

Ph.D. THESIS

**AGE- AND NUTRITIONAL STATE-RELATED ALTERATIONS IN THE
METABOLIC EFFECTS OF LEPTIN AND ALPHA-MSH**

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Introduction

Along with progressive aging of the population, the two characteristic trends observed in long-term body weight regulation, i.e. the **obesity of the middle-aged** (30-60 years of age) (Scarpace *et al.*, 2000a; Cameron *et al.*, 2003) and the **anorexia of aged individuals** (above 75) leading to sarcopenia (Morley 2001; Di Francesco *et al.*, 2007) present ever increasing grave population-wide health-related consequences. The importance of age-related alterations in body composition is underlined by the fact that both the obesity of the middle-aged and the sarcopenia of aging markedly increase morbidity and mortality. Although a number of complex social and psychological factors also contribute to obesity and anorexia, but **alterations of the regulatory processes of energy balance** also play an important role in their development. Observations that show similar tendencies in age-related body composition changes not only in human but also in animal studies also support their putative role.

1. Energy balance

The maintenance of energy balance is a complex process that depends on food intake (FI), metabolic rate (MR) and heat loss (HL). Balance between the food intake and metabolic rate determines the feeding state (hunger, satiety) on the short run and is responsible for the nutritional state (anorexia, obesity) on the long run. Core temperature (T_c) depends on the balance between the metabolic rate (heat production) and heat loss.

Neural and humoral factors participate in the long-term regulation of body weight and energy balance, contribute to the metabolic adaptation to fasting and food intake and also play a role in thermoregulation. They form a complex interactive regulatory system. A strong/close relationship between homeostatic systems is further supported by the identification of more and more common central mediators participating in the regulation of food intake and that of body temperature at the same time. Several peptides exert coordinated food intake – metabolic – thermal effects. Neuropeptide Y (NPY) for example has coordinated anabolic effects (energy conservation): it enhances food intake (orexigenic) and at the same time it induces hypometabolism and hypothermia. As a result of these mechanisms, body weight increases (Morley 1987; Balaskó *et al.*, 1999; Székely and Balaskó, 1999; Székely *et al.*, 2004). On the other hand, the proopiomelanocortin/melanocortin (POMC/MC) system show coordinated catabolic effects. Endogenous agonist of the MC system, the alpha-melanocyte stimulating hormone (alpha-MSH), decreases body weight via suppression of food intake (anorexigenic) and enhancement of metabolic rate (Vergoni *et al.*, 1986).

On the other hand proopiomelanocortin/melanocortin (POMC/MC) system has a coordinated catabolic effect.

The balance between orexigenic and anorexigenic mediators plays an important role in body weight regulation. Dominance of anorexigenic substances results in a fall in body weight. On the other hand, activation of orexigenic effects leads to weight gain. According to previous studies (Kmieć, 2011) the efficacy of components of both short-term and long-term body weight regulation has shown alterations in the course of aging. Individual components of these complex regulatory systems may change at different rates, what may explain age-related changes in energy balance, body weight and body composition.

2. Leptin

Among the peripheral mediators of metabolism leptin has a pivotal role on the long-term regulation of food intake. Inadequate leptin effect is one of the most important causes of

obesity. It has coordinated catabolic effects: it decreases food intake and body weight in addition to increasing the metabolic rate (Pelleymounter *et al.*, 1995). Its main target is the hypothalamic arcuate nucleus: here it inhibits the release of orexigenic peptides (e.g. NPY, agouti-related peptide – AgRP, endocannabinoids) and stimulates the expression of anorexigenic ones (e.g. POMC, alpha-MSH, cocaine-amphetamine-regulated transcript – CART) (Sahu, 2004). Some research groups have described a decreased responsiveness to leptin with aging, which might be attributed to the aging processes themselves (Gabriely *et al.*, 2002). On the other hand, other observations of the literature have emphasized the role of body composition and age-related obesity in the development of leptin-resistance (Scarpace *et al.*, 2000b).

3. Alpha-MSH

The main target of leptin is the MC system which is a major regulator of energy balance and body weight (Adan *et al.*, 2006). Its endogenous agonist, the POMC-derived peptide alpha-MSH, exerts its coordinated catabolic effects by stimulation of the hypothalamic melanocortin receptor-4 (MCR4) and melanocortin receptor-3 (MCR3) (Tatro and Entwistle, 1994; Marks *et al.*, 2003). In addition to its food intake reducing effect, alpha-MSH also has an important influence on metabolic processes via activating the sympathetic nervous system-related thermogenesis (Zimanyi and Pelleymounter, 2003), through MCR3 activation it increases lipolysis (Marks *et al.*, 2001) and via the overexpression of uncoupling proteins (UCP) in the brown adipose tissue it leads to enhanced heat production, (Haynes *et al.*, 1999). Alpha-MSH production is activated by leptin. Since the plasma level of leptin is proportional with fat mass, high fat mass due to overfeeding results in enhanced leptin production, which activates the POMC neurons and thus food intake decreases, the metabolic rate rises. On the other hand, during starvation besides the decreased fat mass and consequently low leptin level the activation of the MC system also decreases, therefore FI would increase and the metabolic rate decreases (Mizuno *et al.*, 1998; Hagan *et al.*, 1999).

In obesity, serum leptin level becomes higher but the inhibition of food intake is not sufficient: leptin-resistance develops. Data of the literature show that in obesity responsiveness to the high endogenous leptin level and also that evoked by centrally applied exogenous leptin is diminished. (Shek and Scarpace, 2000).

In chronic animal experiments in diet-induced obese animals both peripherally and also centrally applied leptin are able to cause significantly lower suppression of food intake and body weight (Rahmouni *et al.*, 2005). Despite leptin-resistance, maintenance of the sensitivity of the melanocortin system may be indicated by the fact that MC agonist (melanotan II) causes more severe anorexia in obese Zucker rats than in the lean control group (Hwa *et al.*, 2001; Cettour-Rose és Rohner-Jeanrenaud, 2002).

Although in animal models age-dependent changes of body weight and body composition (age-dependent obesity) were verified, it is also apparent that the accumulation of fat mass is not so remarkable than that observed in diet induced obese animals or in leptin receptor-deficient (or leptin-deficient) animals. The contradictions found in the literature may originate from the uncertainty whether the nutritional state or the advancing age *per se* is more responsible for the development of leptin resistance. Besides it is also questioned whether the obesity is first, causing leptin resistance, or the other was around, leptin resistance leads to obesity.

Aims

1. From the full spectrum of age-dependent body composition changes, the anorexia of aging may be explained by an aging-associated rise in anorexigenic and a decline in orexigenic tone that become very pronounced in the elderly. However, in order to explain the obesity of the middle-aged, a suppression of the MC system with concomitant strong orexigenic effects should be assumed. According to our hypothesis, age-dependent alterations in the sensitivity of the MC system may contribute to the age-dependent alterations in body composition, therefore in our experiments on male Wistar rats of different age-groups we studied *the dynamics of age-related alterations in the anorexigenic and metabolic effects of alpha-MSH*.

2. Data of the literature have confirmed that leptin-resistance develops in overfed, diet-induced obese animals. However, without any special external influence, aging alone may lead to obesity (age-related obesity). Both diet-induced and age-related obesity result in increased serum leptin level and leptin-resistance. The question is, whether this age-dependent leptin-resistance is based on aging alone (on aging-associated hyperleptinemia), or this phenomenon is only secondary, that is leptin-resistance develops due to aging-associated alterations in body composition. In order to answer this question, in our experiments we tested the influence of aging-associated body composition changes on the anorexigenic and metabolic effects of a central leptin infusion, comparing these effects in 2- and 6-months old ad libitum fed, 6-months old high-fat diet-induced very obese and in calorie-restricted lean animals. Our aim was to determine, *which factor is more important in the development of leptin-resistance: age or body composition?*

3. The main (though not exclusive) activator of the MC system is leptin. According to previous observations the sensitivity of MC system may be maintained in case of leptin-resistance. This suggestion is supported by studies showing that experimental overexpression of alpha-MSH suppresses the obesity/weight gain of animals in leptin deficient (*ob/ob*) or in leptin receptor deficient (*db/db*) mice or in Zucker rats possessing defective leptin receptors (Li *et al.*, 2003; Mizuno *et al.*, 2003; Savontaus *et al.*, 2004). Therefore, in another set of experiments, using the previously applied protocols in genetically intact animals we investigated how (besides the well-known alterations in leptin-sensitivity) the anorexigenic and metabolic effects of alpha-MSH change in 6-months old animals with different nutritional states. Our aim was to answer the question, *whether the sensitivity of the melanocortin system may also be affected by body composition (in addition to age)?*

Materials and Methods

1. Experimental animals

In our experiments male Wistar rats of different age-groups and nutritional states were used. To establish diurnal rhythm, the lights were on between 06:00 and 18:00 h and the dark period lasted from 18:00 to 06:00 h. Tap water was available *ad libitum* for all groups. Following weaning at age 6 weeks, from the age of 2 months 3 groups of different nutritional states were established (via random assignment). The first, *ad libitum* fed (NF=normal fed) group was kept on standard laboratory chow. Calorie-restricted (CR=calorie-restricted) animals received 16g/day of standard powdered rat chow (two thirds of the standard calorie-intake) at the beginning of their active period (at 18:00 h). Finally, the third group was overfed

by a special high-fat (HF=high-fat) diet: a powdered mixture of 90% IPS TestDiet (containing 60% fat-derived calories) and 10% standard powdered rat chow.

For studies of age-dependence, our experiments were carried out in 2, 3-4, 12 és 24 months old NF animals. 2 months (NF2) and 3-4 months (NF3-4) animals refer to the human juvenile and young adult groups, 6 months (NF6) and 12 months (NF12) represent adult/young middle-aged and middle-aged groups and finally the 24 months age-group (NF24) represents old human population. Effects of nutritional state were analyzed in 2 months old NF and 6 months NF, CR, HF groups.

Body weight and food intake were measured manually every day at the same time (09:00 a.m.).

The general rules of the University of Pecs Ethical Committee for the Protection of Animals in Research were strictly observed in all experiments, and we received special approval of the Committee (BA 02/2000–13/2006) for the present experiments. In general, the rules of this Committee and the directives of the European Communities Council (86/609/EEC) are in accord.

2. Measurements of metabolic rate

Complex analysis of the energy balance of freely moving animals was carried out in a biotelemetric system (VitalView Data Acquisition System Series 4000 for Temperature, Activity and Heart Rate[®]) via continuous recording of core temperature (Tc), spontaneous horizontal locomotor activity (ACT), and heart rate (HR). The biotelemetric system registered radio frequency signal produced by the E-mitter (MiniMitter-VMFH, series 4000, Sunriver, OR) every 5 min. Before statistical analysis of the data, a 12-h averaging was applied, which resulted in 2 mean values (Tc, HR, ACT) per day, one corresponding to the the light (inactive) cycle and another one for the dark (active) cycle. The amount of daily food intake (FI) and BW were measured manually (at 09:00 a.m.) in the Minimitter system, as well.

According to observations in the literature (Treuth *et al.*, 1998; Astrand *et al.*, 2004; Brosh, 2007) alterations of HR represent the changes in metabolic rate both in human and animal studies. Its advantage over the widely used metabolic measurement based on oxygen consumption (VO₂), which has to be carried out in closed metabolic chambers lies in the fact, that via monitoring HR we may obtain continuous information on the metabolic rate of freely moving animals among practically natural circumstances. Our research group has also carried out comparative measurements to prove that in addition to VO₂, HR is also an appropriate parameter for the monitoring of changes in metabolic rate. Our data have shown that upon alpha-MSH infusion similar alterations were detected in the VO₂ measured in the OxyMax system and in the HR recorded in the biotelemetric system in all applied age-groups (also used in the present study). Thus, our results confirmed that HR just as well as represented metabolic alterations as did VO₂. (Pétervári *et al.*, 2011)

3. Surgeries

After a habituation of 5–7 days in the biotelemetric system, rats were implanted with an E-mitter intraperitoneally (IP) under IP ketamine (78 mg/kg, Calypsol/Richter)+xylazine (13 mg/kg, Sedaxylan /Eurovet) anesthesia. About 1-1.5 week following transmitter implantation, when daily FI and BW development of animals returned to the normal values of the age-group, registration of the control period has started. Then with the help of a stereotaxic frame using the above mentioned anesthesia, an intracerebroventricular (ICV) cannula has been implanted into the right lateral ventricle using the following parameters: 1 mm posterior from bregma, 1.5 mm to the right from midline and 3.8 mm ventrally from the dura. At the same

time an Alzet osmotic minipump filled with the appropriate solution was implanted under the skin of the nape.

4. Intracerebroventricular infusions

Recombinant leptin (Bachem) or alpha-MSH [acetyl-ACTH (1-13) amide, Bachem] infusion was administered ICV by a flow-rate of 1 $\mu\text{g}/\mu\text{l}/\text{h}$ for 7 days in the treated groups. As a control, solvent pyrogen-free saline (PFS) was used ICV by the same flow-rate (1 $\mu\text{g}/\mu\text{l}/\text{h}$).

5. Intracerebroventricular acute injections

In order to evaluate orexigenic sensitivity in 6 months old animals of different nutritional states (CR6, NF6, HF6), we studied the food intake-related 1-h orexigenic effects following the acute ICV injection of 5 μg NPY (Bachem) or PFS in 5 μl volume.

6. Post mortem examination, assessments of body composition

Following the experiments, the animals were euthanized by an overdose of IP urethane (3-5 g/BWkg), and the injection sites of their brains were checked macroscopically by coronal sections of the removed and fixed brains. The retroperitoneal and epididymal fat pads were removed and weighed simultaneously, along with the tibialis anterior muscle, as indicators of body composition. The weights were related to total body weight. Such assessment was performed also in age groups of control rats not tested with peptides.

7. Statistical analysis

For statistical analysis of the data SPSS 11.0 for Windows program were used. In general, the results are shown as mean \pm SEM. One-way or repeated-measures ANOVA tests, two-way ANOVA and Scheffe's *post hoc* test as well as Student's *t* test were used for statistical analyses, as appropriate.

Results and Discussions

I. Effects of central alpha-MSH infusion in 2-, 3-4-, 12- és 24-months rats

1. Results

1.1. Changes in body weight and food intake

An ICV infusion of alpha-MSH caused a significant delay in body weight gain of the juvenile 2 month-old rats, as compared with the controls. The suppression was relatively greater in this group than the also significant suppression observed in the 3–4 month-old young adult animals. In 12 month-old middle-aged rats the alpha-MSH-induced weight loss hardly reached the level of statistical significance. This age-related attenuation of the weight-reducing effect of alpha-MSH was not continued with further aging, but the peptide-induced fall in body weight became maximal again in the 24 month-old rats.

Taking all age-groups into consideration, two-way ANOVA analysis showed a significant influence of age on alpha-MSH action. According to Scheffe's *post hoc* test the results of 24 month-old animals differed from those of all the other age-groups. In addition, a significant difference was also found between the 12 and 2 month-old animals.

Suppression of food intake was limited to the first 4 days of alpha-MSH infusion in the 2 month-old group, and lasted until day-5 of the infusion in the age-group of 3–4 month-old rats. In case of the 12 month-old rats it was not significant. Similarly to alpha-MSH effects regarding body weight, following a transient age-related attenuation of the effect at age 12 months, the suppression of food intake was maximal and long-lasting (until day-7) in the 24 month-old animals.

1.2. Changes in core temperature

During ICV infusions of alpha-MSH, the daytime (inactive phase) Tc minima of the circadian rhythm exhibited a definite elevation in all four groups, the extent of which was however, also found to vary with age. It was minimal, but significant in 2 month-old animals. In the 3–4 month-old age-group the Tc minima were elevated throughout the infusion period, at day-1 even some nighttime (active phase) Tc maxima were different in control vs. alpha-MSH-treated animals. Both Tc minima and Tc maxima exhibited massive increases all along the alpha-MSH infusion in 12 month-old rats. At the age of 24 months the infusion of the peptide resulted in a significant increase of Tc minima (but not the Tc maxima) for the whole infusion period.

1.3. Changes in heart rate

Age-related differences of the effects of alpha-MSH infusion on HR (used as an indicator of metabolic rate) were also very pronounced. In 2 month-old rats a moderate rise in HR minima, as well as in HR maxima were observed. No significant effect was found in the 3–4 month-old group. Both values were greatly increased in the 12 month-old group: HR minima throughout the infusion period and HR maxima until day-5 of the infusion. In the oldest group the HR minima were elevated throughout the infusion period, while the HR maxima increased for 4 days.

1.4. Changes in spontaneous horizontal locomotor activity

As compared with controls, no changes in ACT were observed in any of the groups upon alpha-MSH infusion.

1.5. Changes in body composition during aging

In 6–9 week-old rats the ratio of both the epididymal and the retroperitoneal fat pad was lower than in all other animals. By the age of 3–4 months the fat ratios doubled, while the muscle ratio remained the same. The highest fat ratios were found in the 12- and 18 month-old groups. The epididymal fat pads of these groups showed higher values than those of the other four groups. The retroperitoneal fat pads were largest in the 18 month-old group – statistical significance was reached in comparison with the two youngest groups of rats. In the oldest group the fat ratios did not rise any more or even decreased somewhat, the epididymal fat pads of old animals were significantly lower than those of 18-months-old rats.

The muscle ratio exhibited a gradually developing significant decline in adult animals. The amount of m. tibialis anterior was significantly lower in 24 months old animals than in the four younger groups.

2. Discussion

Our results confirmed the well-known complex catabolic effects of alpha-MSH (it decreases food intake, increases metabolic rate, and therefore decreases the body weight). At the same time, our data proved that both the anorexigenic and the hypermetabolic effect of alpha-MSH depend on age but the changes of these parameters show different dynamics (pattern) in the course of aging.

The anorexigenic effect of alpha-MSH is strong in the young, while it is mild, not significant in the middle-aged group, and finally in old animals it becomes very pronounced again. Interestingly the alterations of anorexigenic and metabolic effects are not concordant. The most pronounced metabolic effect was seen in 12 months-old rats, although this age-group showed the smallest anorexigenic effect (body weight reduction just reached the level of significance and food intake did not vary significantly).

Concerning metabolic effects of alpha-MSH our conclusions are based on changes in Tc and HR. During the course of the alpha-MSH infusion no change of ACT was observed in any of the age-groups, therefore alterations of Tc may be derived from changes in metabolic rate independent of activity. Observed rises in daytime Tc minima are not likely to be of febrile nature, since in cases of a febrile rise in Tc, the associated sickness behavior usually includes suppression of ACT. On the other hand, the lack of rise in ACT suggests that the temperature elevating hypermetabolic effects of the peptide were due to increased tissue metabolism (brown fat and/or other tissues) — a suggested enhancement of sympathetic activity might contribute to this, and it might also explain the elevation of HR. (Song *et al.*, 2008).

Zhang *et al.* (2004) have reported that both melanocortin agonist melanotan-II and antagonist SHU9119 influenced food intake as well as metabolic responses in leptin-resistant aged-obese rats, similarly to the effects observed in young animals of the same strain. In our experiments, in which (in addition to the two endpoints of aging) the responsiveness of the middle-aged population was also simultaneously analyzed, we have found that the non-linear age-related changes of metabolic and anorexigenic effects show significant differences. In the young the anorexigenic effect of alpha-MSH was strong but the metabolic effects were mild. By middle-age (12 months old) the anorexigenic effect became minimal but at the same time the metabolic effects grew pronounced. In the old animals both the anorexigenic and the metabolic effects of alpha-MSH were strong.

Our data show that the metabolic and anorexigenic effects of alpha-MSH changed disparately with aging. In the background, activation of different intracellular signal transduction pathways may be suggested for the metabolic or anorexigenic effects, furthermore different age-related modifications affecting the activation of these pathways may be assumed. Observations of other research groups focusing on melanocortins have indicated disparate signal transduction pathway activation associated with different parameters of energy balance (Chen *et al.*, 2009): e.g. mutation of G(s)alpha did not affect food intake but diminished metabolic effects.

In addition to the decreased orexigenic effects previously described as the main factor in the development of aging anorexia and sarcopenia (Akimoto-Takano *et al.*, 2005) the increased anorexigenic tone confirmed by our experiments may also have an important contribution.

However, in the background of age-related changes in alpha-MSH responsiveness not only alone age but also body composition may have an important role. Typical age-dependent body composition changes were also confirmed by the autopsies of male Wistar rats in our studies. Our data show that the amount of body fat increases with aging. As compared with body weight the highest fat ratio was found in the middle-aged population, later in old

animals this ratio slightly decreased. The results of autopsies also confirmed the appearance of sarcopenia in old rats.

II. Effects of central leptin infusion in different age groups (2- and 6 months) and nutritional states

1. Results

1.1. Changes in body weight and food intake

Prior to leptin administration, HF6 rats had the highest initial BW, followed by NF6, CR6, and NF2 rats. By the end of a 7-day leptin infusion, BW decreased significantly in NF2 and in NF6 groups, it did not change significantly in HF6 animals and there was no weight loss in CR6 rats either when comparing leptin-treated animals with their own controls.

The leptin-infusion-induced decrease in FI appeared to be more pronounced in NF2 or NF6 than in HF6 rats and it was completely absent in CR6 animals, in which group the FI remained unaltered (the rats ate all 16 g of food despite leptin infusion).

1.2. Changes in core temperature

In response to leptin infusion, the mean daytime (inactive period, the nadir of the circadian rhythm) Tc increased significantly in all four groups. In NF2 animals, the rise was very pronounced (even the Tc-maxima increased), but it was transient and Tc returned to control values by the end of the 7-day-long leptin infusion. In contrast, in NF6 and HF6 rats, the elevation of Tc-minima was much smaller (although statistically significant), but more prolonged, while the Tc rise was the most pronounced (ca. 0.8–1.00°C), long lasting, and progressively increasing in CR6 rats, affecting both nadirs and peaks of Tc.

The increases in daytime minimum Tc values detected in the HF6 group were very small (ca. 0.1–0.15°C by the fourth day of the infusion) but due to the small variability within this group, they proved to be statistically significant. However, the physiological significance of such a small rise is questionable.

Besides daytime minimum Tc alterations, NF2 and CR6 animals also showed nighttime Tc elevations. These additional findings may be suggestive of very high leptin sensitivity in these two groups, not observed in older NF or HF rats.

1.3. Changes in spontaneous horizontal locomotor activity

The elevation in daytime minimum Tc cannot be explained by enhanced locomotor activity, considering that the mean daytime ACT failed to show a significant rise in any of the four groups of rats. The nighttime ACT increased exclusively in the CR6 animals.

1.4. Changes in heart rate

In contrast to ACT, daytime Tc elevations could be explained by a rise in metabolic rate as shown by the increased daytime HR. This high HR was observed across all four groups, with a marked initial increase followed by return to control values halfway through the 7-day infusion in NF2 rats, with a smaller but sustained rise in NF6 and an even more moderate but also sustained increase in HF6 rats. A very significant, sustained, and progressive acceleration of HR was, however, observed in CR6 rats. The highest leptin sensitivity in CR6 animals was indicated by the highest peak and the longest-lasting elevation of not only the daytime minimum but also the nighttime maximum values.

1.5. *Changes in body composition*

Indicators of body composition have shown that the fat ratio in both regions was higher in NF6 than in NF2 animals, and it further increased excessively in HF6 rats. The fat pads were smallest or minimal in the CR6 group (particularly the retroperitoneal fat).

The retroperitoneal fat pads practically disappeared by the end of the leptin infusion in the NF2, NF6, and CR6 groups (although in CR6 rats it was not statistically significant due to the very low initial values). Leptin was least effective in the HF6 group in which only 30–40% of the retroperitoneal fat was lost (although the fall was significant even in this group: $p < 0.001$).

Leptin also decreased the amount of epididymal fat; the order of efficacy was NF2>NF6>HF6>CR6.

The muscle ratio was smallest in HF6, but there were no significant differences in various groups either, and the amount of the m. tibialis anterior was not influenced even by a 1-week leptin infusion.

1.6. *Effect of NPY in different nutritional states*

Due to the chronic fasting state, in CR6 controls spontaneous daytime FI was higher even without NPY than in the NF6 and HF6 groups. In NF6 the FI was significantly enhanced by NPY, but the increase was more pronounced in CR6 animals, while the NPY-induced FI rise was completely suppressed in HF6 rats.

2. Discussion

According to the observations of the literature, serum leptin level increases with aging. The higher the serum leptin level is, the smaller the biological responsiveness becomes (Jackson and Ahima, 2006). A significant difference of the serum leptin levels can be confirmed already between the 3- and 6-months-old animals (Mooradian *et al.*, 2000). Our results also confirm this finding, because the anorexigenic effect of leptin was already less pronounced in the NF6 group, than in the NF2 animals, which may indicate decreased leptin sensitivity and the appearance of mild leptin-resistance. However, in addition to their age the body composition of 6-months animals were also different from that of younger ones. Even normally fed 6-months-old rats had significantly higher fat ratios than NF2 animals (of course, a high-fat diet increased this amount of fat enormously).

During a 7 day-long leptin infusion, the effectiveness of leptin was significantly decreased in HF6 animals. A HF diet leads to obesity (its extent varies in different strains) and consequent leptin-resistance. In diet-induced obese (DIO) animals both peripheral and central effects of leptin are diminished when compared with those of non-obese ones (Halaas *et al.*, 1997; Lin *et al.*, 2000). Corresponding to these data, our results also showed slight, not significant BW decrease during ICV leptin infusion in the HF6 group. Leptin infusion failed to reduce either BW or the fat mass of obese animals to a similar extent that has been confirmed in other groups. However, despite leptin-resistance the food intake of HF6 rats was markedly lower than that of the control group during the infusion period. This finding is in contrast with the previously described leptin-resistance also affecting food intake in DIO C57BL/6J mice (Enriori *et al.*, 2007).

Regarding the anorexigenic effects of leptin, the lack of effects were best seen in calorie-restricted animals: neither body weight nor food intake was altered during the whole infusion period (in fact during leptin infusion they ate more than the NF2 or NF6 animals). This result coincided with the data of Gabriely *et al.* (2002) on resistance to leptin-induced

anorexia in old calorie-restricted rats. However, this paper analyzed anorexia exclusively, and the authors did not measure other metabolic parameters of the energy balance.

The metabolic responses to leptin exhibited a different pattern from those previously seen in connection with the effects on FI and BW. In young rats the increase of Tc and HR nadirs were high but transient, while in NF6 animals this was only slight, but somewhat more prolonged. The smallest effect was seen in the HF6 group, which also confirms previous observations regarding the development of leptin-resistance due to obesity. Simultaneous recording of the anorexigenic and metabolic parameters leads to the most interesting findings in CR6 animals. While anorexigenic parameters suggested leptin-resistance in CR6 rats, registration of metabolic parameters shows the most pronounced leptin effect (higher than that in NF2 and NF6) in this group: both Tc minima and Tc maxima were elevated during leptin infusion. Similar results were seen during the registration of HR in CR6 group.

We have evaluated this leptin-resistance affecting the anorexigenic effects as a virtual one, in the background of which enhanced orexigenic tone of calorie-restricted animals may be assumed. This was further confirmed by the enhanced orexigenic response seen upon central NPY administration in our experiments. Other data of the literature also indicate enhanced NPY synthesis and secretion in fasting or energy deficient states (Inui, 2000).

Diminished leptin sensitivity in 6-month-old high-fat diet-induced obese animals with high fat mass and the very characteristic changes in calorie-restricted rats as compared with leptin responsiveness of normally fed animals of the same age-group emphasize that at the age of 6 months body composition appears to be a more dominant factor (determining leptin-sensitivity) than age itself.

Disparate alterations within the HF6 group showing resistance concerning body weight and metabolic rate with maintained food intake-reducing effects may also indicate that possibly disparate intracellular signal transduction pathways are involved in these effects. These results draw our attention to the need for further investigations concerning these pathways.

Other studies also report varying alterations in the anorexigenic and metabolic effects, e.g. chronic induced hyperleptinemia in non-obese animals leads to resistance to the anorexigenic effects of leptin after 25 days, whereas metabolic resistance to leptin did not develop before the 83rd day (Scarpace *et al.*, 2002). In a 19-week HFD-induced obesity in mice, impairment of the IP3K pathway was described with maintained STAT3 activity (Metlakunta *et al.*, 2008).

III. Effects of central alpha-MSH infusion in various age-groups (2-months and 6 months) and in 6-month-old rats of different nutritional states

1. Results

1.1. Changes in body weight and food intake

The 7-day long central alpha-MSH infusion efficiently decreased BW development in all groups. This suppression of BW was especially significant in juvenile NF2 rats of rapid growth. In the HF6 group, although the comparison of daily data did not show big differences from day-4, repeated-measures ANOVA that focus on the long-term tendencies in BW changes still exhibited a weak but statistically significant difference for the 7-day period.

Regarding FI, centrally applied alpha-MSH caused a significant suppression in the NF2 group during the first 4 days, in the HF6 group during the first 6 days, whereas, in the NF6 and CR6 rats, the effect was significant throughout the infusion period.

1.2. *Changes in core temperature*

Mean daytime Tc increased significantly in all groups during the course of the alpha-MSH infusion. While in the NF2, NF6 and HF6 groups only the daytime Tc minima were significantly elevated, in CR6 rats – besides the Tc minima – the Tc maxima were also increased for 4 days.

1.3. *Changes in heart rate*

HR values showed a somewhat similar pattern to that of Tc. Significant elevations of mean daytime HR were observed in the NF2, NF6 groups during the infusion, and for a shorter period (only for 3 days) also in the HF6 groups, while the HR rise was most pronounced in the CR6 rats. Mean nighttime HR increased for 5 days only in the NF2 group.

1.4. *Changes in local horizontal locomotor activity*

All for groups failed to show any significant alterations in ACT, day or night.

1.5. *Changes in body composition*

Autopsy following the alpha-MSH infusion revealed a decrease in the fat content of the NF6 and HF6 groups with higher initial values of both fat mass indicators. The masses of both retroperitoneal and epididymal fat pads decreased. In NF2 and CR6 animals alpha-MSH infusion failed to decrease their smaller initial fat pads.

Muscle mass indicated by the tibialis anterior muscle remained unchanged in all four groups.

2. Discussion

The main activator of the MC system that plays a central role in the maintenance of energy balance and in body weight regulation is leptin that in turn shows a close correlation with the nutritional state. Previous data (Zang *et al.*, 2004; Wolden-Hanson *et al.*, 2006) indicate that the sensitivity of the MC system may be maintained despite leptin-resistance, while other studies suggest the participation of more and more leptin-independent pathways in MC activation. (Ibrahim *et al.*, 2003; Blouet *et al.*, 2009)

In our experiments that were also performed according to the same protocols as our previous study, we have found several differences regarding the central leptin- and alpha-MSH responsiveness of rats of various nutritional states.

Alpha-MSH infusion was able to suppress body weight of animals in all groups, while the leptin infusion decreased the body weight to a significant degree only in the NF2 and NF6 groups. Concerning food intake, we have also seen a pattern of alpha-MSH sensitivity that was different from that of leptin. During the course of the alpha-MSH infusion food intake of rats was suppressed in all groups, it was significantly suppressed even in the calorie-restricted rats, as well, although a similar leptin infusion did not influence the food intake of this group at all.

Regarding metabolic parameters, similarly to the effects of leptin, the most pronounced metabolic effect was seen in CR6 animals. Somewhat smaller responses were observed in the NF2 and NF6 groups. Contrary to the results of experiments investigating leptin effects where Tc-minima were elevated by only about 0.1 °C (negligible), the alpha-MSH infusion influenced the metabolism of rats to a significant extent: it was able to increase significantly both the Tc-minima and transiently also the HR in HF6 rats.

Regarding body composition alpha-MSH infusion was able to decrease the fat content in the NF6 and HF6 animals with higher initial fat masses, but did not change muscle mass in any of the groups, while leptin abolished the retroperitoneal fat in all animals except HF6. These data show that body composition has an effect not only on leptin responsiveness but also on alpha-MSH responsiveness, i.e. it may influence the sensitivity of the MC system. In calorie-restriction an enhanced MC responsiveness (similarly to that seen regarding leptin), while in obesity a significant catabolic effect (contrary to those of leptin) were observed.

The present results confirm that in the presence of leptin-resistance the sensitivity of the MC system is maintained. It is a very important finding because – contrary to previous opinions according to which only simple effects concerning the energy homeostasis were attributed to the MC system – today many more complex effects, including its role in liver-, muscle- and fat metabolism, actions inducing pancreatic insulin release and peripheral insulin sensitivity are emphasized (Mountjoy *et al.*, 2010). So the role of the MC system in the development of obesity and diabetes mellitus and in their potential therapeutic measures is increasingly recognized.

Maintained MC sensitivity, in spite of leptin-resistance, call our attention to the need for a better understanding of leptin-independent activation of the MC system. However, the efficacy of these pathways – similarly to those of leptin signaling – might depend on the nutritional state, as well. Blood glucose level may play an important role in the leptin-independent activation of the MC system. In the hypothalamic arcuate nucleus about 50% of POMC neurons are able to sense an elevation of blood glucose level, which may lead to an increase in alpha-MSH secretion. As a result of a 20-week HF diet this mechanism is also impaired (Parton *et al.*, 2007).

Summary

1. Responsiveness to alpha-MSH depends on age, but the effects on food intake and metabolic factors do not change parallel with aging. While in young animals the anorexigenic effects of alpha-MSH are dominant, in the middle-aged population the metabolic effects are the most important, and finally in the old age-groups both the anorexigenic and the metabolic effects are pronounced, which might play a role in the development of sarcopenia in elderly.
2. With aging the body composition also shows characteristic age-related changes. The fat content increases until 18 months (late-middle aged), afterwards in old animals it decreases. Additionally, the muscle mass decreases in the course of aging.
3. Signs of a mild leptin-resistance seem to appear at 6 months of age in normally fed animals. But these 6-month-old animals have a significantly higher fat ratio compared with young animals. The effects of leptin in 6 month-old rats were more moderate regarding either food intake or metabolism than in young, 2 months old animals.
4. Regarding leptin sensitivity in 6-months-old rats, the nutritional state seems to be more important than age. Obesity leads to the appearance of leptin-resistance, although the anorexigenic effects appear to be maintained. Calorie-restriction results in enhanced leptin-sensitivity, at least the most pronounced metabolic effects were seen in this CR group. In this group the leptin-resistance regarding food intake (that were previously described in the literature by Gabriely *et al.*, 2002) we considered to be a virtual leptin-resistance due to a high orexigenic tone presumably caused by chronic starvation – which does not affect the pronounced metabolic effects in these animals.

5. The nutritional state also affects MC sensitivity in 6-month-old rats. Despite leptin-resistance that develops due to changes in body composition (obesity), central melanocortin-sensitivity is maintained in 6 month-old animals, moreover the most pronounced anorexigenic effects were seen in this group.

Perspectives

The animal models used in our experiments will be useful later for the studies of the intracellular signal transduction pathways assumed in the background of age- and body composition-dependent alterations, and for the identification of the presumably independent pathways associated with the metabolic and anorexigenic effects, and with their age-related alterations.

The results of our previous studies confirmed that leptin-sensitivity may be ameliorated using calorie-restriction. Through future investigations we would like to clarify, whether age-related leptin-resistance may also be prevented in later age-groups, or on the other hand how long can we delay the appearance of leptin-resistance with the help of calorie-restriction.

Previous observations indicate that a 3-month calorie-restriction in old (20 month-old) animals suppressed the age-related increase in leptin levels, i.e. it was able to restore serum leptin to the level corresponding to that of a 7 month-old animal, while at the same time SOCS-3 upregulation (responsible for the development of leptin-resistance) was also diminished (Peralta *et al.*, 2002).

It remains to be clarified, what effects this long-term calorie-restriction used by us has on the appearance of insulin-resistance and on the age-related changes in muscle mass, i.e. whether this long-term calorie-restriction is able to improve insulin-resistance without reducing muscle mass. Further opportunities lie in the simultaneous application of calorie-restriction and physical training: physical activity may also have an important role in the enhancement and even restoration of insulin-sensitivity and/or leptin-sensitivity.

Concerning leptin-resistance seen in obese animals, our results show that in the 6-month-old age-group it is still rather a consequence of body composition than that of age. According to other data of the literature, obesity may enhance the progression of aging. The question arises, whether the exaggerated age-dependent anorexigenic tone seen in the first set of our experiments would appear earlier in excessively obese animals.

In our animal models the effects of other central (e.g.: CRF) and peripheral (insulin, CCK) peptide mediators (neurotransmitters) may be studied, the anorexigenic and metabolic effects of which may also play a role in the age-related changes in body composition. The age- and body composition-dependent changes of central insulin effects appear to be particularly exciting. Insulin (similarly to leptin) is able to pass the blood-brain-barrier and primarily acts in the hypothalamus (partly via common intracellular pathways with leptin). It decreases the production of orexigenic neuropeptides (NPY/AgRP system) and enhances the synthesis of the anorexigenic ones (POMC/CART). Thus it decreases food intake, increases the sympathetic tone and heat production. It leads to weight loss and at the same time – due to the relative starvation state – it causes enhanced protein catabolism and finally sarcopenia.

Centrally applied insulin infusion also affects the peripheral glucose metabolism, e.g.: glucose release from the liver decreases, insulin production in the pancreas increases. Chronic high serum leptin level (due to obesity or aging) also decreases central insulin sensitivity. In animal models the restoration of central leptin-sensitivity improves the peripheral insulin-sensitivity, as well (Marino *et al.*, 2011). These data emphasize the importance of the investigations aimed at the maintenance of central leptin-sensitivity.

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