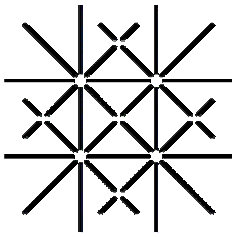
The role of melanocortins and cytokines in human adipose tissue and adipocytes

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel



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Basel, 2007

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
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Basel, den 19. Dezember 2006

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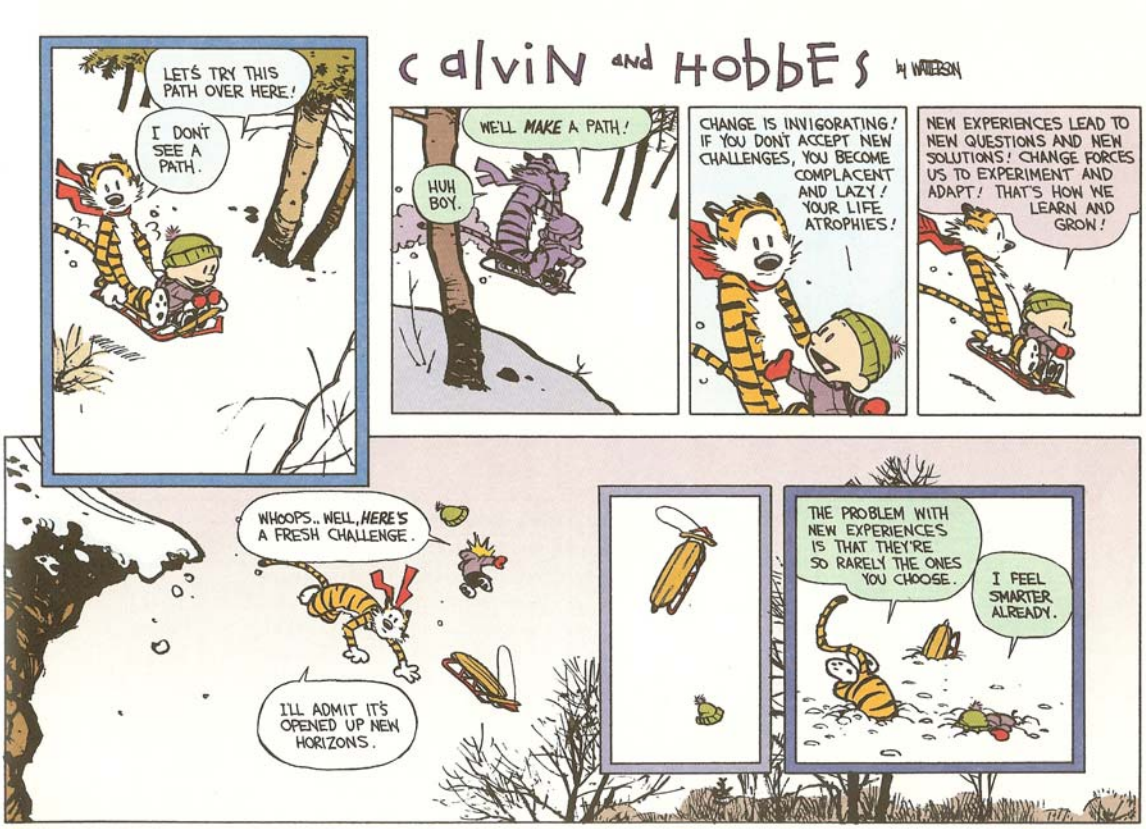

From Calvin and Hobbes: 10th Anniversary Book, Waterson, 1999.

The Economist, 2001.

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Abbreviations

ACTH	adrenocorticotropic hormone
AgRP	agouti-related protein
α-MSH	α-melanocyte-stimulating hormone
ASIP	agouti signaling protein
BAT	brown adipose tissue
BMI	body mass index [kg/m^2]
BPD/DS	biliopancreatic diversion/duodenal switch
cAMP	cyclic adenosine mono phosphat
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CLIP	corticotrophin-like intermediate lobe peptide
CNS	central nervous system
DEX	dexamethasone
DNA	deoxyribonucleic acid
GLP-1	glucagon-like peptide 1
GPRCs	G-protein-coupled receptors
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
LPS	lipopolysaccharide
MC1-R	melanocortin receptor-1
MC4-R	melanocortin receptor-4
MSCs	mesenchymal stem cells
NDP-MSH	[Nle^4, D-Phe^7]-α-MSH
NPY	neuropetide Y
POMC	pro-opiomelanocortin
PYY	peptide YY
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
TNF-α	tumor necrosis factor-α
UCP-1	uncoupling protein-1
WAT	white adipose tissue

Summary

During the last decades obesity has become a major health problem in developed countries, and more recently also in the developing countries it is seriously affecting parts of the populations. Since obesity is an important risk factor for various, partly life-threatening diseases, including heart diseases, stroke, diabetes type 2, atherosclerosis or some type of cancers, the elucidation of the molecular as well as integrated causation of obesity is urgently needed. With this general aim and in close collaboration with the St. Claraspital we wanted to address some crucial unsolved questions of obesity.

The melanocortin system is critical in the regulation of energy homeostasis and feeding. It includes the melanocortins, i.e. peptide hormones derived post-transcriptionally from the POMC gene product (e.g. α-MSH, β-MSH, γ-MSH, ACTH), as well as the five melanocortin receptors (MC1-R, MC2-R, MC3-R, MC4-R, MC5-R) through which the melanocortins signal into target cells. It has been shown that α-MSH produced in the hypothalamus (arcuate nucleus) reveals a potent anorectic effect via the MC4-R, also present in this brain region. Furthermore, MC4-R mutations are the most common monogenic cause of morbid obesity in humans to date.

Laparoscopic gastric banding has been shown to efficiently reduce excessive body weight. However, some patients develop complications (e.g. insufficient weight loss) after the operation, which necessitate a re-operation. We intended to verify in the patient group of the St. Claraspital whether patients requiring re-operation have a higher MC4-R mutation/polymorphism rate. If so, the sequencing of MC4-R prior to the operation could be used as prediction marker for the outcome of the operation. Therefore, the complete MC4-R gene of 37 patients that developed complications after the operation was sequenced. In 95% of the patients we found a normal, unmutated MC4-R gene. However, one novel silent mutation Ile198Ile (C594T) and one polymorphism Ile251Leu (A1144C) were found. This polymorphism had previously been shown to lead to a fully functional receptor. To summarize, we could not confirm the observation previously published that MC4-R defects are associated with a higher complication rate following laparoscopic gastric banding.

Since α-MSH also circulates at low levels in the bloodstream, and its level is elevated in the obese state, there might be a potential role of the melanocortin system in the periphery. Possibly, there even exists a direct feedback loop of the melanocortins from the adipose tissue to the brain

or vice versa. However, the importance of the melanocortin system in obesity, the expression of the melanocortin receptors in human adipose tissue has never been clearly investigated. Therefore, we analyzed the expression of the five melanocortin receptors, and of POMC, together with additional obesity-relevant genes (AgRP, leptin, leptin receptor, UCP-1) in human subcutaneous versus omental adipose tissue. Furthermore, we compared the expression levels in the obese to normal-weight subjects. Of the five melanocortin receptor subtypes, only MC1-R mRNA was substantially expressed and its expression was slightly elevated in the obese subject group. Since we obtained no POMC mRNA transcripts, an auto/paracrine action of α-MSH is doubtful. Fluorescent immunohistochemistry for detection of the MC1-R in human adipose tissue revealed high protein expression on macrophages and to a lesser extent on adipocytes. Human MSC-derived adipocytes were used as *in vitro* model to analyze the functionality of the MC1-R. Thereby, the cAMP production was dose-dependently increased upon stimulation with the potent MC1-R agonist NDP-MSH, suggesting that MC1-R in human adipocytes are functional. Furthermore, we tried to elucidate the function of MC1-R in undifferentiated MSCs as well as in adipocytes derived from these cells. We found a significant anti-proliferative effect of NDP-MSH on undifferentiated MSCs. This finding implies a role of α-MSH in regulating the *de novo* buildup of fat cells and subsequently the development of obesity. However, in adipocytes we were unable to find an effect of NDP-MSH on lipolysis, metabolic rate and inflammation.

In the last years the concept has emerged that obesity is characterized by a chronic mild inflammation. Several cytokines associated with inflammation are elevated in obesity, which may lead to the well known co-morbidities of obesity (e.g. hypertension, atherosclerosis, diabetes type 2). Nevertheless, it is still controversial which cell types in the adipose tissue secrete which cytokines. We analyzed the protein secretion and mRNA expression of the cytokines TNF-α, IL-6, IL-8 and IL-10 in human adipose tissue and in adipocytes, which were either derived from preadipocytes or MSCs. Whereas the adipose tissue secreted all four cytokines into the medium, in the supernatants from adipocytes no TNF-α and IL-10 was detectable (even upon stimulation with highest endotoxin (LPS) concentrations). Adipocytes secreted IL-6 and IL-8 in large quantities. Further investigations on the mRNA expression of cytokines revealed also high expression rates for IL-6 and IL-8. In contrast, TNF-α was expressed only transiently and at low levels after inducing of inflammation with LPS. When we analyzed co-cultures of macrophages isolated from buffy coats, either stimulated or unstimulated with LPS, together with adipocytes, we found substantial amounts of TNF-α protein. We obtained much more TNF-α from the LPS-stimulated cultures. Moreover, when we investigated the biological effects of exogenously

administered cytokines on adipocytes, only TNF-α showed an increase in lipolysis, glucose uptake and IL-6 mRNA expression. To summarize, IL-6 and IL-8 are secreted from adipocytes. Whereas adipocytes express no IL-10, TNF-α is only transiently and weakly expressed. Nevertheless, the adipocytes respond to exogenous TNF-α. Thus, TNF-α secreted from adipose tissue seems to be derived from cells of the stromovascular fraction (probably macrophages). In addition we found further evidence for a cross-talk between macrophages and adipocytes, which results in an elevated inflammation state. Thus, elimination of this macrophage-adipocyte cross-talk might be a future target in order to prevent augmented inflammation in the obese state, and probably avoiding the developing of the co-morbidities of obesity.

To conclude, these studies elucidated the expression of melanocortin receptors in human adipose tissue and adipocytes. We found MC1-R to be expressed in the adipose tissue on macrophages and adipocytes. Further analyzes confirmed the presence of the protein and the functionality of the MC1-R on adipocytes. Whereas MC1-R seems to play role in the regulation of proliferation in undifferentiated MSCs, in adipocytes we found no effect on lipolysis, metabolic rate and inflammation. Further investigations on cytokines in the adipose tissue revealed a very weak TNF-α production, suggesting that TNF-α in the adipose tissue is derived from macrophages rather than from adipocytes. However, human adipocytes respond to administration of exogenous TNF-α, indicating the presence of a cross-talk between adipocytes and macrophages in the adipose tissue.

With this work another tessera could be obtained that can be placed on the huge mosaic called the pathophysiology of obesity. A mosaic, which hopefully will be once completed in the future.

Chapter 1: General introduction

Obesity an epidemic disease

Today, obesity is the most common metabolic disease in developed countries, reaching epidemic dimension. It is estimated that 5% of US adults are morbidly obese (BMI > 40), 30% are obese (BMI > 30), which is more or less a doubling of the percentage in the last 20 years. Another 35% of the US population is overweight (BMI > 25). What is even of greater concern is the percentage of children and adolescents that are obese (~15% in the US). While in Switzerland 22% of women and 39% of men are overweight, 4.5% of women and 5.8% of men are obese (1993). Obesity is a major risk factor for various life-threatening diseases, including diabetes type 2, stroke, heart attack and some types of cancer (e.g. breast, colon). Every year approximately 300,000 people die of obesity-related diseases in the US [1]. Thus, after smoking it is the second cause of preventable premature death in the US. In the year 2003 obesity accounted for 9.1% of total US medical expenses, which costs every US tax payer staggering 175$ [2]. The reason for this pandemic is an increased availability of high caloric food and decreased physical activity. Thus, there exists an imbalance between energy intake and energy expenditure. Additionally, genetic predisposition contributes to the development of the disease by amplifying the effects of the environmental changes in Western society [3].

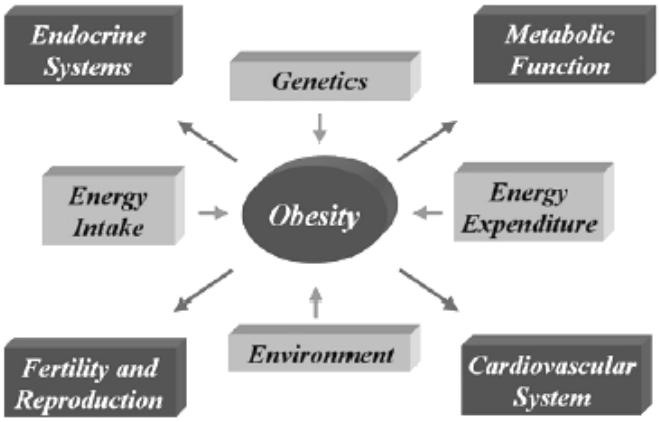


Fig. 1. Obesity, a multi-faceted disease. The size of body mass fat is the result of the balance between energy intake and energy expenditure. An imbalance can be caused by genetic and environmental factors. Adipose tissue is not only a energy depot, but also an active endocrine organ, which has an influence on cardiovascular and metabolic function, fertility and endocrine system. From Hofbauer, 2002 [3].

The melanocortins and their receptors

The melanocortins are all derived from the prohormone pro-opiomelanocortin (POMC)

The melanocortins were first recognized as a physiological regulator of pigmentation of many vertebrate species. Besides their effects on melanocytes the melanocortins possess a wide array of effects, including improvement of memory and attention, facilitation of nerve regeneration, alteration in motor and sexual behavior, pain, anti-inflammatory and lipolytic actions and inhibition of food intake [4-6]. All melanocortins are derived from a common precursor protein termed pro-opiomelanocortin (POMC) from which seven mature peptide hormones with different physiological effects are derived via post translational cleavage by various prohormone convertases (PC1, PC2). These seven hormones are: adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH, corticotrophin-like intermediate lobe peptide (CLIP), β-lipotropin and β-endorphin [7] (Fig. 2). The POMC processing occurs in a tissue-specific manner. POMC is mainly synthesized in the pars intermedia and pars distal of the pituitary and in the CNS, but also in some peripheral tissues such as gut, placenta and pancreas.

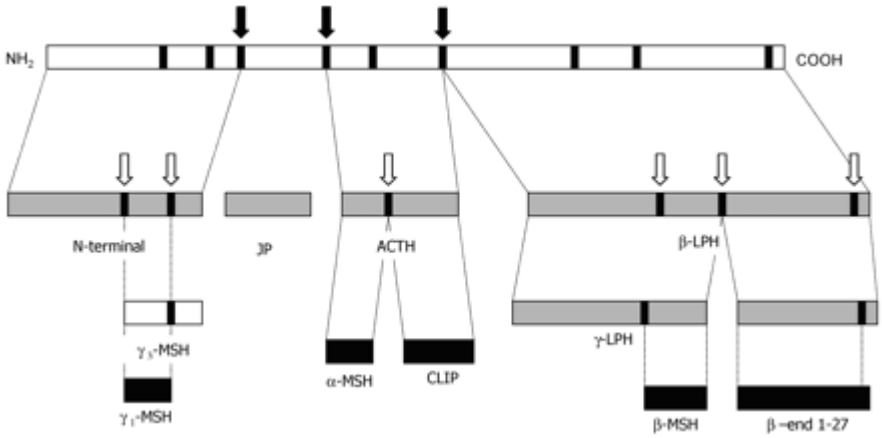


Fig. 2. POMC posttranslation processing. Melanocortin peptides (ACTH and α-, β-, and γ-MSH) are derived from post-translational processing of POMC by PC1 (*black arrow*) and PC2 (*clear arrow*) at dibasic cleavage sites (*solid line*). POMC is also the precursor for opioid peptides and CLIP (corticotropin-like intermediate lobe peptide). Tissuespecific expression results in a different range of peptides produced in the anterior pituitary (▩) compared with the hypothalamus (■). From Eberle, 2000 [8] and Coll, et al, 2004 [9].

Whereas the 12 amino acid long γ-MSH is derived from the N-terminal fragment of POMC, β-MSH is processed from β-lipotropin [7]. γ-MSH can induce several types of effects on the cardiovascular system. For example in rats intravenously injected γ-MSH elevates blood pressure (reviewed in [10, 11]). γ-MSH has been detected in the adrenal medulla and in neurons of the

intestines. β-MSH is mainly formed in the CNS and in the pituitary, where also the precursor protein β-lipotropin is found [7]. In the anterior lobe of the pituitary gland, POMC is processed to ACTH, a 39-amino acid peptide, which acts on the adrenal cortex to stimulate the production of corticoidsteroids. ACTH is further processed to α-MSH, a 13-amino acid peptide [7]. Rare POMC gene mutations in human give rise to deficiency in ACTH and α-MSH, which results in these patients with red hair, adrenal insufficiency and obesity [12]. In Table 1 the amino acid sequence of the physiologically most important peptides are depicted. The melanocortins all share the His-Phe-Arg-Trp core sequence, which could be shown to be crucial for the receptor binding [8].

ACTH[a]	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
α-MSH[b]	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
β-MSH[a]	H-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
γ-MSH[b]	H-Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2
MSH, melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone.	

Table 1. Amino acid sequence of the melanocortins α-, β-, and γ-MSH and ACTH. The core melanocortin amino acid sequence His-Phe-Arg-Trp is underlined. From Eberle, 2000 [8].
[a] human, [b] mammals

α-MSH (α-melanocyte-stimulating hormone)

Already early in the 20th century, a factor later to be known as α-MSH was shown to induce skin darkening in amphibian, which gave the hormone its name. α-MSH is mainly produced in the pars intermedia of the pituitary gland, but it has also been found at numerous other sites, including skin where it is produced in several cell types; in stomach, kidney, intestines, testis, ovaries, adrenal medulla and pancreas [6]. Mammalian α-MSH is N-terminally acetylated and C-terminally amidated [6]. The discovery that the sequence is preserved in many different species with only minor variations, led to the assumption that α-MSH occurred in an early phase of evolution. α-MSH exerts several different physiological functions. Studies in the 1970s indicated that α-MSH might be a trophic factor during fetal development [6]. In addition, effects on the immune system, gonads, eye and cardiovascular system have been reported. It has also been demonstrated that α-MSH improves memory and positively influences nerve regeneration, induces penile erection and, depending on the cell type, exerts either proliferative or anti-proliferative effects [6, 13-15]. Whereas α-MSH protects from ultraviolet radiation-induced apoptosis and DNA damage in human melanocytes [16, 17], it induces cell death in mast cells, presumably via NF-κB [18]. In recent years, the role of α-MSH in the control of feeding behavior and energy expenditure and its consequential link to obesity has attracted considerable attention [1].

Effects of α-MSH on the immune system

In animal models central administration of α-MSH could inhibit fever and other effects induced by proinflammatory molecules (e.g. IL-1β, TNF-α, LPS) [19]. Thereby, it is thought that endogenous α-MSH released within the brain contributes to physiological control of fever. It could be demonstrated that α-MSH down-regulates the synthesis and release of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α as well as the production of proinflammatory nitric oxide (NO) and neopterin in macrophages [19-21]. NF-κB participates in the regulation of many inflammatory genes, including those for cytokines. It has been shown that α-MSH inhibits NF-κB activation in TNF-α and LPS stimulated U937 monocytic cell line [22]. Similar results were obtained in human glioma cells and whole mouse brains stimulated with LPS. In cultured and peripheral human blood monocytes, α-MSH increased the production of IL-10, which reduces the production of cytokines in macrophages [23]. Additionally, in whole blood samples stimulated with LPS, α-MSH inhibited TNF-α and IL-1β production [24]. α-MSH also inhibited LPS-induced TNF-α secretion in the THP-1 human monocyte/macrophage cell line [25], as well as the NO production in the RAW264.7 mouse macrophage cell line stimulated by LPS plus interferon-γ (IFN-γ) [26]. In Table 2 and 3 the various immunomodulating effects of α-MSH are summarized.

Table 2. **Immunomodulating effects of α-MSH**. From Luger et al, 2003 [20].

- Down-regulation of proinflammatory cytokines: IL-1β, IL-6, TNF-α
- Down-regulation of immunomodulating cytokines: IL-2, IL-4, IL-13, IFN-γ
- Up-regulation of IL-10
- Down-regulation of costimulatory molecules: CD40, CD86
- Down-regulation of MHC class I expression
- Down-regulation of adhesion molecule expression: ICAM-1, VCAM-1, E-selectin
- Down-regulation of IgE production
- Down-regulation of NO production

Table 3. Anti-inflammatory effect of melanocortins and the corresponding site of action. From Catania et al, 2004 [21].

Effect	*Target Cell, Tissue, or Organ*
Reduced production/expression of proinflammatory cytokines and chemokines	Macrophages, endothelial cells, keratinocytes, fibroblasts, whole blood, liver
Nitric oxide (NO)	Macrophages, microglia, melanocytes, keratinocytes
Oxygen peroxide,	Keratinocytes, melanocytes
adhesion molecules (ICAM, VCAM)	Endothelial cells, kidney, liver, heart
Inhibition of white cell migration	Skin, lung, heart, kidney, liver, joints

The five melanocortin receptors (MC1-5-R)

During the years 1992-1993 five different melanocortin receptors (MC1-5-R) were cloned, which boosted the research on the melanocortins and their receptors. The five melanocortin receptors are seven transmembrane spanning proteins coupled to G-proteins (G-protein-coupled receptors (GPCRs)) which, when activated by a ligand, stimulate adenylyl cyclase to increase intracellular cAMP. The five subtypes are about 42-67% identical at the amino acid level [5, 7]. The melanocortin receptors belong to the smallest GPCRs (296 to 361 amino acids) [27], with short amino- and carboxyl-terminal ends and a very small second extracellular loop (Fig. 3). They possess the highest homology to the cannabinoid receptors. All melanocortin receptors contain the conserved amino acid Asp-Arg-Tyr (DRY) motif and a C-terminal Cys that may function as a fatty acid acylation site as well as several potential N-glycosylation sites at their N-terminal domain [28]. Whereas the MC4-R is the most conserved receptor subtype with an interspecies homology in the range of 74-94%, the MC1-R is the least. α-MSH has highest affinity for MC1-R but binds also to all other melanocortin receptors, except MC2-R to which it has only low activity [5]. MC2-R is expressed on the adrenal cortex and mediates ACTH-stimulated adrenal steroidogenesis, and trophic effects on the adrenal cortex. In rat and rabbit but not human ACTH induces lipolysis [5, 29].

Two endogenous antagonists of the melanocortin receptors, agouti (also termed agouti signaling protein; ASIP) and agouti-related protein (AgRP) are known, which are the only naturally occurring antagonists of GPCRs discovered to date [30, 31]. Human agouti (ASIP) is a closely homologous protein to rodent agouti and is a competitive antagonist at melanocortin receptors, showing high affinities for MC1-R and MC4-R. However, agouti binds also to MC3-R, as well as MC5-R, but with much lower affinity [32]. Previously, it has been shown that ASIP is expressed in human adipose tissue [33].

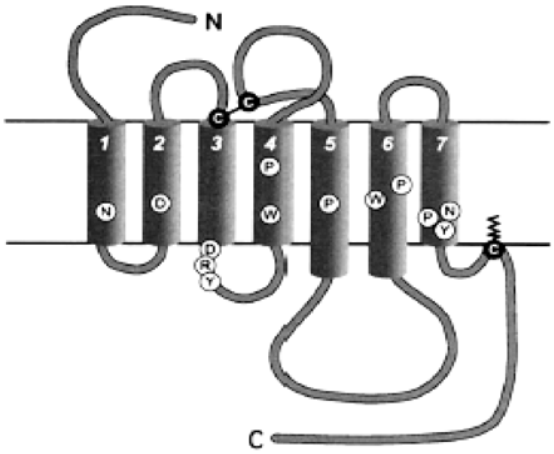


Fig. 3. The structure of the melanocortin receptors. The MCRs belong to the class A of G protein-coupled seven transmembrane receptors (rhodopsin/β_2-adrenergic-like family), which also includes biogenic amine, cannabinoid, melatonin, chemokine, and several other receptors. MCRs share many features with other G protein-coupled receptors: they have several potential *N*-glycosylation sites in their amino-terminal domains, consensus recognition sites for protein kinases C and/or A, which indicate that they may undergo regulation by phosphorylation, and conserved cysteines in their carboxyl termini, potential sites for fatty acid acylation, anchoring the C-terminal end to the plasma membrane. From Catania et al, 2004 [21].

Table 4. The five melanocortin receptor subtypes: their binding affinities to the melanocortins, tissue distribution and physiological function. Adapted from Catania et al, 2004 [21], MacNeil et al, 2002 [7] and Getting, 2006 [34].

MCR subtype	*Ligand affinity*	*Prevalent Tissue Expression*	*Functions*
MC1-R	α-MSH ≥ ACTH > β-MSH> γ-MSH	Melanocytes, immune/inflammatory cells, keratinocytes, endothelial cells; glial cells, fibroblasts	Pigmentation, anti-inflammatory, anti-pyretic
MC2-R	ACTH	Adrenal cortex	Steriodogenesis
MC3-R	γ-MSH = ACTH = β-MSH ≥ α-MSH	CNS, brain, heart, Macrophages	Energy homeostasis, cardiovascular, anti-inflammatory
MC4-R	α-MSH ≥ ACTH > β-MSH > γ-MSH	CNS, brain	Control of feeding and energy homeostasis; erectile disfunction, anti-pyretic
MC5-R	α-MSH ≥ ACTH > β-MSH > γ-MSH	CNS, brain	Control of feeding and energy homeostasis; erectile disfunction, anti-pyretic

The melanocortin receptor-1 (MC1-R)

The MC1-R was the first cloned melanocortin receptor and was first identified in melanoma and melanoma cell lines (e.g. D10, HBL, B16F1), where it is expressed at high level. However, it is also expressed in normal melanocytes and keratinocytes and a number of other tissues and cell types, including macrophages, monocytes, THP-1 cell line, pituitary, testis, placenta, endothelial cells, fibroblasts, glioma cells, astrocytes, and adipose tissue [5, 35, 36]. There are various single nucleotide polymorphisms in the coding region of the MC1-R. It is assumed that 75% of the Northern European population show such allelic variants, which are associated with red hair and higher prevalence for melanoma [37-39]. Thereby, loss-of-function mutations in the MC1-R gene sensitize human melanocytes to the DNA damaging effects of UV radiation, which may increase skin cancer [39]. Various variants of the MC1-R gene result in reduction of the receptor signaling and therefore relate to red or blond hair, lighter skin types and less ability to tan [4, 40]. So far, the MC1-R gene is the only known gene to explain physiological variation of human pigmentation [41]. MC1-R expression is thought to be 10-20-fold higher in malignant cells compared to normal melanocytes [42]. MC1-R is overexpressed in many melanoma cell lines and in solid melanoma tumors, suggesting the possibility of using MC1-R as antigen for tumor targeting in melanoma diagnosis and treatment (e.g. with radiolabelled α-MSH analogs [43]).

In melanocytes binding of α-MSH to MC1-R triggers a signal cascade that activates adenylyl cyclase, increases intracellular cAMP, which induces activity of tyrosinase, the rate-limiting enzyme in melanogenesis. α-MSH and endothelin-1 both increase MC1-R mRNA expression. Furthermore, MC1-R expression appears to be regulated by the microphtalmia-associated transcription factor (MITF), which promotes transcription of certain genes, including tyrosinase, TRP1 and TRP2. MITF mRNA expression is regulated by cAMP responsive element binding protein (CREB) [4]. Increased cAMP levels lead to activation of PKA, which mediates phosphorylation of CREB followed by binding if CREB to the CRE and the cis-activation of the MITF promoter (Fig. 4) [21]. TRP2 was suggested to be involved in cell proliferation effects and protection of melanocytes from apoptosis [44].

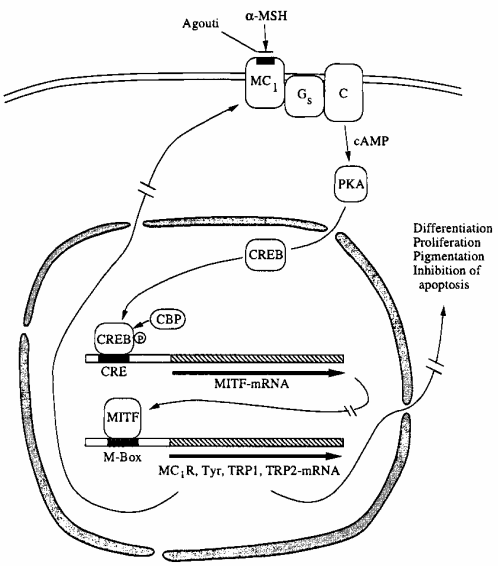


Fig. 4. Model for the regulation of the expression of MC1-R receptor gene and other genes in melanocytes and melanoma cells. Melanocortic peptides bind to the receptor, which stimulates adenylyl cyclase (C) and hence the production of cAMP. PKA is activated by cAMP leading to the phosphorylation of CREB. MITF mRNA expression is achieved only after binding of CBP to the CREB. MITF binds to the M-Box and induces the expression of MC1-R, tyrosinase (Tyr), TRP1, TRP2 and probably other genes as well. These proteins in turn influence differentiation, proliferation, formation of pigment and inhibit apoptosis. The increased MC1-R production causes an increased sensitivity to melanocortic peptides. Agouti acts as inverse agonist on MC1-R. From Wikberg et al, 2000 [4].

It is thought that MC1-R is the major melanocortin receptor involved in the anti-inflammatory influence of α-MSH since virtually all cell types responsive to the anti-inflammatory effect of melanocortins express MC1-R, including monocytes, macrophages, neutrophils, mast cells, fibroblasts, dendritic cells, astrocytes, and microglia [21]. However, MC3-R and MC5-R have also been suggested to be involved in the anti-inflammatory effects of the melanocortins. In human monocytes MC1-R binding sites are up-regulated by the endotoxin lipopolysaccharide (LPS) and some cytokines [45]. The anti-inflammatory effects of α-MSH are listed in Table 2 and 3.

The melanocortin receptor-2 (MC2-R)

The MC2-R consists of 297 amino acids and is only activated by ACTH. By binding to the MC2-R, ACTH regulates the release of steroids in the adrenal cortex via activation of PKA [34]. MC2-R is mostly expressed in cells of the adrenal cortex, in a region presumed to be the production site of glucocorticoids [46]. MC2-R mRNA has also been detected in human skin [47] and murine adipocytes [48]. The mRNA expression of MC2-R is sensitive to cAMP, which is the reason for the receptor up-regulation by binding of ACTH [34]. It is thought that in mice MC2-R present in adipocytes is responsible for stress-induced lipolysis mediated by ACTH released from the pituitary [29]. However, Chhajlani [35] did not succeed in detecting MC2-R in human adipose

tissue. Moreover, freshly isolated mature human adipocytes did not show any lipolytic action stimulated by ACTH [49]. Thus, ACTH seems not to induce lipolysis in human. Interestingly, MC2-R is expressed in the spleen and adrenal gland of chicken, indicating potential other functions of the receptor in birds [50]. Rare mutations in the MC2-R gene in humans are associated with familial glucocorticoid deficiency [51]. Noteworthy, mutations in other genes can also lead to similar disease conditions.

The melanocortin receptor-3 (MC3-R)

The MC3-R is expressed throughout the brain (mainly in the hypothalamus), placenta, gut and heart of mammals but was also found in murine macrophages, thymus, human monocytes and at lower levels in testis, ovary, muscle and kidney [4, 7, 21]. To note, MC3-R is the only melanocortin receptor activated by γ-MSH, in fact with similar potency as the other melanocortins [52]. Like all melanocortin receptors the MC3-R gene is intronless encoding for a protein of 361 amino acids. MC3-R has been demonstrated to participate in modulation of inflammation, feeding and energy homeostasis [53, 54]. The latter was elucidated in MC3-R knock-out mice, which leads to increased fat mass, reduced lean mass, and higher ratio of weight gain to food intake [55]. Furthermore, MC3-R activation reveals a protective effect in ischaemic-reperfusion injury in the heart [56]. Thus, it is speculated that MC3-R is some kind of a fine tuner of inflammation, cardiovascular functions and energy homeostasis [57].

The melanocortin receptor-4 (MC4-R)

Like MC3-R the MC4-R is expressed virtually in all brain regions. In contrast to MC3-R, its expression in the CNS is wider and in the periphery it is essentially absent. However, Chagnon et al [58] found some traces of MC4-R mRNA by RT-PCR in human adipose tissue (epiploon), although this finding could not be confirmed by others [35]. In chicken the MC4-R is expressed in many peripheral tissues [59]. MC4-R consists of 332 amino acids encoded by a single exon of 999 nucleotides. It seems that MC4-R has an important role during the embryonic phase. Possibly, MC4-R is involved in neuronal developments. Activation of MC4-R exerts many different functions including pain [60], penile erection, sexual behavior [61]. However, the primary interest for the pharmaceutical industry at present is its role in controlling food intake and energy expenditure. It could be shown that α-MSH inhibits food intake via MC4-R activation. MC4-R knock-out mice become obese, with hyperphagia, hyperinsulinemia and hyperglycinemia. These mice do not respond to the anorectic effects of α-MSH and have enhanced caloric efficiency [62].

In humans about 60 different MC4-R gene mutations have been described, which are linked to obesity. Mutations in the MC4-R gene occur, depending on the study, in 1-6% of morbidly obese individuals. Thus, it represents the most common monogenic mutation causing obesity [63].

The melanocortin receptor-5 (MC5-R)

The MC5-R consists of 325 amino acids and is ubiquitously expressed in peripheral tissues including adrenal gland, kidney, liver, lung, lymph nodes, bone marrow, thymus, mammary glands, testis, ovary, pituitary, testis, uterus, esophagus, stomach, duodenum, skin, skeletal muscle, and exocrine glands [21]. Thereby, highest expression levels of MC5-R are found in exocrine tissues. In mice MC5-R was also found in adipocytes [48], but not in human [35]. Thus, MC5-R appears to participate in the regulation of exocrine gland function and certain immune responses. Both ACTH and α-MSH are able to up-regulate the MC5-R expression. Buggy et al [64] suggested the participation of α-MSH in the immune regulation in B-lymphocytes via MC5-R binding and activation of the Jak/STAT pathway. However, the different functions of the MC5-R in the various tissues remain to be elucidated.

Role of melanocortins in the control of feeding

The regulation of food intake and energy homeostasis is mediated by both peripheral and central signals. In the last two decades various studies could demonstrate that there is an integrated control of appetite, fat metabolism and hence fat reserves, which links the melanocortin system to the levels of the adipocyte-derived hormone leptin. It has been shown that leptin plays an important role in the maintenance of energy homeostasis [65]. Leptin is released into the blood and is able to cross the blood-brain barrier in the arcuate nucleus, the master center of food intake. In the arcuate nucleus leptin binds to the leptin receptors that leads to the production of α-MSH. α-MSH on its part binds to the MC4-R also present in the arcuate nucleus [1]. By a not yet clearly understood mechanism the binding of α-MSH to the MC4-R decreases appetite and food intake. However, studies in mice suggested an involvement of the brain-derived neurotrophic factor (BNDF) expressed in the ventromedial hypothalamus in regulating energy balance downstream of the MC4-R signaling [66]. Very recently, one case of an 8-year-old girl with hyperphagia and severe obesity, impaired cognitive function, and hyperactivity was reported that was haploinsufficient for BNDF, providing evidence for the role of BNDF in human energy homeostasis [67]. In the

following paragraphs the mode of action of leptin and the melanocortins in the regulation of food intake and body weight will be explained in more details.

Leptin

In the 1970s two spontaneous mutations in mice were discovered, which caused hyperphagia and morbid obesity (*ob* (obese) and *db* (diabetes)) [68]. Only in the year 1994 it was first discovered that disruption of the leptin gene is the genetic cause of the obese phenotype in *ob/ob* mice. By injecting leptin into *ob/ob* mice the animals' obesity could be cured. Additionally, it was found that *db/db* mice have a mutated leptin receptor gene. As expected, these mice do not respond to leptin treatment [69]. Leptin is a 16 kDa non-glycosylated protein produced predominantly by white adipose cells [70]. It induces weight loss by decreasing appetite while at the same time inducing energy expenditure (Fig. 5). Circulating leptin levels are proportional to adipose tissue mass. Thus, leptin signals its energy reserves to the body. Leptin levels are higher in female than males and its synthesis is greater in subcutaneous compared to omental adipose tissue. Furthermore, leptin seem to possess a role in reproduction since both *ob/ob* and *db/db* mice are infertile [71]. In *ob/ob* mice the fertility can be restored by injection of exogenous leptin. It has been speculated that leptin is important in the onset of puberty in these mice [72]. However, by now there is no study showing direct effects of leptin on reproductive function in healthy humans [65]. Leptin can also be considered as a pro-inflammatory cytokine. During acute infection, inflammation and sepsis leptin production is increased [73]. Moreover, its secretion is stimulated by insulin.

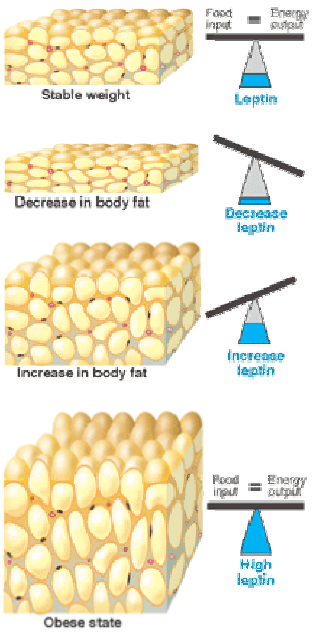


Fig. 5. Long-term body weight control by leptin.
From Marx, 2003 [1].

Unfortunately leptin did not become the attempted "magic bullet" against obesity due to the fact that obese humans turned out to have higher leptin blood levels than normal weight individuals [1]. However, some rare cases of mutations in the leptin production were identified leading to early-onset morbid obesity. Children with one of these mutations could successfully be treated with leptin [74]. Thus, there seem to be some kind of leptin resistance in obese humans, comparable to insulin resistance also seen in obesity. The reason for this resistance to leptin is not yet clearly understood. One hypothesis is a decreased ability of leptin to cross the blood-brain barrier by an impaired leptin transport across the endothelial cells. Another potential cause of leptin resistance could be a reduced leptin-receptor cell signaling [75]. Recent studies in mice suggest that overexpression of suppressor of cytokine signaling (SOCS)-3 in POMC neurons in the hypothalamus induces leptin resistance [76, 77]. To summarize, leptin's main role seems to be the protection against weight loss in times of deprivation rather than against weight gain in times of abundance [1].

The leptin-melanocortin axis

Leptin secreted by adipose tissue crosses the blood-brain barrier and binds to the leptin receptor in the hypothalamus, thereby increasing the POMC mRNA expression [78], which leads to the production of α-MSH. Leptin receptors are highly expressed in the arcuate nucleus in the hypothalamus, known to be the master center of appetite regulation, both for the short and long-term. There are two different neurons highly expressing leptin-receptors in the arcuate nucleus. These neurons are the NPY/AgRP and POMC/CART neurons, which possess opposing effects. Whereas activation of the NPY/AgRP neurons by NPY (neuropeptide Y) and AgRP (agouti-related peptide) stimulates appetite, while reducing metabolism, the activation of POMC/CART neurons causes the release of α-MSH that reduces food intake (Fig. 6) [1, 75]. Thus, after shrinking of fat stores and the consequential decrease of leptin, the NPY/AgRP neurons are activated and the POMC/CART neurons are coevally inhibited, leading to a decrease of α-MSH, resulting in weight gain. By contrast, increased fat mass and resulting elevated leptin levels cause an inhibition of the NPY/AgRP neurons and activation of the POMC/CART neurons leading to weight loss. Upon activation the NPY/AgRP and POMC/CART neurons project their signals to other brain regions (e.g. paraventricular nucleus, zona incerta, perifornical area) involved in the regulation of food intake and energy homeostasis [1, 75].

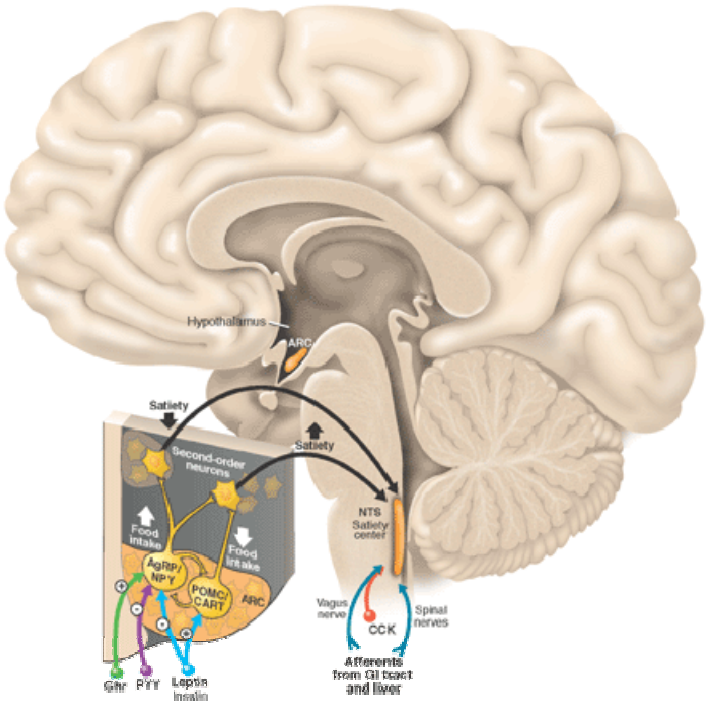


Fig. 6. The arcuate nucleus: the master center of food control. The arcuate nucleus (ARC) contains two sets of neurons with opposing effects. Activation of the AgRP/NPY neurons increases appetite and metabolism, whereas activation of the POMC/CART neurons has the opposite effect. Apart from AgRP and NPY, PYY, ghrelin, leptin and insulin act also on these neurons. From Marx, 2003 [1].

Intracerebroventricular injection of α-MSH and the superpotent α-MSH agonist NDP-MSH inhibits feeding in mice [79]. The natural production of α-MSH in arcuate nucleus implies the presence of melanocortin receptors in that region that exert the anorectic actions of the peptide. In fact, MC3-R, MC4-R and MC5-R are expressed in the brain. Whereas MC3-R expression is largely restricted to the hypothalamus and MC5-R to the cerebral cortex and cerebellum, respectively, MC4-R is widely distributed throughout the brain, including the arcuate nucleus [80]. The anorectic effect of α-MSH is blocked by the melanocortin MC3-R and MC4-R non-selective antagonist SHU9119 [81, 82]. This finding implies that the activation of MC3-R and MC4-R in the arcuate nucleus reduces food intake. Further observations in various mouse models support the same idea. MC4-R knock-out mice display an identical obesity phenotype as the agouti (A^y) mice, which ubiquitously express agouti (see next paragraph). Importantly, heterozygous mice with a disrupted MC4-R display an intermediate obesity phenotype. MC3-R knock-out mice do not show significant overweight, but exhibit a late onset increase in adipose mass (ca. 50-60%) [83]. However, recent data suggest that MC3-R is more important in modulating energy expenditure than on direct food intake. It is noteworthy that MC3-R and MC4-R double knock-out mice display an even more obese phenotype than single knock-out mice [55].

Agouti is known to be an inverse agonist of the MC1-R, but it also binds to MC3-R, MC4-R and MC5-R. Agouti expression is normally restricted to the skin [7]. In the *agouti lethal yellow (A^y)* mouse agouti is ubiquitously expressed, which leads to a yellow fur and a hyperphagic and obese phenotype. In this mutant mouse model ectopically expressed agouti in the skin binds to MC1-R, which results in a shift of the production of the black eumelanin to the yellow pheomelanin [6]. The obese phenotype has been shown to be due to the competitive binding of agouti at the MC4-R in the brain.

In the last decade different melanocortin receptor agonists and antagonist were developed. MTII is a non-selective melanocortin receptor agonist, which potentially decreases food intake in mice and rats. The first MC4-R selective antagonist was the cyclic HS014 [84]. Further compound optimization resulted in the discovery of the super-selective HS028 [85] and the super-potent HS024 [86].

While it is well accepted that mutations in the MC4-R gene are the most common monogenic cause of human obesity [87], the relevance of MC3-R mutations is not yet clear. Several studies with a large cohort group failed to identify any mutations in the MC3-R gene from patients with type 2 diabetes and morbid obesity [87].

The role of the melanocortins in anorexia and cachexia

It is well known that stress influences the feeding behavior in mammals. Thereby, the duration and type of stress has an important impact. Exposure of animals to severe stressful conditions can induce anorexia leading to severe body weight loss. It was shown that blockage of MC4-R signaling can reduce stress-induced anorexia. However, this reduction is only partial, implying the participation of some other mediators than MC4-R [88].

Cachexia is common in infectious diseases, cancer and AIDS that lead to loss of appetite and weight loss, muscle atrophy, weakness, fatique and anorexia. It is thought that cachexia is induced by interleukins and other cytokines activating POMC/CART neurons leading to the production of α-MSH. In a study with tumor-bearing MC4-R knock-out mice it could be demonstrated that the activation of MC4-R takes part in development of cachexia. Therefore, blockade of the MC4-R might be a good target to treat and/or prevent cachexia [88, 89].

Other appetite regulating neuropeptides

Besides α-MSH other neuropeptide effector molecules released in specific areas in brain upon leptin stimulation include agouti-related protein (AgRP), melanin-concentrating hormone (MCH), corticotropin releasing hormone (CRH), galanin, glucagon-like peptide 1 (GLP-1), neurotensin and cocaine- and amphetamine-regulated transcript (CART). Much attention in the last years attracted the finding of the hormone ghrelin, produced mainly in the stomach, peptide YY (PYY) and oxyntomodulin both produced in the so called L-cells in the intestine [90-93]. Ghrelin exhibits potent orexigenic properties and its blood plasma levels rise before meal and decreases following feeding. Thus, ghrelin seems to serve as a meal initiator [94]. By contrast, PYY plasma levels rises following food intake and signals satiety. Peripheral administration of PYY in rodents and humans inhibits food intake and reduces weight gain [95]. Insulin has also been found to inhibit production of appetite-stimulating NPY in the arcuate nucleus [96]. Mice, in which the insulin-receptor is knocked-out in the arcuate nucleus, overeat and become obese. Another mentionable satiety hormone is cholecystokinin (CCK) produced in the intestine [97]. In Table 5 the most important neuropeptides in appetite control are listed. Some of these peptide hormones are described in more details in the following section.

Table 5. Major neuropeptides involved in appetite control. Adapted from Arora and Anubhuti, 2006 [92].

Neuropeptides	**↑ food intake (orexigenic)**	**↓ food intake (anorexigenic)**
Central	• NPY • MCH • Orexins/hypocretins • AgRP • Galanin • Endogenous opoids • Endocannabinoids	• Melanocortins (derived from POMC) • CART • GLP-1 • Corticotropin releasing factor (CRF) • Insulin • Serotonin • Neurotensin
Peripheral	• Ghrelin	• PYY • CCK • Leptin • Amylin • Insulin • GLP-1 • Bombesin • Oxyntomodulin

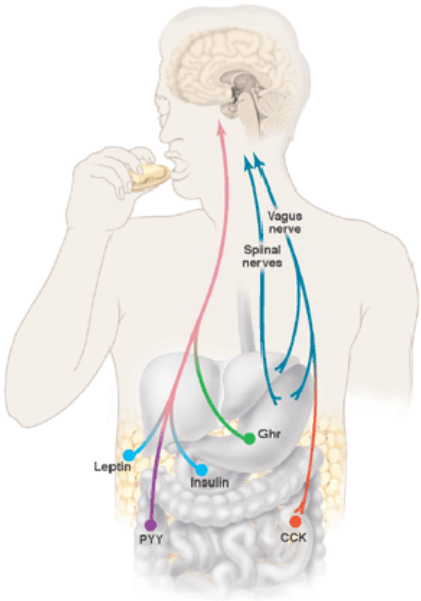


Fig. 7. Appetite controlling neuropeptides. Hormones produced by the body in different organs act through the brain to regulate short- and longterm appetite and also the body's metabolism. The indicated hormones are now under intense investigation. From Marx, 2003 [1].

Agouti-related peptide (AgRP)

AgRP (132 amino acids) exhibits less than 20% identity with agouti and is almost exclusively expressed in the arcuate nucleus and is a potent endogenous antagonist of MC3-R and MC4-R with high affinity [98]. In contrast, agouti's expression is restricted to skin playing an important role of rodent pigmentation and binds to MC1-R and MC4-R. Low plasma levels of AgRP were reported both in rats and humans and its concentration changes after meal consumption. Thus, it was suggested that AgRP may be a satiety factor [92]. Icv administration stimulates acutely food intake in mice. Overexpression of AgRP in mice leads to hyperphagia and obesity [28] and its stimulatory effect can be reversed by α-MSH.

Table 6. Pharmacological profile of agouti and AgRP on melanocortin receptors. From Yang and Harmon, 2003 [28].

Receptor	Antagonism by Agouti	AgRP
MC1-R	++++	-
MC2-R	+++	-
MC3-R	+	++++
MC4-R	++++	++++
MC5-R	++	++

Peptide YY (PYY)

PYY is a 36-amino acid peptide, which belongs to the NPY family (including NPY, PP and PYY). They all share high sequence homology and are rich of tyrosines [92]. The main production site of PYY is the intestine (in the L-cells) with highest tissue concentrations in distal segments of the gastrointestinal tract [99, 100]. PYY is released into the blood and exists in two major forms: PYY_{1-36} and PYY_{3-36}. Thereby PYY_{3-36} is the peripherally active form, exerting the anorectic action. PYY_{1-36} is cleaved by dipeptidyl peptidase IV (DPP-IV) to form PYY_{3-36}. PPY_{3-36} represents the major form of PYY in the circulation. Like NPY, PYY also binds to Y receptors, with highest affinity for Y_2 and low affinity for Y_1 and Y_5 receptors [92, 101]. PYY is released into the circulation upon food intake and plasma levels peak after 1-2 h. PYY blood plasma levels are proportional to meal-energy intake. Thereby, PYY concentrations increase more after fat-intake as compared to carbohydrates or proteins [102]. Administration of PYY causes a delay in gastric emptying as well as a delay in secretions from the pancreas and stomach. Additionally, it increases the absorbance of fluids and electrolytes from the ileum after a meal. It has been reported that peripheral administration of PYY_{3-36} to rodents inhibits food intake and reduces weight gain [95, 103]. It has been proposed that PYY_{3-36} binds to the Y_2 receptor in the arcuate nucleus, which leads to an inhibition of the NPY/AgRP neurons and stimulation of the POMC/CART neurons. These anorectic effects of PYY_{3-36} are induced at physiological concentrations, which suggests the importance of PYY in the everyday food intake control. Infusions of PYY_{3-36} to healthy normal-weight human subjects has a massive impact on appetite, resulting in a 30% reduction in food intake [95]. Interestingly this effect persists for 12 h after the termination of the infusion, despite the circulating PYY_{3-36} are back on basal levels. Thus, PYY_{3-36} could be an important satiety signal. The anorectic action of PYY_{3-36} is also present in obese subjects and by now there is no resistance reported after several PYY_{3-36} infusions. Therefore, long-term administration of PYY_{3-36} could be an effective obesity therapy [92, 95]. At the moment a PYY nasal spray is under clinical investigation. However, very recently Degen et al [104] reported that administration of higher PYY concentrations often lead to nausea, while lower concentrations were ineffective in reducing food intake. Thus, the therapeutic window of PYY seems to be very narrow.

Cholecystokinin (CCK)

It has been demonstrated that endogenous CCK controls the meal size and is therefore some kind of meal terminator. CCK is found in the gastrointestinal tract and the brain. It is rapidly released into the circulation in response to nutrients and remains elevated for up to 5 h [105]. CCK exerts several other important physiological functions like stimulation of pancreatic secretion, intestinal motility, gall bladder contraction, memory enhancement and inhibition of gastric motility [92].

Ghrelin

Ghrelin is a 28-amino acid peptide, which is very potent in stimulating appetite (orexigenic) and seems to participate in the adaptive response to weight loss. Ghrelin is a short-acting satiety factor like CCK, GLP-1, glucagons, amylin and bombesin. In addition, ghrelin regulates body adiposity and energy balance [106]. Main sites of production are the endocrine cells in the stomach (gastric mucosa). However, it is also found in many other tissues (e.g. immune cells, placenta, ovary, testis, kidney and some tumors) [107, 108]. Ghrelin binds to its receptor, which is predominantly expressed in the pituitary and at lower levels in other tissues (e.g. hypothalamic nuclei, stomach, heart, lungs, kidneys). Since ghrelin and its receptor are widely distributed, ghrelin has multiple biological effects. For instance it stimulates the release of growth hormone in the pituitary, where it binds to the secretagogue receptor (GHS-R). Additionally, it induces the release of ACTH and cortisol into the circulation [108, 109]. Ghrelin stimulates the production of NPY and AgRP in the arcuate nucleus, thereby antagonizing the leptin-induced inhibition of food intake, leading to an increase in food intake [92, 110]. Moreover, ghrelin increases plasma glucose concentrations [108]. *In vitro* ghrelin stimulates the differentiation of rat preadipocytes to adipocytes and antagonizes lipolysis. Thus, it has been proposed that ghrelin plays a role in rat adipogenesis [111]. Tschöp et al [106] could demonstrate that icv and peripheral administration of ghrelin in mice caused a dose-dependent increase in food intake and body weight. Ghrelin levels are preprandially sharply increased before scheduled meals (1-2 h) and decrease afterwards (~20 min-1 h after meal initiation, Fig. 8) [94]. Studies in humans confirmed the findings in rodents. In one study administration of ghrelin caused a 46% increase in the perception of hunger and resulted in an average of 28% higher caloric intake at a buffet meal [112]. Interestingly ghrelin levels are decreased in obese subjects. Furthermore, ghrelin levels also seem to be influenced by behavioral parameters (e.g. binge eating) [113]. High ghrelin levels are reported in obese individuals with the Prader Willi syndrome [91, 114].

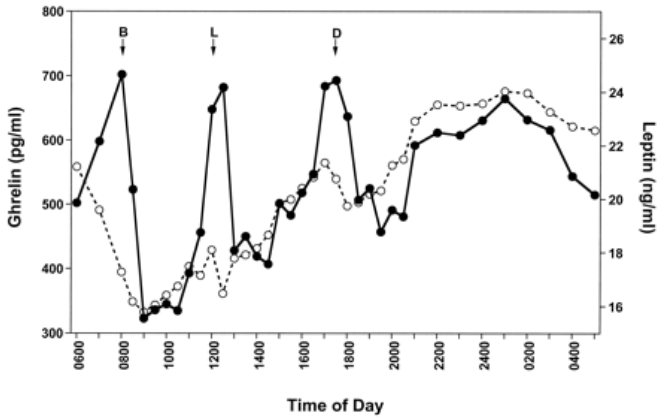


Fig. 8. Overlaid average plasma ghrelin (•) and leptin (○) concentrations during a 24-h period in 10 human subjects consuming breakfast (B), lunch (L), and dinner (D) at the times indicated. From Cummings et al, 2001 [94].

Very recently, a novel peptide called obestatin has been identified, which is derived from the same gene as ghrelin; it appears to oppose the effect of ghrelin on energy homeostasis and gastrointestinal function in rats [115]. At the moment many questions around obestatin remain unanswered.

Glucagon-like peptide 1 (GLP-1)

GLP-1 is derived from the larger precursor molecule proglucagon. This post-translational modification takes place in the pancreas, intestinal cells and the CNS. GLP-1 suppresses food intake in animals and humans [116]. It is of note that icv administration of a GLP-1 antagonist increases food intake [117]. However, GLP-1 receptor deficient animals reveal no alteration in mass of body fat. GLP-1 is released by both nutrient and neurohumoral stimulation in the small intestine. The predominant function of GLP-1 is the stimulation of insulin secretion during meal. GLP-1 is a very labile and short lived peptide. It is degraded in the plasma within 2 minutes, mainly by the enzyme dipeptidyl-peptidase IV (DPP-IV), yielding the inactive analogs of GLP-1 [91]. Noteworthy, DPP-IV inhibition has become a drug target to treat type 2 diabetes as well as more stable GLP-1 agonists. Despite the short half-life of GLP-1, peripherally administered GLP-1 can cross the blood-brain barrier and act in the hypothalamus. However, the contribution of GLP-1 on food intake within the CNS is controversial [91].

Adipose tissue as an endocrine organ

In the last decades the traditional view of the adipose tissue as passive reservoir for energy storage has shifted to an active and tightly regulated metabolic and endocrine organ, which interacts extensively with other organs controlling metabolism. Already in the year 1984 the adipose tissue was identified as a major site for metabolism of sex steroids. In 1994 leptin has been characterized and established adipose tissue as an endocrine organ (for details on leptin see above) [118]. Besides leptin, adipose tissue has been found to secrete various other endocrine factors including other cytokines (e.g. IL-1β, IL-6, IL-8), adiponectin, monocyte chemoattractant protein 1 (MCP-1), complement components (e.g. adipsin, acylation-stimulating protein), plasminogen activator-1 (PAI-1), proteins of the renin-angiotensin system and resistin (Table 7) [118, 119]. All these bioactive peptides secreted from adipose tissue are termed as adipokines and can act either locally (autocrine/paracrine) or systemically (endocrine). Important to note, adipose tissue does not only contain adipocytes but it also consists of the so-called stroma-vascular fraction (SVF), which

consists of endothelial cells, preadipocytes and leukocytes (including macrophages) [120]. Fain et al [120] reported that the majority of the adipose tissue-derived cytokines originate from non-fat cells (SVF fraction), with the exception of leptin and adiponectin.

Table 7. Factors produced by WAT. Adapted from Ahima, 2006 [71].

Secreted proteins	**Receptors**	**Enzymes and transporters**
Leptin	*Peptide and glycoprotein*	*Lipid metabolism*
Adiponectin	Insulin	Lipoprotein lipase
Resistin (in rodents)	Glucagon	Apolipoprotein E
Angiotensinogen	Thyroid stimulating hormone	Cholesterol ester transfer protein
IL-1β	Growth hormone	Adipocyte fatty acid binding protein
IL-6	Angiotensin-II	CD36
IL-8	Gastrin/cholecystokinin B	
Adipsin	Adiponectin	*Glucose metabolism*
Acylation stmulating protein		Insulin receptor substrate 1,2
Fasting-induced adipose factor	*Cytokine*	Phosphatidylinositol 3-kinase
PAI-1	IL-6	Protein kinase B (Akt)
Tissue factor	TNF-α	GLUT4
MCP-1	Leptin	Protein kinase ζ
Tranforming growth factor-β		Glycogen synthase kinase-3α
Visfatin	*Nuclear*	
Vaspin	PPARγ	*Steroid metabolism*
Retinol binding protein-4	Glucocorticoid	Aromatase
	Estrogen	11β-hydroxysteroid dehydrogenase type 1
	Progesterone	17β-hydroxysteroid dehydrogenase
	Androgen	
	Thyroid	
	Vitamin D	
	Nuclear factor-κB	

WAT, white adipose tissue; TNF, tumor necrosis factor; IL, interleukin; MCP, monocyte chemoattractant peptide, PPAR, peroxisome proliferator-activated receptor; GLUT4, glucose transporter 4.

Inflammation-related adipokines

Adiponectin

Adiponectin is an approximately 30 kDa polypeptide and is exclusively expressed in differentiated adipocytes. It circulates at high levels in the bloodstream (500-30'000 µg/l), accounting for 0.01% of total plasma protein. In human obesity the plasma levels of adiponectin are decreased. The expression of adiponectin is higher in subcutaneous than visceral adipose tissue (reviewed in [121, 122]). The most prominent action of adiponectin seems to be the modulation of insulin sensitivity and therefore its link to the development of type 2 diabetes [123]. It has been suggested that adiponectin together with leptin work together to sensitize peripheral tissues to insulin [65]. Adiponectin levels are low in insulin resistance (e.g. due to obesity) and rise after weight loss, which consequently improves insulin sensitivity. Moreover, several polymorphism of the adiponectin gene were identified that are associated with obesity and insulin resistance. In addition, the hormone exerts vascular functions and possesses anti-inflammatory actions [124]. Two adiponectin receptors have been described: Adipo R1 and Adipo R2. The adiponectin receptors are primarily expressed in muscles (Adipo R1) and liver (Adipo R2). Whereas adiponectin in the liver enhances insulin sensitivity, it stimulates glucose use and β-oxidation in the muscle. To summarize, adiponectin is a unique adipocyte-derived hormone with antidiabetic, anti-inflammatory and antiatherogenic effects [118] (Fig. 9).

Tumor necrosis factor-α (TNF-α)

TNF-α is a multipotential pro-inflammatory cytokine, which is a 26-kDa cell surface transmembrane protein that undergoes cleavage to produce the 17-kDa soluble biologically active TNF-α. TNF-α is linked to obesity and insulin resistance. Neutralization of TNF-α in obese rat improves insulin resistance. However, this effect could not be confirmed in human obese subjects [118]. While some studies reported a clear correlation between TNF-α plasma levels and obesity and insulin resistance in humans, other studies failed to confirm. TNF-α stimulates lipolysis, apoptosis, inhibits lipogenesis and regulates the adipokine expression on adipose tissue [125]. The expression of TNF-α is higher in subcutaneous than omental adipose tissue [120]. In humans TNF-α is expressed and secreted by adipocytes and stromo-vascular cells. Weight loss decreases TNF-α levels [126]. Two TNF-α receptors were reported (type 1 (p55) and type 2 (p75)) [71]. TNF-α secreted from adipose tissue can act paracrine/autocrine, as well as endocrine when it is secreted into the bloodstream. In the liver, on one hand TNF-α suppresses genes involved in

glucose uptake, metabolism and β-oxidation. On the other hand it increases genes involved in *de novo* synthesis of cholesterol and fatty acids. Additionally, TNF-α impairs insulin signaling [118].

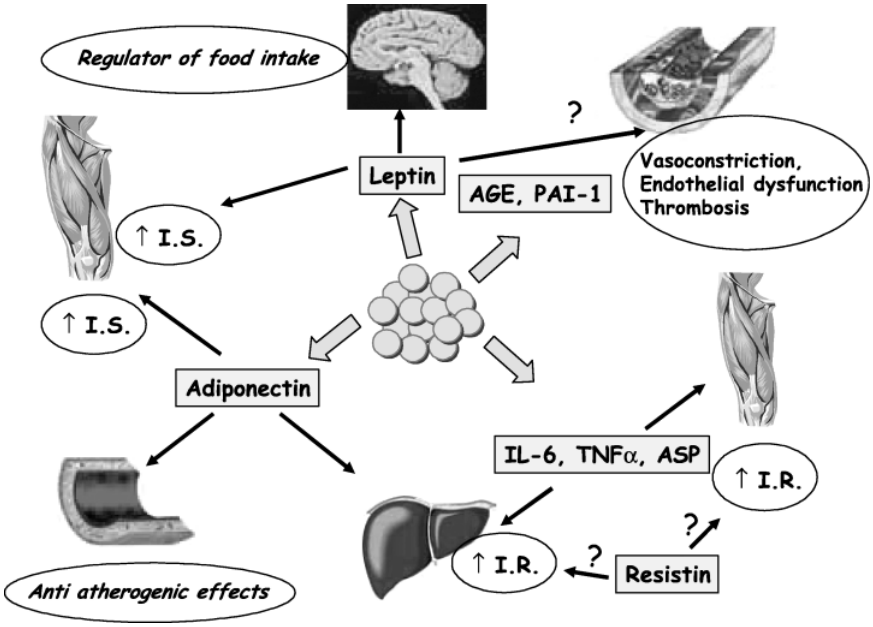


Fig. 9. Adipokines implicated in energy homeostasis, insulin sensitivity (IS), insulin resistance (IR) and atherothrombosis. Excessive production of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), acylation-stimulating protein (ASP) deteriorates insulin action in muscle and/or in liver, whereas increased angiotensin (AGE) and PAI-1 secretion favours hypertension, endothelial dysfunction and thrombosis. The role of resistin on insulin resistance is still not clear. Leptin regulates energy balance and exerts an insulin sensitizing effect. Adiponectin increases insulin action in muscle and in liver exerts an anti atherogenic effect. From Ronti et al, 2006 [119].

Interleukin-6 (IL-6)

IL-6 is another cytokine closely linked to obesity and insulin resistance. It could be demonstrated that IL-6 expression and circulating IL-6 levels are positively correlated with obesity and insulin resistance [127]. High IL-6 plasma concentrations are a prediction marker for type 2 diabetes as well as cardiovascular disease. Moreover, IL-6 inhibits adipogenesis and decreases adiponectin secretion. Unlike TNF-α, leptin and adiponectin, IL-6 expression is higher in omental fat than subcutaneous adipose tissue [128]. Weight loss leads to a significant reduction in IL-6 levels in adipose tissue and circulation. However, it was suggested that only about 10% of total IL-6 are

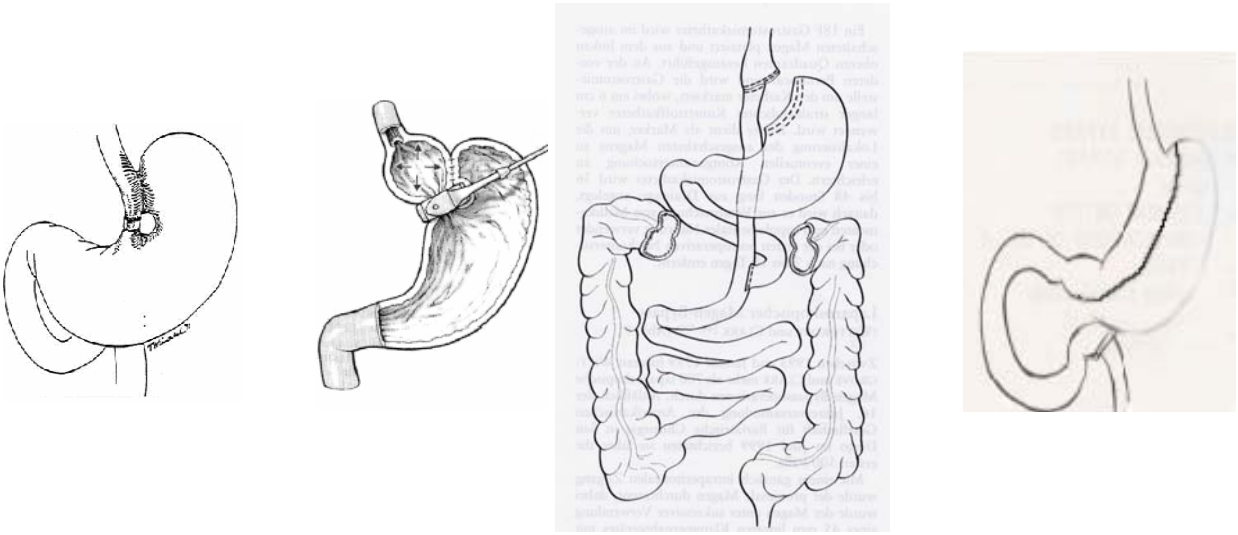
produced by adipose tissue [129]. There are two types of IL-6 receptors (IL-6R). One receptor type exists as membrane-bound form (~80 kDa), the other as soluble form (~50 kDa) [118]. Interestingly, mice with a disrupted IL-6 gene develop mature-onset obesity and reveal metabolic abnormalities. This phenotype could be reversed by administration of IL-6. Thus, IL-6 somehow protects from the conditions mentioned above rather than causing it and there seem to be a difference in the action of IL-6 in the periphery and the CNS [130].

Steroid hormones

Adipose tissue is able to metabolize sex steroids and glucocorticoids. However, they are both not *de novo* synthesized by the adipocytes. Whereas estrogens secreted from the adipocytes stimulate adipogenesis in the subcutaneous tissue, androgens promote central obesity, which is associated with development of insulin resistance and diabetes type 2, dyslipidemia, hypertension and coronary artery diseases [71].

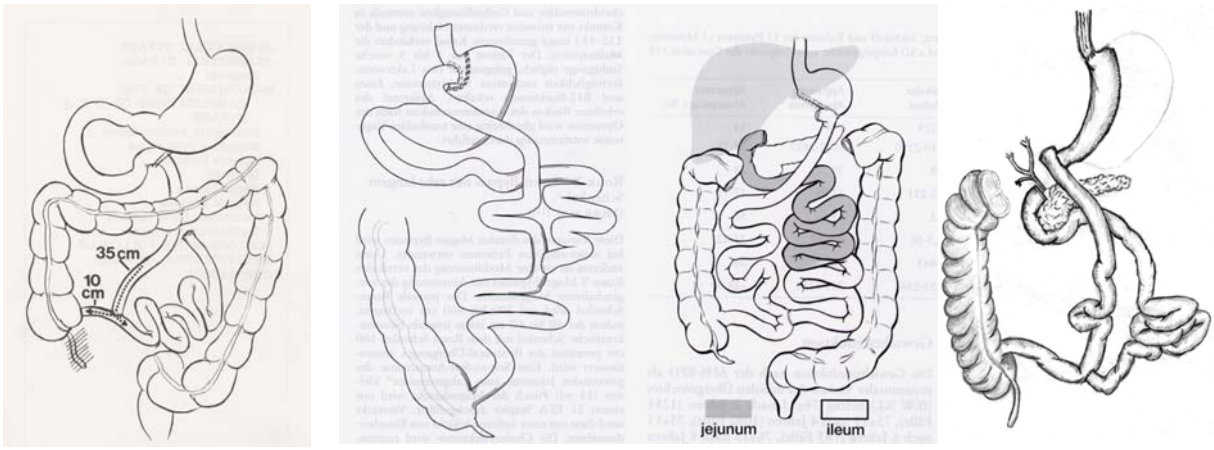
Bariatric surgery

Obesity is reaching epidemic proportions in the developed world. In morbidly obese patients only surgical treatment (bariatric operations) leads to a sustained weight loss (Fig. 12) and cure of co-morbidities in the majority of patients. There exist a number of different operations resulting in either a restrictive and/or malabsorptive effect, accompanied by a humoral effect which is caused by changes of the different gastrointestinal hormones. Bariatric surgery is a rapidly growing discipline throughout the western world, with increasing number of interventions performed every year (reviewed in [131]). There exist various types of operations developed in the past 50 years. Purely restrictive operations lead to a reduction of food intake by a small gastric pouch (gastroplasty (Fig. 10a), gastric banding (Fig. 10b), sleeve-gastrectomy (Fig. 10d)). Other interventions combine this effect with a malabsorption of micronutrients (proximal gastric bypass (Fig. 10c)) and/or macronutrients (distal gastric bypass, bilio-pancreatic diversion (BPD) (Fig. 11c,d)). Purely malabsorptive procedures, such as the jejuno-ileal bypass, have been abandoned due to severe side effects.



a b c d

Fig. 10. Mainly restrictive bariatric procedures: a: vertical banded gastroplasty, b: gastric banding, c: proximal Roux-Y-gastric bypass, d: sleeve-gastrectomy.



a b c d

Fig. 11: Mainly malabsorptive bariatric procedures: a: jejuno-ileal bypass, b: distal gastric bypass, c: bilio-pancreatic diversion with horizontal gastrectomy (Scopinaro [132]), d: bilio-pancreatic diversion duodenal switch (BPD/DS) with sleeve-gastrectomy (Marceau [133]).

Besides restriction and/or malabsorption, these operations appear to induce also humoral mechanisms affecting weight loss and especially diabetes by changing the secretion of gastrointestinal hormones. That this may depend on the type of operation is demonstrated by the observation that in bypass procedures diabetes is better cured compared to purely restrictive operations, even before weight loss begins. Although in the past few years considerable progress

has been made in our understanding on how the brain regulates energy homeostasis in response to hormonal signals from the adipose tissue and the gastrointestinal tract (recently reviewed by Stanley et al [116]), there is only scattered knowledge on the role of the different gastrointestinal hormones known to affect food intake in pathophysiological situations in general and after bariatric surgery in particular. For example, the reports on changes of PYY and ghrelin levels after Roux-Y gastric bypass surgery, vertical banded gastroplasty or gastric banding demonstrate differences between the different types of surgery but the published observations from different studies are partly controversial [134]. At present, there is no detailed study simultaneously analyzing all the different gastrointestinal hormones before and after bariatric surgery in a systematic way and with the same patient group.

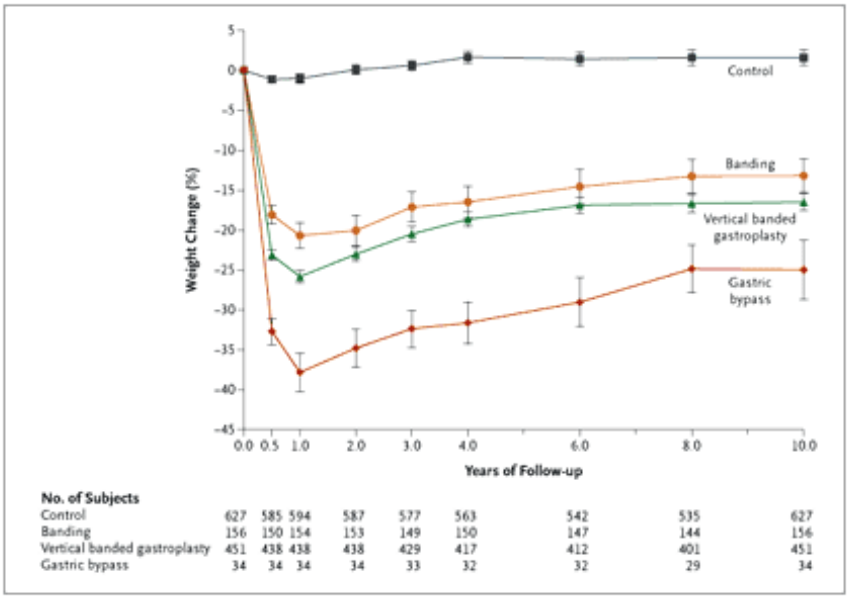


Fig. 12. Weight changes among subjects participating in the Swedish Obese Subjects (SOS) study over a 10-year period. From Sjöström et al, 2004 [135].

Aim of the thesis

The melanocortin receptors have a broad distribution pattern and exert various important different physiological functions. Recently, the interest in the melanocortins and its receptors lighted up when it was discovered that MC4-R, expressed in the master center of appetite control, plays a pivotal role in the development of human obesity. In fact, 1-6% of the obese individuals carry a mutated MC4-R gene. Furthermore, preliminary data suggested a correlation of MC4-R mutations/polymorphisms with the emerging of complications after gastric banding operation. Since α-MSH is also found in the circulation, there might be an integrated control regulation system of food intake and metabolism by the melanocortins, thereby, linking the central control center (brain) with the periphery (adipose tissue). However, the expression of the five melanocortin receptors and their potential physiological role in human adipose tissue has never been clearly elucidated. Over recent years, many findings suggested that obesity represents a chronic mild inflammation, leading to the known co-morbidities associated with obesity. Besides adipocytes, adipose tissue consists of stromovascular and immune cells, including macrophages. To date, it is still controversial, which cytokine is produced in adipocytes or in other cell types (i.e. macrophages).

The aims of the thesis can be summarized as follows:

1. Sequencing of the complete MC4-R gene in obese patients that underwent gastric banding operation and developed complications or did not lose enough weight. Do these patients exhibit a higher mutation rate and can the pre-operational sequencing of the MC4-R gene be used as a prediction marker for a successful outcome of the gastric banding operation?

2. Evaluation of the gene expression of the five melanocortin receptor subtypes and several obesity-related genes in human subcutaneous compared to omental adipose tissue in morbidly obese patients versus normal weight subjects.

3. Elucidation of the protein expression, functionality and function of the melanocortin receptor expressed in human adipose tissue, adipocytes and precursor cells of adipocytes.

4. Analyzing the expression and secretion of the obesity-related cytokines TNF-α, IL-6, IL-8 and IL-10 in human adipose tissue and *in vitro* differentiated adipocytes. Investigation of biological effects of exogenous administered cytokines, which would imply the presence of a cross-talk between adipocytes and non-fat cells in the adipose tissue.

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Chapter 2: Melanocortin-4 receptor gene and complications after gastric banding[1]

Abstract

Objective: Mutations of the melanocortin-4 receptor (MC4-R) gene are associated with up to 5.8% of monogenetic causes of obesity. Correlations between defects in MC4-R and complications after laparoscopic gastric banding have recently been reported. The aim of this study was to investigate whether in our patient population band-associated complications can be correlated with MC4-R defects, which in turn could be a contraindication for gastric banding.

Methods: Of 370 morbidly obese patients operated between December 1996 and May 2004 with laparoscopic gastric banding (LapBand®), 37 required re-operation, by re-banding or bilio-pancreatic diversion duodenal switch (BPD/DS) for band-associated complications. Genomic DNA was extracted from leukocytes of these 37 patients using standard methods. The entire MC4-R gene was amplified by PCR and sequenced on an ABI Prism 3100 automated DNA sequencer. Any detected mutation or polymorphism was verified utilizing a high-fidelity proofreading polymerase.

Results: No mutation was seen in 35 patients (95%). The polymorphism Ile251Leu (A1144C) which was found in one patient is known not to be associated with obesity. A silent mutation Ile198Ile (C594T) was found in another patient. Based on published data, ~12 patients of the 37 would have been expected to carry one of the known obesity-associated MC4-R mutations, but none of these was found.

Conclusion: In the patient group of the St. Claraspital, we could not confirm the observation previously published that MC4-R defects are associated with a higher complication rate following laparoscopic gastric banding. Thus, we do not recommend general screening for MC4-R defects routinely before laparoscopic gastric banding operation.

[1] Data of this chapter are published in: Peterli R, Peters T, von Flue M, Hoch M, Eberle AN 2006 Melanocortin-4 receptor gene and complications after gastric banding. Obes Surg 16:189-95

Introduction

The homeostatic circuit controlling energy balance involves a number of different neuropeptides and receptors [1]. A prominent member is the melanocortin system, now known to play a crucial role in the regulation of the hypothalamic appetite-control centers [2]. The nutritional state of an organism is signaled to the hypothalamus by adipocyte-derived leptin, which stimulates POMC neurons in the arcuate nucleus and triggers the release of α-MSH. This POMC-derived peptide activates MC4-R in the paraventricular nucleus and the lateral hypothalamic area/parafornical area, thus mediating the anorectic properties of α-MSH [3]. Leptin also inhibits the production of orexigenic agouti gene-related protein (AgRP) which, like agouti protein, is an inverse agonist of α-MSH blocking the MC4-R [3, 4]. In animal models, lack of α-MSH, overexpression of AgRP or functional defects of the MC4-R gene all lead to obesity [2, 3, 5]. In man, defects in the POMC gene resulting in complete lack of α-MSH are rare, but several cases with very early-onset obesity have been reported [6, 7]. More frequent are mutations in the MC4-R gene that may also lead to the development of obesity [8-10]. Recent reports associate MC4-R mutations with up to 5.8% of the monogenetic causes of obesity [11].

The laparoscopic adjustable gastric banding with the LapBand® is the least invasive, and fully reversible surgical procedure for the treatment of morbid obesity. It is effective, and early complications are very rare [12-14]. However, at a later stage after the gastric banding operation, a number of patients require re-operation. Preoperative parameters for patients with unfavorable outcome (insufficient weight loss, complications) would be very helpful, but could not yet be defined. Patients who undergo restrictive bariatric procedures, such as the gastric banding, will only reduce food intake and consequently lose weight when satiety signals are generated efficiently. Any defect in the correct signaling of satiety, e.g. mutations in the MC4-R gene [15], may represent a cause of gastric banding failure or complications. In a recent study analyzing 300 patients following gastric banding, patients with MC4-R mutations had significantly more band-associated complications as compared to patients without MC4-R mutations [16].

In this study we investigated whether band-associated complications in patients who underwent gastric banding can be associated with MC4-R mutations and, hence, whether defects in the MC4-R gene may represent a contraindication for this operation.

Gastric banding surgery and its impact on appetite hormones

Adjustable gastric banding is a strictly restrictive technique. Its principle is to limit the food intake volume (approximately a 20-ml pouch). The banding complicates the emptying of the remaining stomach. However, with this operation the absorption surface of the digestive tubes remains intact. According to recent data, patients lose 47.5% of their excess body weight after gastric banding additionally most of the co-morbidities are improved [17]. However, long-term studies suggest a gradual weight regain and return of co-morbidities [18]. The effects of the gastric banding on various appetite hormones are partly controversial (reviewed in Diniz et al [19]). Like in diet-induced weight loss and other bariatric surgeries, leptin levels decrease after gastric banding. Most of the studies reported an increase in ghrelin levels following gastric banding surgery [20, 21]. However, Mariani et al [21] reported only a transient increase in ghrelin plasma levels, suggesting a role in weight regain following this procedure. It could be demonstrated that gastric banding increases the basal and postprandial GLP-1 levels. In contrast, no change on circulating PYY levels were reported [22]. Nutrient deficiencies after gastric banding surgery are rare since it does not have a malabsorptive component [23].

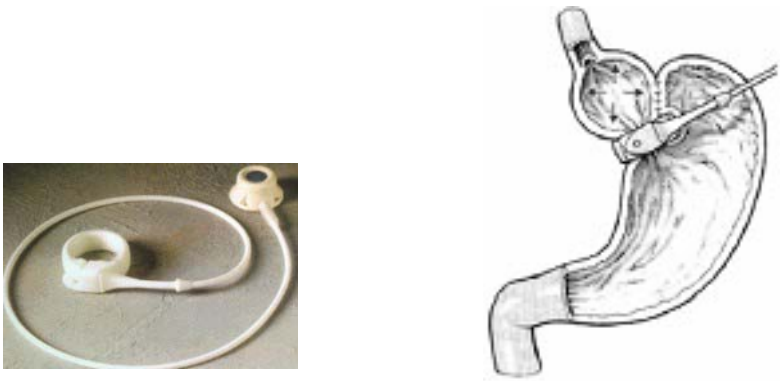

Fig. 1. The LapBand® system.

Mutations/polymorphisms in the MC4-R gene

The MC4-R gene is an intronless gene, located on the chromosome 18q22 encoding a 332 amino acid G protein-coupled receptor protein. It is expressed throughout the brain, particularly in the hypothalamus implicated in appetite and body weight regulation [24]. Like for all melanocortin receptors, binding of MC4-R leads to stimulation of adenylyl cyclase promoting the formation of intracellular cAMP [25]. Maybe other, by now unknown, signaling pathways are involved in MC4-R signaling [26]. Whereas the melanocortin α-MSH, a peptide cleaved from the precursor POMC, was shown to be the endogenous MC4-R agonist, AgRP, like agouti protein, proved to be

a potent endogenous MC4-R inverse antagonist [27]. α-MSH decreases appetite and increases energy expenditure (in mice but not in human), via binding to MC4-R expressed in the arcuate nucleus in the hypothalamus. To date, the downstream signal cascade of MC4-R stimulation is still not clearly elucidated. Shinyama et al [28] reported that in HEK-293 and GT1-7 transfected cells, MC4-R undergoes desensitization and internalization in response to agonist. Very recently, bioluminescence energy transfer analyses of transfected HEK-293 cells suggested that MC4 receptors exist as constitutive homodimer, which is interestingly not regulated by interaction with α-MSH and AgRP [29]. Whether this observation is also true for MC4-R expressed in neurons (and at lower numbers) awaits clarification.

It was shown that MC4-R deficiency leads to increased appetite, height, lean mass and bone mineral density and hyperinsulinemia [11]. To date, in human more than 60 MC4-R mutations have been discovered and, depending on the study, such mutations were found in 1 to 6% of morbidly obese adults or children with early onset obesity. It is of note that the mutation rate is clearly dependent on the ethnic composition of the population samples [10, 11, 30-39]. Thus, MC4-R mutations represent the most common monogenetic cause of obesity so far reported. It is estimated that carriers of functionally relevant MC4-R gene mutation have a 4.5-fold higher risk of becoming obese relative to non-carriers [40]. Very recently, Lubrano-Berthelier et al [41] found very similar MC4-R mutation rates in patients developing obesity in childhood (2.83%) compared to patients with a later onset of the disease (2.35%). Most of the patients carry heterozygous missense mutations [42]. Noteworthy, homozygosity leads to a more severe obesity phenotype [11].

MC4-R mutations occur throughout the coding sequence. Though, the third cytoplasmatic loop of the receptor, which couples directly to G_s seems to have fewer functional relevant mutations. It was shown by Schiöth et al [43] that N-terminal part of the MC4-R is not important for ligand binding. By now no mutation of the MC4-R promoter region has been described to be associated with obesity [44]. There are four different classes of mutations proposed by Tao and Segaloff [45] and nicely reviewed and adapted by MacKenzie [25]. Class 1 mutations result in truncated non-functional receptors due to missense substitutions or frameshifts. Another type of disruption leads to partial or complete intracellular retention of the protein (class 2), which leads to impaired cell surface expression. In class 3 mutations the ligand binding of MC4-R or its signal transduction is altered, including alterations of basal receptor activity. The last class (class 4) did not show any alteration or remains to be investigated (Table 1).

Table 1. MC4-R mutations and mutation classes.

Mutation	*Class*	*Reference*
Thr5Thr	4 ND	[36]
Arg7His	3	[46]
Thr11Ser	3	[32]
Thr11Ala	3 ND	[36]
16:ins 'G'	1	[32]
Trp16Stop	1	[37]
Arg18Cys	3	[32]
Arg18His	3	[47]
Arg18Leu	3	[47]
Ser30Phe/Gly252Ser	2,3	[10, 26, 48]
Tyr35Stop/Asp37Val	1	[10]
Pro48Ser	4	[45]
Val50Met	4	[39, 45]
Phe51Leu	4 ND	[36]
57:del 'C'	1	[31]
Ser58Cys	2	[39, 45, 48]
Asn62Ser	3	[38]
Pro78Leu	2	[26, 45, 48]
88:del 'GTGGCTG ATATGCTG	3	[49]
Asp90Asn	3	[15]
Asn97Asp	3	[38]
Gly98Arg	2	[45]
Ile102Ser	2,3	[48]
Ile102Thr	4 ND	[31]
Val103Ile	4	[50, 51]
Leu106Pro	3	[38]
112:ins 'A'	1	[38]
Thr112Met	2,4	[26, 50]
Ile125Lys	2	[38]
Ser127Leu	3	[48]
Ile137Thr	3	[50]
Thr150Ile	3	[32]
Tyr157Ser	3	[45]
Arg165Gln	2,3	[26]
Arg165Trp	2	[26, 48]
Ile170Val	2,3	[32]
Ala175Thr	3	[38]
Met200Val	4 ND	[36]
Phe202Leu	4 ND	[31]
211:del 'CTCT'	1	[8]
Ile226Thr	4	[35]
Pro230Leu	3	[40]
Asn240Ser	4 ND	[31]
Ala244Glu	4	[48]
246:ins 'GATT'	1	[9]
250:del 'GA'	1	[52]

Leu250Gln	3	[26, 32]
Ile251Leu	4	[32]
Ser30Phe/Gly252Ser	2,3	[10, 26, 48]
Val253Ile	4	[48]
Cys271Tyr	2	[38, 48]
Asn274Ser	4 ND	[30]
278:ins 'GT'	1	[53]
Tyr287Stop	2	[38]
Pro299His	2	[48]
Ile301Thr	3	[32]
Ile316Ser	3	[38]
Ile317Thr	2	[26, 48, 54]

Class 1: Truncated, non-functional receptor. Class 2: Intracellular retention of the receptor. Class 3: Alteration in receptor binding, signaling, gain-of-function mutation. Class 4: No alteration or not yet determined (ND). From MacKenzie et al, 2006 [25].

A decreased constitutive activity was found to be the most common defect in obesity-associated MC4-R mutations in adults. The MC4-R missense variants Val103Ile and Ile251Leu are the most common polymorphisms [42, 53] and are not associated with obesity since they are found at similar frequencies among obese and normal-weight subjects. It was demonstrated that 80% of childhood obesity-associated MC4-R variants are partially or totally intracellularly retained (class 2) [48]. Decreased cell surface expression may therefore be an important mechanism underlying the increased risk for obesity of individuals carrying a MC4-R mutation, also supported by another study [26]. Recently, Boston et al and Potoczna et al [16, 36] suggested that all MC4-R mutation carriers are binge eaters. However, this finding was contradicted by other studies [41, 55, 56].

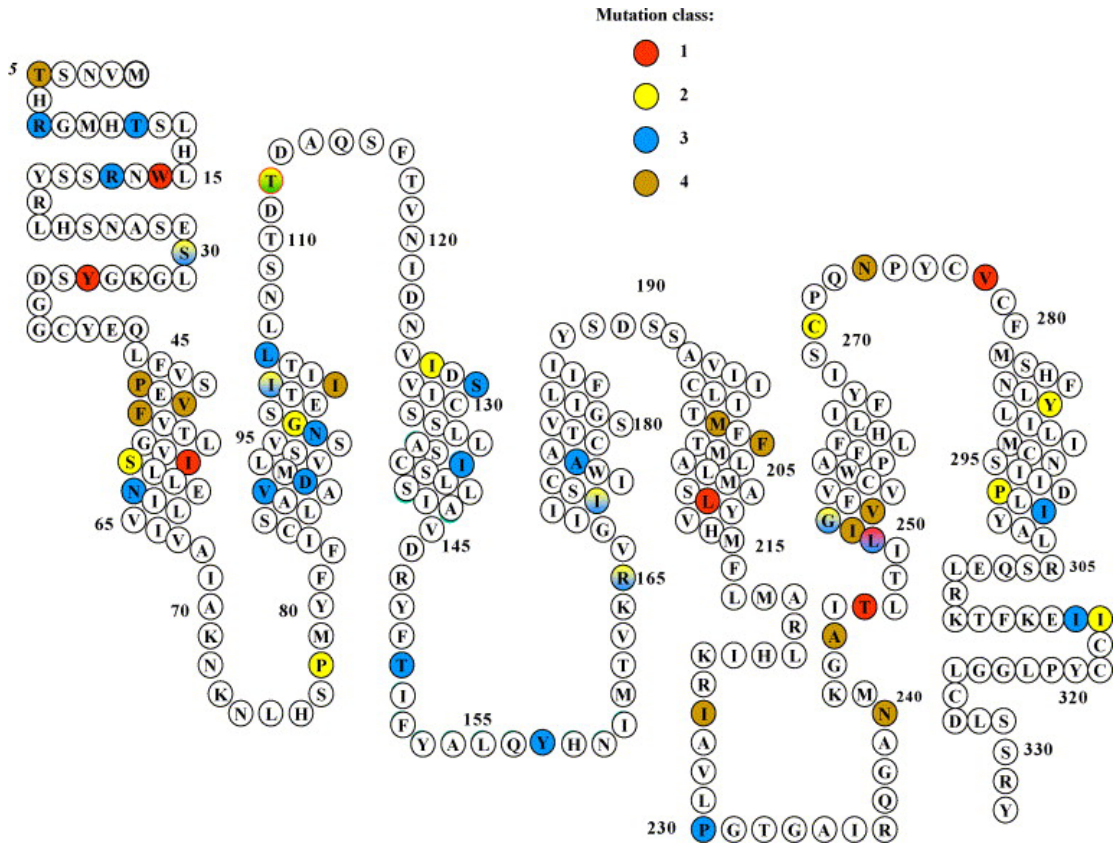

Fig. 2. MC4-R mutations. Amino acid residues are color-coded according to the mutation effects on MC4-R function. *Class 1 mutations*: truncated non-functional receptors due to missense substitutions or frameshifts. *Class 2 mutations*: partial or complete intracellular retention of an apparent full-length protein. *Class 3 mutations*: effects on ligand binding or signal transduction including alterations of basal activity. *Class 4 mutations*: no impairment or remain not yet tested (*Class 4 ND*). Mutations of more than one class are multi-colored. From MacKenzie et al, 2006 [25].

To find a reliable marker for the outcome of the gastric banding surgery is of great economical importance. Potoczna et al [16] reported that patients carrying a MC4-R mutation needed more often a re-operation after a gastric banding operation than non-carriers. In this study we wanted to investigate, whether in our patient group of the St. Claraspital (n=300) band-associated complications are associated with MC4-R mutations and, hence, whether defects in the MC4-R gene may represent a contraindication for this surgery.

Material and Methods

Subjects

Between December 1996 and March 2004 a total of 370 morbidly obese patients were operated by laparoscopic gastric banding (LapBand®, INAMED, Santa Barbara, CA). Their average weight was 125 (87-250) kg, BMI 44.6 (35-75) kg/m^2, age 41 (18-66) years. We selected patients requiring re-operation for band associated complications, such as pouch enlargement, esophageal motility disorder, secondary band intolerance or lack of satiety with insufficient weight loss, for genetic analysis.

Genomic DNA extraction

Genomic DNA was extracted from leukocytes using a method described by Miller et al with some minor modifications [57]. Briefly, 3 ml of whole blood collected in EDTA vials were mixed with 15 ml erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.4) and kept on ice for 30 min. The lysate was centrifuged at 2000 rpm for 10 min and the pellet washed twice with SE buffer (75 mM NaCl, 25 mM Na_2EDTA; pH 8.0). The pellet was resuspended in SE buffer containing 400 µg/ml proteinase K and 1% SDS and incubated at 37°C for 16 h. Then 3 ml of 3 M NaCl was added, vortexed vigorously and centrifuged at 3500 rpm for 10 min. The supernatant containing the DNA was carefully transferred to a fresh 50 ml Falcon tube and centrifuged again. This step was repeated twice, yielding a total clear supernatant free of cellular proteins. The DNA was precipitated with ice-cold ethanol and the resulting pellet was washed with ethanol, briefly dried, and resuspended in 300 µl DNase-free water.

Direct nucleotide sequencing of the MC4-R gene

The entire MC4-R gene was amplified by PCR using two primers described by Farooqi et al [53]. Approximately 1 µg of genomic DNA was amplified in a volume of 50 µl, containing 1x HotStarTaq master mix (Qiagen, Basel, Switzerland) and 0.5 µM forward and reverse primer. The reaction was carried out under standard conditions and consisted of an initial incubation at 95°C for 15 min, followed by 35 cycles at 94°C for 30 s, 60° C for 30 s and 72°C for 1 min, and one final extension at 72°C for 10 min. In order to check the integrity of the PCR products they were subjected to electrophoresis on 1% agarose gel. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Basel, Switzerland).

The resulting PCR product was sequenced on both strands using six nested primers, as described by Farooqi et al [53]. Big Dye terminator chemistry v3.0 (Applied Biosystems, Foster City,

California) was employed and then electrophoresed on an ABI Prism 3100 automated DNA sequencer. Any detected mutation or polymorphism was verified utilizing the high-fidelity proofreading ProofStart polymerase (Qiagen, Basel, Switzerland). The sequences were analyzed using Sequencher (GeneCodes, Ann Arbor, Michigan).

Results

Of the 370 patients originally operated, 37 needed a re-operation after an average of 32 (3-68) months due to band-associated complications (Table 2). Compared to the original population, the re-operated patients were younger (37 vs. 42 years, $P < 0.007$), less heavy (BMI 42.5 vs. 44.5, $P < 0.04$), and more often females (92% vs. 78%, $P < 0.05$). The frequency of binge eating disorders was approximately the same (43% of re-operated patients vs. 48% in the original population, $P = 0.53$).

In 57% of the patients, the first re-operation was a laparoscopic re-gastric banding due to slippage. In 35%, biliopancreatic diversion/duodenal switch (BPD/DS), and the remaining 8% of the patients requested that the band be laparoscopically removed. The reasons were secondary band intolerance (27% of re-operated patients, of which 30% in addition had esophageal motility disorder), or lack of satiety with insufficient weight loss or weight regain (Table 2). Re-operations for minor complications, i.e. port and/or tube disconnection, dislocation, or leaks were required in 7% of all patients. Of the re-operated patients, the total follow-up time was 65 (34-93) months after the primary operation. The percent excess weight loss (%EWL) was 66% (-7 to 113%), corresponding to an average final BMI of 30.1 (21.4 - 46.1) kg/m^2. One patient, after laparoscopic band removal without any further bariatric operation, regained weight exceeding the preoperative weight (%EWL: -7%).

Genetic analysis revealed that 35 patients (95%) showed no mutation in the MC4-R gene (Table 3). Only the polymorphism Ile251Leu (A1144C) was found in one patient (patient number 29). This polymorphism had previously been shown to be fully functional [53]. One silent mutation Ile198Ile (C594T) was found in patient number 5 (Table 3, Fig. 3). The clinical data of these patients, however, did not differ from those with wild type MC4-R (Table 2).

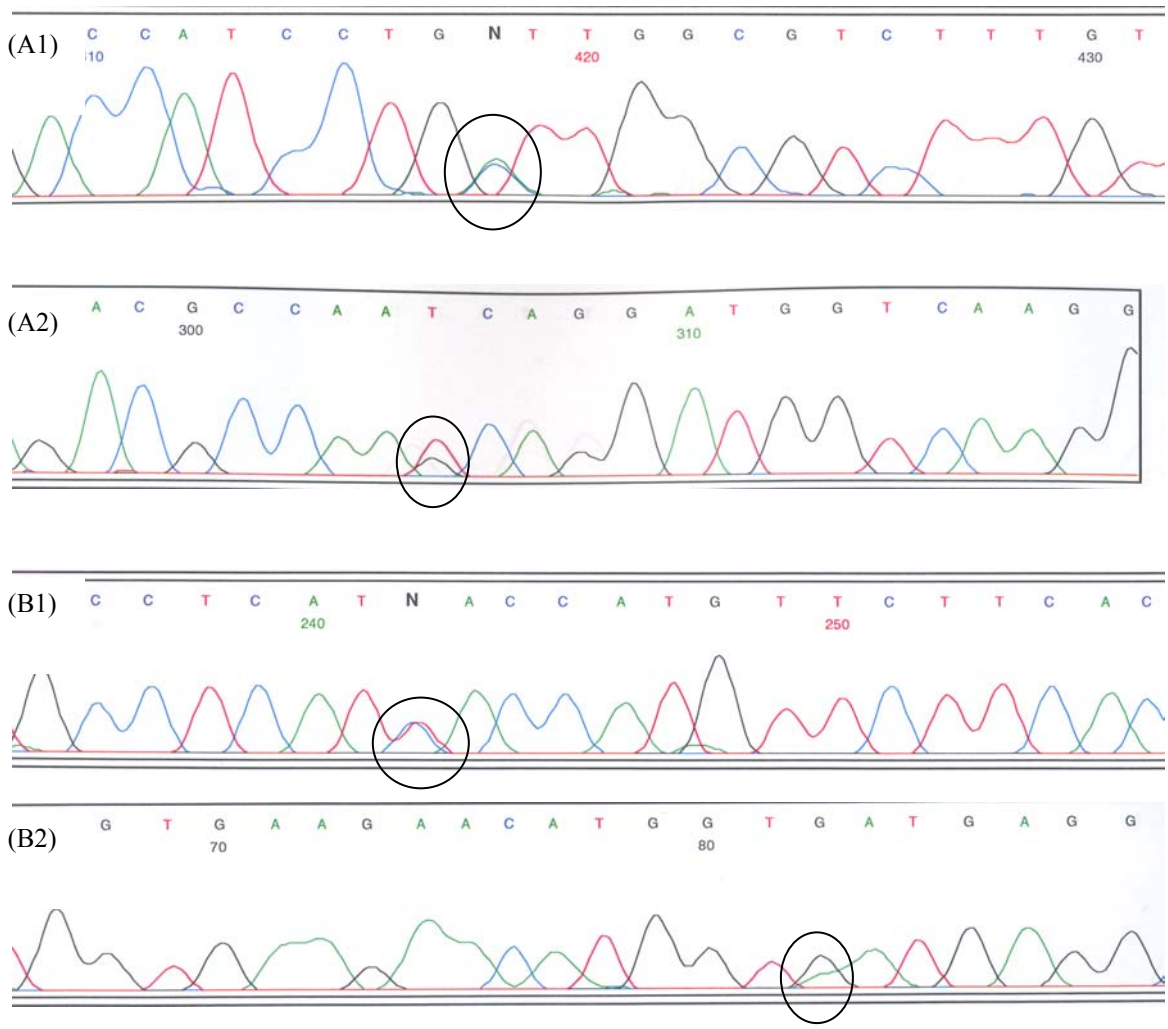

Fig. 3. Electropherogram of the two mutated MC4-R sequences. (A1) Forward sequencing of patient number 29. Nucleotid at position 418 could be an A or C. (A2) Reverse sequencing of patient number 29. Nucleotid at position 305 could be a T or G. Both mutations are at the same position of the MC4-R gene (A1144C, Ile251Leu, polymorphism). (B1) Forward sequencing of patient number 5. Nucleotid at position 242 could be C or T. (B2) Reverse sequencing of patient number 5. Nucleotid at position 82 could be a G or A. Both mutations are at the same position of the MC4-R gene (C594T, Ile198Ile, heterozygous silent mutation).

Table 2: Clinical data of the re-operated patients.

Primary Lap-Band				Co-morbidity							Binge eating
Pat#	Gender	Age	BMI0	Cardiovascular	Diabetes	Hyperlipidemia	Hyperuricemia	Sleep apnea	Joint/back pain	Gastroesophageal reflux	
1	F	37	55.1				+		+		+
2	F	36	43.7			+	+		+		
3	M	37	38.7	+					+		
4	F	40	38.9						+	+	
5	F	22	43.9	+					+	+	
6	M	31	41.9	+					+		
7	F	40	42.2		+				+		
8	F	38	41.8	+					+		
9	F	37	38.5	+					+	+	
10	F	37	48.4	+	+				+	+	+
11	F	28	40.0	+		+	+	+	+		
12	F	41	36.7	+					+		+
13	F	26	34.3			+			+		+
14	F	38	42.3	+					+	+	
15	F	30	41.2			+			+		
16	F	33	45.2	+					+		
17	F	52	39.5	+		+	+		+	+	+
18	F	27	36.7						+	+	+
19	F	48	43.3	+		+			+	+	+
20	F	33	41.2	+					+	+	
21	F	32	41.4	+			+		+	+	+
22	F	35	44.6						+	+	
23	F	37	44.7						+	+	+
24	F	37	40.5						+	+	+
25	F	34	45.9	+					+		
26	F	32	35.3						+		+
27	F	55	43.7	+					+		
28	F	29	43.6						+		+
29	F	46	36.3						+	+	+
30	F	38	51.1	+					+	+	
31	F	35	46.9	+				+	+		+
32	F	37	48.1	+				+	+	+	
33	F	42	47.9	+				+	+	+	+
34	F	37	40.5	+		+			+	+	
35	F	54	48.8						+		
36	F	29	44.4	+		+		+	+		+
37	M	43	34.3	+	+	+	+		+	+	
Mean		37	42.5								
Min		22	34.3								
Max		55	55.1								
Stdev		7	4.7								

Table 3: Re-operations and MC4-R status.

Pat #	1st reoperation				2nd reoperation				at last follow-up			
	What *	Dg †	Time	BMI1	What *	Dg †	Time II	BMI2	Time ‡	BMIact	%EWL §	MC4Rec **
1	Reban	1	32	34.2	BPD	1	5	33.8	70	34.9	65	wt
2	Reban	1	10	36.2	BPD	2,3	75	41.5	88	31.2	64	wt
3	Reban	1	13	29.6	BPD	2,3,4	33	35.3	58	22.8	113	wt
4	Reban	1	37	30.1					66	33.8	34	wt
5	Reban	1	19	31.2	BPD	2,4	40	35.8	70	23.7	102	SM
6	Reban	1	12	28.9					34	28.9	76	wt
7	Reban	1	50	33.8	BPD	2,3	37	36.7	90	27.5	80	wt
8	Reban	1	27	31.3					38	31.6	55	wt
9	Reban	1	12	33.8					88	35.8	19	wt
10	Reban	1	25	41.6					53	45.9	10	wt
11	Reban	1	8	29.0					53	30.5	59	wt
12	Reban	1	3	32.7					55	28.7	63	wt
13	Reban	1	10	30.8	BPD	3,4	83	32.2	93	32.2	21	wt
14	Reban	1	37	27.9	BPD	2,3,4	20	32.1	69	21.4	109	wt
15	Reban	1	38	32.5					41	30.7	56	wt
16	Reban	1	63	26.0					75	26.0	89	wt
17	Reban	1	14	30.1					86	30.5	56	wt
18	Reban	1	26	27.3					73	29.4	58	wt
19	Reban	1	49	30.1					50	32.0	58	wt
20	Reban	1	26	26.9	BPD	2,3,4	8	35.9	76	32.6	50	wt
21	Reban	1	19	25.2	BPD	2,3,4	17	37.6	78	32.7	49	wt
22	B'rem	2	11	31.8					38	46.1	-7	wt
23	B'rem	2	25	45.5	BPD	3	12	52.2	47	33.6	52	wt
24	B'rem	2,3	39	34.8	BPD	3	14	43.7	70	31.6	54	wt
25	BPD	2	50	37.4					69	25.7	94	wt
26	BPD	2,4	59	31.7					78	24.5	103	wt
27	BPD	2	47	38.1					69	25.2	90	wt
28	BPD	3	39	39.0					50	25.5	93	wt
29	BPD	2,4	44	33.2					74	26.3	83	PM
30	BPD	2	47	43.8					69	27.7	87	wt
31	BPD	3	27	44.0					45	24.5	87	wt
32	BPD	3	37	44.9					67	28.8	77	wt
33	BPD	3	18	48.6					75	42.7	22	wt
34	BPD	2	26	28.7					40	24.0	100	wt
35	BPD	2	68	36.3					70	29.4	79	wt
36	BPD	3	36	36.5					49	27.5	82	wt
37	BPD	2,4	65	34.9					85	29.7	48	wt
Mean			31.6	34.0			28.8	37.3	64.8	30.2	65.7	
Min			3	25.2			5.0	26.8	34	21.4	-7	
Max			68	48.6			82.6	52.2	92	46.1	113	
Stdev			17.4	5.9			25.0	6.4	16.4	5.7	28.8	

* reban = rebanding, b'rem = laparoscopic band removal, BPD = bilio-pancreatic diversion duodenal switch
† reason for re-operation: 1=pouch dilatation/slippage, 2=band intolerance, 3=lack of satiety with insufficient weight loss, 4=esophageal motility disorder
‡ time since last operation in months
§ EWL = % excessive weight loss – [(operative weight – follow-up weight)] / [operative excess weight] x 100.
II follow-up time since primary operation (months)
** wt = wild type, SM = silent mutation, PM = polymorphism

Discussion

Laparoscopic gastric banding is a safe and effective bariatric procedure for the treatment of morbid obesity, with very low early morbidity and an average loss of 50-60% of excessive weight 5 years postoperatively and, as a consequence, a reduction in obesity-associated co-morbidity [12-14]. Re-operations may be more frequent compared to other bariatric operations but, usually, can be performed again with little morbidity [13, 58].

Nevertheless, preoperative selection of patients through identification of specific parameters that indicate a higher risk of band-associated complications would be of great importance. Because satiety plays a major role in the functioning of all bariatric procedures, in particular after laparoscopic gastric banding, pathologies in the satiety signaling by the melanocortins could represent such a parameter. MC4-R defects are the most frequent monogenetic cause of obesity, with a prevalence of up to 5.8% in obese patients [11], and, therefore, carriers with an MC4-R mutation could be at higher risk for failure of gastric banding. Indeed, in a series of 300 gastric banding patients 6.3% were MC4-R mutation carriers [16]. These patients were all binge eaters, lost less weight and had five-fold more gastric complications than non-carriers. Outcome was poorest in MC4-R variant carriers, better in non-carriers with binge eating disorder and best in non-carriers without binge eating disorder [16, 36]. In this series of 300 gastric banding patients, the actual number of patients requiring re-operation was 33 of which 11 (33%) were carriers of a MC4-R mutation [16].

In our study, we have restricted the MC4-R sequencing to patients with complications following gastric banding, as the likelihood to find MC4-R mutations in this patient group was expected to be five-fold higher than in patients without complications (see above). From our 370 gastric banding patients, 37 patients developed band-associated complications; both the total number of patients and the number of complications compare well with those of the study published by Potoczna et al [16]. By contrast, in our study 35 of the re-operated patients (95%) showed no MC4-R mutation. We only found one polymorphism Ile251Leu (A1144C), which is known not to be associated with obesity [32], and a novel silent mutation Ile198Ile (C594T). Very recently, a silent mutation Ile198Ile (C593T) was described [41]. Since at nucleotide position 593 of the MC4-R gene there is a T instead of a C, this obviously seemed to be a mistake made by the authors. This was personally confirmed by the senior author Prof. Christian Vaisse via e-mail after having contacted him. Thus, in fact this group found the same silent mutation as we did. Whether this silent mutation may lead to an altered MC4-R expression, as has been demonstrated for "silent" mutations of other receptors genes and mRNA [59], is not yet known. Nevertheless, our

results are in contrast to the published data since the MC4-R gene of at least 36 of the 37 patients with band-associated complications coded for a fully functional receptor, whereas approximately 12 carriers of a MC4-R mutation would have been expected [16].

Although the number of re-operated patients in both the published study (n=33) [16] and in this study (n=37) was not large enough for a precise statistical analysis, we believe that the data are clear enough for the conclusion that sequencing of the MC4-R gene of all bariatric patients prior to gastric banding is of little value for the prediction of complications. It appears that other feedback mechanisms were not functioning properly in the patients of our group, which will be the focus of future studies.

At the St. Claraspital a "two-stage-concept" was followed in the treatment of morbid obesity with gastric banding (Lap-Band®) being the primary operation in all patients followed by BPD/DS in cases of failure [58, 60]. Thus, patients with binge eating disorder were also primarily treated with gastric banding. The frequency of binge eating disorder did not differ between the re-operated patients compared to the original population (43% vs. 48% respectively). Band-associated complications (slippage, concentric pouch dilatation, esophageal motility disorder or band intolerance) seem to occur more often in patients with binge eating disorder. It is speculated that these patients do not generate a proper signal from the periphery and, consequently, overfill their pouch above the band that may provoke these complications, possibly aggravated by additional structural weakness of the stomach wall or insufficient propulsive motility of the esophagus [16, 36, 61, 62]. In line with the literature, the surgeons of St. Claraspital have abandoned gastric banding as routine primary operation in patients who have binge eating disorder [63-65]. The surgeons now prefer a bypass procedure (either laparoscopic Roux-Y-gastric bypass or BPD/DS).

In conclusion, based on our data we could not confirm the observation that MC4-R defects are associated with a higher complication rate following gastric banding [16]. Therefore, we do not recommend general screening for MC4-R defects routinely prior to gastric banding.

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Chapter 3: Expression and localization of melanocortin-1 receptor in human adipose tissues of severely obese patients[2]

Abstract

Objective: The melanocortin system is a key regulator in the hypothalamus of energy intake and expenditure, frequently linked with obesity and apparently modulating sympathetic outflow to white adipose tissues. The role of the melanocortins within adipose tissues however is not entirely clear. This study was aimed at determining the quantitative expression of the five melanocortin receptors (MC1-R to MC5-R) in subcutaneous and omental fat of obese patients and non-obese subjects.

Methods: Expression of MC1-R to MC5-R, pro-opiomelanocortin (POMC), agouti signaling protein (ASIP), leptin, leptin receptor and uncoupling protein-1 (UCP-1) was investigated in human fat samples by quantitative RT-PCR. MC1-R expression was also studied in preadipocytes, adipocytes and monocytic THP-1 cells, and by immunohistochemical localization in adipose tissues.

Results: Notable expression was found for MC1-R whereas no mRNA for MC2-R and MC3-R was detected; MC4-R and MC-5-R mRNA was occasionally detectable but at very low levels. MC1-R mRNA in subcutaneous fat was increased in obese patients as compared to controls; omental fat of both groups had slightly higher MC1-R expression than subcutaneous fat and did not differ between patient groups. Immunohistochemical analysis of the MC1-R in adipose tissue sections showed that MC1-R expression was higher in macrophages but also present in adipocytes.

Conclusion: The expression of MC1-R and the lack of MC2-R in human adipose tissues indicate that the melanocortins may regulate cell proliferation and/or inflammatory signals rather than lipolysis. Also, the increased expression of MC1-R in subcutaneous fat of obese subjects may reflect one aspect of the pathophysiology of obesity.

[2] Data of this chapter are published in: Hoch M, Eberle AN, Wagner U, Bussmann C, Peters T, Peterli R 2007 Expression and localization of melanocortin-1 receptor in human adipose tissue of severely obese patients. Obesity (Silver Spring) 15:40-9

Introduction

White adipose tissue (WAT) serves as energy storage depot, produces and releases a wide range of adipokines (bioactive peptides and cytokines) which influence energy homeostasis, glucose and lipid metabolism, vascular homeostasis and immune responses [1, 2]. As obesity is now recognized as a state of mild inflammation [3], the expression of adipokines may differ between obese and non-obese subjects. Also, in obesity the adipose tissue function is frequently disturbed, as for instance lipolysis may be down-regulated, the sensitivity of lipolysis to insulin reduced, and the adipose tissue blood flow dysregulated. The most prominent member of the adipokines is leptin which is secreted into the bloodstream by white adipocytes and signals to the control center of body-weight regulation in the hypothalamic arcuate nucleus [4].

Leptin signaling in the brain is intimately associated with the activation of melanocortins, via activation of pro-opiomelanocortin (POMC)/CART neurons, causing the release of α-MSH, a POMC-derived peptide [4, 5]. Simultaneously, leptin inhibits neurons that secrete agouti gene-related protein (AgRP), the endogenous antagonist of α-MSH in the brain [6]. Both AgRP and α-MSH competitively interact with the MC4-R, the type 4 of the five melanocortin receptors, which mediates appetite-inhibiting signals [7]. Hence, at the level of the central nervous system, leptin and the melanocortin system play a pivotal role in the control of energy balance [4]. Defects of both leptin and POMC biosynthesis and secretion, or of leptin receptor function, or of MC4 receptor function may all lead to obesity in animals and man [8-10]. In fact, mutations in the MC4-R gene are the most common monogenic cause of obesity in humans with a prevalence of up to 5.8% in obese patients [11]. Mutations of the MC4-R have also been associated with the outcome of bariatric surgery [12] although in a study with our own patient group, we could not confirm these findings [13]. Finally, in addition to the MC4-R-mediated regulation of food intake, central melanocortins appear to modulate directly sympathetic outflow to WAT, resulting in changes in lipid mobilization, as recently demonstrated for the Siberian hamster [14].

Similar to MC4-R, the MC3-R is expressed mainly in the brain and also represents an important switch for energy homeostasis [15]. By contrast, the MC1-R is a peripheral melanocortin receptor found in many human tissues [16], with highest abundance in melanocytes and melanoma cells where it triggers α-MSH-induced melanogenesis [5, 17]. Lower levels of MC1-R occur in adipocytes [18], endothelial cells [19], macrophages and monocytes [20]. Through regulation of the latter, MSH is thought to mediate anti-inflammatory reactions. The MC2-R corresponds to the ACTH receptor, which is expressed in the adrenal cortex and exhibits high affinity for ACTH but relatively low affinity for α-MSH [21]. In rodents the MC2-R is also expressed on adipocytes and

plays an important role in lipolysis. In a recent study, Smith et al [22] demonstrated expression of MC2-R in subcutaneous human fat as well as human adipocytes and mesenchymal stem cells (MSCs); higher expression in the same tissues and cells were noted for MC1-R which was also found in preadipocytes and adipocytes [20]. These authors also demonstrated a functional role for MC1-R in the regulation of fat cell proliferation. The MC5-R is expressed in many peripheral tissues, including skin, a variety of exocrine glands and at very low levels in several brain regions [19]; its role in man is still unclear. The agouti signaling protein (ASIP), the human homolog of the rodent agouti protein [23], interacts with MC1-R as an inverse agonist of α-MSH [24], conferring yellow pigmentation [25]. ASIP is expressed in the adipose tissue and at lower levels in testis, ovary and heart [23]. In patients with type 2 diabetes, ASIP expression was found to be elevated [22].

As subcutaneous adipose tissue differs manifold from visceral adipose tissue, e.g. sensitivity to stimulation of lipolysis or secretion of hormones, and also fat depot-specific variation in mRNA expression [26-28], our study was based on the analysis of subcutaneous and omental adipose tissue from morbidly obese subjects compared to fat from normal-weight persons. Several regional differences in the mRNA expression and secretion of different proteins have been reported and are summarized in Table 1.

Protein	*Difference in subcutaneous as compared with visceral adipose tissue*	*References*
Leptin	mRNA ↑ / protein secretion ↑	[29-31]
PAI-1	Controversial	[29]
TNF-α	no differences in three out of four reports	[27, 29]
Angiotensinogen	mRNA ↑	[29, 32]
ASP	mRNA ↓ of the precursor adipsin	[27, 29, 32]
UCP-1	mRNA ↓	[33]
IL-6	protein secretion ↓	[29, 31, 34]
Visfatin	no differences in mRNA expression / protein secretion ↓	[35]

Table 1. Regional differences in mRNA expression and protein production by human adipose tissue.

To date, it is still controversial which MC receptors are expressed in human adipose tissue as no quantitative analysis has been performed. Also, no report exists whether MC receptor expression differs between normal-weight and obese persons. Therefore, the present study was conducted to investigate the mRNA expression profile of the five melanocortin receptors and their ligands (precursors) POMC and ASIP as well as three additional obesity-related genes, leptin, leptin receptor and uncoupling protein-1 (UCP-1). The latter plays an important role in energy homeostasis in rodents by dissipating energy through heat in brown adipose tissue (BAT). In humans UCP-1 is also expressed in human WAT [36]. Finally, we studied the localization of MC1-R in human adipose tissue sections.

Material and Methods

Subjects and adipose tissue sample preparation

The study was approved by the local ethics commission. All subjects gave their written consent to participate in the study. Abdominal subcutaneous and omental adipose tissue samples of about 5 grams were obtained from 22 morbidly obese patients undergoing bariatric surgery following a two-stage-therapy concept with primary laparoscopic gastric banding followed by bilio-pancreatic diversion duodenal switch (BPD/DS) in case of failure [37, 38]. The mean BMI of these patients was 42.3 ± 7.3 kg/m^2. The clinical characteristics of the subjects are shown in Table 2. As control, abdominal subcutaneous and omental fat samples were taken from 9 subjects. The control group had a mean BMI of 22.4 ± 2.5 kg/m^2. Immediately after surgical removal of the tissue, the samples were frozen in liquid nitrogen and stored at -75° C.

RNA extraction from adipose tissue, RT-PCR

Total RNA was isolated using TriReagent (MRC, Cincinnati, OH). In brief, frozen fat samples were ground with mortar and piston in liquid nitrogen. Approximately 50 mg of ground fat tissue was then transferred to 1 ml TriReagent. The following RNA extraction was done according to the manufacturer's protocol. Subsequently the RNA was treated with DNAse I in order to completely remove genomic DNA using the DNA-free kit from Ambion (Austin, TX). The integrity of the total RNA was checked on a 1.5% agarose gel. 1 µg total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega, Madison, WI) using oligo(dT)$_{15}$ primers (Promega). A reaction from which the reverse transcriptase was omitted served as control for any residual DNA in the RNA sample.

Isolation of leukocytes and RNA extraction

Leukocytes were isolated from 10 ml heparinized whole blood from healthy volunteers by density gradient centrifugation (400 *g*, room temperature, 20 min) using a lymphocyte separating medium (Lymphodex; *Inno-Train*, Kronberg, Germany). After extensive washing (3x) with PBS, cells were centrifuged at 440 *g* for 5 min and the supernatant was aspirated. Dry pellets were frozen immediately and stored at –75°C. Total RNA from leukocytes was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Dueren, Germany) following the instructions provided by the manufacturer. The subsequent RT-PCR was conducted as described above.

Cultivation of THP-1, D10 and HBL cell lines and mRNA extraction

The human monocytic cell line THP-1 was cultivated in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Invitrogen, Paisley, UK). The differentiation of the THP-1 cells to macrophages was initiated by addition of 10 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO) to the medium for three days. Human D10 and HBL melanoma cell lines were cultivated in minimum essential medium (MEM) in the presence of heat-inactivated 10% FCS, 2 mM L-glutamine, 1% MEM vitamin solution and 1% MEM non-essential amino acids, penicillin (100 units/ml) and streptomycin (100 µg/ml). Total RNA of D10, HBL and THP-1 cells was extracted with the Nucleospin RNA II kit (Macherey-Nagel). Subsequently the reverse transcription was performed as mentioned above.

Cultivation and differentiation of preadipocytes and RNA extraction

Preadipocytes were extracted from adipose tissue and cultured as described by Linscheid et al [39]. Adipogenic differentiation was induced by adding D-MEM/Nutrient Mix F12 (Invitrogen) containing 3% FCS, 1 µM dexamethasone (Sigma), 0.1 mM L-ascorbic acid (Sigma), 250 µM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 5 µM transferrin (Calbiochem, La Jolla, CA), 0.2 nM 3,3,5-triiodo-L-thyronine (T3; Sigma), 100 nM Actrapid insulin (Novo Nordisk, Bagsvaerd, Denmark) and 1 µM rosiglitazone (gift from GlaxoSmithKline, Worthing, UK). The medium was exchanged every 3 days until at least 70% of the cells were differentiated into adipocytes (16-18 days). The adipocytes expressed adipocyte-specific mRNA for leptin and were full of visible fat droplet, verified to contain triglycerides by oil red O staining. For the total RNA extraction cells were washed with PBS, lysed in TriReagent (MRC) and processed as mentioned above.

Real-time TaqMan PCR

Quantitative real-time TaqMan PCR was performed employing predeveloped primers and MGB-probes (Assay-on-Demand; the exact sequences of the primers and probes were not provided by the manufacturer) and the TaqMan Universal PCR Master Mix on a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Each TaqMan reaction contained 10 ng of sample cDNA in a total reaction volume of 20 µl. All samples were measured in duplicates. TaqMan standard two-step PCR conditions were applied (2 min at 50°C, 10 min at 95°C, 45 cycles with 15 s at 95°C and 1 min at 62°C). Genomic DNA encoding MC1-R to MC5-R (all intronless) served as positive control for testing the different TaqMan PCR systems: substantial and robust amplification with minimal standard deviation of duplicates was observed for all MC receptors. Human GAPDH, the recommended endogenous control gene for preadipocytes and adipocytes

[40], was chosen as internal reference, following our own comparison of GAPDH vs. 18S rRNA expression in 22 fat samples of different origins: whereas 18S rRNA displayed marked variation between patients and fat depots, GAPDH expression exhibited no significant variation (Fig. 1). The relative expression was determined by the comparative threshold method as described in the ABI Prism 7700 User Bulletin #2 (P/N 4303859) from Applied Biosystems.

Immunohistochemistry

Adipose tissues were obtained either from obese patients undergoing bariatric surgery or from normal weight subjects undergoing obesity-irrelevant surgeries. Right after the excision the samples were formalin fixed, paraffin embedded and 4 μm sections were obtained. Fluorescent immunostaining was performed using Alexa Fluor 488 coupled to streptavidin (Invitrogen). First the paraffin sections were deparaffinized and rehydrated. For antigen retrieval the sections were incubated for 30 min at 95°C in citrate buffer, 0.05% Tween-20, pH 6.0. Unspecific binding sites were blocked with blocking solution (2% normal goat serum, 1% BSA, 0.1% Triton X-100 and 0.05% Tween-20) for 30 min. Endogenous biotin was blocked using the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). The sections were incubated with anti-hMC1-R primary antibody (1:50; Acris, Hiddenhausen, Germany) at room temperature for 1.5 h. This antibody had been raised in rabbits and was directed to the 2nd cytoplasmic loop of the human MC1-R, specific for this MC receptor subtype; its specificity for MC1-R immunostaining was assessed with human melanoma tumor sections. As secondary antibody a biotinylated anti-rabbit IgG (H+L) (1:500; Vector Laboratories) was used, followed by incubation with Alexa-488 coupled to streptavidin (1:1000; Invitrogen). All antibodies were diluted in 0.01 M PBS containing 1% BSA. Sections were then counterstained with DAPI (Sigma). For wash steps in between PBS/0.1% Tween-20 was used. As negative control, rabbit IgG (Vector Laboratories) at the same concentration as the first antibody was added instead of the MC1-R primary antibody. All slides were assessed by an experienced pathologist.

Some adipose tissue sections were stained with anti-CD68 (clone KP1; Dako, Glostrup, Denmark) to detect macrophages. These slides were incubated with a biotinylated secondary antibody coupled to horseradish peroxidase. The procedure was performed using the automated NexES IHC staining module together with the iView DAB detection kit (Ventana, Illkirch, France). Slides were counterstained with hematoxylin, followed by dehydration.

Statistical analysis

All values are presented as the mean ± SEM if not stated differently. For statistical analysis, the Student's unpaired *t* test was used to compare levels of mRNA expression from subcutaneous and omental adipose tissue and one-way ANOVA for the comparison of all four data groups. A value of $P < 0.05$ was regarded as statistically significant. All analyses were performed using Prism 4.0 (GraphPad, San Diego, CA).

Results

Evaluation of hGAPDH as a suitable endogenous control for quantitative real-time PCR

Human GAPDH is the reference gene recommended for work with preadipocytes differentiated to adipocytes. It was shown that expression of GAPDH did not change during the adipogenic differentiation [40]. However, to be assured that mRNA expression of hGAPDH does not vary in subcutaneous and omental adipose tissue in morbidly obese subjects and normal weight patients, respectively, we analyzed the relative gene expression of hGAPDH versus 18S rRNA in 22 samples of 11 different subjects (11 subcutaneous, 11 omental, 4 lean subjects, 7 obese subjects, 6 female, 5 male). Data shown in Fig. 1 demonstrate higher variations for 18S rRNA expression compared to hGAPDH. Therefore, the usage of hGAPDH proved to be suitable for conducting our intended gene expression study.

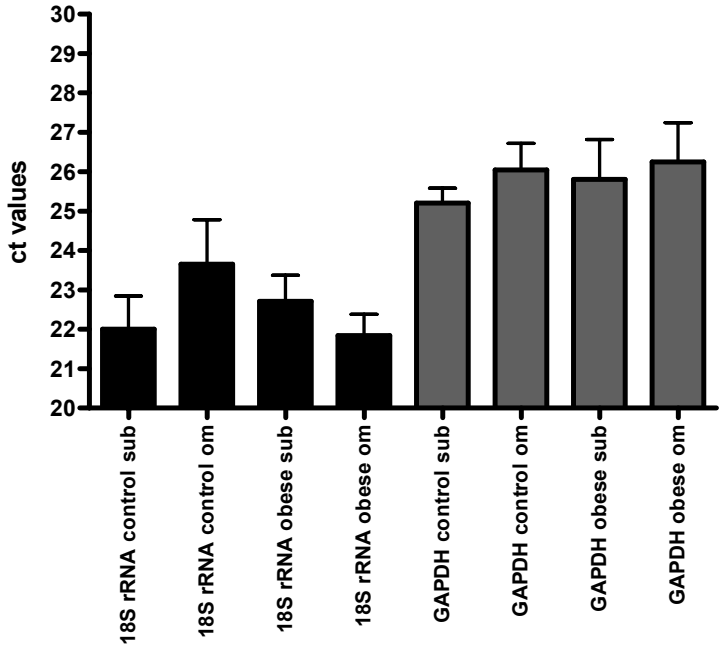

Fig. 1. Comparison of ct values of 18S rRNA vs. GAPDH of the real-time TaqMan PCR of the different fat depots and study groups (control sub, om: n=4; obese sub, om: n=7). The gene expression of 18S rRNA and GAPDH was analyzed simultaneously in duplicates. sub = subcutaneous; om = omental.

Expression of the melanocortin receptors and POMC mRNA in subcutaneous and omental adipose tissues

The clinical characteristics of the study subjects are listed in Table 2. Quantitative real-time TaqMan PCR revealed that of the five melanocortin receptor subtypes, only MC1-R was expressed in all biopsies of human adipose tissue (Fig. 2a). mRNA of all other melanocortin receptors could either not be identified (MC2-R, MC3-R) or was detectable in only very few samples (MC4-R, MC5-R) and near the detection limit (data not shown). In subcutaneous adipose tissue, morbidly obese patients tended to express higher amounts of MC1-R mRNA compared to control subjects but owing to individual variations in gene expression, this difference was not statistically different. In omental fat, MC1-R mRNA expression was very similar in both study groups. In addition, there was no gender difference in the MC1-R expression levels (data not shown).

Table 2. Clinical characteristics of study subjects

Characteristics	Obese (n=22)[a]	Non-obese (n=9)[a]
Sex (n)	F : M = 15 : 7	F : M = 5 : 4
Age (yr)	39.6 ± 9.7	47.8 ± 20.7
Weight (kg)	120.7 ± 24.9	64.1 ± 10.4
BMI (kg/m^2)	42.3 ± 7.3	22.4 ± 2.5
No. of subjects with diabetes type II	1	0
No. of subjects with hypertension	6	0
No. of subjects with dyslipidemia	13	0

[a] Values are means ± SD

POMC mRNA was found at very low levels, similar to MC4-R and MC5-R (data not shown). Detailed analysis demonstrated that in both subcutaneous and omental fat tissue POMC mRNA was found in obese patients but not in control subjects and at a ratio relative to MC1-R mRNA of approximately 1:200 (Fig. 2b).

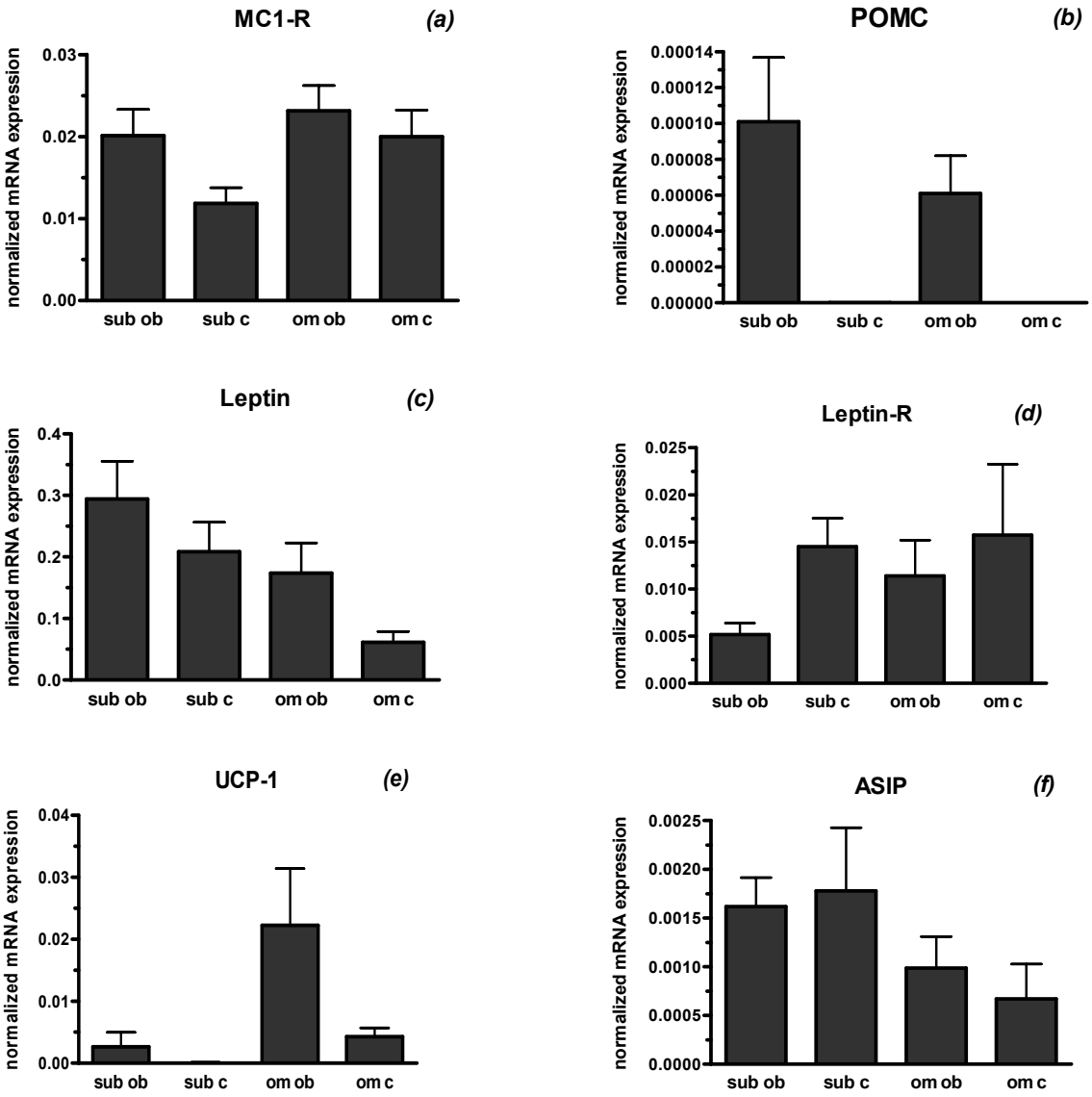

Fig. 2. Gene expression in obese and non-obese subjects of *(a)* MC1-R, *(b)* POMC, *(c)* leptin, *(d)* leptin-R, *(e)* UCP-1, *(f)* ASIP. RNA was extracted from subcutaneous (sub) and omental (om) fat tissue either from normal weight (c; n=9) or obese subjects (ob; n=22). The corresponding cDNA was subjected to real-time TaqMan PCR normalized to hGAPDH. Data are means ± SEM.

Expression of leptin, leptin receptor, ASIP and UCP-1 mRNA in subcutaneous and omental adipose tissues

We selected leptin and leptin receptor as representatives of well-expressed obesity-related genes in adipose tissues of normal and obese subjects. Their determination served the purpose to relate the expression of the melanocortin receptors to these well-studied markers. Quantitative real-time TaqMan PCR revealed a high expression of leptin mRNA in subcutaneous and omental adipose tissue of both subject groups (Fig. 2c), reaching 3- to 15-fold higher values than MC1-R mRNA. The ratio of leptin expression in subcutaneous to omental fat was 2.9 ± 0.6 for obese subjects and 2.1 ± 0.9 for control subjects. This difference was not statistically significant and there was no correlation between the BMI and the level of leptin mRNA. However, in both fat depots leptin expression was significantly higher in women than in men ($P < 0.05$) (data not shown). Also, the subcutaneous-to-omental ratio in women was slightly elevated as compared to men (3.3 ± 0.8 vs. 2.3 ± 0.9, n.s.). The expression of leptin receptor mRNA was in the same range as that reported for MC1-R (Fig. 2d). There was no statistically significant difference of leptin receptor expression in the two subject groups or fat depots.

UCP-1 mRNA was detected in both fat depots of obese patients, in the range of that of MC1-R mRNA. As expected, UCP-1 mRNA expression in omental fat was higher than in subcutaneous fat. In contrast to a previous report [41], in our patient groups morbidly obese individuals showed markedly increased expression of UCP-1 mRNA as compared to control subjects (Fig. 2e). In the former group, the subcutaneous-to-omental ratio for UCP-1 was 0.3 ± 0.003 ($P < 0.05$). ASIP mRNA was expressed at about 10-fold lower levels than MC1-R mRNA but at 10-20-fold higher levels than POMC (Fig. 2f). Although there was a tendency of higher expression of ASIP mRNA in subcutaneous fat than omental fat, this difference was again not statistically significant.

MC1-R mRNA expression in preadipocytes and adipocytes

In order to investigate whether MC1-R in adipose tissue was expressed primarily on adipocytes or on other cells, we determined the MC1-R mRNA expression in isolated human preadipocytes, before and after differentiation to adipocytes. As shown in Fig. 3, differentiation leads to a 3-fold increase in MC1-R mRNA ($P < 0.001$) but differentiated adipocytes still have a 1.5- to 3-fold lower expression level than human adipose tissue. It appeared therefore that substantial amounts of MC1-R mRNA in human adipose tissue may not originate from adipocytes.

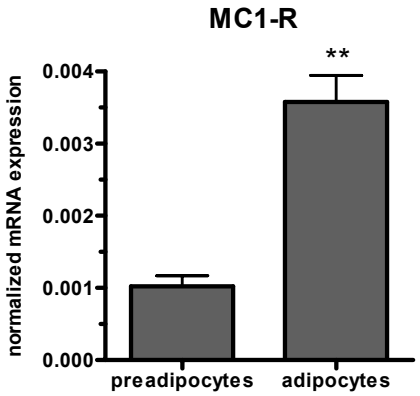

Fig. 3. MC1-R mRNA expression in undifferentiated human preadipocytes (n=4) and differentiated adipocytes (n=4). MC1-R mRNA gene expression was normalized to hGAPDH from two independent experiments. Data are means ± SEM. ** $P < 0.001$.

Evaluation of the specificity and usability of the MC1-R antibody for immunohistochemistry

It is crucial to have a specific, suitable first antibody to work with, in order to be able to localize the desired protein in tissue sections. Not all antibodies fulfil these criteria, especially for immunohistochemistry on paraffin embedded tissue sections. In addition, adipose tissue revealed to be a rather difficult tissue to cut into thin enough sections. To evaluate the usability of the first antibody raised against human MC1-R, we tested the antibody on different human melanoma sections. Thereby, as expected the melanoma and macrophages were positively stained (Fig. 4). Surrounding non-melanoma tissues did not show any staining, except a slight signal for erythrocytes. It is known that erythrocytes can lead to false positive signals due to their endogenous pseudo peroxidase activity. Thus, we considered this to be an artefact. Unfortunately with this commonly performed immunohistochemistry method we could not clearly predict whether adipocytes were MC1-R positive or not. However, there seemed to be a faint staining of the adipocytes stronger than the background signal. We therefore decided to perform the more sensitive fluorescence immunohistochemistry method.

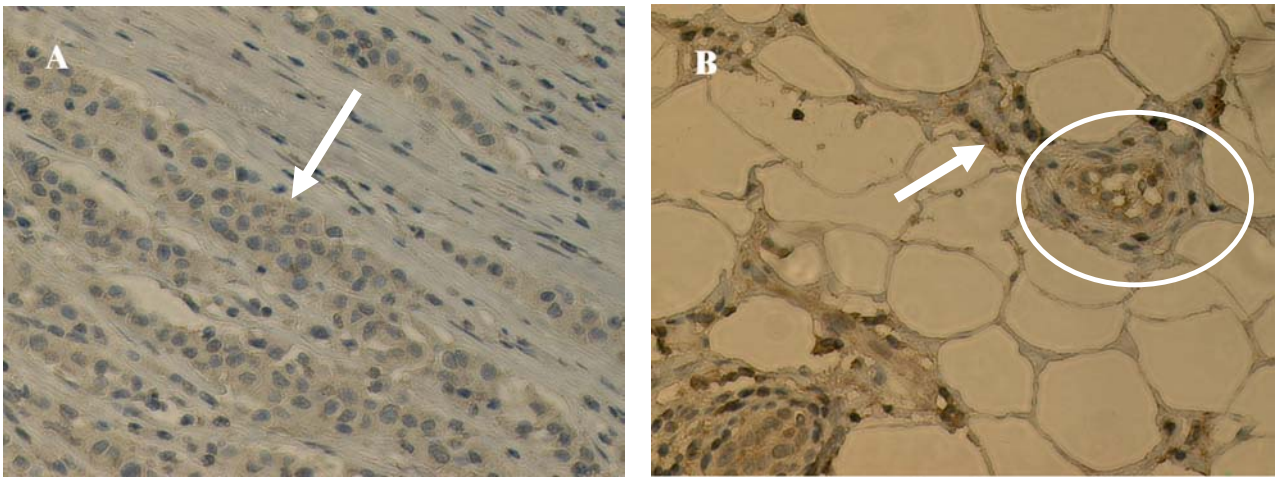

Fig. 4. Immunohistochemistry of MC1-R in melanoma sections. (A) Melanoma cells (white arrow) were MC1-R positive (brown colour) but not the fibers aside. Magnification 20x. (B) In the same section beyond the melanoma cells, macrophages (white arrow) were stained strongly MC1-R positive. In blood vessels (circle) erythrocytes exerted a faint positive signal, which we predicted to be an artifact. Although there were some indications, we could not definitely determine whether the adipocytes express MC1-R or not. Magnification 20x.

Localization of MC1-R on adipose tissue sections

Paraffin embedded sections of adipose tissue were analyzed for their expression of the MC1-R protein by fluorescence immunohistochemistry. An intense staining was found for monocytes and macrophages present in blood vessels, clustered around lipolytic adipocytes and unequally distributed in between adipocytes (Fig. 5). Characteristically macrophage accumulations were encircling small foci of lipolytic or necrotic adipocytes. Cytoplasmatic lipid droplets corroborate their phagocytic activity. These lesions were found more frequently in obese subjects. The distribution pattern of the MC1-R positive monocytes/macrophages was very similar to the CD68 staining of corresponding sections (Fig. 6). We made the observation that in non-obese subjects with a history of severe inflammation the number of lesion in fat tissue can also be elevated (unpublished observation).

The adipocytes, which made up the major body of cells in the adipose tissue sections, were only slightly positive for MC1-R staining (Fig. 5), and hence, MC1-R in human adipose tissue sections appears to be more abundant in cells of the immune system than in adipocytes where its expression is distinct but small.

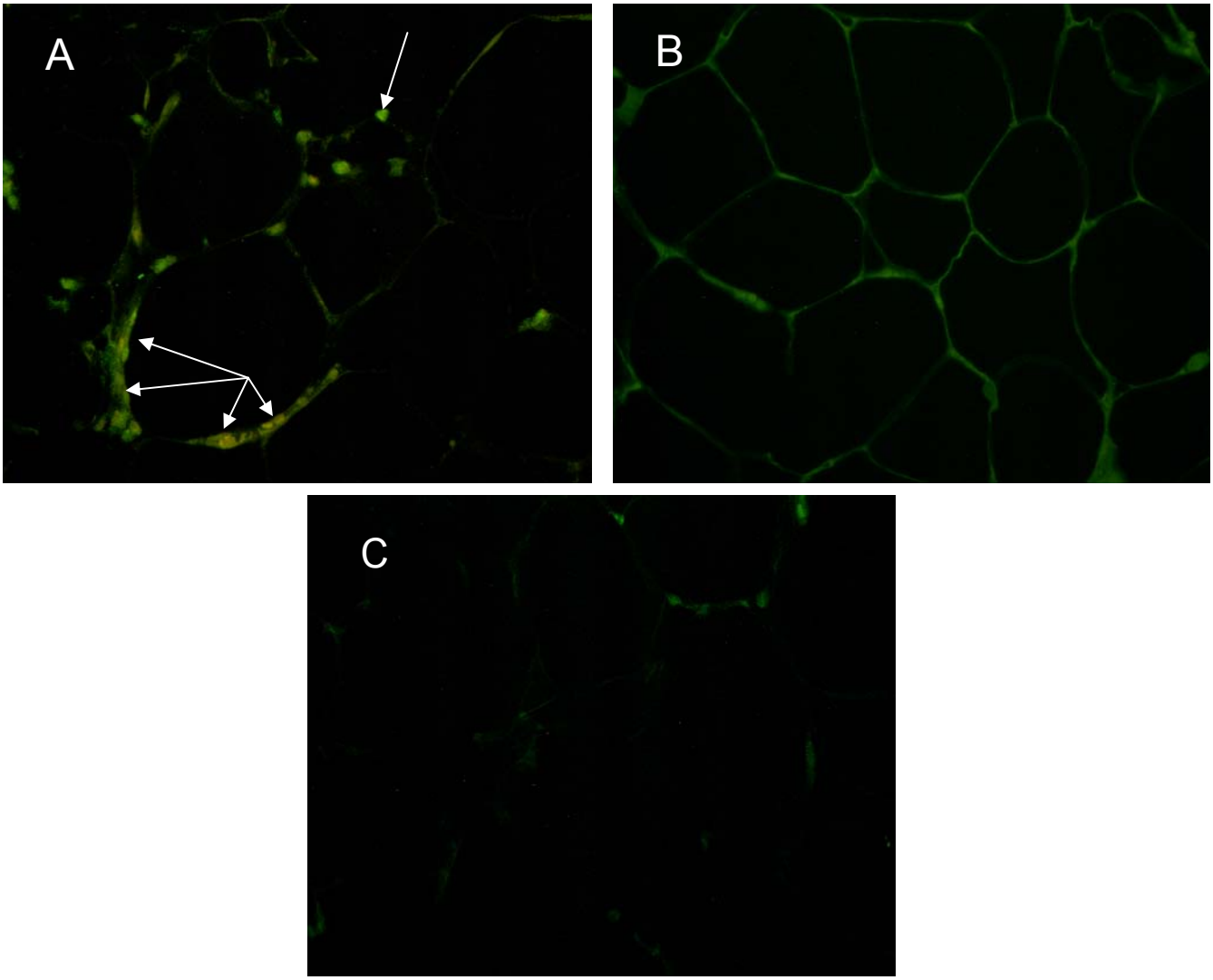

Fig. 5. Immunohistochemical expression analysis of MC1-R in adipose tissue. Immunofluorescent staining with biotinylated Alexa-488. (A) Clustered MC1-R positive macrophages surrounding a lipolytic focus (white arrows). Additional unevenly distributed macrophages. (B) Adipocytes with a faint membranous staining. (C) Negative control (rabbit IgG instead of the 1st antibody). Magnification 20x.

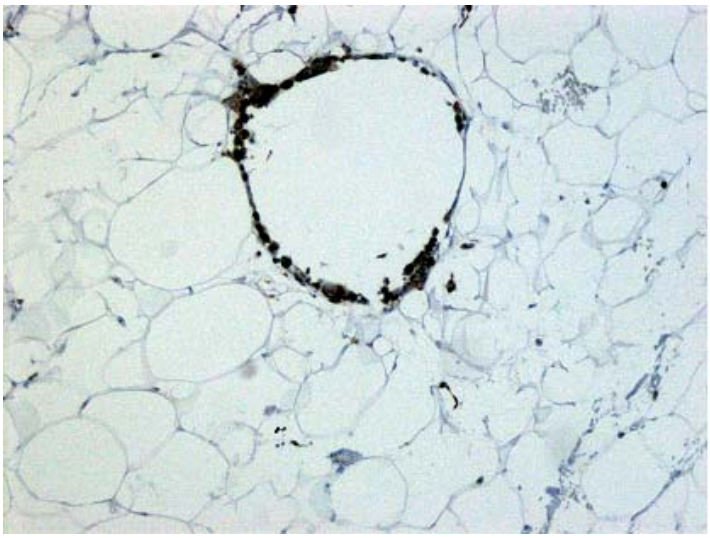

Fig. 6. Immunohistochemical detection of CD68 protein. DAB staining of paraffin embedded adipose tissue. Staining pattern of the CD68 positive macrophages is identical to the fluorescent staining of the MC1-R. Adipocytes are CD68 negative.

Estimation of MC1-R numbers by comparison of mRNA expression in human fat tissues, preadipocytes, THP-1 macrophages and leukocytes with human melanoma cells

Based on the assumption that MC1-R mRNA content may reflect the order of MC1-R protein expressed, we compared mRNA expression of MC1-R by a human macrophage cell line, THP-1 (unstimulated and stimulated with PMA), by human leukocytes, human preadipocytes and adipocytes, and human adipose tissue with that of well characterized human melanoma cell lines [42, 43]. For example, human HBL melanoma cells have been reported to express between 6000 and 12,000 MC1-R per cell. As shown in Fig. 7a, the lowest MC1-R mRNA content was found in unstimulated THP-1 cells; the value was about 170-fold lower than that determined in HBL cells (Fig. 7b). The highest amount in Fig. 7a was noted for leukocytes whose value was about 6-fold lower than that of HBL cells. As binding analysis with cells expressing MC1-R numbers lower than 200/cell is not precise, we estimate that MC1-R numbers on the cells displayed in Fig. 7a ranges from a few dozen for unstimulated THP-1 cells and preadipocytes to 100-200 for adipocytes and to 1000-2000 for leukocytes.

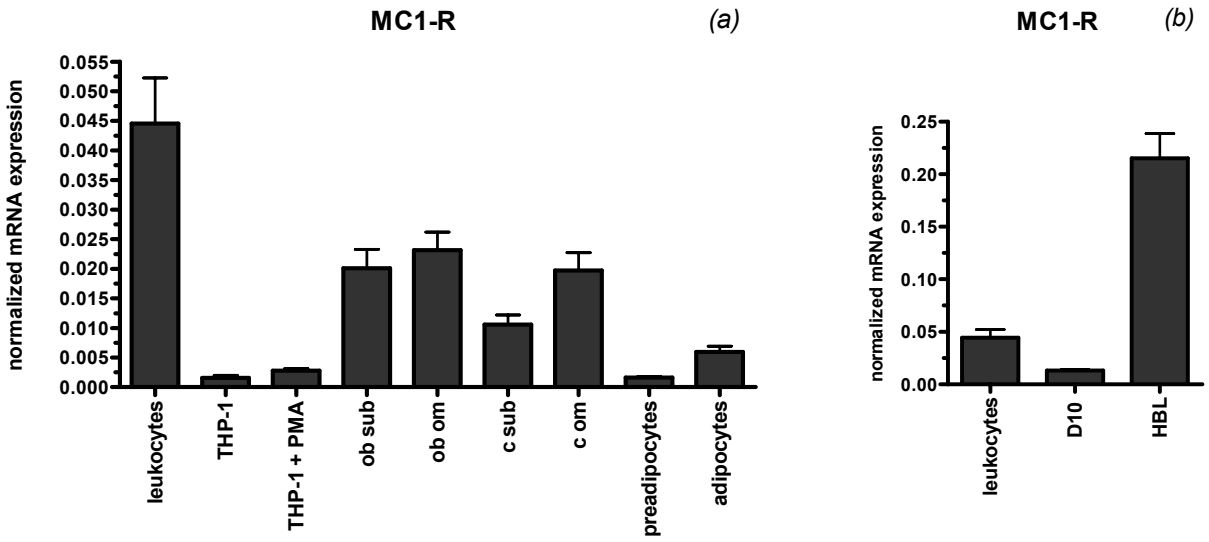

Fig. 7. Comparison of the MC1-R mRNA expression. *(a)* MC1-R mRNA expression by non-activated and activated THP-1 human monocytes (n=4), leukocytes (n=9), adipose tissue (ob = obese, c = control, sub = subcutaneous, om = omental), undifferentiated preadipocytes (n=4) and differentiated to adipocytes (n=4), and *(b)* by D10 (n=4) and HBL (n=4) human melanoma cells, compared to leukocytes (n=9). Data are means ± SEM.

Discussion

In the past few years several studies were performed on the identification of genes and their expression in adipose tissues in order to find a relation to the pathogenesis of obesity. The aim of the present study was to investigate the depot-specific mRNA expression of melanocortin receptors in comparison to some known obesity-related genes. Subcutaneous and omental adipose tissues were separately investigated because it is now well known that they possess different properties. For example, the rate of lipolysis is higher in subcutaneous than in omental adipose tissue [44]. Also, the anti-lipolytic effect of insulin is elevated in subcutaneous fat [45, 46]. Different studies showed that accumulation of fat in the omental, visceral region is more problematic for the health condition than in other regions [47-50].

Whereas in murine adipocytes and 3T3-L1 cells, MC2-R and MC5-R are expressed [18, 21], previous reports on the occurrence of the different melanocortin receptors in human fat tissues are inconsistent: Chhajlani [16] discovered MC1-R and MC5-R in omental fat, Chagnon et al [51] also detected MC4-R, and finally Smith et al [22] reported expression of MC1-R in subcutaneous adipose tissue samples, in preadipocytes (abundant), adipocytes (less abundant) and mesenchymal stem cells (MSCs) differentiated to adipocytes. These authors found no expression of MC3-R, MC4-R and MC5-R but distinct expression of MC2-R in all these cells and tissues, except for preadipocytes. By contrast, Kiwaki and Levine [52] detected MC2-R mRNA in mouse fat tissue but not in human fat tissue, and ACTH(1-39) induced lipolysis only in minced mouse fat but not in human fat. Our own findings based on quantitative RT-PCR and investigating subcutaneous and omental adipose tissues from 31 patients are consistent with those of Smith et al [22] with respect to MC1-R expression as this receptor was expressed in obese and non-obese subjects and fat-depots at similar levels. The lack of MC2-R expression in our patients contradicts the findings by Smith et al [22] but is in agreement with the report by Kiwaki and Levine [52]. MC3-R, MC4-R and MC5-R were found not to be expressed or at only very low levels in some of the fat sample, which would support the findings by Smith et al [22].

In our adipose tissue samples, only a very weak mRNA expression of the POMC gene was found in the obese subjects and no POMC was detectable in lean controls. Since the mRNA signals for POMC were near the detection limit, a physiological role for this low amount of POMC in fat tissue is questionable and needs further investigation, in particular whether it could at all represent a source for melanocortin peptides. It has been demonstrated that plasma levels of α-MSH are elevated in obese subjects as compared to lean controls, suggesting some peripheral involvement of melanocortins in energy balance regulation [53, 54]. Recently, Hoggard et al [54] observed no

difference to basal levels prior to fast after a weight loss of about 5% in the obese and normal weight subject groups. However, it is important to note that the level of circulating α-MSH is very low (approximately 16 pmol/l in obese subjects [54]) and its physiological role in humans remains to be determined. Similar weak expression was noted for ASIP and no significant difference in the mRNA levels neither in the two fat-depots nor the subject groups was found. Our results for ASIP expression in adipose tissue suggest that ASIP is not part of the obese phenotype. Smith et al [22] also noted no increase of ASIP expression in obese subjects, but elevated levels in patients with type 2 diabetes.

Leptin, leptin receptor and UCP-1 mRNA were determined in this study to relate the expression analysis of the melanocortin system with well known obesity-related genes of fat tissue. We found higher leptin expression in the subcutaneous than in the omental adipose tissue in both subject groups, which is in agreement with other recently published studies [26, 27, 30, 55-57]. In women the ratio of subcutaneous to omental expression of leptin was higher. This confirms the results previously reported by Montaque et al [57]. In contrast, our results do not confirm a relationship between BMI and leptin mRNA expression that may be explained by the fact that in our study several fat samples from patients undergoing a second bariatric surgery were included; the first operation (mostly gastric banding) may have altered leptin mRNA expression.

As expected we found higher UCP-1 mRNA expression in the omental fat than in subcutaneous fat. Moreover, we obtained a slightly stronger signal in the obese group than in the normal weight control group. These findings are however opposed to previously published results by Esterbauer et al [41] who analyzed the UCP-1 mRNA expression of 153 morbidly obese patients and obtained significantly lower UCP-1 mRNA levels in the obese subject group. Possibly an elevated expression rate of UCP-1 in morbidly obese subjects is part of a counter-regulation to loosing weight. Recently, leptin was shown to induce UCP-1 expression in BAT in rats [58] and in addition also in WAT in mice [59]. In our human samples we could not find any correlation between leptin mRNA expression and UCP-1 expression. Possibly, in humans additional factors may influence UCP-1 expression.

Since macrophages are known to express MC1-R, we hypothesized that MC1-R mRNA determined in human fat tissue may be derived from tissue macrophages. To verify this hypothesis we measured the MC1-R mRNA in freshly isolated leukocytes and in the THP-1 cell line and compared it with MC1-R expression in preadipocytes and adipocytes. MC1-R expression in leukocytes was about two-fold higher than the signal from adipose tissue whereas in preadipocytes and adipocytes it was lower. This was confirmed by immunohistochemical analysis of adipose tissue sections where macrophages stained more intensely with MC1-R antibodies than fat cells.

Thus, it appears that the MC1-R mRNA found in the adipose tissue originates in part from macrophages. In order to estimate MC1-R protein expression in the different cell lines and tissues, MC1-R expression was compared with that of well characterized melanoma cells: assuming similar rates of translation in the different systems, the ensuing rough estimation would predict a few dozen of MC1-R for THP-1 cells and preadipocytes, a slightly higher expression in mature adipocytes and in leukocytes values as found in human melanoma cell lines.

In conclusion, we demonstrated that human adipose tissue expresses MC1-R but not MC2-R and that the MC1-R protein is more abundant on macrophages but is also present on adipocytes. As a next step, functional studies with cultivated human preadipocytes, adipocytes, macrophages and human fat tissue samples should clarify the question whether MC1-R mediates lipolysis or more likely plays a role in the regulation of cell proliferation or inflammatory signals.

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Chapter 4: Melanocortin receptor-1 function in human adipose tissue and adipocytes[3]

Abstract

Objective: Recently, we could demonstrate that the melanocortin receptor-1 (MC1-R) is the only subtype of the melanocortin receptors that is substantial expressed in human adipose tissue [1]. The protein is expressed not only on macrophages present in the adipose tissue but also on adipocytes. Here we evaluated the functionality and function of the MC1-R on human adipocytes derived from mesenchymal stem cells (MSCs), on undifferentiated MSCs and human adipose tissue explants.

Methods: MC1-R protein was detected in MSC-derived adipocytes by immunohistochemistry. In order to analyze the functionality of the MC1-R the cAMP production upon stimulation with NDP-MSH was measured. Furthermore, we investigated the function of MC1-R by analyzing the effect of NDP-MSH on lipolysis, cytokine mRNA expression (TNF-α, IL-6, IL-8) and secretion (TNF-α, IL-6, IL-8, IL-10), metabolic rate (MTT assay) and cell proliferation (BrdU assay) either in adipose tissue, adipocytes derived from MSCs or undifferentiated MSCs.

Results: Adipocytes derived from MSCs were found to express MC1-R on their cell membrane. Furthermore, in MSC-derived adipocytes MC1-R could be dose-dependently stimulated with NDP-MSH to produce cAMP. Neither an effect of NDP-MSH on lipolysis, nor on cytokine release and mRNA expression of IL-6, IL-8 and TNF-α upon LPS stimulation could be seen on MSC-derived adipocytes. Additionally, there was no effect of NDP-MSH on the metabolic rate in undifferentiated MSCs. When looking at the proliferation rate NDP-MSH showed a significant anti-proliferative effect on undifferentiated MSCs.

Conclusion: Human MSC-derived adipocytes express functional MC1-R. MC1-R stimulation leads to inhibition of cell proliferation on undifferentiated MSCs but does not regulate lipolysis and inflammation on MSC-derived adipocytes and human adipose tissue. Thus, the anti-proliferative effect of α-MSH on adipocyte precursor cells via the MC1-R might regulate the development of additional adipocytes that possibly leads to obesity when the MC1-R signaling is disrupted.

[3] Data of this chapter are in preparation for publication in: Hoch M, Eberle AN, Linscheid P, Martin I, Peters T, Peterli R NDP-MSH mediates an anti-proliferative effect on undifferentiated human MSCs but does not control lipolysis or inflammation.

Introduction

The discovery of satiety-inducing effects of melanocortin peptides by Vergoni et al [2] some twenty years ago was followed by numerous studies into the (patho-)physiology of the pro-opiomelanocortin system with respect to its role in body weight regulation (reviewed in [3, 4]). It is now well established that hypothalamic neurons secreting melanocortins play a central role in the control of energy homeostasis because they mediate the anorectic effects of leptin [5]. Five melanocortin receptors (MC1-R – MC5-R) have been cloned and have shown to mediate a variety of physiological signals (reviewed in [3, 4, 6, 7]). MC4-R and, to some extent, MC3-R are involved in the regulation food intake and energy expenditure, although the details of downstream signaling for these two effects are yet to be elucidated [8]. Hypothalamic MC4-R activation includes the regulation of central sympathetic outflow to white adipose tissue, resulting in changes of lipid mobilization [9]. In addition, melanocortin peptides (ACTH, α-MSH) exert potent lipolytic activity in the adipose tissue of several species (reviewed in [10-12]). Accordingly, the presence of functional melanocortin receptors (MC1-R, MC5-R) has been demonstrated in murine adipose tissue and in 3T3-L1 adipocytes [13]. Controversy exists regarding the specific roles of MC-R subtypes in human adipose tissue [14-17].

We recently reported the expression of MC1-R in human subcutaneous and omental adipose, which contrasted the absence MC2-R and MC3-R mRNAs [1]. MC4-R and MC-5-R mRNAs were occasionally detectable but at very low levels. MC1-R mRNA in subcutaneous fat was increased in obese patients as compared to lean controls. Immunohistochemical analysis showed that MC1-R expression was higher in macrophages of adipose tissue but was also present in adipocytes [1]. The present report addresses the question of the functional role of MC1-R in human adipose tissues which appears to be restricted to an anti-proliferative effect on undifferentiated mesenchymal stem cells (MSCs).

Material and Methods

Culture of human adipose tissue

The study was approved by the local ethic committee and informed written consent of the subjects was obtained. Abdominal subcutaneous and omental adipose tissue samples of about 50-100 grams were obtained from five morbidly obese women undergoing gastric banding or gastric bypass surgery. The mean BMI of these patients was 41.6 ± 1.7 kg/m^2. Initially, the connective tissue and blood vessels were removed by dissection. The fat tissue was minced into small pieces (approximately 5 mg) and extensively washed with PBS. To further wash the fat pieces they were incubated in D-MEM/Nutrient Mix F12 (Invitrogen, Basel, Switzerland) supplemented with 0.25% bovine serum albumin fraction V (BSA; Sigma, St. Louis, MO), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Invitrogen) for 1 h in the incubator with periodical mixing. The fat fragments were separated from the medium with a nylon mesh (250 µm). A total of 300 mg of minced tissue fragments were placed into 3 ml fresh medium (for lipolysis D-MEM/Nutrient Mix F12 without phenol red was used). Hormones of different concentrations for measuring lipolysis or cytokines released were supplemented and the explants were incubated for 24 h in the incubator.

Cultivation of human mesenchymal stem cells (MSCs)

MSCs from bone marrow were isolated from two separate healthy donors (18-63 years), following approval by the local committee and after informed consent, as described previously [18]. In brief, MSCs were obtained from 20-40 ml bone aspirates after routine orthopaedic surgical procedure. Nucleated cells were isolated from the aspirate by Ficoll density gradient centrifugation (Histopaque1, Sigma). MSCs were thereafter selected within the nucleated cells in culture on the basis of adhesion and proliferation on the plastic surface. The cells were expanded in D-MEM, supplemented with 10% fetal calf serum (FCS; Invitrogen, Basel, Switzerland) and 5 ng/ml basal FGF (Invitrogen) until they reached confluence. Adipogenic differentiation was induced by incubation in D-MEM/Nutrient Mix F12 (Invitrogen) containing 3% FCS, 1 µM dexamethasone (Sigma), 0.1 mM L-ascorbic acid (Sigma), 250 µM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), 5 µM transferrin (Calbiochem, La Jolla, CA), 0.2 nM 3,3,5-triiodo-L-thyronine (T3) (Sigma) and 100 nM Actrapid insulin (Novo Nordisk, Kuesnacht, Switzerland). The medium was changed every 3 days until at least 70% of the cells were differentiated into adipocytes.

Before the experiments were conducted, the cells were incubated for 24-48 h in D-MEM/F12 supplemented with 3% FCS.

Cultivation of mouse 3T3-L1 cells

Mouse 3T3-L1 cells were cultured in D-MEM (high glucose), supplemented with 10% FCS (heat inactivated), 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen) until 2 d after they reached confluence. The differentiation of 3T3-L1 cells into adipocytes was initiated by the addition of 25 µM dexamethasone (Sigma), 0.5 mM IBMX (Sigma) and 1 µg/ml insulin (Sigma) to the culture medium for 2 days, followed by the cultivation of the cells in basal medium containing 1 µg/ml insulin for an additional 4 days. Then the medium was withdrawn and the cells were kept in basal medium until they were fully differentiated after a total of 10-14 d.

RNA extraction, RT-PCR

Total RNA of MSCs was isolated using TriReagent (MRC, Cincinnati, OH) according the manufacturer's protocol. Since MC1-R is an intronless gene, any contaminating DNA would affect the analysis. Therefore the RNA was treated with DNAse I to remove genomic DNA using the DNA-free kit from Ambion (Austin, TX). First-strand cDNA was synthesized from 1 µg total RNA with M-MLV reverse transcriptase (Promega, Madison, WI) using oligo$(dT)_{15}$ primers (Promega). Negative controls without reverse transcriptase were run in order to confirm the absence of genomic DNA contamination in the RNA sample.

Real-time TaqMan PCR

Quantitative real-time TaqMan PCR was performed employing pre-designed primers and MGB-probe (Applied Biosystems, Foster City, CA) for either MC1-R, IL-6, IL-8 or TNF-α and 2x Universal PCR Master Mix (Applied Biosystems). The analysis was run on a 7500 Fast real-time PCR system (Applied Biosystems). Each TaqMan reaction contained 10 ng of sample cDNA in a total reaction volume of 20 µl. As endogenous control human GAPDH was used, which is stably expressed in preadipocytes, and throughout the differentiation to adipocytes [19]. The usability of GAPDH as reference gene was evaluated by comparing the different threshold values (ct values) treated versus untreated cells. The relative expression was determined by the comparative

threshold method as described in the ABI Prism 7700 User Bulletin #2 (P/N 4303859) from Applied Biosystems.

Lipolysis assay

Human adipocytes derived from MSCs cultured in 6-well plates were washed with PBS and incubated in D-MEM/F12 without phenol red with hormones of different concentrations for 24 h at 37°C. The fat tissue explants were treated in the same way, except that they were incubated in the medium mentioned above. All hormones were purchased from Sigma, except [Nle^4,D-Phe^7]-α-MSH (NDP-MSH), which was obtained from Bachem (Bubendorf, Switzerland) and ACTH(1-24), which was a gift from Novartis Inc, Basel. The β-adrenergic agonist isoproterenol (Sigma) and the adenylate cyclase stimulant forskolin (Sigma) served as positive controls. In order to stop any enzymatic reaction the collected supernatants were heated in a water-bath at 80°C for 15 min. Glycerol released into the medium was determined as an indicator for lipolysis. Glycerol content of the medium was determined using the UV-method kit from R-Biopharm (Darmstadt, Germany) according manufacturer's protocol.

Detection of IL-6, IL-10 and TNF-α

MSCs were differentiated to adipocytes as described above. After the end of the differentiation phase, the cells were washed with PBS and the medium was changed to D-MEM/Nutrient Mix F12, 3% FCS. After a pre-incubation with NDP-MSH (10^{-6}-10^{-12} M) for 24 h, the adipocytes were stimulated with 1 ng/ml lipopolysaccharide (LPS, from E. coli, serotype 055:B5; Fluka, Buchs, Switzerland) for an additional 24 h. The glucocorticoid dexamethasone (1 μM), which is known to reduce inflammation, served as control. The supernatants were collected and analyzed for Il-6, IL-10 and TNF-α. All cytokines were measured with commercially available ELISA kits from eBioscience (San Diego, CA). The adipose tissue explants were treated in the same way as the adipocytes, except that they were stimulated with 1 μg/ml LPS without pre-incubation of NDP-MSH. In order to analyze the mRNA expression of the cytokines TNF-α, IL-6 and IL-8, MSC-derived adipocytes were pre-incubated with NDP-MSH for 24 h. LPS (1 ng/ml) or IL-1β (PeproTech, London, UK; 20 units/ml) were added for 3 h to stimulate inflammation. Again dexamethasone (1 μM) served as control for an anti-inflammatory effect on the cytokine mRNA expression.

cAMP assay

In order to analyze the functionality of the MC1 receptors on MSC-derived adipocytes, intracellular cAMP concentrations were measured after the stimulation with NDP-MSH; stimulation with isoproterenol served as positive control. The adipocytes derived from MSCs were incubated in MEM (Biochrom, Berlin, Germany), 0.1 mM IBMX (Sigma) and 0.2% BSA (Sigma) supplemented with hormones of different concentrations for 30 min. The intracellular cAMP was measured using the cAMP Biotrak enzymeimmunoassay (EIA) system from Amersham Biosciences (Piscataway, NJ) according the manufacturer's protocol.

Cell proliferation

The mitochondrial metabolic rate was determined with the 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Undifferentiated cells were grown in 6-well plates to 90% confluence; then the medium was changed and NDP-MSH of different concentrations was added for 24 h. After a PBS washing step the cells were incubated with 0.5 mg/ml MTT in D-MEM/F12 without phenol red at 37°C in the cell incubator for 1 h. The medium was removed and the converted purple dye was dissolved in 1 ml DMSO. Subsequently, 100 µl were transferred to a 96-well plate and the absorption was measured at 450 nm using a Spectramax 190 (Molecular Devices, Sunnyvale, CA).

For the proliferation assay with the 5-bromo-2'-deoxyuridine (BrdU) method, approximately 2,000 undifferentiated MSCs were dispersed in each well of a 96-well plate and incubated for 2 days in basal medium until the cells were ~80% confluent. The medium was changed and NDP-MSH of different concentrations was added and incubated for 24 h. Then BrdU was supplemented to a final concentration of 10 µM and the cells were re-incubated for 24 h. The incorporation of BrdU during proliferation was measured by applying the colorimetric BrdU ELISA cell proliferation kit from Roche (Basel, Switzerland).

Immunocytochemistry

MSC-derived adipocytes were kept in chamber slides (Nalge Nunc, Rochester, NY). Cells were washed with PBS and fixed with 4% formaldehyde in PBS for 30 min at room temperature. The cells were extensively washed with PBS. Unspecific binding sites were blocked with blocking solution (2% normal goat serum, 1% BSA, 0.1% Triton X-100 and 0.05% Tween-20) for 30 min.

Endogenous biotin was blocked using the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). The sections were incubated with anti-hMC1R primary antibody (Acris, Hiddenhausen, Germany, 1:50) at room temperature for 1.5 h. Beforehand, the antibody's specificity und usability was evaluated on different human melanoma sections, where MC1-R is often overexpressed. A biotinylated anti-rabbit IgG was used as secondary antibody (Vector Laboratories, 1:500). All antibodies were diluted in 0.01 M PBS containing 1% BSA. To visualize the biotinylated second antibodies bound to the primary antibody against MC1-R, the Vectastain ABC kit (Vector Laboratories) together with 3-amino-9-ethyl carbazole (AEC) as substrate (DakoCytomation, Glostrup, Denmark) was used. Finally the slides were counterstained with hematoxylin according to Mayer (Fluka) and assessed with light microscopy. As negative control, rabbit IgG in the same concentration was added instead of the MC1-R primary antibody.

Statistical analysis

All values are presented as the mean ± SEM. For all statistical analysis, the Student's unpaired *t* test was used. A value of $P < 0.05$ was regarded as statistically significant. All analyses were performed using Prism 4.0 (GraphPad, San Diego, CA).

Results

Expression of the melanocortin 1 receptor

First we evaluated the usability of the human GAPDH mRNA expression for the normalization of the gene expression. We obtained only little differences in the GAPDH expression in the MSC-derived adipocytes untreated versus treated with LPS (1 ng/ml) or LPS (1 ng/ml) together with NDP-MSH (10^{-6} M) (Fig. 1). Thus, the usage of GAPDH is suitable for normalization of the gene expression.

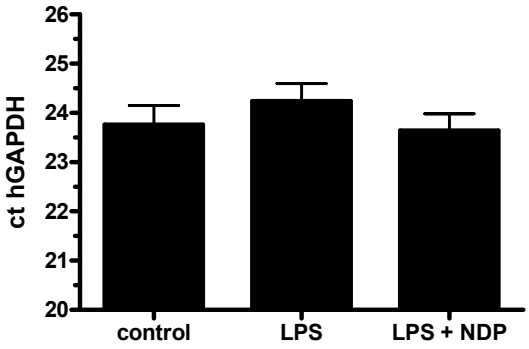

Fig. 1. Human GAPDH expression in treated vs. untreated MSC-derived adipocytes. MSC-derived adipocytes were incubated with LPS (1 ng/ml) or LPS plus NDP-MSH (NDP; 10^{-6} M) for 3 h. The corresponding cDNA was subjected to real-time TaqMan PCR (n=9). The incubation with LPS and LPS + NDP-MSH had no effect on the hGAPDH mRNA expression (ct values). Thus, hGAPDH is a suitable endogenous gene for the normalization of the gene expression.

Both undifferentiated MSCs and MSC-derived adipocytes revealed MC1-R mRNA expression (Fig. 2). Following differentiation to adipocytes, MC1-R expression was 1.9-fold increased but this difference was not significant.

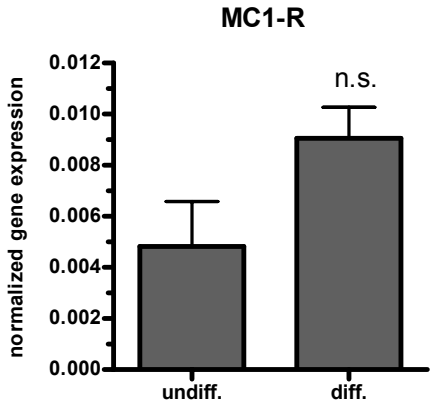

Fig. 2. MC1-R mRNA expression in undifferentiated MSCs and MSC-derived adipocytes. In both cell types MC1-R mRNA was expressed. However, the expression was increased after differentiation. cDNAs were subjected to real-time TaqMan PCR normalized to hGAPDH (n=6). Data are means ± SEM. undiff. = undifferentiated MSCs, diff. = MSC-derived adipocytes, n.s.= not significant.

Incubation of MSC-derived adipocytes with 10^{-6} M NDP-MSH for 24 h had no effect on the MC1-R mRNA expression (Fig. 3).

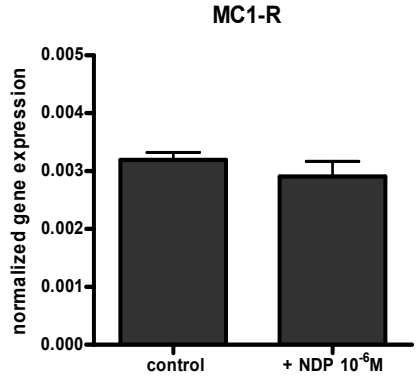

Fig. 3. MC1-R mRNA expression after stimulation with NDP-MSH. MSC-derived adipocytes were incubated with 10^{-6} M NDP-MSH for 24 h. The corresponding cDNA was subjected to real-time TaqMan PCR normalized to hGAPDH (n=4). The incubation with NDP-MSH had no effect on the MC1-R mRNA expression.

In order to verify that the MC1-R protein was indeed expressed on the adipocyte membranes. Immunocytochemistry for the MC1-R with AEC staining was performed. The MSC-derived adipocytes stained positively for MC1-R with an intensive red color (Fig. 4a). However, the fibers

showed a slightly more intensive staining than the rest of the cell. The negative control with rabbit IgG replacing the first antibody revealed only a faint staining of the fat vacuoles (Fig 4b).

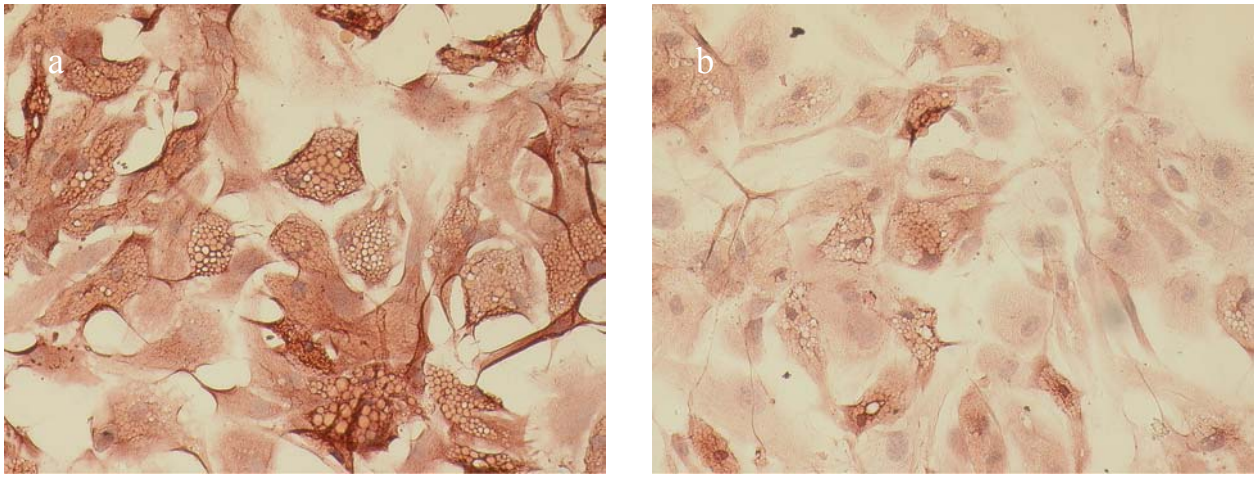

Fig. 4. Immunocytochemistry of MSC-derived adipocytes of MC1-R. Adipocytes were strongly positively stained (a). In the negative control, rabbit IgG of the same concentration instead the first antibody was added (b), whereby only slight reddish background was visible. Cells were incubated with a primary antibody against the human MC1-R, followed by incubation of a second biotinylated anti-rabbit IgG. Ampflification: 10x.

cAMP production

Binding of the MC1-R results in the activation of the adenylyl cyclase, which converts ATP to cAMP. Stimulation of MC1-R with the potent agonist NDP-MSH for 30 min was followed by the determination of intracellular cAMP content. Thereby, a NDP-MSH dose-dependent increase of cAMP was obtained (Fig. 5). However, the stimulation was relatively small, most likely because of the low number of MC1-R expressed on the human adipocytes. Control experiments performed with isoproterenol showed a several-fold cAMP induction (data not shown).

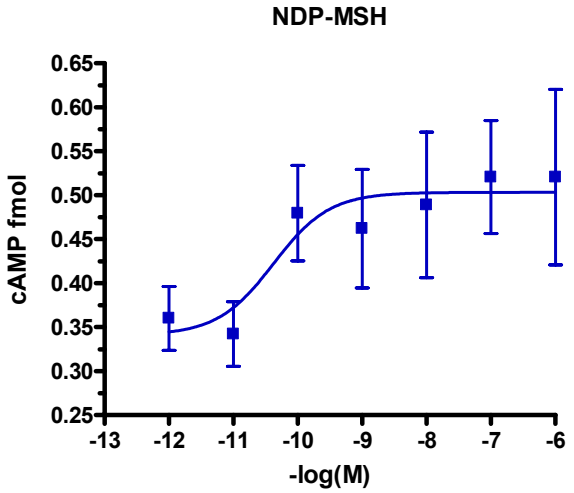

Fig. 5. Dose-dependent response of NDP-MSH stimulation on cAMP production in MSC-derived adipocytes. Cells were split and differentiated in 6-well plates. The cells were incubated in NDP-MSH from 10^{-6} to 10^{-12} M for 30 min at 37°C in incubation medium containing 0.1 mM IBMX. cAMP levels were determined by EIA. Each value is a mean ± SEM of 7 samples in duplicates from two independent experiments.

Lipolysis

We aimed to clarify the possible induction of lipolysis by MC1-R-activation. Therefore, glycerol content was assessed in supernatants of MSC-derived adipocytes and adipose tissue explants subjected to NDP-MSH and ACTH(1-24), respectively. As expected, isoproterenol and forskolin caused an increase in lipolysis, which in the MSC-derived adipocytes was 2-fold higher with isoproterenol ($P < 0.0001$) and 4-fold higher with forskolin ($P < 0.0001$), respectively (Fig. 6a). In the adipose tissue explants, isoproterenol caused a 2-fold increase in glycerol release ($P < 0.001$) (Fig. 6b). In contrast, addition of NDP-MSH and ACTH(1-24) to the MSC-derived cells had no effect on lipolysis (Fig. 6a). Also, on adipose tissue explants NDP-MSH showed no effect on lipolysis (Fig. 6b).

Furthermore, we analyzed the effect of NDP-MSH on murine 3T3-L1 adipocytes on lipolysis. Thereby, we obtained a dose-dependent increase in the glycerol release by NDP-MSH (Fig. 7), which is consistent with the findings of Boston et al [13].

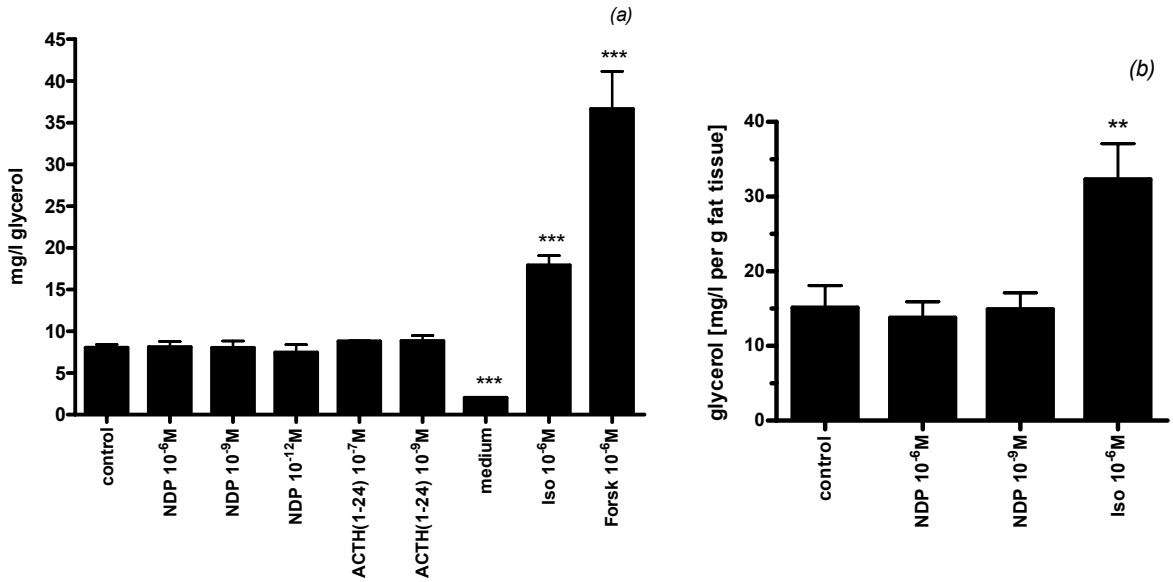

Fig. 6. Lipolysis in MSC-derived adipocytes and human adipose tissue. (a) Lipolysis of MSC-derived adipocytes. Cells were grown and differentiated in 6-well plates. Before the start of the experiment the medium was changed, NDP-MSH (NDP) with increasing concentration (10^{-6} – 10^{-12} M), 10^{-6} M isoproterenol (Iso) or 10^{-6} M forskolin (Forsk) was added and re-incubated for 24 h at 37°C. (*** $P < 0.0001$; n=6). (b) Lipolysis in human tissue explants. The minced and extensively washed human adipose tissue explants (300 mg in 3 ml medium) were incubated with D-MEM/F12 medium without phenol red supplemented either with NDP-MSH (NDP) or isoproterenol (Iso) for 24 h at 37°C (** $P < 0.001$; n=10, NDP-MSH, control; n=5, Iso). All values are means ± SEM.

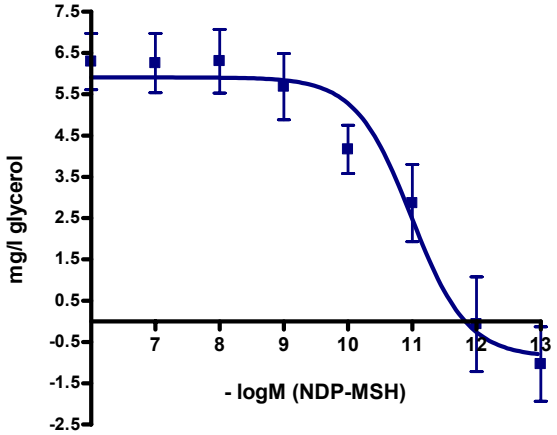

Fig. 7. Lipolysis in murine 3T3-L1 adipocytes. Cells were grown and differentiated in 6-well plates. For the experiment the medium was changed to D-MEM without phenol red supplemented with NDP-MSH with increasing concentration (10^{-6} – 10^{-13} M) and incubated for 90 min at 37°C (n=6). NDP-MSH dose-dependently increased lipolysis. All values are means ± SEM over basal glycerol release.

Cytokine release and expression

Recently, different studies [20-23] could demonstrate that α-MSH displays anti-inflammatory effects on monocytes, macrophages and dentritic cells via MC1-R stimulation. Therefore, we analyzed the potential influence of MC1-R on the inflammatory status in MSC-derived adipocytes and adipose tissue explants by inducing inflammation with LPS. In MSC-derived adipocytes there was a low basal IL-6 secretion (~0.75 ng/ml) but no IL-10 and TNF-α was detectable after a 24-h incubation. TNF-α and IL-10 secretion could not be induced by the addition of LPS, even up to a concentration of 1 µg/ml (data not shown). The addition of 1 ng/ml LPS led to a 27-fold increase in the IL-6 secretion compared to basal values ($P < 0.0001$). In addition, dexamethasone (1 µM) decreased the LPS-stimulated (1 ng/ml) IL-6 secretion approximately 7-fold ($P < 0.0001$) (Fig. 8).

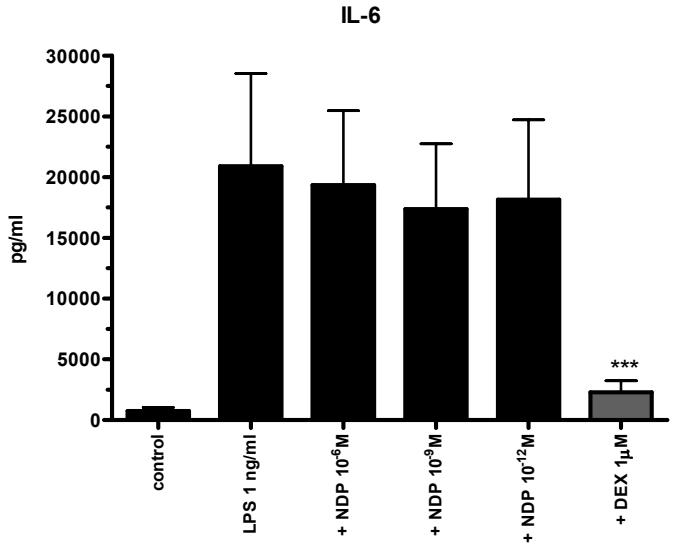

Fig. 8. IL-6 secretion in MSC-derived adipocytes. Adipocytes in 6-well plates were stimulated with 1 ng/ml LPS and co-administered with NDP-MSH (NDP) or 1 µM dexamethasone (DEX); incubated for 24 h at 37°C. Values are given the mean ± SEM. *** $P < 0.0001$ vs. LPS; n=9 LPS, NDP; n=6, control.

In adipose tissue explants, we found substantial amounts of IL-6 (Ø 226 ± 56 pg/ml per mg fat tissue) and lower amounts of TNF-α (Ø 0.098 ± 0.021 pg/ml per mg fat tissue) and IL-10 (Ø 0.188 ± 0.04 pg/ml per mg fat tissue) at basal conditions. After stimulation of the adipose tissue explants with 1 µg/ml LPS, we obtained a 2.6-fold increase for IL-6 to 592 ± 104 pg/ml per mg adipose tissue ($P < 0.005$), 11-fold increase for TNF-α to 1.109 ± 0.011 pg/ml per mg fat ($P < 0.0001$) and 6.6-fold increase for IL-10 to 1.246 ± 0.188 pg/ml per mg fat ($P < 0.001$).

Dexamethasone decreased the secretion of IL-6 by 50% ($P < 0.05$), IL-10 by 36% (n.s.) and TNF-α by 62% ($P < 0.0001$), respectively. Addition of NDP-MSH, at any concentration, did not change the secretion of these three cytokines (Fig. 9).

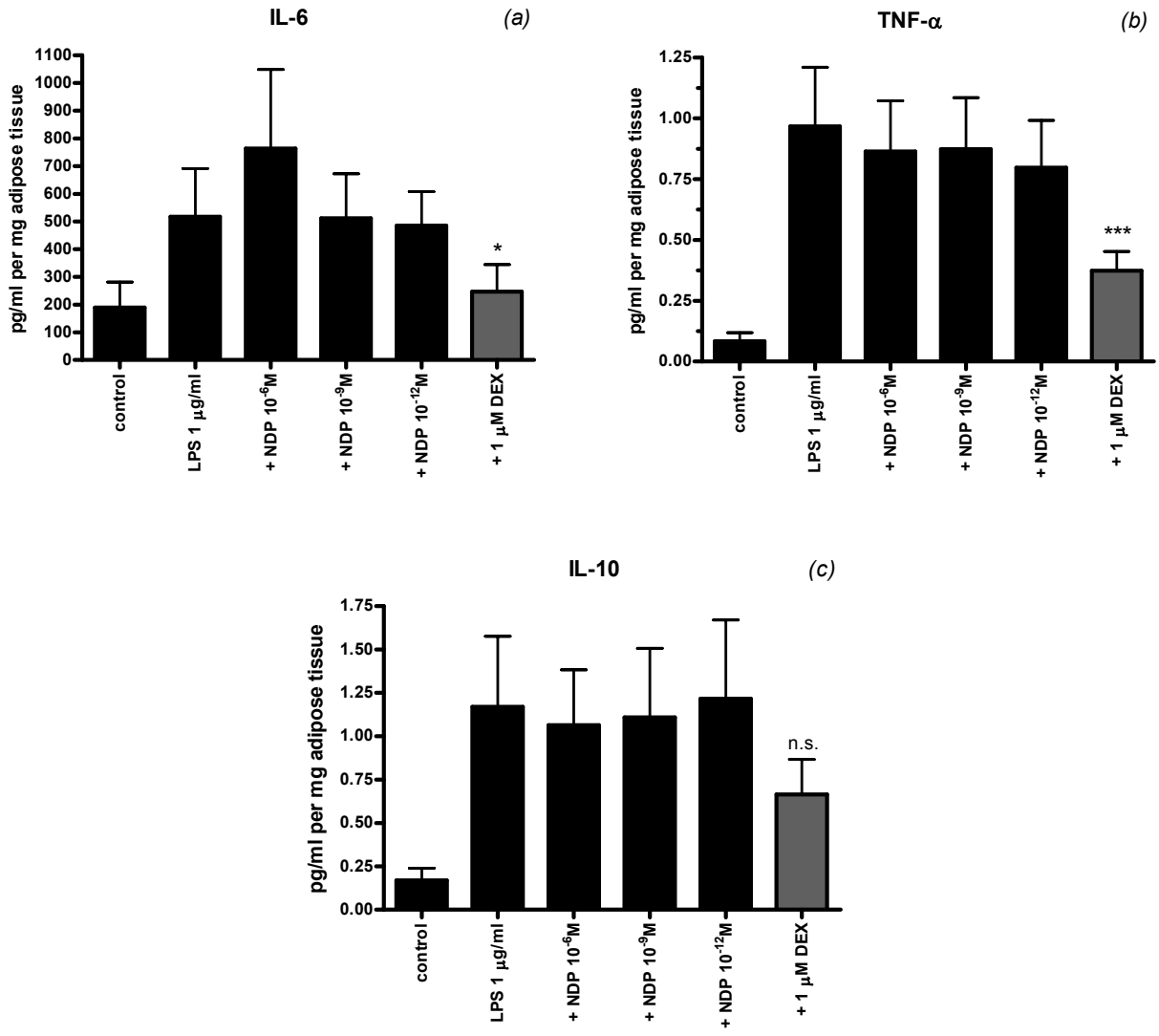

Fig. 9. Secretion of IL-6 (a), TNF-α (b), IL-10 (c) from human adipose tissue explants. Inflammation was induced by 1 µg/ml LPS and NDP-MSH (NDP) of different concentrations or 1 µM dexamethasone (DEX) was supplemented. The explants were incubated for 24 h at 37°C. Values are means ± SEM (n=18, NDP-MSH 10^{-9} and 10^{-12} M; n=9, NDP-MSH 10^{-6} M). n.s. = not significant.

Studying mRNA expression after a 3-h LPS stimulation (1 ng/ml) of the MSC-derived adipocytes, only low basal IL-6 and IL-8 expression was obtained. Interestingly, in the basal state very little TNF-α-mRNA, near the detection limit, was detectable. The 3-h stimulation with LPS resulted in a several-fold increase of the mRNA expression of IL-6, IL-8 and TNF-α ($P < 0.0001$). Whereas very high expression rates were obtained for IL-8 and IL-6, comparatively low expression was seen for TNF-α. Dexamethasone caused a robust decrease of the mRNA expression of all cytokines (Fig. 10).

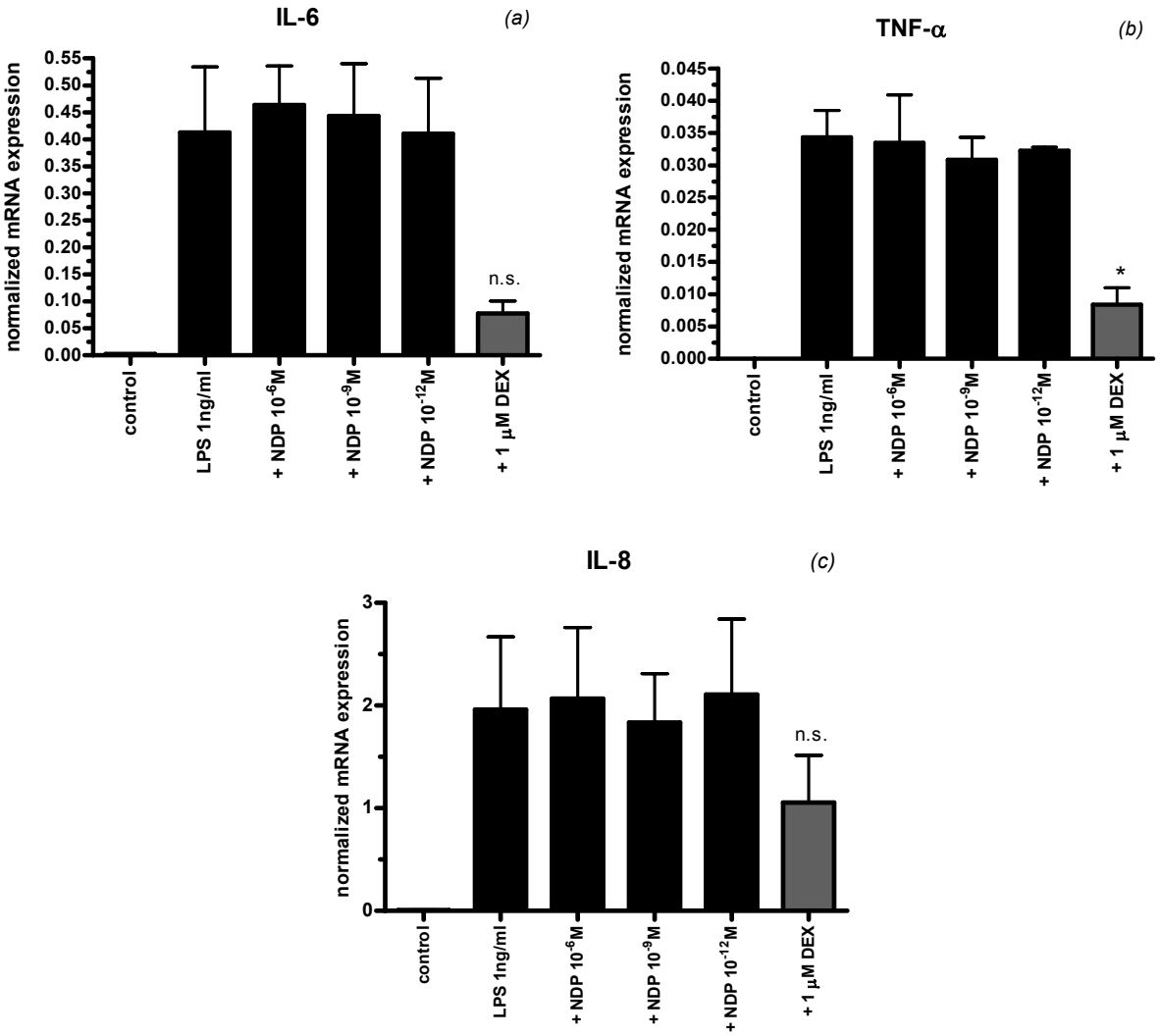

Fig. 10. mRNA gene expression of MSC-derived adipocytes of IL-6 (a), TNF-α (b) and IL-8 (c) after stimulation with LPS. MSC-derived adipocytes were treated with 1 ng/ml LPS and co-administered with NDP-MSH of different concentrations or 1 µM dexamethasone (DEX). The cDNA was analyzed with real-time TaqMan PCR. The results are given as means ± SEM of mRNA gene expression relative to hGAPDH (n=9, LPS; n=6, NDP-MSH, control; n=3). (* $P < 0.05$). n.s. = not significant.

On the other hand, there was no significant difference in the mRNA expression of IL-6, IL-8 and TNF-α when we compared the LPS + NDP-MSH stimulated samples versus the samples without addition of NDP-MSH (Fig. 10). Apart from LPS, in some experiments we administered IL-1β (20 units/ml) to accomplish stimulation of inflammation. Previously, it has been demonstrated that IL-1β potently increases inflammation in different cell types. As expected, IL-1β induced several fold the mRNA expression of TNF-α, IL-6 and IL-8. Though, the inflammatory induction by IL-1β was lower than with LPS (1 ng/ml). Dexamethasone decreased potently the IL-1β induced mRNA expression of TNF-α (by 50%), IL-6 (by 80%), and IL-8 (by 73%), respectively. However, co-administration of NDP-MSH had no effect on the IL-1β induced cytokine mRNA expression (Fig. 11).

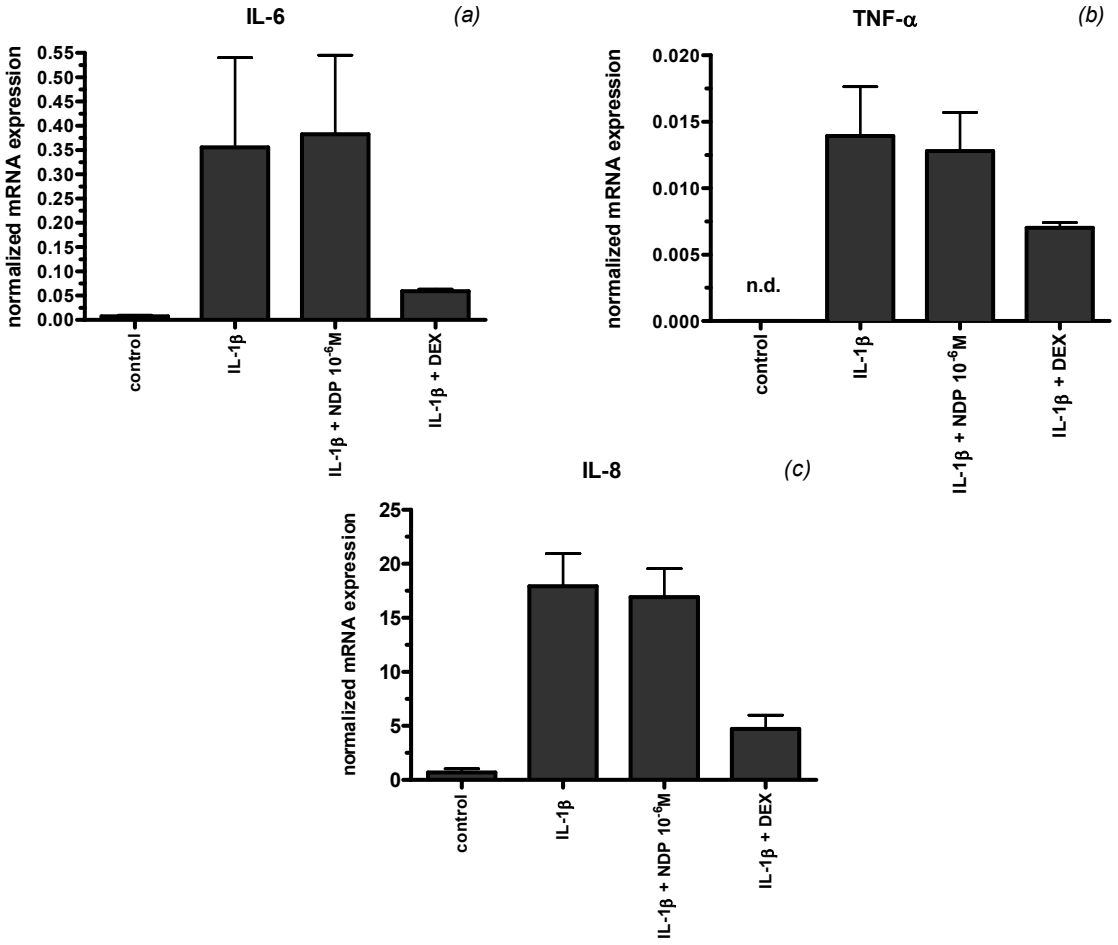

Fig. 11. mRNA gene expression of MSC-derived adipocytes of IL-6 (a), TNF-α (b) and IL-8 (c) after stimulation with IL-1β. MSC-derived adipocytes were treated with IL-1β (20 units/ml) and co-administered with NDP-MSH (NDP) of different concentrations or 1 μM dexamethasone (DEX). cDNA was analyzed with real-time TaqMan PCR. The results are given as means ± SEM of mRNA gene expression relative to hGAPDH (n=3). n.d. = not detectable.

To summarize, NDP-MSH did not show any effect on inflammation in adipose tissue explants as well as in MSC-derived adipocytes.

Cell viability (MTT assay)

The undifferentiated MSCs were analyzed for effects of NDP-MSH on proliferation and toxicity. The metabolic rate of the mitochondria was tested by using the colorimetric MTT assay. However, we found no differences between the controls and the NDP-MSH stimulated cells (Fig. 12).

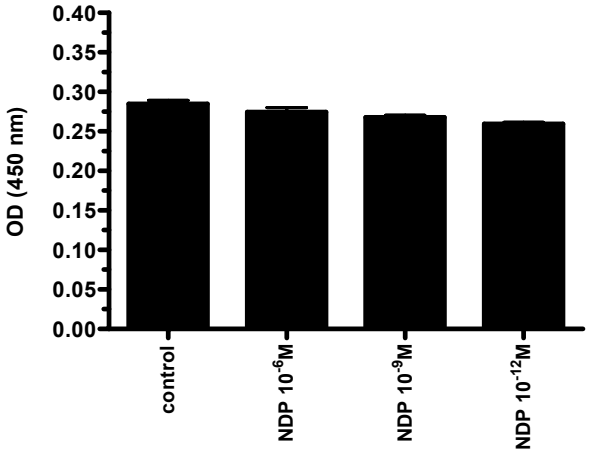

Fig. 12. Effect of NDP-MSH on the metabolic rate (MTT assay) on undifferentiated MSCs. Cells were seeded in 6-well plates and grown to be ~90% confluent. Medium was changed and NDP-MSH (NDP) was added for 24 h at 37°C. After an additional incubation time of 1 h in the presence of 0.5 mg/ml MTT the formed crystallized formazan was dissolved in 1 ml DMSO. Of the obtained solution 100 µl was transferred to wells of a 96-well plate and absorbance was measured in duplicates at 450 nm. Means ± SEM, n=6, from two separate experiments.

Cell proliferation (BrdU assay)

The proliferation rate of the undifferentiated MSCs was analyzed by measuring the incorporation of BrdU during DNA replication (n=16, from three independent experiments). The addition of NDP-MSH resulted in a significantly lower BrdU incorporation in the DNA compared to controls and was highest at the concentration of 10^{-6} M and lowest for 10^{-12} M. The incubation with 10^{-6} M NDP-MSH resulted in a decrease in proliferation of 24% ($P < 0.0001$). This anti-proliferative effect was seen even at the lowest concentration of 10^{-12} M NDP-MSH ($P < 0.005$) (Fig. 13).

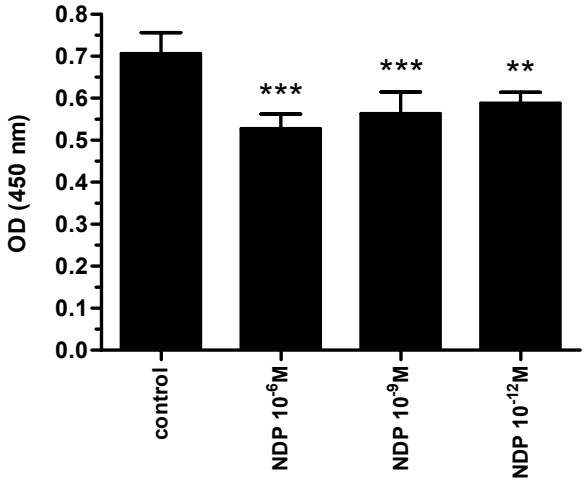

Fig. 13. Anti-proliferative effect of NDP-MSH on undifferentiated MSCs (BrdU incorporation). MSCs (2000 cells/well) were seeded into wells of a 96-well plate and grown to ~80% confluence. NDP-MSH (NDP) of different concentrations was added and incubated for 24 h at 37°C. BrdU (10 µM) was supplemented and the cells were re-incubated for 24 h, the cells were fixed and incubated with an anti-BrdU antibody coupled to POD, which was then visualized with TMB as substrate. The absorbance was measured at 450 nm ($n \geq 16$, from three separate experiments with cells from two different MSC donors). (*** $P < 0.0001$, ** $P < 0.005$).

Discussion

The aim of the present study was to analyze the functionality and role of MC1-R in MSC-derived adipocytes, adipose tissue and undifferentiated MSCs. Comparison of MC1-R mRNA expression levels in undifferentiated MSCs with that of MSCs differentiated to adipocytes showed that the expression level was higher in differentiated cells. This could reflect the importance of MC1-R in the adipocytes. MC1-R protein on the plasma membrane on the MSC-derived adipocytes was identified by immunocytochemistry. The receptor appeared equally distributed throughout the plasma membrane. To further investigate the functionality of the MC1-R on these adipocytes, cAMP production was determined after exposure of the cells to the potent agonist NDP-MSH. Although only a low increase in cAMP production was obtained, the effect was dose-dependent. The low level of cAMP increase may be explained by the very low copy number of MC1-R per cell, which we assumed to be limited to a few hundred receptors per cell [1] and which contrasts with the higher expression of adrenergic receptors on MSCs [24] and the ensuing markedly higher cAMP production induced by isoproterenol. Nevertheless, it appears that functional MC1-R are expressed on human adipocytes.

The next step was to elucidate the biological role of MC1-R on human adipocytes. MC1-R, together with MC2-R, was the first MC receptor subtype that was cloned [25, 26]. Its main physiological function is to mediate the induction of melanogenesis by α-MSH in melanocytes [10] and melanoma cells [27], and – in some species such as rabbits and mice – the lipolysis in adipocytes. However, in a majority of experimental animal species, this latter activity is more often mediated by MC2-R, the receptor for ACTH [10]. Data obtained in humans are controversial [14-17]. Therefore, we studied a possible effect on lipolysis of NDP-MSH in MSC-derived adipocytes and in human adipose tissue: NDP-MSH did not increase lipolysis in MSC-derived adipocytes, nor in adipose tissue explants; in addition no lipolytic effect of ACTH(1-24) was observed in the latter. Similarly, Bousquet-Mélou et al [28] reported that ACTH and α-MSH, even at high doses (1 μM), had very little effect on lipolysis on freshly isolated primate and human adipocytes. Furthermore, MC2-R is not expressed in human adipose tissue [1, 14, 17]. Since it is well known that ACTH binds to MC1-R with much lower affinity than α-MSH and that α-MSH has very weak affinity to MC2-R [10, 29], the data obtained in our study indicate that MC1-R does not mediate lipolysis in those types of human adipocytes or fat tissue used; whether MC2-R may occur in some specialized human fat depots remains to be elucidated.

Recently, different articles described an anti-inflammatory effect of α-MSH and NDP-MSH via activation of MC1-R on monocytes, macrophages and THP-1 cell line (reviewed in [30]), which is thought to be exerted through inhibition of NF-κB-mediated transcription [31]. Bhardwaj et al [32] showed that human peripheral blood monocytes and cultured human monocytes increased production and expression of the anti-inflammatory cytokine IL-10 upon α-MSH stimulation. Another study demonstrated an inhibition by α-MSH on TNF-α and IL-1β production in LPS-stimulated whole blood samples [21]. Thus, the melanocortin receptors are thought to be a good target to modulate inflammation [30]. However, in our experimental system, LPS-induced mRNA expression of TNF-α, IL-6 and IL-8 mRNA abundances were not affected by NDP-MSH. Accordingly, the LPS-mediated secretion of IL-6 was unchanged in the presence of NDP-MSH. Interestingly, after a 24-h incubation with LPS no TNF-α and IL-10 was detectable in these cells. In human adipose tissue explants, concentrations of TNF-α, IL-6 and IL-10 were not altered by the addition of NDP-MSH, indicating that in this tissue α-MSH has no anti-inflammatory activity.

Although adipocytes represent the main cellular component of adipose tissue (~50-70%), it consists also of stromal-vascular (SV) cells (~20-40%; i.e. fibroblasts, preadipocytes, non-differentiated MSCs), and infiltrated macrophages (~1-30% [33, 34]), which contribute significantly to the production of inflammatory factors by adipose tissue [35]. Recently, it has been suggested that macrophages accumulate in adipose tissue in obese mice and man [36, 37]. This accumulation is probably due to overexpression of the macrophage attracting protein MCP-1 in adipose tissue [38]. The rate of macrophage infiltration in adipose tissue appears to correlate with obesity and insulin resistance [39]. Important to note is that IL-8 also possesses a macrophage attracting potential [36].

Since macrophages express MC1-R and NDP-MSH is effective in lowering inflammation in peripheral macrophages and monocytic cell lines, we expected to obtain a decrease in cytokine secretion in adipose tissue. An explanation for the lack of effect could reside in the relatively low number of macrophages present in human adipose tissues compared to other cell types. Another possibility might be that the effect of NDP-MSH on macrophages infiltrated in the adipose tissue is only marginal or inexistent owing to their altered gene expression profile and/or activation status compared to macrophages in the blood circulation. Nevertheless, we obtained a significant increase in inflammation by addition of LPS in human adipose tissue and MSC-derived adipocytes. Furthermore, addition of dexamethasone revealed the expected effect of lowering

inflammation, indicating sensitivity of the system to anti-inflammatory mediators. Our findings suggest that MC1-R on adipocytes and possibly also on adipose tissue-infiltrated macrophages does not take part in the inflammatory signaling pathway.

We then analyzed the cell viability after addition of NDP-MSH in undifferentiated MSCs by performing the MTT assay, where MTT is formed by a mitochondrial enzyme to an insoluble purple substance which is an indication for the metabolic rate of the mitochondria or for toxicity of a substance, respectively. Again, we could not see any change upon NDP-MSH stimulation at any concentration. Therefore, we believe that NDP-MSH does not alter the mitochondrial metabolic rate nor exert a cytotoxic effect on human MSCs.

In order to analyze cell proliferation of undifferentiated MSCs, we performed the BrdU incorporation assay, a non-radioactive proliferation assay where incorporation of BrdU into DNA during DNA replication is measured. We obtained a significant effect of NDP-MSH on the MSCs on cell proliferation. Thereby, 10^{-6} M NDP-MSH had the highest and 10^{-12} M NDP-MSH the lowest but still significant effect. Smith et al [15] reported similar findings for α-MSH studying mouse and human preadipocytes. They demonstrated that agouti, at a 10-fold excess, can inverse the action of α-MSH on cell proliferation and that this inverse agonist of α-MSH promotes the differentiation of preadipocytes to mature adipocytes [15]. Our observation that in human adipose tissue and adipocytes only MC1-R is substantially expressed [1], supports the hypothesis that MC1-R is responsible for the anti-proliferative effects of NDP-MSH on undifferentiated MSCs. *De novo* adipocytes develop from MSCs infiltrating the adipose tissue where these stem cells eventually differentiate into preadipocytes and adipocytes (reviewed in [40]). If NDP-MSH inhibits proliferation via MC1-R on preadipocytes or MSCs under physiological conditions, this would be of importance for the stimulation of (pre-)adipocyte generation and differentiation, eventually causing obesity. It is interesting to note that blood plasma samples of α-MSH are elevated in human obesity [41, 42] but still remain at very low levels (approximately 16 pmol/l in obese subjects [42]). Thus, there might be a counter regulation of the organism against the *de novo* adipogenesis, which normally accompanies obesity. However, the origin of elevated circulating α-MSH is not yet known; whether adipose tissue could be the source is questionable because we found very low POMC mRNA expression in human subcutaneous and omental adipose tissue [1]. Yet, POMC mRNA was only detectable in obese subjects [1] which could be an indication that α-MSH may in fact be secreted from adipocytes, thus exerting a paracrine

action on adipocyte precursor cells. However, further studies are needed to clarify the source and role of α-MSH.

In summary, MC1-R protein is expressed in MSCs and in MSCs differentiated to adipocytes and MC1-R reveals functional activity. There was no effect of NDP-MSH on lipolysis and inflammation in MSC-derived adipocytes and in adipose tissue, respectively. Whereas there was no modulation of the metabolic rate, we observed a profound anti-proliferative effect of NDP-MSH on undifferentiated MSCs, likely mediated via the MC1-R. Thus, the anti-proliferative effect of α-MSH on adipocyte precursor cells via the MC1-R might regulate the development of additional adipocytes that possibly leads to obesity when the MC1-R signaling is disrupted.

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Chapter 5: TNF-α, IL-6, IL-8 and IL-10 in human adipose tissue and adipocytes[4]

Abstract

Objective: TNF-α, IL-6, IL-8 and IL-10 are cytokines known to be associated with obesity. We investigated their secretion and mRNA expression in inflammatory induced human adipose tissue explants and adipocytes. In addition, the effect of these cytokines on lipolysis, glucose uptake and IL-6 mRNA expression was analyzed.

Methods: In order to induce inflammation human adipose tissue explants, mesenchymal stem cell (MSC)-derived adipocytes and adipocytes derived from preadipocytes were incubated with different concentrations of endotoxin (LPS) at different time points. The cytokine protein concentrations in supernatants were determined after a 24-h LPS exposure with commercially available ELISA kits. Gene expression of the cytokines was determined with quantitative real-time PCR. Lipolysis was analyzed by measuring glycerol released into the medium. Glucose uptake was evaluated by measuring the uptake of tritium labelled glucose by the cells.

Results: Administration of LPS to adipose tissue explants increased the secretion of all tested cytokines. Co-administration of dexamethasone caused a robust decrease. Only in short term cultures (> 6 h) low levels of TNF-α mRNA was expressed in LPS-exposed MSC-derived adipocytes and adipocytes derived from preadipocytes. IL-6 and IL-8 mRNA expression peaked after 6 h and declined continuously afterwards. IL-10 mRNA was not detectable at any time point. Dexamethasone revealed a strong decreasing impact on mRNA expression of all analyzed cytokines. Large amounts of IL-6 and IL-8 protein were detected when adipocytes were stimulated with LPS for 24 h. TNF-α and IL-10 protein was not detectable at any LPS concentration. Of the four cytokines only TNF-α showed an effect on lipolysis, glucose uptake and IL-6 mRNA expression.

Conclusion: Our results suggest that in adipose tissue TNF-α and IL-10 are secreted by macrophages rather than adipocytes. However, exogenous TNF-α can modulate lipolysis, glucose uptake and IL-6 mRNA expression in human adipocytes. IL-6 and IL-8 secreted from adipocytes might modulate or activate inflammation of non-adipose cell types including macrophages, possibly leading to the known co-morbidities of obesity.

[4] Data of this chapter are submitted for publication in: Hoch M, Eberle AN, Peterli R, Peters T, Seboek D, Keller U, Muller B, Linscheid P LPS induces interleukin-6 and interleukin-8 but not tumor necrosis factor-α in human adipocytes, in revision, Int J Obes

Introduction

Studies performed in several rodent models have provided evidence on adipose tissue-derived, obesity-induced TNF-α production [1]. Obesity-related insulin resistance was improved by TNF-α neutralization and, at a cellular level, TNF-α was shown to interfere with insulin receptor signal transduction [2, 3]. Subsequently, it became clear that both rodent and human obesity are accompanied by a substantial inflammatory response. In fact, circulating plasma levels of several immuno-modulatory peptides including TNF-α [4], IL-6 [5], IL-8 [6], IL-10 [7], IL-18 [8] and C-reactive protein [9] were found to be elevated in human obesity. Accordingly, TNF-α production was elevated in adipose tissue explants from obese as compared to lean human subjects [10]. Moreover, adipose tissue IL-6 content correlates with glucose uptake-related insulin resistance both *in vivo* and *in vitro* [11]. The catabolic and insulin resistance-inducing properties attributed to many of these signaling molecules indicate they may be responsible for generation of type 2 diabetes [12].

TNF-α and IL-6 are two extensively studied adipose tissue-derived cytokines with presumed roles in obesity-associated disorders. Nevertheless, their relevance in the etiology of human type 2 diabetes remains equivocal. While several authors confirmed obesity-associated increased serum TNF-α levels [13-15], others did not [16]. In contrast to rodents, TNF-α neutralization by specific antibodies did not improve insulin sensitivity in humans [17]. In another study, obesity was associated with circulating IL-6, but insulin resistance was independent of both IL-6 and TNF-α and type 2 diabetes [18]. Moreover, it has been demonstrated *in vivo* that human subcutaneous adipose tissue releases IL-6 but not TNF-α [19]. TNF-α-induced metabolic effects are well established, and IL-6 is often grouped together with TNF-α as a so-called "pro-inflammatory" cytokine. However, recent evidence suggests a more balanced, under specific conditions even anti-inflammatory and TNF-α-opposing activity spectrum of IL-6 [20, 21].

While IL-6 production is well documented in several adipocyte models [22-24], a relevant expression of TNF-α in triglyceride-storing adipocytes may be questioned. In fact, a detailed study in collagenase-digested adipose tissue fractions demonstrated high IL-6 secretion by mature adipocytes, which contrasted the low and transient TNF-α secretion by the same cells [25]. Therefore, it may be hypothesized that several adipose tissue-derived factors, including TNF-α, are mostly produced by non-fat cells within the adipose tissue, likely to be macrophages [26]. This notion is supported by the increased macrophage infiltration identified in obese mice [27, 28] and humans [29]. In numerous reports and review articles TNF-α has been referred to as an adipocyte-secretion factor. However, to

the best of our knowledge, TNF-α production has never been unequivocally demonstrated in an adipocyte model, including murine 3T3-L1 cells.

In view of these controversial data, we aimed at clarifying the basal and endotoxin-induced expression and secretion of cytokines known to be elevated in obesity, including TNF-α, IL-6, IL-8 and IL-10 in human adipose tissue explants, in human adipocytes as well as in macrophage-adipocyte co-cultures. In order to identify potential autocrine effects, gene expression, lipolysis induction and insulin-mediated glucose uptake were assessed in MSC-derived adipocytes exposed to the four inflammatory mediators.

Material and Methods

Human adipose tissue cultures

The study was approved by the local ethical committee, and informed written consent of the subjects was obtained. Abdominal subcutaneous and omental adipose tissue samples of approximately 50-100 grams were obtained from five morbidly obese women undergoing gastric banding or bypass surgery. The mean BMI of these patients was 41.6 ± 1.7 kg/m^2. Initially, the connective tissue and blood vessels were dissected. The tissue was minced into small pieces (approximately 5 mg) and washed in PBS (Invitrogen AG, Basel, Switzerland). The adipose explants were incubated for 1 h with periodical mixing in D-MEM/F12 (Invitrogen) supplemented with 0.25% BSA fraction V (Sigma, St. Louis, MO), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Invitrogen). The adipose fragments were separated from the medium with a nylon mesh (250 µm). For experiments, 300 mg aliquots were placed for 24 h into 3 mL fresh medium with supplements.

Human ex vivo differentiated adipocytes

Human mesenchymal stem cells (MSCs) from bone aspirates (20-40 ml) were obtained from healthy donors (18-63 years) during routine orthopaedic surgical procedures. Nucleated cells were isolated from the aspirate by Ficoll density gradient centrifugation (Histopaque1, Sigma). MSCs were thereafter selected within the nucleated cells in culture on the basis of adhesion and proliferation on the plastic surface. The cells were expanded in D-MEM, supplemented with 10% fetal calf serum (FCS) and 5 ng/ml basal fibroblast growth factor (bFGF, Invitrogen) until they reached confluence. Alternatively, preadipocytes were obtained by centrifugation from collagenase-digested adipose tissue (200 g) and cultured like MSCs. Adipogenic differentiation was induced by adding D-MEM/F12 (Invitrogen) containing 3% FCS (Invitrogen), 1 µM dexamethasone (Sigma), 0.1 mM L-ascorbic acid (Sigma), 250 µM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), 5 µM transferrin (Calbiochem, La Jolla, CA), 0.2 nM 3,3,5-Triiodo-L-thyronine (T3) (Sigma) and 100 nM insulin (Actrapid, Novo Nordisk, Kuesnacht, Switzerland). The medium was changed every 3 days. After 15 to 18 days differentiation medium was removed by washing with PBS. Adipocytes were kept for 24 h in basal medium before experiments.

RNA isolation and RT-PCR

Total RNA from cell cultures was isolated using TriReagent (MRC, Cincinnati, OH) according the manufacturer's protocol. First strand cDNA was synthesized from 1 µg total RNA with M-MLV reverse transcriptase (Promega, Madison, WI) using oligo$(dT)_{15}$ primers (Promega). PCR was performed on a conventional thermal cycler (TGradient, Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen). Human gene-specific, intron spanning primers were as follows: adiponectin (102 bp): 5'-TGGGCCATCTCCTCCTCA-3'' (sense), 5'-AATAGCAGTAGAACAGCTCCCAGC-3' (antisense); leptin (121 bp): 5'-TGCCCATCCAAAAAGTCCA-3' (sense), 5'-GAAGTCCAAACCGGTGACTTTCT-3' (antisense); PPARγ2 (580 bp): 5'-GCGATTCCTTCACTGATAC-3'' (sense), 5'-GCATTATGAGACATCCCCAC-3' (antisense); GLUT4 (319 bp): 5'-CCCCCTCAGCAGCGAGTGA-3'' (sense), 5'-GCACCGCCAGGACATTGTTG-3' (antisense);

Real-time TaqMan PCR

Quantitative real-time TaqMan PCR was performed with commercial pre-designed primers and FAM dye-labelled TaqMan minor groove binder (MGB)-probes (Applied Biosystems, Foster City, CA) for IL-6, IL-8, IL-10 and TNF-α and 2x Universal PCR Master Mix (Applied Biosystems). The analysis was run on a 7500 Fast real-time PCR system (Applied Biosystems). Each TaqMan reaction contained 2 µl of 1:10 diluted cDNA solution in a total reaction volume of 20 µl. As endogenous control human GAPDH was used, which is the recommended reference gene to normalize in adipocytes [30]. The relative expression was determined by the comparative threshold method as described in the ABI Prism 7700 User Bulletin #2 (P/N 4303859) from Applied Biosystems.

Cytokine determinations in culture supernatants

Adipose tissue explants in 35 mm dishes were subjected to 1 µg/ml LPS (055:B5; Fluka, Buchs, Switzerland) for 24 h. The glucocorticoid dexamethasone (1 µM) is known to reduce NF-κB-induced gene expression and was co-administrated in some wells as a control. Supernatants were collected and subjected to IL-6, IL-8, TNF-α and IL-10 analysis by ELISA. Kits were from eBioscience (San Diego, CA) except for IL-8 (Orgenium Laboratories, Helsinki, Finland). MSC-derived adipocytes were stimulated with different LPS concentrations between 10 pg/ml and 1 µg/ml. The supernatants were collected and analyzed as described above.

Lipolysis

MSC-derived adipocytes kept in 6-well plates were washed with PBS and the medium changed to phenol red free D-MEM/F12 (Invitrogen), 3% FCS. 24 h later, fresh medium was provided with supplements: TNF-α (10 ng/ml), IL-6 (10 ng/ml), IL-8 (100 ng/ml) or IL-10 (20 ng/ml) (all from PeproTech EC Ltd, London, UK). As positive control isoproterenol (10^{-6} M) and forskolin (10^{-6} M) were administrated. Supernatants were collected after 24 h and heated in a water-bath (80° C) for 15 min. Glycerol content of the medium was determined using the UV-method kit from R-Biopharm (Darmstadt, Germany) according the manufacturer's protocol.

Adipocyte-macrophage co-cultures

Adipocytes and macrophages were co-cultured as previously described [31]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from Buffy Coats by Ficoll gradient separation and kept in cell culture inserts with 0.4 μm pore size (BD Falcon, Bedford, MA) for five days. PBMC-derived macrophages were exposed to LPS (1μg/ml) for 2 h. Thereafter, cell culture inserts containing the activated macrophages were washed three times and kept in co-culture with MSC-derived adipocytes for an additional 24 h. Supernatants were collected for TNF-α analysis.

Glucose uptake

Glucose uptake experiments were performed as previously described [32]. Briefly, MSC-derived adipocytes were washed three times in warm PBS and kept in D-MEM/F12 containing 5 mM glucose and 3% FCS. After 24 h supplements were added. On the next day, fresh medium with supplements was provided. After 2 h 100 nM insulin was added to half of the wells and incubated for 20 min. 1 μCi deoxy-D-glucose, 2-[^{3}H(G)] (PerkinElmer, Boston, MA) was added to all wells and incubated for 15 min. Cells were washed three times in ice cold PBS and lysed in 0.1% SDS. Radioactivity was measured in a scintillation counter.

Statistical analysis

All values are presented as means ± SEM. For statistical analysis one way ANOVA was used and subjected posthoc to Bonferroni's multiple comparison tests. Two groups of data were compared with Student's t-test (Fig. 4B). A value of $P < 0.05$ was regarded as statistically significant. All analyses were performed using Prism 4.0 (GraphPad, San Diego, CA).

Results

Cytokine secretion by LPS-exposed adipose tissue explants

In basal adipose tissue explant-conditioned media (24 h) cytokine concentrations were as follows: IL-6 (226 ± 56 pg/ml/mg adipose tissue), TNF-α (0.098 ± 0.021 pg/ml/mg), IL-8 (1954 ± 519 pg/ml/mg) and IL-10 (0.19 ± 0.04 pg/ml/mg) (Fig. 1). LPS administration evoked significant increases in IL-6 (5.5-fold, $P < 0.01$), TNF-α (19.5-fold, $P < 0.01$), IL-8 (3.5-fold, $P < 0.05$) and IL-10 (12.5-fold, $P < 0.01$) secretion. Dexamethasone co-administration (1 μM) reduced the LPS-induced secretions by 62% (IL-6, $P < 0.01$) and 63% (TNF-α, $P < 0.01$). Under the same conditions LPS-induced IL-8 and IL-10 secretions were also reduced, even though statistical significance was not reached.

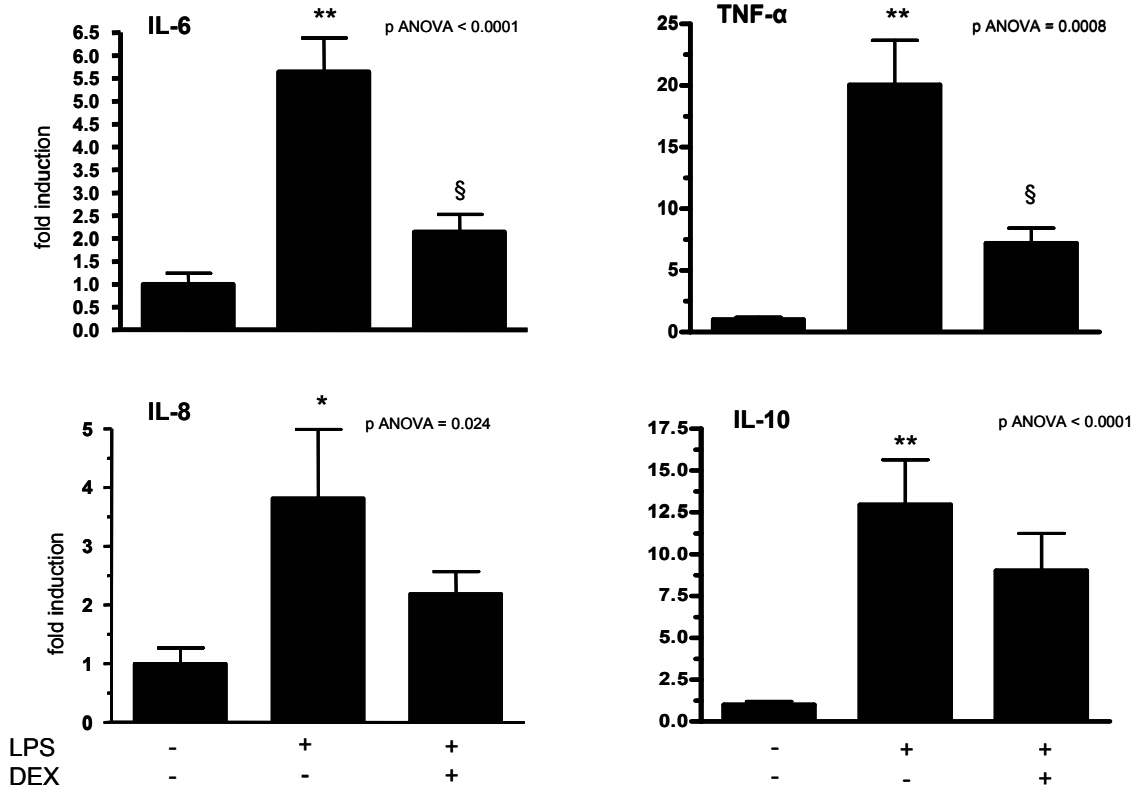

Fig. 1. Cytokine secretion by human whole adipose tissue. Adipose tissue explants were kept in culture medium and subjected to LPS and LPS-dexamethasone treatments for 24 h. Basal concentrations were 226 ± 56 pg/ml/mg adipose tissue (IL-6), 0.098 ± 0.021 pg/ml/mg (TNF-α), 1954 ± 519 pg/ml/mg (IL-8), and 0.19 ± 0.04 pg/ml/mg (IL-10). Data are means ± SEM from triplicate experiments using n=5 donors, except for IL-8 (n=3). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, as compared to untreated controls. § indicates $P < 0.01$ as compared to LPS alone.

LPS-induced cytokine production by adipocytes

Typically, at least 90% of MSC had visible lipid droplets after exposing the cells to adipogenesis-inducing medium (Fig. 2A). The adipocyte phenotype of MSC-derived adipocytes was confirmed by the presence of adipocyte-specific mRNAs including adiponectin, leptin, glucose transporter 4 (GLUT 4), and PPARγ2 (Fig. 2B).

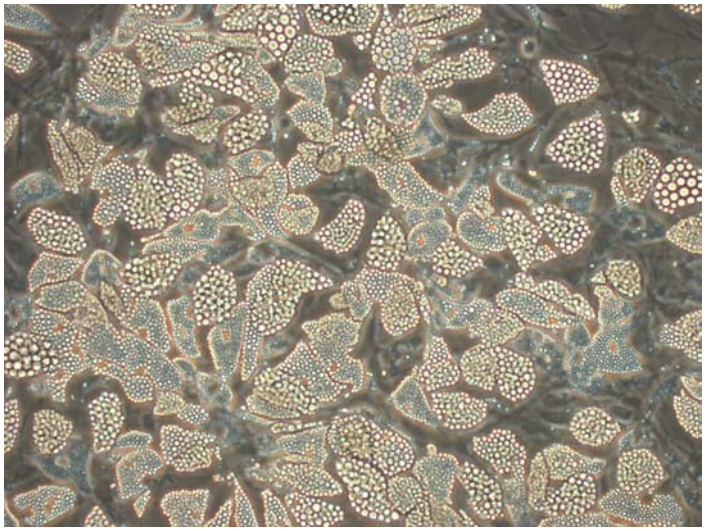

Fig. 2A. Characterization of MSC-derived adipocytes: lipid accumulation. *Ex vivo* differentiated adipocytes had visible lipid droplets. Magnification 10x.

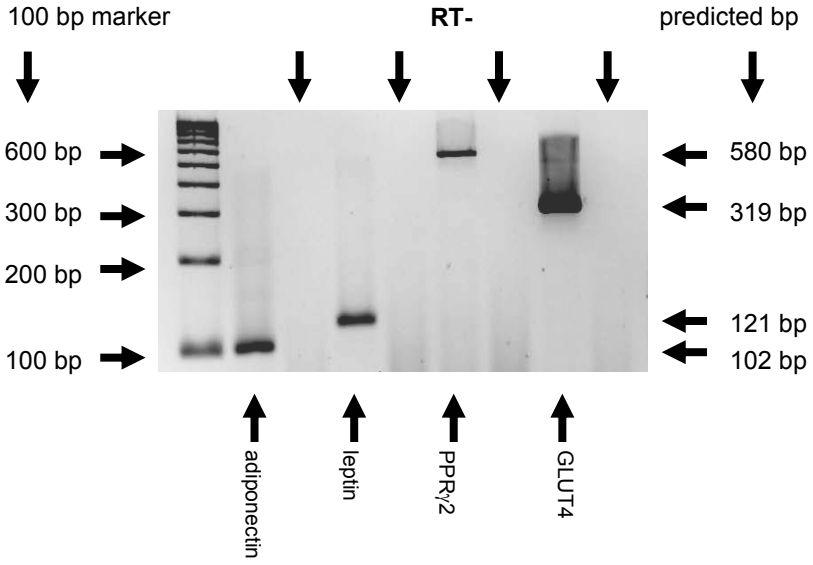

Fig. 2B. Characterization of MSC-derived adipocytes: adipocyte specific gene expression. Expression of adipocyte-specific mRNAs was verified by RT-PCR. "RT-" indicates negative controls lacking reverse transcriptase.

In addition, we verified that glucose transport was dose-dependently activated by insulin (Fig. 2C). Finally, lipolysis was inducible by isoproterenol- and forskolin-administration (10^{-6} M), respectively (Fig. 2D). Identical results were obtained using adipose tissue-derived preadipocytes as adipocyte precursor cells (not shown).

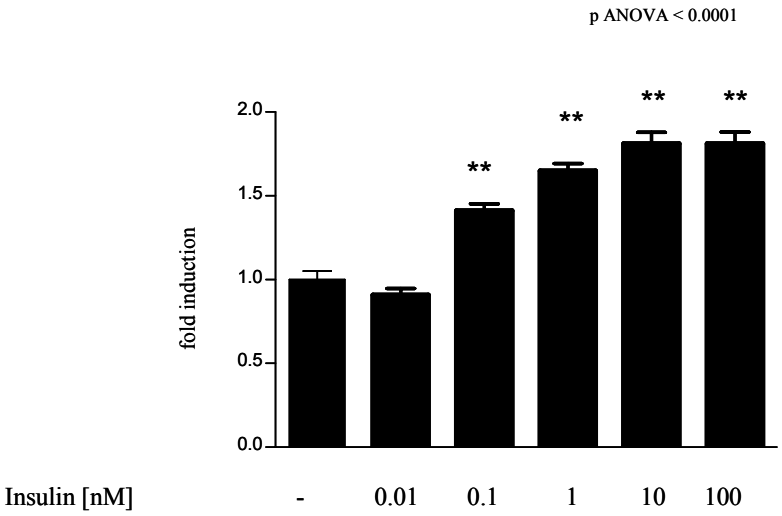

Fig. 2C. Characterization of MSC-derived adipocytes: glucose uptake. Glucose uptake was measured in the presence of increasing insulin doses. Data are means ± SEM from n=6 wells. ** indicates P < 0.01 as compared to controls.

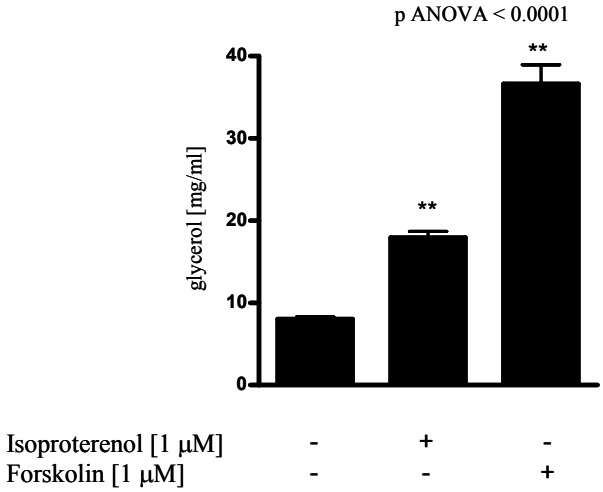

Fig. 2D. Characterization of MSC-derived adipocytes: lipolysis. Forskolin- and isoproterenol-induced lipolysis, respectively, was assessed by measuring glycerol release. Data are means ± SEM from n=6 wells. ** indicates P < 0.01 as compared to controls.

Using both MSC- as well as preadipocyte-derived adipocytes, we determined the kinetics of LPS-mediated IL-6, IL-8, TNF-α and IL-10 mRNA inductions. IL-6 and IL-8 mRNAs were rapidly ($t < 3$ h) induced 37- and 62-fold, respectively (Fig. 3) and remained elevated for until at least $t = 6$ h. Co-administration of dexamethasone reduced the LPS-induced mRNA levels by 59% for IL-6 ($P < 0.01$) and 33% for IL-8 ($P < 0.01$) at $t = 3$ h. At 24 h the transcript abundances slightly declined and reached approximately 22% and 41% of the respective 6 h levels after 48 h. In striking contrast, LPS-administration evoked only a weak (as compared to other cytokines) and transient TNF-α mRNA induction in relation to GAPDH. In fact, TNF-α transcript was detected after 3 h, but almost completely disappeared after 6 h and was undetectable after 24 h and 48 h. IL-10 mRNA was not induced at any time point.

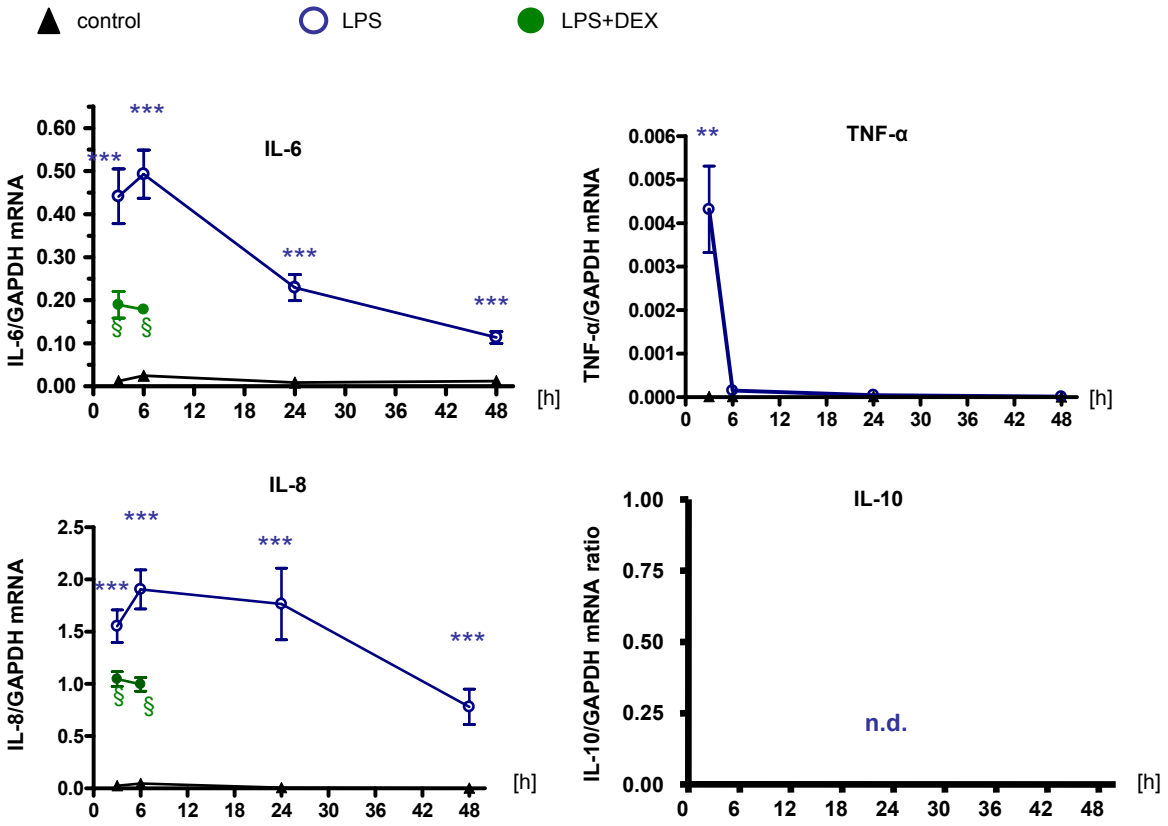

Fig. 3. Time-dependent cytokine mRNA induction. At $t = 0$ LPS and LPS-dexamethasone, respectively, were administrated to MSC- (n=6) and preadipocyte-derived (n=3) adipocytes. RNA was extracted at indicated time points and subjected to quantitative real-time PCR analysis for TNF-α, IL-6, IL-8 and IL-10. Results from both MSC- and preadipocyte-derived adipocytes were combined and presented as means ± SEM. ** and *** indicate $P < 0.01$ and $P < 0.001$ respectively. § indicates $P < 0.01$ as compared to LPS alone. "n.d." indicates not detected. DEX = dexamethasone.

Cytokine secretion by MSC-derived adipocytes was assessed in the presence of increasing LPS concentrations. Between 0.01 to 1 ng/ml LPS, IL-6 and IL-8 productions were dose-dependently induced. At higher doses ranging from 10 ng/ml to 1 µg/ml IL-6 and IL-8 reached plateau levels of 28 ± 1.1 ng/ml and 210 ± 13 ng/ml, respectively (Fig. 4). LPS-induced (10 ng/ml) IL-6 and IL-8 secretions were reduced by the addition of dexamethasone by 87% ($P < 0.001$) and 68% ($P < 0.001$). In contrast to IL-6 and IL-8, both TNF-α and IL-10 remained undetectable even at the highest LPS dose. In a few samples, minimal amounts around the detection limit (4 pg/ml) of TNF-α were detected in short-term (< 6 h) stimulated cells. Conversely, in supernatants from 24 h co-cultured adipocytes and macrophages TNF-α was present in basal (0.80 ± 0.042 ng/ml) and in LPS-treated cells (4.4 ± 0.2 ng/ml) (Fig. 5).

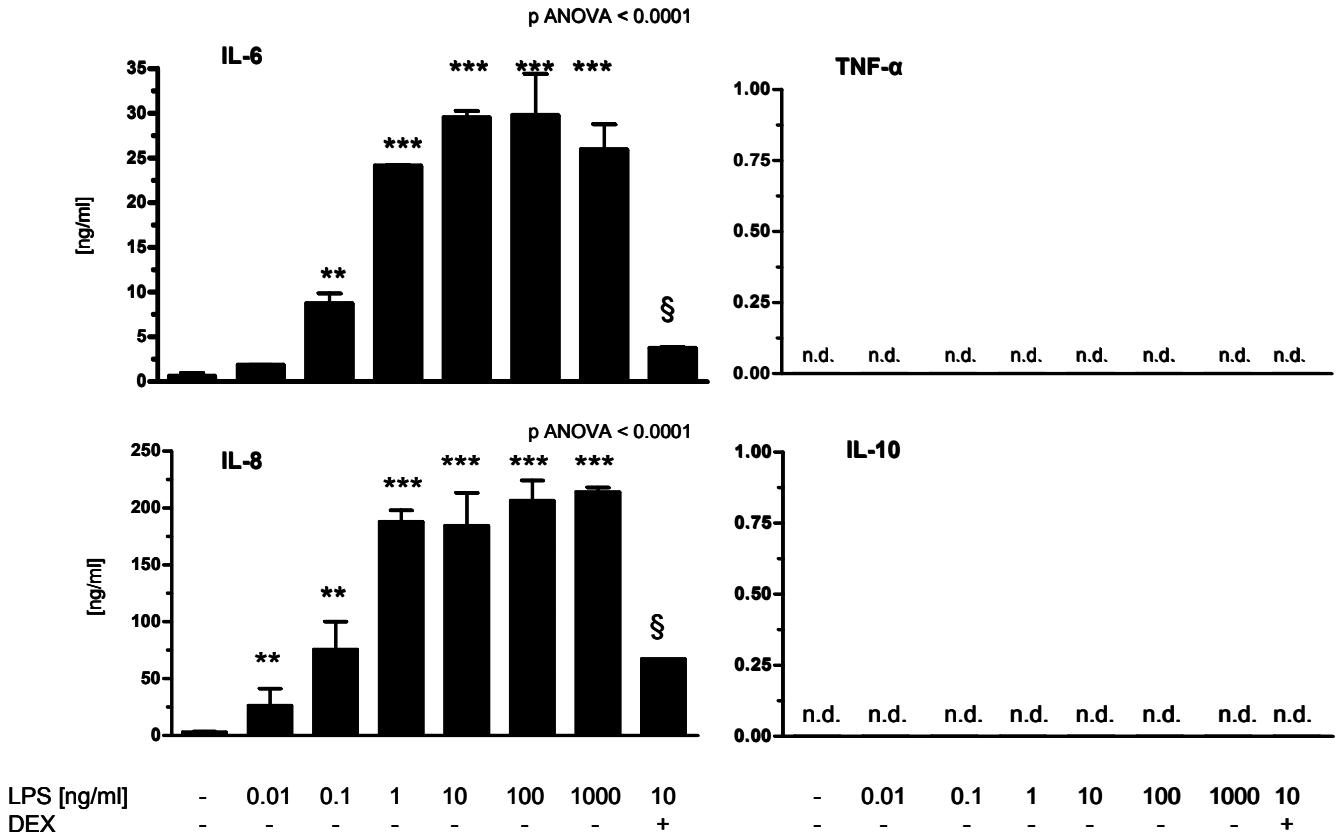

Fig. 4. Dose-dependent LPS-induced IL-6 and IL-8 productions. MSC-derived adipocytes were exposed for 24 h to LPS concentrations ranging from 10 pg/ml to 1 µg/ml and TNF-α, IL-6, IL-8 and IL-10 protein concentrations were determined in supernatants after 24 h. Data are from triplicate experiments from at least n=3 donors and from n=3 wells. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, as compared to controls. § indicates $P < 0.001$ as compared to LPS (10 ng/ml) alone. DEX = dexamethasone.

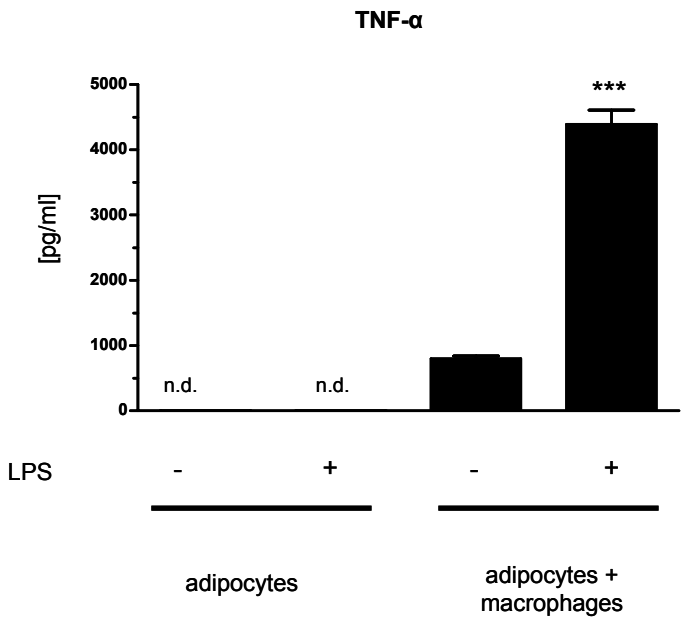

Fig. 5. TNF-α secretion from adipocyte-macrophage co-cultures. Supernatants from adipocytes and adipocyte-macrophage co-cultures were subjected to TNF-α analysis. Data are from n=3 wells. *** indicates $P < 0.001$ as compared to controls. "n.d." indicates not detected.

Effects of IL-6, IL-8, TNF-α and IL-10 on adipocytes

The induction of lipolytic activity was assessed by measuring glycerol release. TNF-α (10 ng/ml) administration evoked a 2-fold ($P < 0.01$) induction in glycerol release (Fig. 6A). In contrast, lipolysis was not affected by exposure to IL-6 (10 ng/ml), IL-8 (100 ng/ml) and IL-10 (20 ng/ml), respectively.

Glucose transport was assessed in both, absence and presence of insulin. Basal glucose uptake was increased 1.7-fold ($P < 0.01$) by TNF-α (Fig. 6B). In contrast, none of the other cytokines influenced glucose uptake. Insulin-induced glucose uptake rates were increased approximately 2.5-fold above basal uptake and were not affected by cytokine administrations.

In order to determine the dose-dependence of TNF-α on adipocyte functions, IL-6 mRNA was assessed in the presence of different TNF-α concentrations. In the presence of 0.1 ng/ml TNFα, IL-6 mRNA was elevated 11.3-fold as compared to untreated controls (Fig. 6C). Increasing TNF-α up to 100 ng/ml had only modest additional effects on IL-6 mRNA induction. In analogy with both lipolysis and glucose uptake data, IL-6 mRNA was not affected by IL-6, IL-8 and IL-10 administrations.

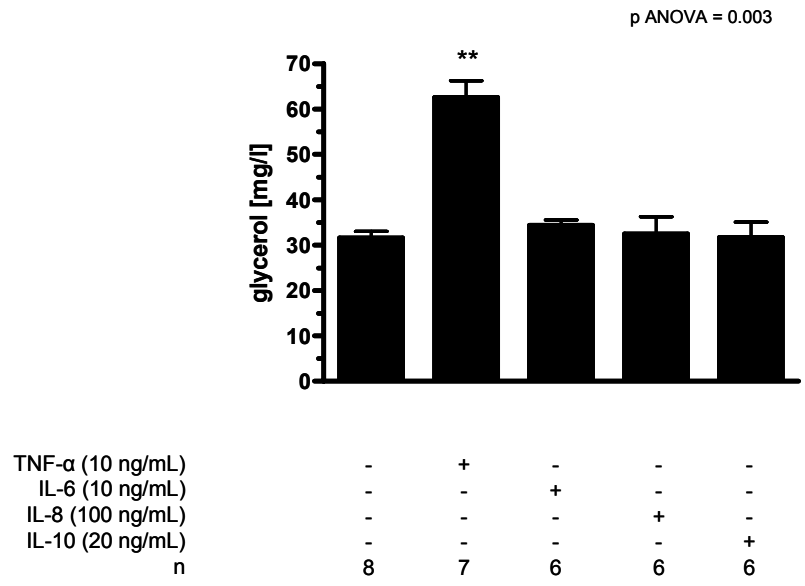

Fig. 6A. TNF-α-induced effects in adipocytes: lipolysis. MSC-derived adipocytes were exposed to TNF-α, IL-6, IL-8 and IL-10 as indicated. Lipolysis was assessed by measuring the 24 h release of glycerol. Data are means ± SEM from 6 or from indicated number of wells (n). ** indicates $P < 0.01$ as compared to controls.

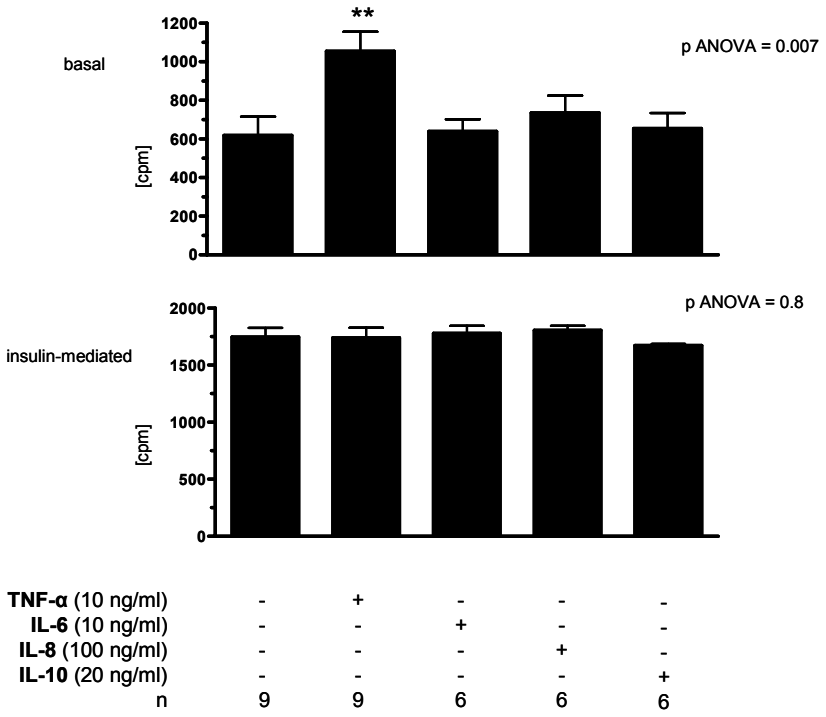

Fig. 6B. TNF-α-induced effects in adipocytes: glucose uptake. Basal and insulin-mediated glucose uptake was monitored for 15 min. Data are means ± SEM from 6 or from indicated number of wells (n). ** indicates $P < 0.01$ as compared to controls.

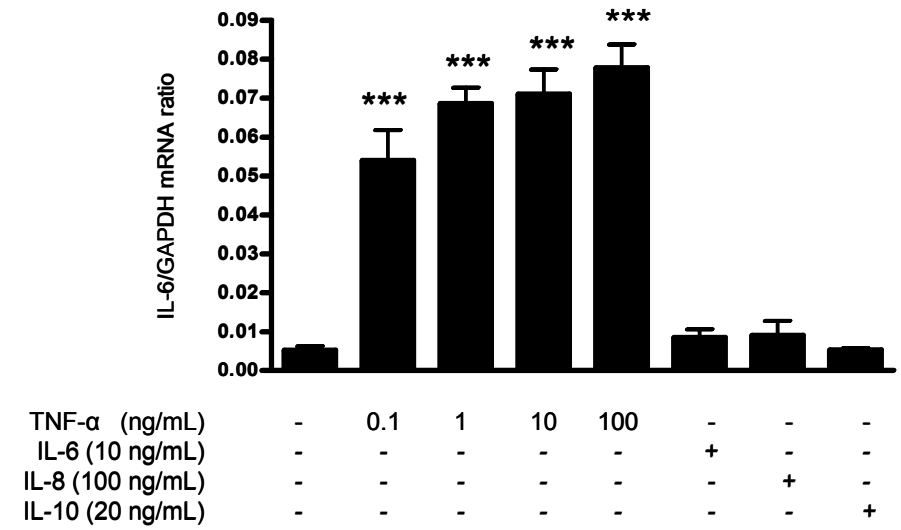

Fig. 6C. TNFα-induced effects in adipocytes: IL-6 mRNA expression. Dose-dependent IL-6 mRNA induction was measured in MSC-derived adipocytes by real-time PCR. IL-6 expression was normalized to GAPDH. Data are means ± SEM from 6 wells from two independent experiments. *** indicates $P < 0.001$ as compared to controls.

Discussion

Obesity is accompanied by low chronic inflammation within adipose tissues. Moreover, increasing evidence points to active roles of inflammatory cytokines in translating obesity to related pathologies including atherosclerosis and type 2 diabetes. However, the involved molecular mechanisms are not well understood and the specific physiological roles of these mediators in human disease remain under intense debate.

Herein we analyzed the expression dynamics and potential autocrine/paracrine effects of selected cytokines including TNF-α, IL-6, IL-8 and IL-10 in human adipose tissue. LPS-exposed explanted adipose tissue biopsies produced and secreted all four cytokines. As this model contains all cell types which compose adipose tissue it closely mimics *in vivo* conditions. However, it does neither allow determining which cells are targeted by LPS nor does it define the specific cytokine-secreting cells. While LPS-induced TNF-α production was previously shown in similar experiments [26, 33], we are not aware of analogous demonstrations for IL-6, IL-8 and IL-10.

Ex vivo differentiated adipocytes obtained either from adipose tissue-derived preadipocytes or from bone marrow-derived MSC represent an interesting alternative to the fragile and short-lived culture of primary mature adipocytes. The demonstration of triglycerides in this adipocyte cell model, the induction of several adipocyte-specific mRNAs, the increase in glucose uptake by insulin as well as the inducible lipolytic activity confirmed the adipocyte phenotype and functionality at several levels. Upon LPS-exposure high IL-6 and IL-8 concentrations were detected in supernatants. These data are in line with several reports which demonstrate IL-6 production by human [22, 23, 25], murine [24] and porcine [34] adipocytes. Adipocyte IL-8 production has also been described [25, 35]. It is of interest to note that adipocytes responded to LPS concentrations as low as 10 pg/ml. Similar concentrations are required for macrophage activation [36], which suggests that adipocytes in a physiological environment can easily activate IL-6 and IL-8 genes. Expression and function of Toll-like receptor-4 has been reported in 3T3-L1 adipocytes and might therefore mediate the herein described LPS effects [37, 38]. Being a chemotaxis inducing cytokine [39], adipocyte-derived IL-8 may contribute to the increased monocyte / macrophage accumulation observed in obesity [28].

In striking contrast to IL-6 and IL-8, neither TNF-α nor IL-10 were detectable in supernatants of adipocytes after 24 h even at the highest LPS dose (1 μg/ml). The fact that we found trace amounts of

TNF-α in a few supernatants after short incubation (< 6 h) but never after 24-h incubation was mirrored by short-lived TNF-α mRNA expression; this was possibly attributable in part to adipocyte-mediated TNF-α clearance [26]. This weak and transient TNF-α expression is in accordance with recent work by Fain et al showing low TNF-α secretion during the first 4 h, but not during the subsequent 44 h of incubation of freshly isolated mature human adipocytes [25, 26]. The authors estimated that approximately 5% of adipose tissue-derived TNF-α may be attributable to the adipocyte fraction. The herein described transient TNF-α mRNA expression is not restricted to LPS-mediated induction as we previously observed the same phenomenon in adipocytes exposed to IFNγ-LPS or IFNγ-IL-1β-LPS [40]. TNF-α release by freshly isolated human mature adipocytes has been reported [33]. However, we and others have observed that stromal cells (e.g. endothelial cells, macrophages) adhere quite firmly to adipocytes obtained by collagenase digestion and might therefore lead to false results [40, 41]. Increased accumulation of adipose tissue macrophage was initially described in mice [27, 28] and subsequently confirmed in humans [29]. The substantial TNF-α production we measured in supernatants from adipocyte-macrophage co-cultures supports the hypothesis that macrophages but not adipocytes represent the major source of TNF-α in adipose tissue.

TNF-α-administration increased both lipolysis and basal glucose uptake. Moreover, IL-6 mRNA was dose-dependently induced by TNF-α (> 0.1 ng/ml) reaching a plateau around 1 ng/ml. These data clearly indicate that the adipocytes were responsive to TNF-α. However, in the present setting no insulin resistance-inducing effects were observed after a 24-h exposure to 10 ng/ml TNF-α, as insulin-mediated glucose levels remained unaffected.

In contrast to TNF-α, exogenous IL-6 had no influence on adipocyte lipolysis and glucose uptake. These data are in opposition to previous reports, which however were performed in murine cell line models (i.e. 3T3-L1, 3T3-442A). For example, IL-6 was shown to induce basal glucose transport, to decrease IRS-1 expression and to reduce insulin-mediated glucose uptake [42-44]. In human adipocytes, membrane-located IL-6 receptors were shown by immunofluorescence [11]. Moreover, mature as well as *in vitro* differentiated adipocytes express IL-6 receptor-specific mRNAs [23]. However, we are not aware of previous studies clearly showing IL-6- or IL-8-mediated metabolic effects in human adipocytes. The results presented results here suggest that adipocytes secrete large amounts of both IL-6 and IL-8, but that these factors have no measurable autocrine action on adipocytes. It may be hypothesized that these two factors are released in order to activate or modulate non-adipose cell types including macrophages. This view is supported by the fact that, in contrast to

TNF-α and IL-1β, IL-6 and IL-8 administrations do not reduce adipocyte-specific leptin production in explanted adipose tissue samples [45].

About 35% of obesity-related elevation of serum IL-6 levels have been estimated to be adipose tissue-derived [19]. The present as well as previous data suggest that a significant portion of IL-6 is produced in adipocytes [25, 26]. Several authors have proposed that elevated IL-6 serum levels are associated with obesity [5] and may predict the development of type 2 diabetes [46]. However the causative role of IL-6 in this process may be questioned, and based on recent observations it is conceivable that IL-6 production represents an attempt to counter-regulate low grade inflammation caused by other mediators [20, 21, 47].

IL-10 has been described as a cytokine with anti-inflammatory properties. However, this description appears somewhat simplistic in view of the pleiotropic functions ascribed to the cytokine in different target cells [48]. While we show that IL-10 is actively produced by LPS-activated explanted adipose tissue, IL-10 gene appears to remain completely silent in LPS-treated adipocytes. We hypothesize that antigen-presenting cells produce obesity- and inflammation-related IL-10. The role of obesity-related increased circulating IL-10 concentrations is also obscure.

In conclusion, based on our findings we propose that TNF-α should be viewed as an adipose tissue- but not as an adipocyte-derived factor, in contrast to IL-6 and IL-8. The fact that TNF-α but neither IL-6, IL-8 nor IL-10 modulate metabolic activity of adipocytes underlines the yet to be understood interplay between and adipocytes and non-fat cells present in adipose tissue.

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Chapter 6: Final discussion

Key findings

The aim of the thesis was to investigate the mRNA expression, localization, functionality and function of the melanocortin receptors in human subcutaneous and omental adipose tissue from morbidly obese patients and normal weight subjects, as well as in MSC-derived adipocytes. Since α-MSH also circulates in the periphery, and its levels are elevated in obesity, there might be some auto/-paracrine or even endocrine action of the melanocortins in adipose tissues, eventually signaling to the arcuate nucleus and consequently modulating food intake and energy expenditure. We tried to elucidate the presence of such a potential feedback loop of the melanocortin system from the adipose tissue to the brain. Obesity is now recognized as a chronic low grade inflammation condition leading to the well known co-morbidities of obesity. Thus, it is of great importance to understand which cells of the adipose tissue produce which cytokines and whether there exists a cross-talk between adipocytes and tissue-infiltrated macrophages. Therefore, we analyzed the mRNA expression and secretion of different obesity-related cytokines as well as their metabolic effect in adipose tissue and adipocytes. Furthermore, we evaluated a possible correlation between the development of complications after gastric banding surgery and MC4-R gene mutations or polymorphisms, respectively.

The key findings are the following:

- Out of 37 patients we found one novel silent mutation Ile198Ile (C594T) and the polymorphism Ile251Leu (A1144C). However, we could not find any correlation between the rate of MC4-R gene mutations and complications after gastric banding surgery.

- Of the five melanocortin receptors only the MC1-R is substantially expressed in human adipose tissue and it is slightly increased in obese compared to lean subjects.

- By immunohistochemistry it could be shown that MC1-R protein is expressed in human adipose tissue on macrophages and adipocytes.

- MSC-derived adipocytes express functional MC1-R since stimulation with NDP-MSH increases cAMP production.

- NDP-MSH reveals a significant anti-proliferative effect on undifferentiated MSCs. However, NDP-MSH has no effect on lipolysis, metabolic rate and inflammation on MSC-derived adipocytes and/or adipose tissue.

- Adipose tissue-derived TNF-α is mainly produced from non-fat cells. However, exogenously administered TNF-α leads to increased lipolysis, glucose uptake and IL-6 mRNA expression in MSC-derived adipocytes.

The melanocortin system in human adipose tissue and obesity

The melanocortin system has been shown to be crucial for appetite control. Leptin produced by the adipose tissue induces the production of α-MSH in the arcuate nucleus, which binds to the MC4-R, causing a reduction in appetite and food intake. In our studies we sequenced the complete MC4-R gene of 37 gastric banding patients that developed complications after operation (e.g. insufficient weight loss, slippage, concentric pouch dilatation, esophageal motility disorder or band intolerance). We hypothesized that the development of these complications is caused by a disruption of the MC4-R. Based on a previously published study [1] we would have expected 12 instead of two carriers of a MC4-R mutation (32%). However, we were unable to detect a higher rate of MC4-R variants in our subject group, suggesting there is no correlation between MC4-R gene mutations or polymorphisms and gastric banding complications. Why we could not confirm the finding of Potoczna et al [1] with the subject group of the St. Claraspital is unclear. To our knowledge, the only difference between our subject group and the subject group of Potoczna is the usage of another gastric band. Nevertheless, they reported the same gastric banding failure and re-operation rate as the St. Claraspital.

Despite the importance of the melanocortin system in food control and energy expenditure (at least in rodents), it has never been clearly investigated which melanocortin receptor subtypes are expressed in human adipose tissue and whether there is a difference in the expression in the subcutaneous compared to omental fat, respectively, between obese versus normal weight subjects. The only melanocortin receptor subtype we found to be expressed was the MC1-R. Moreover, by immunohistochemistry we could demonstrate that MC1-R protein is expressed on the macrophages and adipocytes in adipose tissue. Stimulation of MSC-derived adipocytes with NDP-MSH resulted in a dose dependent cAMP production, indicating that the MC1-R is virtually functional.

The lack of substantial POMC transcripts in the adipose tissue indicates that there seems to be no auto-/paracrine nor endocrine action of the melanocortins derived from human adipose tissue. Therefore, it is also doubtful whether adipose tissue-derived α-MSH ever reaches the bloodstream and finally the arcuate nucleus, creating a direct feedback loop modulating food intake. However, it was shown that α-MSH circulates in the periphery at low levels. It is important to note that α-MSH circulates at approximately 16 pmol/l in obese subjects, which is

below the EC_{50} values for the melanocortin receptors [2]. Though of interest, its plasma level is increased in obesity. The physiological role of the increased plasma α-MSH levels in obesity remains to be determined. Previously it has been demonstrated that α-MSH regulates leptin and insulin expression and secretion. Furthermore, it inhibits the effects of cytokines and apoptosis, stimulates the release of corticosterones, and regulates thermogenesis [2, 3]. The source of the increased plasma α-MSH in obese subjects remains to be elucidated. Maybe it originates from the hypothalamus or from the increased number of immune cells, which are associated with a greater fat mass. In humans these immune cells have been shown to secrete α-MSH [4]. However, our findings suggest that α-MSH does not originate from adipose tissue. Another question is whether peripheral α-MSH indeed crosses the blood-brain barrier, as hitherto no active transporters for α-MSH have been described. Additionally, the size and low lipophilicity of α-MSH make it unlikely that it crosses the blood-brain barrier easily. Yet, recently it has been reported that peripherally administered NDP-MSH increases resting metabolic rate in wild type *C57BL/6* and *ob/ob* mice [5], suggesting that at least in mice some NDP-MSH crosses the blood-brain barrier. Whether this applies also for humans is not known. Noteworthy, Hallschmid et al [6] reported that prolonged intranasal administration, which bypasses the uptake into the bloodstream, of MSH (MSH/$ACTH_{4-10}$) induced a slight weight loss in healthy volunteers but interestingly not in overweight subjects.

We could demonstrate the presence of functional MC1-R protein on the cell surface of adipocytes, which implies that circulating α-MSH could in principle have a physiological effect on adipocytes. Proliferation analysis revealed a significant anti-proliferative effect of the super potent MC1-R agonist NDP-MSH in undifferentiated MSCs. Since of the five melanocortin receptors only MC1-R was expressed in these cells, we think that the obtained effect was mediated via MC1-R or maybe via a not yet identified receptor. The anti-proliferative effect was significant at even the lowest concentration of 10^{-12} M NDP-MSH, which is not very surprising since in different previous reports it was noted that even at very low concentrations of α-MSH or NDP-MSH an anti-inflammatory effect was seen. For example Taherzadeh et al [7] reported a significant decrease in TNF-α secretion in LPS-stimulated human monocytic/macrophage THP-1 cells after 24 h preincubation and co-administration of α-MSH at a concentration of 10^{-14} M. The anti-proliferative effect of α-MSH on adipocyte precursor cells, which we observed, might regulate the growth of these cells that eventually become adipocytes. And therefore, the development of further adipose tissue could be suppressed by melanocortins, which could serve

as a novel drug target in the future. Another future target for the treatment of obesity could be the blocking of the differentiation process of the adipocyte precursor cells to mature adipocytes.

Our findings are in accordance with results previously reported by Smith et al [8]. They reported that α-MSH inhibits proliferation of murine and human preadipocytes. In addition, they found that agouti blocks the anti-proliferative effect of α-MSH and that agouti enhances the differentiation of murine and human preadipocytes in vitro. Probably the regulation of the proliferation of preadipocytes is more important in the development of white adipose tissue right after birth or even during fetal development instead in adults.

Apart from the anti-proliferative effect of NDP-MSH on undifferentiated MSCs we could not find any influence on inflammation and lipolysis in adipose tissue explants and MSC-derived adipocytes. Moreover, administration of NDP-MSH did not change the metabolic rate.

Previously, it has been demonstrated that α-MSH has a potent anti-inflammatory effect in human monocytic cell lines and in freshly isolated macrophages. Furthermore, after α-MSH injections a beneficial influence could be shown in patients with septic shock, fever and HIV [9]. However, we could not see any influence of NDP-MSH on the inflammation state induced by LPS, neither in adipose tissue explants nor in adipocytes. Very recently, it has been reported that in mice and humans adipose tissue macrophage numbers increase in obesity and participate in inflammatory pathways that are activated in adipose tissues of obese individuals. Interestingly, the number of infiltrated macrophages correlates closely with the body mass [10]. Since macrophages also express MC1-R, the reported accumulation of macrophages in the obese patients might explain the higher MC1-R mRNA expression which we obtained in our obese patient group. Nevertheless, it remains unclear why we did not see any effect of NDP-MSH on the release of cytokines in the LPS-exposed adipose tissue explants. We would have expected to obtain a decrease in the secretion of cytokines due to the macrophages present in the adipose tissue. Possibly, NDP-MSH could not penetrate the explants and therefore did not reach the macrophages within the tissue. However, in additional experiments we co-administered the glucocorticoid dexamethasone and, as expected, reduced the LPS-induced secretion of all analyzed cytokines, including TNF-α and IL-10. To remind, we were unable to detect neither TNF-α nor IL-10 in the supernatants from adipocytes. Thus, dexamethasone seemed to target directly the macrophages in the adipose tissue, resulting in a decrease of cytokine production. Thus, we speculate that the adipose tissue infiltrating macrophages could have a different

expression pattern than the circulating macrophages and therefore act differently on the stimulation with NDP-MSH. Induction of inflammation with IL-1β instead of LPS revealed the same results upon NDP-MSH co-administration. The same picture was seen in experiments omitting the LPS. In future experiments, inflammation could be induced by administration of different cytokines including TNF-α or IFNγ. While we could clearly demonstrate that macrophages in adipose tissue and, at lower levels, adipocytes express MC1-R, we were unable to elucidate its biological function in adipocytes and adipose tissue. An explanation for our failure might be a too small effect on the output signal that consequently was below the detection limit.

Adipose tissue inflammation

As already mentioned several times, in the last years a new hypothesis has emerged that describes obesity as a chronic low grade inflammatory condition, which may lead to the well known co-morbidities of obesity (i.e. atherosclerosis, diabetes, cancer). Recently, it has been demonstrated that the majority of the adipose tissue-derived cytokines originate from non-fat cells, including tissue infiltrated macrophages, which seem to play a prominent role of forcing the adipose tissue into a pro-inflammatory state [11]. Previously, it could be shown in humans and rodents that there is a correlation between the number of macrophages in the stroma-vascular fraction and the BMI, the adipocyte size and the total fat mass [10, 12, 13]. It is important to note that most of these macrophages in the adipose tissue (85%) are bone marrow-derived cells [10]. Adipocytes as well as macrophages produce the chemokine monocyte chemoattractant protein 1 (MCP-1) that attracts blood monocytes into the tissue. Subsequently, these cells become activated macrophages. Moreover, it could be shown that MCP-1 production is enhanced in obesity. Another monocyte attracting cytokine is IL-8, which is also secreted by the adipocytes and elevated in obesity. Based on our own observation we hypothesize that physical disruption after banging the belly occurs more often in obese subjects, maybe due to the unprotected exposure of the fat tissue to the environment. This disruption may lead to single necrotic cells that attract macrophages. We speculate that this is another reason why obese subjects possess more macrophages in the adipose tissue than normal weight persons.

We could demonstrate that adipose tissue produces higher amounts of IL-8 and IL-6 than adipocytes, supporting the finding of Fain et al [11]. In contrast to Fain et al, we were unable to

detect TNF-α and IL-10 proteins in supernatants from adipocytes derived either from preadipocytes or from MSCs without administration of LPS. Only in a few supernatants after short incubation with LPS (< 6 h) we found some traces of TNF-α. Fain et al suggested that approximately 5% of adipose tissue-derived TNF-α may be attributable to the adipocyte fraction. Whereas in our experiments the mRNA expression of TNF-α was transiently induced by LPS, IL-10 mRNA transcripts could never be detected. In the study of Fain, TNF-α secretion was found in freshly isolated mature adipocytes. It is well known that the collagenase treatment for the isolation of adipocytes leads to inflammation. Additionally, there are always contaminating non-fat cells within the adipocyte fraction. Thus, the reported TNF-α might origin from those contaminating non-fat cells.

Despite the low or even absent expression and release of TNF-α, respectively, we could show that the adipocytes act on exogenously administered TNF-α. Thereby, lipolysis, glucose uptake and IL-6 mRNA expression is enhanced. Neither IL-6, nor IL-8, nor IL-10 had an effect on the adipocytes. Very recently, Permana et al [14] reported that in murine 3T3-L1 adipocytes incubated with media conditioned by RAW264.7 macrophages (for 18 h), the expression of various inflammation-related genes, including MCP-1, ICAM-1 and IL-6, is increased. The macrophage-conditioned media were analyzed and they found TNF-α (1 nM) and IL-1β (0.006 nM) to be present. Interestingly, incubation of the adipocytes with the same concentrations of TNF-α and IL-1β resulted in a less pronounced effect than with the macrophage-conditioned media, implying that some additional factors are responsible for induction of inflammation.

To summarize, our findings provide further evidence for the presence of a cross-talk between adipocytes and macrophages in the adipose tissue. This orchestra modulates the inflammatory condition and generates a vicious circle leading to the co-morbidities of obesity. IL-8, MCP-1 and IL-6 secreted from adipocytes attract and activate macrophages, which on their part secrete TNF-α. The adipocytes subsequently react on TNF-α with higher IL-6 production and so on. This model is also in accordance with the previously reported correlation of body mass and the number of macrophages in adipose tissue [10]. Therefore, blocking of this adipocyte-macrophage cross-talk might be a future drug target for treatment or prevention of diabetes type 2 or atherosclerosis.

Conclusion

Human adipocytes express functional MC1-Rs on their cell membrane. In human adipose tissue, apart from low expression on adipocytes, MC1-R is highly expressed on macrophages. Binding of NDP-MSH reveals a potent anti-proliferative effect on undifferentiated MSCs. The function of MC1-R in human adipose tissue and adipocytes remains unclear. However, it does not seem to play a role in lipolysis and modulation of mitochondrial metabolic rate or inflammation. Another potential effect of MC1-R signaling could be an involvement in apoptosis. Further work has to be done to address this question. The lack of substantial POMC mRNA expression in human adipose tissue suggests that there is no auto-/paracrine action of α-MSH, as well as no direct feedback signal from the fat to the brain. However, α-MSH released from the hypothalamus into the blood circulation might reach the adipose tissue and exert an anti-proliferative effect on preadipocytes preventing the building of additional fat tissue.

We found that TNF-α is only transiently expressed at low levels in human adipocytes, while IL-10 does not seem to be expressed in adipocytes. In contrast, IL-6 and IL-8 are secreted in large amounts by adipocytes. In addition, explants of human adipose tissue secrete TNF-α, IL-6, IL-8 and IL-10, implying that adipose tissue-derived TNF-α and IL-10 are mainly produced by non-fat cells, presumably macrophages. However, adipocytes respond to exogenous TNF-α by inducing lipolysis, glucose uptake and IL-6 mRNA expression. Taken together, we found additional evidence for a cross-talk between macrophages and adipocytes in adipose tissue, which leads to inflammation and the development of the well known co-morbidities of obesity. Further elucidation of the network of the different cell types in adipose tissue may help to find new drug targets for treating or preventing type 2 diabetes and atherosclerosis.

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Acknowledgements

This work was supported by the Hirzbrunnen-Stiftung, Basel, the Novartis Foundation for Biomedical Research, Basel, and by a Universiy Children's Hospital Basel research grant.

I must say that I very much enjoyed working on my thesis the last few years. During this period I made many valuable experiences and I could immensely broaden my horizon. But in order to complete such a broad work I also relied on the help of many people who supported and mentored me in different ways.

First of all, I would like to thank Prof. Alex N. Eberle for giving me the opportunity to work in his lab on this very interesting project. He always supported and encouraged me in pursuing new ideas and techniques. I appreciated his open-mindedness and our amicable contact. Moreover, I admired the interdisciplinary communication with Prof. Thomas Peters and Dr. Ralph Peterli from the St. Claraspital. At this point, I'd like to thank them for our enjoyable, pleasant and easy-going collaboration. Furthermore, they, together with Prof. Alex Eberle, enabled me to attend various very interesting symposia. By their aid, I could also experience an extremely interesting and instructive week in the lab of Prof. M. Rydén at the Karolinska Institute in Huddinge, Sweden. During my PhD thesis, I met many interesting people from all over the world. I always enjoyed the scientific and social exchange with people with various backgrounds. With some of them a real friendship has evolved. Unfortunately, there is not enough space to thank all these persons individually.

Special thanks go to Dr. Philippe Linscheid for teaching me various fat cell and fat tissue preparation and analyzing methods, as well as supervising me in the adipose tissue inflammation part of my thesis. Additional thanks go to the other members of the group of Prof. U. Keller, especially Käthi Dembinski, Michael Eberhardt, Dalma Seboek and Susi Vosmeer. Thanks also to Dr. Urs Wagner, Dr. Christian Bussmann and Andrea Carlen from Viollier AG for their support in the immunohistochemistry and for providing me with perfect fat tissue sections. Many thanks also to the nurses of the St. Claraspital for the delivery of the fat and blood samples and the group of Prof. Ivan Martin for providing us the mesenchymal stem cells.

Moreover, the members of the endocrinology lab contributed greatly to my thesis. Therefore, many thanks go to: Jean-Philippe Bapst, Jean Burckhardt, Martine Calame, Vreni Jäggin, Steven Knecht, Peter Lindinger, Gabi Mild, Kurt Müller, Heidi Tanner, Olivier Wenger and Kerstin Wunderlich. I would also like to mention Monique Sauter and thank her for performing the comet assay and for the supply of many delicious home-made cakes and cookies. Additionally, I am indebted to Michèle Attenhofer for showing me the gDNA isolation method from whole blood and Dr. Heike Gutmann for her support in real-time PCR.

Furthermore, I would like to give special credits to all patients, who were selflessly willing to donate blood, fat tissue or bone marrow aspirates. Without their "sacrifices" it would not have been possible to realize my PhD thesis. Thanks a lot!

Like every PhD student, I went through different ups and downs. I have to mention many people, who supported me morally even in difficult periods, especially my parents, sister and dear friends.

Last but not least, I would like to thank Prof. K. Hofbauer, who agreed to join my thesis committee as a co-referent.

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Hoch, M., Linscheid, P., Peterli, R., Peters, T., Eberle, AN. NDP-MSH has an anti-proliferative effect on undifferentiated human MSCs but causes no change in lipolysis and inflammation on MSC-derived adipocytes, in preparation.

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Other papers

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December 3-7, 2006 IDF 2006 Cape Town, South Africa
19th World Diabetes Congress
Poster: Philippe Linscheid, Matthias Hoch, Ulrich Keller, Beat Müller, Ralph Peterli, Thomas Peters, Alex N. Eberle. "Mesenchymal stem cell-derived adipocytes secrete large amounts of IL-6 but no measurable TNF-α"

November 10, 2006 SGED 06 Bern, Switzerland
Annual Meeting of the Swiss Endocrine and Diabetologia Society (SGED)
Oral presentation: "TNF-α targets human adipocytes but is not a major adipocyte secretion factor, in contrast to IL-6 and IL-8"

June 24-27, 2006 ENDO 06 Boston, USA
The Endocrine Society's 88th Annual Meeting
Poster: Matthias Hoch, Alex N. Eberle, Urs Wagner, Christian Bussmann, Thomas Peters, and Ralp Peterli. "Expression and localization of melanocortin-1 receptor in human adipose tissue of morbidly obese patients"

March 12-15, 2006 Basel, Switzerland
10th Swiss Receptor Workshop
Poster: Matthias Hoch, Alex N. Eberle, Urs Wagner, Christian Bussmann, Thomas Peters, and Ralp Peterli. "Expression and localization of melanocortin-1 receptor in human adipose tissue of morbidly obese patients"

September 30, 2005 FORC 2005 Fribourg, Switzerland
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Attendance

November 18, 2004 Bern, Switzerland
Pädiatrie Forschertag für junge Forscher
Oral presentation: "Quantitative analysis of gene expression of the melanocortin system in omental and subcutaneous adipose tissue of morbidly obese patients"

September 9, 2004 Basel, Switzerland
Pädiatrischer Forschertag des Universitäts Kinderspitals beider Basel
Poster: Matthias Hoch, Thomas Peters, Alex N. Eberle, Ralph Peterli
"Quantitative analysis of gene expression of the melanocortin system in omental and subcutaneous adipose tissue of morbidly obese patients"

June 16-19, 2004 ENDO 04 New Orleans, USA
The Endocrine Society's 86th annual meeting
Poster: Matthias Hoch, Thomas Peters, Alex N. Eberle, Ralph Peterli
"Quantitative analysis of gene expression of the melanocortin system in omental and subcutaneous adipose tissue of morbidly obese patients"

Additional Trainings

Tutor of a Biology Tutorial (Einführung in die Biologie) at the **University of Basel**, Winter Semester 2006/07

8-day Graduate course: **"Key Issues in Drug Discovery & Development"**, Autumn 2005, ETH Zurich and University of Basel in collaboration with Novartis and Hoffmann-La Roche

Practical lab training in the group of Prof. M. Rydén and P. Arner, Department of Medicine, Karolinska Institute, Karolinska University Hospital at Huddinge, Stockholm, January 17-21, 2005

Create Switzerland's Entrepreneurship Course, Summer Semester 2004, University of Basel