$

$$LBM(\text{men}) = (0.32810 \times BW) + ((0.33929 \times \text{height}) - 29.5336)$$

In these formulas, BW is body weight in kg and height is in cm.

Liver and mediastinal bloodpool (MBP) SUV were considered as reference golden standard and should not be influenced by differences in body weight or any other confounder (21). Blood glucose concentrations did not influence liver SUL and MBP SUL. BAT was considered active if the mean SUL (SUV corrected for LBM) of BAT was at least more than 3SD above the mean of the maximal SUL in the subcutaneous WAT depot of all subjects.

Meteorological data

Meteorological data from Rotterdam were retrieved from the Royal Dutch Meteorological Institute (KNMI) (<https://data.knmi.nl/portal/KNMI-DataCentre.html>).

Statistical analysis

Subject characteristics of continuous variables are presented as mean, standard error of the mean (SEM) and range. Categorical variables are presented as percentages.

Between-group differences were calculated with chi-square statistics for categorical variables and independent sample T-tests for continuous variables. A Mann-Whitney U test or Wilcoxon signed rank test was used in case of a non-normal distribution. The values of BMI, age, fasting blood glucose level and mean outside temperature (average of one week before the scan) were divided into tertiles. The statistical difference of the percentage of patients with activated BAT in each tertile was assessed using a chi-square test for linear trend. All tests were performed using SPSS (version 21.0, SPSS, Inc., Chicago, IL) and outcome was considered statistically significant at $P < 0.05$.

RESULTS

The aim of our study was to investigate whether men and women have differences in the prevalence of active BAT in a period when BAT is most likely to be activated, *i.e.*, in young individuals during winter. For this, we retrospectively analyzed ^{18}F -FDG PET/CT

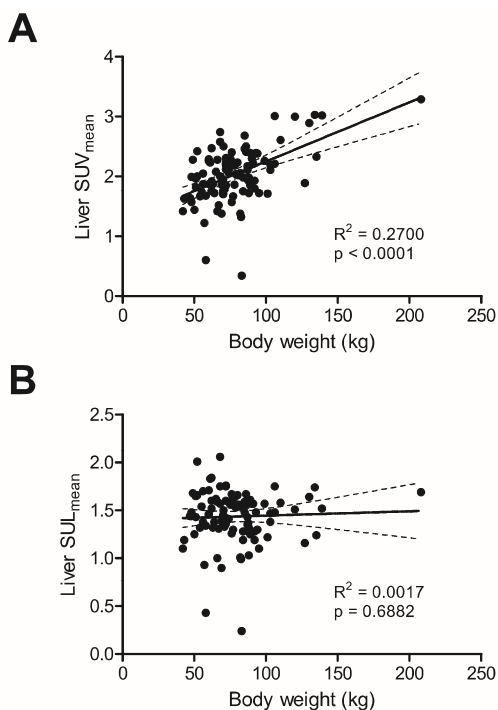


Figure 1. Correlation between BW and liver SUV (a) and SUL value (b). Data are from a retrospective analyses of ^{18}F -FDG PET CT whole-body scans from 53 men and 46 women, aged 20 – 35 years. The scans were performed in the winters (October 1 - April 30) of 2011/2012, 2012/2013 and 2013/2014.

Table 1. Characteristics of the 99 subjects from whom the ^{18}F -FDG PET CT whole-body scans were analyzed. Values are means \pm SEM (range), or number of patients (%). *, $P < 0.05$ vs. men.

	Men (n = 53)	Women (n = 46)
BMI (kg/m^2)	24.8 ± 0.9 (16.6 – 52.0)	25.7 ± 1.0 (17.7 – 46.4)
LBM (kg)	59.5 ± 1.4 (41.2 – 106.6)	48.9 ± 1.3 (31.3 – 71.9) *
Age (years)	29.0 ± 0.7 (20 – 35)	29.2 ± 0.6 (20 – 35)
Fasting blood glucose (mM)	5.1 ± 0.1 (3.6 – 8.6)	5.1 ± 0.1 (3.2 – 10.0)
Beta-blocker use (N (%))	4 (7.7%)	6 (13%)

whole-body scans of 53 young men and 46 young women. These men and women did not differ with respect to body mass index (BMI), age, fasting blood glucose concentration and the use of beta-blockers that can potentially inhibit BAT activity (22) (Table 1). Sugawara *et al.* (21) reported that SUL is a more appropriate method to quantify ^{18}F -FDG uptake since it avoids overestimation of glucose utilization in obese patients while SUV is a weight-dependent index for ^{18}F -FDG uptake. This is especially relevant when studying adipose tissues, since this tissue normally hardly accumulates ^{18}F -FDG (23). To determine whether SUL is a weight-independent index for ^{18}F -FDG uptake in our popu-

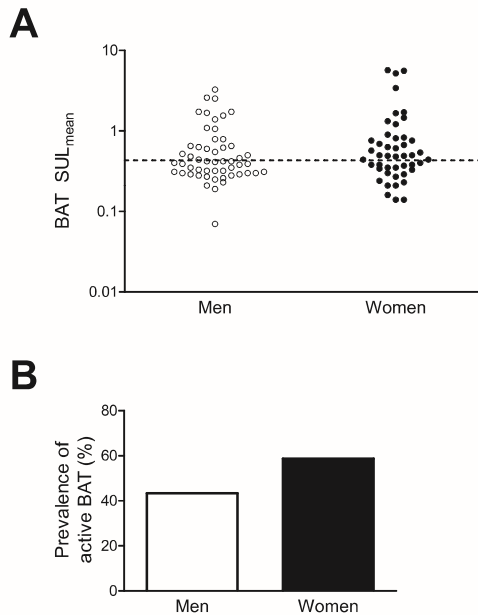


Figure 2. Individual SUL values for the location where BAT can be found in the supraclavicular area for either the 53 men or the 46 women. The dotted line represents the cut-off value for activated that that is set as 3SD above the mean of the maximal SUL in the subcutaneous WAT depot of all scanned subjects (a). Prevalence of active BAT in the supraclavicular area calculated using the cut-off value described above (b).

lation, we first determined SUV and SUL of the liver, an organ in which ^{18}F -FDG uptake is less likely to correlate with BW and LBM than BAT (Figure 1). Indeed, liver SUL was not correlated with BW while liver SUV had a positive correlation with BW. Therefore, we choose SUL to quantify BAT activity in the supraclavicular area.

It is evident that BAT SUL shows a wide variation and that it is not statistically significant different between men and women: 0.95 ± 1.34 vs. 0.67 ± 0.67 , women vs. men, NS (Figure 2a). Fifty of our 99 subjects (50.5%) had active BAT (i.e., BAT SUL was at least more than 3SD above the mean of the maximal SUL in the subcutaneous WAT depot of all subjects) with a slightly higher prevalence in women compared to men: 58.7% vs. 43.4%, women vs. men, NS (Figure 2b).

We next determined the association of prevalence of active BAT with parameters of metabolism. Due to the small study population, these parameters were not distributed normally, hence we divided them in tertiles. BAT was most frequently activated in subjects in the lowest BMI tertile but no associations were found between the prevalence of active BAT and age, fasting blood glucose or mean outdoor temperature the week before scanning (Figure 3). Remarkable, analyzing the prevalence of active BAT over BMI tertiles per sex revealed that the prevalence of active BAT was higher in women than in men in all tertiles (Figure 4). However, this sex differences did not reach statistical significance.

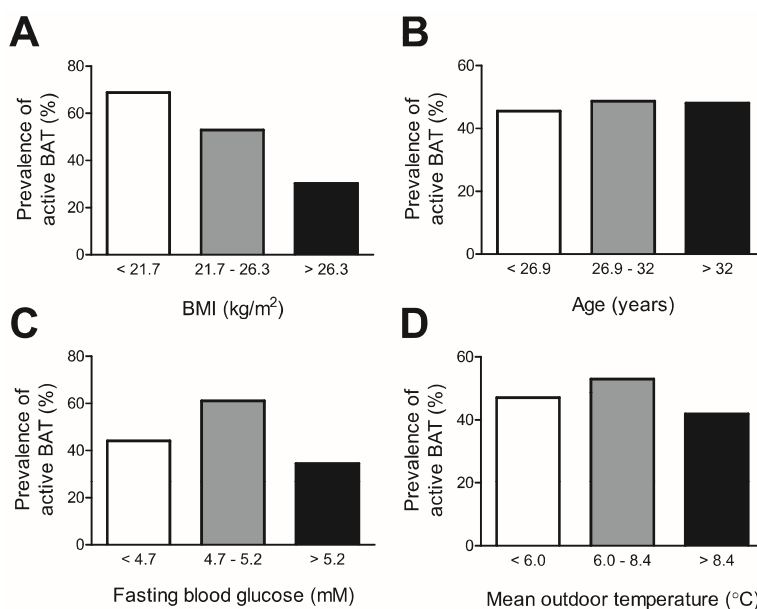


Figure 3. Prevalence of active BAT in the supraclavicular area in all 99 subjects divided by tertiles according to BMI (a), age (b), fasting blood glucose (c) and mean outdoor temperature the week before scanning (d).

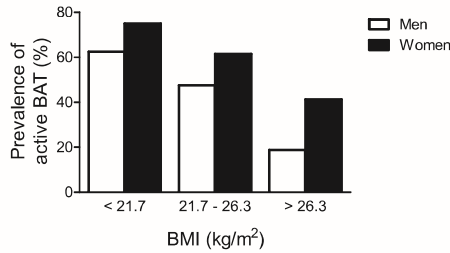


Figure 4. Prevalence of active BAT in the supraclavicular area divided by tertiles according to BMI and by sex.

DISCUSSION

In the present study, we investigated whether sex differences in the prevalence of active BAT were present in subjects with likely the highest BAT activity: young individuals in the winter. For this, we retrospectively analyzed ¹⁸F-FDG PET/CT whole-body scans. In addition, we tried to elucidate possible confounding factors which might have influenced BAT activity and prevalence of active BAT on ¹⁸F-FDG PET scans.

Before analyzing BAT activity, we first determined whether we should use SUV or SUL and thus corrected the ¹⁸F-FDG uptake by the liver for either total body weight or LBM, respectively. Although Tahari *et al.* (24) recently published a new algorithm for the calculation of LBM, which especially results in differences in LBM for subjects with a high BMI, we used LBM calculation suggested by Hume *et al.* (20) since we did not have many people in the higher BMI range. In agreement with previous studies (21), liver SUV had a positive correlation with body weight, which disappeared when liver SUL was used instead. Thus, SUL is more appropriate to quantify ¹⁸F-FDG uptake when studying subjects within a (broad) range of body weights.

Using SUL, we observed that women only have a tendency to have higher ¹⁸F-FDG uptake in the supraclavicular area than men. This may be due to the large variation within the groups, similar as reported by Cypess *et al.* (3). In our study, the women only had a tendency towards a prevalence of active BAT than men. This is in contrast to the findings published by Pfannenber *et al.* (16) and Ouellet *et al.* (12), who showed that both BAT activity and BAT mass were significantly higher in women than in men. Several other studies also reported a significantly higher prevalence of active BAT in women than in men (3,7,14,15). Thus in general the probability of having activated BAT is greater in women than in men.

The absence of a clear sex difference in prevalence of active BAT in our subjects suggests that the probability of having activated BAT is already higher in colder seasons and therefore eliminates a potential sex difference. When the outdoor temperature is relatively high, men may not have activated BAT yet, while women have already activated

their BAT. However, a recent publication in which the authors analyzed 1060 ^{18}F -FDG PET scans made in the winter of 2014/2015 of 1031 patients did report a higher prevalence of active BAT in the female population compared to males (25). Thus, it is likely that we failed to see a difference due to the small sample size of in our study. Another explanation for the contrasting results could be the lower cut-off point for active BAT used in our study compared to many other studies. Nevertheless, the high prevalence of active BAT in our study is in line with the study by van Marken Lichtenbelt *et al.* (1) who found that 24 of the 25 (96%) individuals had a positive PET/CT when exposed to a temperature of 16°C and the study by Lee *et al.* (15), who found a prevalence of activated BAT of 64%. An important factor that may partially explain why in other studies a more prominent sex difference in activated BAT was found is the use of SUV rather than SUL values. Since other studies have used SUV, any difference in average BMI between women and men may have contributed to the overall differences in BAT found in these studies.

Active BAT correlated strongly with BMI: more active BAT was found in subjects with lower BMI. This finding is in line with several other studies (1,3,8,12,14-16). In contrast to these studies, we did not find a correlation between the prevalence of active BAT and age, blood glucose levels or outside temperature. We only analyzed ^{18}F -FDG PET scans from patients with a narrow age range (20 to 35 years) during a season with little variation in the outside temperature. For further research, it would be useful to add a group of patients in whom the ^{18}F -FDG PET/CT whole-body scan was made in summer. In conclusion, our study shows that there is a tendency towards more active BAT in females, even when measured in a group with likely very active BAT: young individuals in the winter.

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CHAPTER 8

GENERAL DISCUSSION

The number of publications on the function and regulation of brown adipose tissue (BAT) has increased exponentially over the last 10 years. Since approximately 2007, BAT has become of interest as a possible target in the treatment of obesity. The research described in this thesis contributes to a better understanding of BAT function, especially the role of stress and sex on BAT function. In short, this thesis describes: a) the inhibition of BAT function in mice by the stress hormones glucocorticoids (GCs) which contributes to GC-induced disturbances in lipid metabolism; and b) the greater activity of BAT in women and female mice, which is at least partly contributable to estrogens but might also involve other factors secreted by the ovaries.

GCs are essential for energy homeostasis and the secretion of GCs is thus tightly regulated, predominantly via secretion of adrenocorticotrophic hormone (ACTH), which in turn is decreased by GCs. This balance is not only true for the secretion of these hormones but, as I describe in this thesis, also for BAT activity, since GCs inhibit and ACTH stimulates this tissue. In addition, long term exposure to GCs results in lowering of ACTH secretion, further tipping the balance in favor of the inhibitory effect of GCs. This inhibition of BAT function by GCs can be held partly responsible for the disturbances in lipid metabolism found in glucocorticoid-treated mice, although other organs, such as the liver, are also involved in this process. However, increased BAT activity was at least partly responsible for the alleviated lipid profile after exposure to cold. Also humans with Cushing's syndrome, who are chronically exposed to high levels of GCs have very low BAT activity and have deteriorated lipid metabolism. After removal of the adenomas the lipid profile, and possibly also BAT activity, improves, indicating that BAT activity is negatively influenced by exposure to GCs and lack of ACTH in humans as well.

Another focus of this thesis were the differences of BAT activity between the sexes and the hypothesis that this difference is due to the sex steroid hormones. Indeed we found that in rodents, expression of the factor *Bmp8b*, a growth factor that induces BAT activity, is increased by estrogens. These effects are likely not directly on BAT but probably centrally mediated since treatment of a BAT cell line with estrogens did not change *Bmp8b* expression. These central effects of estrogens might also be responsible for the sex difference in the potency of cold to induce browning of fat. Exposure to low environmental temperatures had greater potential to induce browning of perirenal fat in women than men. This may be due to a central effect of circulating factors that are higher in females, such as estrogens, which may change the setpoint of the thermostat resulting in higher BAT activity in females. This sex-dependent potency of browning however remained *in vitro* in primary cultures of adipocytes from men and women, which suggests that there are sex-specific intrinsic properties of adipocytes that determine their potency of browning, possibly resulting from a difference in expression of X chromosome-related genes or epigenetic differences. To further investigate the

increased BAT activity in women, we compared two groups of patients who underwent an ^{18}F -FDG PET/CT whole-body scan in winter for other purposes than measuring BAT activity. Even in this relatively small cohort, with an already high BAT activity since we chose young patients scanned in winter, women tended to have more and higher activity of BAT.

Although this thesis provides new insights into the role of stress and sex steroid hormones on BAT function, here I would like to suggest some further studies on these two topics and make some recommendations for possible treatment options to increase BAT activity and to improve metabolic health.

GLUCOCORTICOIDS AND BAT REGULATION; FURTHER RECOMMENDATIONS

This thesis shows that GCs inhibit BAT functioning and browning of WAT. GCs inhibit expression of uncoupling protein-1 (UCP1) and diminish the activity of BAT *in vivo* and *in vitro* (Chapter 2 and 3). Moreover, GCs prevent the stimulatory effect of catecholamines on BAT activity in an *in vitro* model, showing that GCs have at least a direct effect on BAT function. How GCs influence intracellular β -adrenergic signaling is not totally clear. The main rodent GC corticosterone has been reported to diminish the number of β -adrenergic receptors and their downstream signaling cascade in adipocytes (1). Suppressed β -adrenergic signaling might contribute to the reduced content and activity of the crucial thermogenic UCP1 in both BAT and WAT. High circulating GC levels might also contribute to diminished BAT function and browning of WAT via other mechanisms. For instance, since macrophages can produce catecholamines to sustain adaptive thermogenesis (2), depletion of these macrophages by GCs (3-5) could lead to diminished BAT activity. Also, browning of white adipocytes partly depends on immune-mediated responses to cytokines secreted by the muscle (6) that promote formation of specific macrophages that stimulate the formation of brown adipocytes in WAT. GCs, however, might induce apoptosis of these cells in WAT (3-5). It would therefore be of interest to study the role of inflammation on BAT function and to investigate how GCs influence this process. The ongoing collection of perirenal adipose tissue from patients with Cushing's syndrome might aid this research. Until now, we have studied two patients with Cushing's syndrome before and after adrenalectomy and collected their peri-adrenal adipose tissue (Chapter 4). More patients have to be included and I suggest that the immune profile and expression of β -adrenergic receptors in their adipose tissue be studied.

Although the *in vitro* studies in chapter 2 clearly show that ACTH and GCs have a direct effect on BAT function, this does not rule out the possibility that (one of) these hor-

mones might affect BAT via a central mechanism. GC release is controlled by specific brain areas in the hypothalamus, such as the paraventricular nucleus (PVN) and the pituitary. The circadian rhythm of GC release, however, is regulated by the suprachiasmatic nucleus (SCN) that is also located in the hypothalamus. The SCN integrates environmental signals such as light to set the circadian rhythm of all cells in the body. The SCN controls activity of the neurons that secrete corticotrophin releasing hormone (CRH) in the PVN. CRH, in turn, stimulates release of ACTH by the pituitary. Finally, ACTH stimulates the secretion and synthesis of GCs by the adrenal gland. Interestingly, the sensitivity of the adrenal gland to ACTH is regulated by the autonomic nervous system, via afferent neurons deriving from the same PVN (7-9). In addition, it is known that central GCs might reduce the sympathetic outflow (10), thus GCs might also reduce BAT activity by a central effect, but this has not been studied yet.

It would be relatively simple to study the relative contributions of direct and central effects of GCs on BAT activity in a mouse model, for instance by sympathetic denervation of BAT in mice in different temperature conditions. The most elegant way to differentiate between direct and central effects of GCs might be unilateral denervation of one of the BAT lobes. Other options might be retroviral tracing studies to find out which brain centers are involved in the regulation of BAT. In addition to these experiments, the central effects of GCs on BAT activity could be studied by intracerebroventricular (ICV) infusion of GCs or knockdown of GC receptors in specific brain areas.

Various studies have addressed the interactions between catecholamines and GCs in the adrenal gland. Animal studies show that corticosterone enhances the production and release of catecholamines by inducing the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH), dopamine- β -hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) in the adrenal gland (11-13). In turn, GC producing cells in the adrenal cortex are dependent on the presence of the catecholamine-producing medullary cells to produce adequate amounts of GCs (14). In line with these findings are clinical data in patients with Addison's disease who have not only diminished levels of GCs but also lower plasma catecholamine concentrations (15). Since this interaction between GC production and catecholamines is present, it would be of interest to study BAT function after inactivation of the sympathetic nerves innervating the adrenals. It would be expected that denervation of the adrenals will result in slightly reduced plasma GC concentrations and hence a moderate increase in BAT activity. With respect to this, it is also of interest to study GC levels in patients with pheochromocytomas who have elevated plasma catecholamine concentrations and find out whether their levels of GCs and catecholamines correlate with BAT activity and metabolic health.

In the *in vivo* studies in which mice were implanted with a corticosterone pellet (Chapter 3) the circadian rhythm of GC release was not taken into account. It is known that BAT thermogenesis also has a circadian rhythm that is regulated by the clock-gene Rev-

erb α , a nuclear receptor and transcriptional repressor (16). If Rev-erb α is not expressed, thermogenesis is increased. In the liver, expression of Rev-erb α mRNA is reduced by GCs (17). It might thus be of interest to see whether long-term GC exposure disturbs the circadian rhythm of Rev-erb α expression in BAT as well and thereby the circadian oscillation of thermogenesis. I would thus propose to study Rev-erb α expression patterns in mice implanted with a corticosterone pellet, in adrenalectomized mice and in adipocytes of humans exposed to high GC concentrations, e.g., patients with Cushing's syndrome. Along these lines, it would be of interest to study the circadian rhythm of body temperature in patients with Cushing's disease or syndrome. If GCs influence expression of clock-genes in BAT, it will be of interest to study BAT activity in subjects with a disturbed and normal circadian rhythm.

SEX-DIFFERENCES AND BAT REGULATION; FURTHER RECOMMENDATIONS

The studies in this thesis show that sex differences are present in BAT regulation, with females having a higher BAT prevalence and activity than males. The exact mechanism behind this difference is not known, but it can be localized at two different levels, namely the brain and in the BAT itself.

Among the usual suspects that likely contribute to the sex difference in BAT activity are of course the sex steroid hormones. Of interest, under normal physiological conditions, women have higher estrogen levels during the last two weeks of the menstrual cycle, a period when the body temperature is also slightly higher (18). This suggests that estrogens induce BAT activity, an effect that is at least partly due to central effects since knock-out of the estrogen receptor α in the thermoregulatory brain centers in the hypothalamus of mice reduced body temperature and resulted in a phenotype with the metabolic syndrome (19). Also Martínez de Morentin *et al.* (20) showed that β -adrenergic signaling to BAT is increased by estrogens. Similar to rodents, it has been found in epidemiological studies that women have higher BAT activity than men (21-23). Since women are more cold sensitive than men (24, 25) it might be possible that due to their differences in thermosensitivity, women have more and higher activity of BAT on PET scans under the same scanning temperature compared to men. It is of interest to study differences in BAT activation between males and females in a temperature-controlled way and determine the temperature at which BAT is activated for each sex. Additionally, I would propose testing whether shivering temperature, as a measurable derivative of the thermostat setpoint, is different between men and women. Furthermore, shivering temperature might be examined in healthy young women during different stages of their menstrual cycle to study the role of sex steroid hormones in regulation of the thermostat and hence BAT activation.

Chapter 6 of this thesis reports that women have more browning of the perirenal fat depot when exposed to low outdoor temperatures. Remarkably, after a couple of weeks in culture, the adipocytes of female origin still expressed higher amounts of UCP1 mRNA. Differences in the expression of genes located on the X or Y chromosomes may be the cause of this difference, although X inactivation normalizes, for the most part, the chromosomal differences between the sexes (26) and the Y chromosome contains very few genes (27). Epigenetic profiles due to earlier exposure to sex-specific factors such as estrogens and testosterone might give a better explanation of sex differences in browning of WAT and even BAT function. In addition, the effects of estrogens and testosterone on the function or differentiation of human and rodent BAT and WAT cell lines might elucidate the differences in response to positive stimuli and available machinery for thermogenesis in male and female BAT.

Since recent literature convincingly shows that higher BAT activity is related to better glucose tolerance, it would be of interest to study glucose tolerance in women and men matched for age and BMI. For example, overexpression of SIRT1, a key modulator of energy expenditure, resulted in an enhanced response of BAT to β -adrenergic stimuli in mice and, hence, improved glucose tolerance and insulin sensitivity (28). In addition, BAT transplantation in mice improved glucose tolerance and insulin sensitivity (29) and even reversed type 1 diabetes mellitus in streptozotocin-treated animals (30). These effects of BAT transplantation were suggested to be mediated via endocrine mediators derived from the BAT transplants. Indeed, it was shown that mice with transplanted BAT had increased levels of IL6 and FGF21 (29). Also more physiologically relevant experiments such as intermittent cold exposure showed a positive association between BAT activity and whole body glucose tolerance and insulin sensitivity (31). Thus, if women have higher BAT activity this should result in better metabolic health and improved glucose tolerance and I would therefore suggest studying glucose tolerance in young healthy males and females and to correlate the results to testosterone and estrogen levels. In addition, it would be of interest to examine whether women have altered circulating endocrine stimuli or BATokines compared with men by using laboratory techniques such as proteomics.

SUGGESTED TREATMENT OPTIONS TO ACTIVATE BAT

Pharmacological approaches

To improve BAT function, it might be helpful to block the effects of GCs in this tissue. GC action and its ability to bind to the glucocorticoid receptor is modulated by 11 β -Hydroxysteroid Dehydrogenase Type 1 (11 β HSD1) that converts inactive cortisone to active cortisol or, in rodents, cortisone to corticosterone. There are several reports showing that inhibition of 11 β HSD1 improves metabolic health by influencing the activity

of GCs in adipose tissue (32). In primary brown adipocytes from mice, overexpression of 11 β HSD1 led to down-regulation of BAT specific genes and pharmacological inhibition of 11 β HSD1 led to increased UCP1 expression and increased oxygen consumption (33). *In vivo*, pharmacological inhibition of 11 β HSD1 led to increased BAT activity, alleviation of obesity and improvement of glucose tolerance in high-fat-diet-fed mice (33). Interesting in the context of this thesis is the finding that estrogens inhibit the function of 11 β HSD1 (34). This effect might however be tissue-specific since it is reported that estrogens inhibit 11 β HSD1 in liver and visceral adipose tissue, but not subcutaneous adipose tissue (35). The effect of estrogens on activity or gene expression of 11 β HSD1 in BAT has not been addressed yet, but we recently found reduced 11 β HSD1 mRNA expression in female BAT compared to male BAT (Grefhorst et al. in preparation). It will be of interest to develop a BAT specific 11 β HSD1 inhibitor to prevent GC inhibition of BAT function but allowing the activation of the GC receptor in other tissues. Other ways to block the effect of GCs are partial antagonists of the GC receptor. Such molecules resemble the glucocorticoid structure but do not fully induce or inhibit expression of GC receptor target genes. This way of modulating receptor function has recently been shown to be successful in reducing the pathogenic glucocorticoid receptor mediated effects such as diet-induced obesity in mice, but keep the beneficial effects of glucocorticoid receptor signaling such as reduction of inflammation (36).

As the data in Chapter 5 suggest, estrogens might regulate the expression of various BATokines, as is exemplified by BMP8b. Since BATokines might be an excellent source to develop new pharmacological strategies to activate BAT due to their paracrine and autocrine nature, BMP8b might be a valuable pharmaceutical target to activate BAT. Another BATokine, fibroblast growth factor 21 (FGF21), is already under investigation as a novel tool to improve metabolism. FGF21 administration to mice results in improved metabolic health and increased energy expenditure (37, 38). This effect is at least partly contributable to activated BAT (39, 40). In humans, high circulating levels of FGF21 are associated with higher BAT activity (41) and chronic treatment with an FGF21 analog improved plasma lipid profiles, decreased fasting insulin levels and reduced body weight in obese patients with type 2 diabetes mellitus (42).

Lifestyle changes

During cold exposure with temperatures just above shivering temperature, resting energy expenditure is increased by 20% in Caucasians (43). This indicates that lowering environmental temperature might, when permanently implemented, contribute to prevention of obesity. This effect might already be reached with only a mild decrease of ambient temperature (44). Interesting in this context is the observation that the increased prevalence of obesity is associated with an increase in room temperature during the last century in both the United States and the United Kingdom (45).

Secondly, long term stress and thereby increased circulating levels of GCs has been shown in this thesis to diminish BAT function in mice and probably also in humans. Acute stress however, as was simulated by ACTH injection in mice, has been shown to increase BAT function. It would therefore be best for BAT function to prevent long term stress, but not to avoid acute stress. Long term stress can be reduced by regular moderate physical exercise. Exercise is an excellent way to lower GC levels in the long term (46) and to increase short term cortisol levels (47) next to the beneficial effects on increased energy expenditure and increased muscle mass. However, we should not exaggerate exercise since extreme endurance training has been reported to increase hair GC concentrations (48) a marker of long-term GC exposure (49).

As discussed earlier, GCs are secreted in a diurnal rhythm which is controlled by the suprachiasmatic nucleus, which is influenced by light. More and more evidence shows that in our current society with increasing amounts of artificial light we disturb our natural day-night rhythm and thus also the circadian rhythm of GCs. For example, in shift workers dysregulation of the day-night rhythm led to increased levels of hair cortisol (50) and the rhythm of clock-genes and consequently GC release can be altered by sleep deprivation (51), change in light-dark exposure (52) or change in calorie intake (53). Of interest for this thesis is that the latter has also been reported to change body temperature (54). Thus changing day-night rhythms might influence BAT activity by changing the circadian rhythm of, amongst others, GCs (55). All these influences and habits interact with our temperature and energy homeostasis. In conclusion, next to a pharmacological approach, modulation of GC levels by changes in lifestyle might be one of the most important tools to increase BAT activity.

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CHAPTER 9

SUMMARY-SAMENVATTING

SAMENVATTING

Gezien de stijging van de incidentie van obesitas hebben we meer middelen nodig om obesitas te behandelen. Bruin vet kan een mogelijk middel van behandeling van obesitas zijn omdat dit weefsel de unieke eigenschap bezit om warmte te produceren en daarmee energie te verbruiken. Daarmee is bruin vet een van de weefsels die belangrijk zijn voor een stabiele lichaamstemperatuur. Als we bruin vet actiever kunnen maken kunnen we mogelijk ook energieverbruik doen stijgen en daarbij obesitas verminderen. Er is nog niet veel bekend over de regelmechanismen waardoor bruin vet onder controle wordt gehouden onder normale fysiologische omstandigheden. Omdat een stabiele lichaamstemperatuur essentieel is voor een goed functioneren van het lichaam, is het aannemelijk dat bruin vet activiteit door verschillende mechanismen wordt geregeld, waaronder verschillende hormonale regulatoren. Dit proefschrift beschrijft het effect van twee verschillende hormonen op bruin vet activiteit: geslachtshormonen en stresshormonen. **Hoofdstuk 1** is de algemene introductie van dit proefschrift en beschrijft dat bruin vet de energie balans kan beïnvloeden door verbruik van glucose en triglyceriden (TG) voor thermogenese. Voor deze thermogenese is het eiwit uncoupling protein 1 (UCP1) essentieel, dit eiwit is ook aanwezig in een 3^e type vet namelijk het beige vet. Dit vet is wit vet wat bruin vet eigenschappen heeft verworven. Verschillen in bruin vet activiteit tussen mannen en vrouwen zijn mogelijk verklaarbaar door verschillen in geslachtshormonen en dientengevolge ook een verschillende afgifte van andere autocriene, paracriene en endocriene factoren die bruin vet activiteit kunnen beïnvloeden.

Stress hormonen en bruin vet activiteit

De hypothalamus-hypofyse-bijnier as hormonen adrenocorticotroop hormoon (ACTH) en glucocorticoiden (GCs) zouden beiden bruin vet activiteit kunnen reguleren. **Hoofdstuk 2** beschrijft de rol van ACTH op bruin vet activiteit in zowel een *in vivo* als *in vitro* model. Het effect van ACTH op bruin vet activiteit werd bestudeerd in zowel bruine als beige gecultiveerde vetcellen aan in muizen door middel van het gebruik van ¹⁸F-deoxyglucose positron emissie tomografie (¹⁸F-FDG PET). ACTH verhoogde de bruin vet activiteit in alle modellen. Echter, ACTH stimuleert ook de afgifte van glucocorticoiden door de bijnier, om alleen het effect van ACTH te kunnen bestuderen dienden we naast ACTH ook RU486 toe wat een antagonist is van de GC receptor in de *in vivo* studies. We vonden dat RU486 de bruin vet activiteit verder deed toenemen. ACTH stimuleert dus bruin vet activiteit en de bruining van wit vet, terwijl GCs precies het tegenovergestelde effect lijken te hebben.

Een nieuwe serie experimenten werd gedaan om de effecten van GCs op bruin vet activiteit beter te bestuderen. **Hoofdstuk 3** beschrijft de lange termijn effecten van hoge

doses corticosterone op verschillende weefsels waaronder bruin vet en de effecten van koudeblootstelling (4° C voor 24 uur) op deze muizen om te bepalen of koude blootstelling de verwachte remming van bruin vet door GCs weer kon opheffen. Zoals verwacht was de bruin activiteit sterk verminderd. Verder vonden we dat corticosterone de plasma concentraties van TG verhoogde, maar deze verhoogde concentratie werd vooral veroorzaakt door een verhoogde uitscheiding van TG rijke very low density lipoprotein (VLDL) deeltjes door de lever na blootstelling aan corticosterone. Koude normaliseerde inderdaad bruin vet activiteit in corticosterone behandelde muizen. De plasma TG spiegels normaliseerden na koude blootstelling, maar dit werd vooral veroorzaakt door een forse daling in de uitscheiding van VLDL deeltjes door de lever na koude blootstelling. We kunnen dus concluderen dat GCs de bruin vet activiteit verminderen, maar dat de bruin vet activiteit weer normaliseert na koude blootstelling, wat de flexibiliteit van het bruine vet benadrukt.

Hierop aansluitend, beschrijven we in dit proefschrift het effect van GCs op bruin vet activiteit en/of bruining van wit vet in mensen. **Hoofdstuk 4** beschrijft bruin vet activiteit in twee patiënten die een GC producerend bijnier adenoom hebben zowel voor als zes maanden na adrenalectomie. ¹⁸F-FDG PET scans lieten zien dat bruin vet was toegenomen na adrenalectomy, dus wanneer de plasma cortisol spiegels waren genormaliseerd. Overeenkomstig met de resultaten gevonden in corticosterone behandelde muizen waren de plasma TG spiegels verlaagd na adrenalectomie.

Samenvattend hebben we aangetoond door middel van celkweken, muismodellen en humane studies dat GCs de activiteit van bruin vet remmen, in ieder geval op de lange termijn.

Geslachtshormonen en bruin vet activiteit

Verschillende studies suggereren dat vrouwen meer en actiever bruin vet en beige vet hebben, maar de oorzaak en gevolgen hiervan zijn niet nog niet beschreven. Dit proefschrift behandelt verschillende aspecten van de geslachts verschillen. In **hoofdstuk 5** worden twee families van groeifactoren met bekende effecten op bruin vet activiteit, namelijk bon morphogenetic proteins (BMPs) en fibroblast growth factors (FGFs), bestudeerd om vast te stellen of deze factoren verschillend tot expressie komen tussen mannetjes en vrouwtjes muizen. In aanvulling hierop werd bestudeerd wat voor effect de gonaden hadden op de expressie van deze factoren. In het kort lieten deze studies zien dat *Bmp8b* was hoger in vrouwtjes dan in mannetjes muizen. Dit geslachts verschil in *Bmp8b* expressie was niet meer aanwezig na ovariectomie, maar toegenomen door diethylstilbestrol (DES) behenadeling. Dit laat zien dat estrogenen de expressie van *Bmp8b* expressie reguleren. *In vitro* studies laten geen effect zien van estrogenen

op *Bmp8b* wat impliceert dat de effecten van estrogeen door het brein gemedieerd worden.

Het perirenale vet depot is in de literatuur beschreven als een wit vet depot wat makkelijk bruin vet eigenschappen kan aannemen. Dit proefschrift beschrijft in **hoofdstuk 6** de geslachtsverschillen in bruin vet eigenschappen in humaan perirenaal vet. Buitentemperaturen van onder de 11°C verhoogde de expressie van UCP1 in perirenaal vet van vrouwen maar niet in mannen. Vanuit stamcellen gedifferentieerde vetcellen die verkregen waren uit vrouwelijk perirenaal vet hadden duidelijk een hogere expressie van *UCP1* gedifferentieerde vetcellen van mannelijke origine. De mate van bruining hangt dus af van geslachts specifieke intrinsieke karakteristieken van cemuïsen en geslachts specifieke reacties op stimuli uit de omgeving zoals temperatuur.

Bruin vet kan worden gemeten in volwassen mensen door middel van ¹⁸F-FDG PET scans, bij deze studies wordt een hogere prevalentie en activiteit van bruin vet gezien in vrouwen ten opzichte van mannen. **Hoofdstuk 7** beschrijft een retrospectieve studie waarin ¹⁸F-FDG PET scans worden bestudeerd gemaakt in volwassen patiënten voor andere diagnostische doelen dan bruin vet onderzoek, gedurende de winter en in een jong cohort. Er werd geen verschil gezien in prevalentie van actief bruin en mate van bruin vet activiteit tussen mannen en vrouwen. Echter in iedere BMI tertile leken de vrouwen een hogere prevalentie te hebben van actief BAT. Er moeten meer patiënten worden geïncludeerd om te bewijzen dat dit verschil inderdaad van statistische significantie is.

Dit proefschrift laat dus zien dat zowel in muizen als mensen, de vrouwen meer potentie hebben om wit vet bruin te laten worden en dat vrouwen een hogere prevalentie hebben van actief bruin vet. Estrogeen zijn hiervoor in ieder geval gedeeltelijk verantwoordelijk.

Hoofdstuk 8 is de algemene discussie. Hier worden suggesties gedaan voor verder onderzoek naar de effecten van GCs op inflammatie in bruin vet, de interactie van GCs en catecholamines en GCs en klokgenen in bruin vet. Andere suggesties betreffende de man vrouw verschillen zijn in bruin vet activiteit zijn temperatuur gecontroleerde metingen van bruin vet in zowel mannen als vrouwen, metingen van rilt temperatuur gedurende verschillende stadia van de menstruatiecyclus, het bestuderen van sexeverschillen in expressie van bruin-vet factoren die bruin vet activiteit stimuleren en het bestuderen van verandering van epigenetische profielen door geslachtshormonen. I suggereer enkele farmacologische studies die bruin vet activiteit kunnen reguleren zoals bruin vet specifieke 11βHSD1 remmers of een partiële antagonist van de GC receptor en toediening van bruin-vet stimulerende factoren zoals FGF21 en BMP8b. Leefgewoontes

zoals regelmatige beweging, het vermijden van gewoontes die de circadiane klok in de war sturen en een regelmatige blootstelling aan kou kunnen ook bijdragen aan hogere bruin vet activiteit en verdienen nadere bestudering.

SUMMARY

Due to the increasing incidence of obesity, more means of treating obesity are necessary. Brown adipose tissue (BAT) is a potential target tissue via which obesity can be treated due to its unique ability to use energy to produce heat. As such, BAT is one of the tissues involved in maintenance of a stable body temperature. If we can increase BAT activity we can potentially enhance energy expenditure and thereby diminish obesity. Not much is known however about the mechanisms via which BAT activity is maintained under physiological circumstances. Since a stable body temperature is one of the most important conditions for a body to function properly, it is thought that BAT activity is very well regulated via different mechanisms, among which are various endocrine regulators. This thesis describes the influence of two different types of hormones on BAT activity: sex hormone steroids and stress hormones.

Chapter 1 is the general introduction of this thesis and describes that active BAT can influence energy metabolism, predominantly via the utilization of glucose and triglycerides (TGs) for thermogenesis. In thermogenesis, uncoupling protein 1 (UCP1) is the essential protein in BAT but it is also present in a third type of adipose tissue: beige fat, which is WAT that gained BAT-like characteristics. Differences in BAT activity between males and females might be due to sex steroid hormones and subsequent release of several autocrine, paracrine and endocrine factors which influence BAT activity.

Stress hormones and BAT activity

The hypothalamic pituitary adrenal axis hormones adrenocorticotrophic hormone (ACTH) and glucocorticoids (GCs) might both regulate BAT activity. **Chapter 2** describes the role of ACTH on BAT activity, using both *in vivo* and *in vitro* models. The effects of ACTH on BAT activity were studied in cultured brown and beige cultured adipocytes and in mice using ^{18}F -deoxyglucose positron emission tomography (^{18}F -FDG PET). ACTH increased BAT activity in all models. ACTH induces GC release from adrenal glands, thus we also administered the GC receptor antagonist RU486 before ACTH administration in the *in vivo* studies and found that this resulted in a further increased uptake of ^{18}F -FDG. ACTH thus activates BAT and browning of WAT while GCs very likely counteract this effect.

A new set of studies was performed to explore the effects of GCs on BAT in more detail. **Chapter 3** describes the long term effects of a high dose of corticosterone on various tissues such as BAT, but also the effects of subjecting these mice to 4°C for 24 hours to determine whether cold could possibly overrule the suspected inhibition of BAT activity by GCs. As expected, BAT activity was strongly reduced by corticosterone. In addition, we found that exposure to corticosterone increased plasma TG concentrations, but increased plasma TG was mainly the result of elevated excretion of TG-rich

very low density lipoprotein (VLDL) particles by the liver upon corticosterone treatment. Plasma TG concentrations were normalized in the corticosterone-treated mice by cold exposure. Cold partially normalized BAT activity in the corticosterone-treated mice, but the normalization of plasma TG concentrations was likely mainly the result of a severe reduction of hepatic VLDL-TG secretion upon cold exposure. Thus, in mice GCs also inhibit BAT activity, but this can be overruled by cold exposure, underscoring the flexibility of this tissue.

Next, this thesis describes the effects of GCs on BAT activity and/or browning of WAT in humans. **Chapter 4** describes BAT activity in 2 patients with a GC producing adrenal adenoma before and 6 months after adrenalectomy. Using ^{18}F -FDG PET scans to determine BAT activity, it was evident that BAT activity was increased after adrenalectomy, thus when plasma cortisol concentrations were normalized. In line with the results found with the corticosterone-treated mice, plasma TG concentrations were decreased after adrenalectomy.

Altogether, using cell cultures, animal models and human data, the studies in this thesis unequivocally show that GCs inhibit BAT activity, at least in the long turn.

Sex steroid hormones and BAT activity

Various studies suggest that females have more active BAT or BAT-like WAT, but the cause and consequences of this have not been addressed, yet. This thesis therefore addresses various aspects of this sex-difference. In **Chapter 5**, two families of growth factors with known effect on BAT functioning, namely bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs), were studied to determine whether they are differentially expressed between male and female mice. In addition, the effect of gonads per se on these factors was also studied. In short, the studies showed that *Bmp8b* was differentially expressed in BAT of male and female mice, with the highest expression found in females. This sex difference in *Bmp8b* expression was blunted by ovariectomy, but increased by diethylstilbestrol (DES) treatment, showing that estrogens regulate BAT *Bmp8b* expression. *In vitro* studies showed that the effects of estrogens are very likely mediated via the brain.

The perirenal fat depot has previously been described as WAT that can gain brown-like features. This thesis described in **chapter 6** sex differences in BAT-like properties of human perirenal adipose tissue. An outside temperature below 11°C increased expressing UCP1 in perirenal adipose tissue of women, but not in men. From stem cells differentiated adipocytes derived from female perirenal adipose tissue expressed significantly more UCP1 than differentiated adipocytes from male origin. Thus, the degree of gaining BAT-like properties depends on intrinsic sex-specific characteristics of cells and sex-specific reactions on environmental triggers such as temperature.

Brown adipose tissue (BAT) is detected on ^{18}F -FDG PET scans in adult humans with a higher prevalence of active BAT in women compared to men. **Chapter 7** describes a retrospective study using ^{18}F -FDG PET scans in adult humans, made for other diagnostic purposes during the winter in a young cohort. No statistical significant difference in prevalence of active BAT and BAT activity were found between men and women. However, women tended to have a higher prevalence of activated BAT in all BMI tertiles. More patients need to be included to proof that this difference is indeed statistically significant.

Thus, this thesis shows that in both rodents and humans females have a higher potency to induce browning of WAT and have a higher prevalence of active BAT. Estrogens are at least partly responsible for higher BAT activity in females.

Chapter 8 is the general discussion. Suggestion for further research are the effects of GCs on BAT inflammation, the central effects of GCs on BAT activity, the interaction between GCs and catecholamines and GCs and clock-genes in BAT are presented. Other research suggestions concerning sex-differences in BAT activity in males and females are temperature-controlled measurement of BAT activity in both males and females, measurement of shivering temperature during different stages of the menstrual cycle, studies to compare BATokines between males and females, and studies to determine the effects of sex steroids on epigenetic profiles. I also suggest investigations into (novel) pharmacological regulators of BAT activity such as BAT specific $11\beta\text{HSD1}$ inhibitors or a partial antagonist of the glucocorticoid receptor, and administration of BATokines such as FGF21 and BMP8b. However, also lifestyle changes such as regular exercise, avoiding disruptors of the circadian clock and exposure to cold might also contribute to a higher BAT activity and require further studies.

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LIST OF PUBLICATIONS

Cold Exposure Partially Corrects Disturbances in Lipid Metabolism in a Male Mouse Model of Glucocorticoid Excess.

van den Beukel JC, Boon MR, Steenbergen J, Rensen PC, Meijer OC, Themmen AP, Grefhorst A.

Endocrinology. 2015 Nov;156(11):4115-28. doi: 10.1210/en.2015-1092. Epub 2015 Sep 15.

Women have more potential to induce browning of perirenal adipose tissue than men.

van den Beukel JC, Grefhorst A, Hoogduijn MJ, Steenbergen J, Mastroberardino PG, Dor FJ, Themmen AP. Obesity (Silver Spring). 2015 Aug;23(8):1671-9. doi: 10.1002/oby.21166. Epub 2015 Jul 14.

Estrogens increase expression of bone morphogenetic protein 8b in brown adipose tissue of mice.

Grefhorst A, **van den Beukel JC**, van Houten EL, Steenbergen J, Visser JA, Themmen AP. Biol Sex Differ. 2015 Apr 3;6:7. doi: 10.1186/s13293-015-0025-y. eCollection 2015.

Direct activating effects of adrenocorticotrophic hormone (ACTH) on brown adipose tissue are attenuated by corticosterone.

van den Beukel JC, Grefhorst A, Quarta C, Steenbergen J, Mastroberardino PG, Lombès M, Delhanty PJ, Mazza R, Pagotto U, van der Lely AJ, Themmen AP.

FASEB J. 2014 Nov;28(11):4857-67. doi: 10.1096/fj.14-254839. Epub 2014 Aug 1.

Interactions between the gut, the brain and brown adipose tissue function.

van den Beukel JC, Grefhorst A.

Front Horm Res. 2014;42:107-22. doi: 10.1159/000358320. Epub 2014 Apr 7. Review.

Morphology and size of stem cells from mouse and whale: observational study.

Hoogduijn MJ, **van den Beukel JC**, Wiersma LC, IJzer J.

BMJ. 2013 Dec 12;347:f6833. doi: 10.1136/bmj.f6833.

PhD PORTFOLIO

Name PhD student: Johanna C. van den Beukel
 Erasmus MC department: Internal Medicine, Endocrinology
 Research School: MoIMed
 PhD period: May 2011- November 2014
 Promotor: A.P.N. Themmen
 Co-promotor: A. Grefhorst

	Year	Workload (ECTS)
1. PhD training		
1.1 General academic skills		
Scientific English Writing and Communication	2012	4
Basiscursus regelgeving en organisatie van klinische trials	2012	1.5
Research Management for PhD students	2012	1
Stralingsbeschermingsdeskundige niveau 5B	2011	0.8
1.2 Research skills		
In Vivo Cellular Imaging	2012	1.8
1.3 (Inter) national conferences, participation and presentations		
Internal medicine days, Antwerpen, België, <i>Women have more potency to induce browning of perirenal fat tissue</i> , Oral	2014	0.8
Molmed Dag, Rotterdam, Nederland, <i>Women have More Potential to Induce Browning of Perirenal Fat than Men</i> , Poster	2014	0.8
Dutch Endocrine Meeting, Noordwijkerhout, <i>Women have more potency to induce browning of perirenal fat tissue</i> , Oral	2014	0.8
95th Annual Meeting of the Endocrine Society, San Fransisco, Verenigde Staten, <i>Women have More Potential to Induce Browning of Perirenal Fat than Men</i> , Poster	2014	1
Dutch Endocrine Meeting, Noordwijkerhout, Nederland, <i>ACTH activates brown adipose tissue and induces browning of white adipocytes in mice</i> , Oral	2013	0.8
European Congres for Endocrinology, <i>ACTH rapidly stimulates BAT and browning of inguinal WAT mice</i> , Oral	2013	1
95th Annual Meeting of the Endocrine Society, San Fransisco, Verenigde Staten, <i>ACTH rapidly stimulates BAT and browning of inguinal WAT mice</i> , Oral	2013	1
Dutch Endocrine Meeting, Noordwijkerhout, <i>ACTH but not glucocorticoids enhance activity of brown adipose tissue in mice</i> , Oral	2012	0.8
Molmed Dag, Rotterdam, Nederland, <i>Adrenocorticotrophin hormone (ACTH) rapidly stimulates brown adipose tissue activity and browning of white adipose tissue in mice</i> , Poster	2012	0.8

EMBO EMBL Symposium Diabetes and Obesity, Heidelberg, <i>Increased activity of the hypothalamus-pituitary-adrenal axis potentiates brown adipose tissue activity via direct effects of adrenocorticotrophin hormone (ACTH) in mice, Poster</i>	2012	1
Wetenschapsdagen, Inwendige Geneeskunde, Antwerpen, België, <i>Pituitary-adrenal axis is involved in the regulation of BAT activity, Poster</i>	2012	0.8
1.4 Seminars and workshops		
Attending seminars of the Department of Internal medicine- Erasmus MC lectures, Rotterdam, The Netherlands	2011-2014	1
course and workshop basic and translational endocrinology	2013	2.2
38e Erasmus Endocrinologie cursus	2012	1
2. Teaching activities		
2.1 Supervising bachelors thesis (2x)		
Sanne van den Berg, medisch student. Project title: Higher prevalence in brown adipose tissue activity in women: due to a different set point of the thermostat?	2014	7.5
Bas Kreeft, medisch student. Project title: Activating brown adipose tissue in mice and human by adrenocorticotrophic hormone and alpha melanocyte-stimulating hormone	2012	7.5
2.2 Teaching medical students		
Practicals endocrinology, ErasmusMC, Rotterdam. Subjects: thyroid and hypercortisolism	2011-2014	2.4
2.3. Other teaching activities		
Supervising junior medschool students: Indira Schouten & Suzanne van Woudenberg	2014	2.5
Supervising junior medschool students: Amir Abdelmoumen & Miliaan Zeelenberg	2013	2.5
3. Grants and Awards		
Best basic abstract award, Dutch Endocrine Meeting	2014	
NVE Goodlife Healthcare travelgrant (endocrine society meeting)	2013	
Women in Endocrinology Young Investigator Award	2013	
Best poster award, Internal medicine days ErasmusMC	2009	
4. Organizational skills		
1st congres of young Dutch endocrine society (jNVE), Amsterdam, The Netherlands	2014	
1st congres of european young endocrine scientists (EYES), Rotterdam, The Netherlands	2013	

LIST OF ABBREVIATIONS

11-βHSD	11 β -hydroxysteroid dehydrogenase
18F FDG PET	18F-fluorodeoxyglucose positron emission tomography
ACTH	adrenocorticotrophic hormone
ADX	adrenalectomy
AMPK	AMP-activated protein kinase
AVP	arginine-vasopressin
BAT	Brown adipose tissue
BMI	body mass index
BMP	Bone morphogenetic protein
cAMP/PKA	cyclic adenosine monophosphate/protein kinase A
CD36,	cluster of differentiation 36
CETP	cholesterol ester transfer protein
CNS	central nervous system
CRH	corticotropin releasing hormone
CT	computed tomography
CYP11α1	Cholesterol side-chain cleavage enzyme
CYP11β1	Steroid 11-beta-hydroxylase
DBH	dopamine β hydroxylase
DES	diethylstilbestrol
DHT	dihydrotestosterone
DNL	de novo lipogenesis
DXA	Dual X-ray absorptiometry
E	epinephrine
E2	estradiol
ERR	estrogen-receptor-related receptor
ERα	estrogen receptor alpha
FFA	free fatty acid
FGF	fibroblast growth factor
GC	glucocorticoid
GDX	gonadectomy
GR	glucocorticoid receptor
HDL	high density lipoprotein
HPA axis	hypothalamic-pituitary-adrenal axis
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSD3β1:	3beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type I
LBM	lean body mass
LDLR	low density lipoprotein receptor

LPL	lipoprotein lipase
MBq	mega bequerel
MC2R	melanocortin receptor 2
MRAP	melanocortin receptor associated protein
MSC	mesenchymal stem cell
MYF5	myogenic factor 5
NE	norepinephrine
OCR	oxygen consumption rate
OGTT	oral glucose tolerance test
PGGC1α	peroxisome proliferator-activated receptor γ coactivator 1 α
PNMT	phenylethanolamine N-methyltransferase
PPARγ	proliferator-activated receptor gamma
PRDM16	PR domain containing 16
PVN	paraventricular nucleus of the hypothalamus
RU486:	mifepristone
SCN	suprachiasmatic nucleus of the hypothalamus
SEM	standard error of the mean
SIRT1	NAD-dependent deacetylase sirtuin-1
SREBP1C	sterol-regulatory element-binding protein-1c
STAR	Steroidogenic acute regulatory protein
SUL	standard uptake value corrected for lean body mass
SUV	standard uptake value
TG	Triglycerides
TH	tyrosine hydroxylase
TLE3	transducin-like enhancer of split 3
UCP1	uncoupling protein 1
VLDL	very low density lipoprotein
WAT	White adipose tissue

ABOUT THE AUTHOR

Johanna Cornelia (Anneke) van den Beukel was born on June 21st 1986 in Rotterdam, The Netherlands. She obtained her gymnasium high school degree at the Wartburg college in 2004. That same year she started medical school at the Erasmus University in Rotterdam and obtained her bachelors degree in 2009. During her medical study she worked as a student assistant on the gynaecology/urology ward. She was selected to participate in a honours program, to obtain a Master of Science degree in Clinical Research at the Netherlands Institute for Health Sciences. During this Master of Science program she spent a month at Harvard School of Public Health in Boston, USA, and attended the summer program of this university. She performed her masters graduation research, entitled "Unacylated ghrelin and brown adipose tissue; Regulation of differentiation and lipid metabolism" at the section of Endocrinology of the Department of Internal Medicine, ErasmusMC, under the supervision of dr. Patric Delhanty and dr. Ir. Jenny Visser. She graduated for her master of science in clinical research in 2009. She continued medical school with her interships from 2009-2011 and graduated for medical school in 2011. In 2011 she returned to research as a PhD, also at the Department of Internal Medicine, Section of Endocrinology with the project entitled "hormonal regulation of brown adipose tissue". In January 2015 she started her specialization in Internal Medicine in Havenziekenhuis, Rotterdam and continued her residency in Maastricht ziekenhuis from June 2016.