Impaired oxidative capacity due to decreased CPT1b levels as a contributing factor to fat accumulation in obesity.

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Running head: Obesity-prone rats have impaired fat oxidation
In order to characterize mechanisms responsible for fat accumulation we used a selectively bred obesity-prone (OP) and obesity-resistant (OR) rat model, where the rats were fed a Western diet for 76 days. Body composition was assessed by MRI scans and as expected the OP rats developed a higher degree of fat accumulation compared to OR rats. Indirect calorimetry showed that the OP rats had higher respiratory exchange ratio (RER) compared to OR rats indicating an impaired ability to oxidize fat. The OP rats had lower expression of carnitine palmitoyltransferase 1b in intra-abdominal fat, and higher expression of stearoyl-CoA desaturase 1 in subcutaneous fat compared to OR rats, which could explain the higher fat accumulation and RER values. Basal metabolic parameters were also examined in juvenile OP and OR rats before and during the introduction of the Western diet. Juvenile OP rats likewise had higher RER values indicating that this trait may be a primary and contributing factor to their obese phenotype. When the adult obese rats were exposed to the orexigenic and adipogenic hormone ghrelin, we observed increased RER values in both OP and OR rats, while OR rats were more sensitive to ghrelin’s orexigenic effects as well as ghrelin-induced attenuation of activity and energy expenditure. Thus, increased fat accumulation characterizing obesity may be caused by impaired oxidative capacity due to decreased carnitine palmitoyltransferase 1b levels in the white adipose tissue, while ghrelin sensitivity did not seem to be a contributing factor.

Keywords: Indirect calorimetry, polygenic obese, adipose tissue, ghrelin, respiratory exchange ratio
Introduction

Obesity and its associated diseases such as diabetes mellitus type 2, cardiovascular diseases and some types of cancer are some of the severe health challenges today. As no effective pharmacological treatment of obesity currently exists, there is a great need of new treatment strategies that can give rise to sustained weight loss.

In order to study human obesity, animal models are highly important to understand the pathophysiology behind obesity and to pursue novel treatment strategies. These include rodent models fed with high-fat diet (DIO rodents) and rodent models with a monogenetic cause of obesity such as leptin deficiency (35, 40). While these animal models may be useful in modeling certain aspects of obesity, they do not mirror human obesity that is caused by a complex interplay of environmental, social, and genetic factors. One animal model, the selectively bred obesity-prone (OP) and obesity-resistant (OR) rat employed in the present study was developed by Barry Levin, in an attempt to mimic both the polygenetic and environmental (diet) aspects of obesity (31). In outbred Sprague Dawley rats, some are genetically predisposed to obesity when placed on a Western diet with relatively high fat and sucrose compared to chow (31.8% fat, 25.7% sucrose), while others are obesity resistant gaining no more weight than chow-fed controls (34). Selective breeding of high and low weight gainers has produced the OP and OR sub-strains (31). When placed on a Western diet, the OP rats are described to have higher energy intake, accumulate more fat, be glucose intolerant, hyperinsulinemic, and hyperleptinemic and defend their body weight once obesity is established (31, 36).

Excessive fat accumulation is a hallmark of obesity and can be attributed to an imbalance between energy consumed and energy expended or alterations in substrate utilization and trafficking. The underlying molecular mechanisms giving rise to
excessive adiposity in both humans and rodent models including the OP rats are incompletely understood. Centrally, the OP rats have higher hypothalamic neuropeptide Y (NPY) mRNA expression (29), as well as more inhibitory synapses onto the anorexigenic proopiomelanocortin (POMC) neurons than OR rats (18) prior to high-fat diet feeding and thus the establishment of obesity. This could contribute to their higher food intake and body weight, but it does not explain the selective fat accumulation.

Ghrelin is a peptide primarily secreted from the endocrine cells of the stomach (28), which stimulates growth hormone release, appetite and fat accumulation (38, 50, 52, 53, 57). Ghrelin is most well described for its orexigenic effects in the arcuate nucleus (ARC) of the hypothalamus, where it increases the appetite-stimulating neuropeptides agouti-related peptide (AgRP) and NPY (9, 27, 47). Importantly, Ghrelin also promotes adiposity independently of its appetite-stimulating effects by upregulating lipogenic genes and downregulating fat oxidative genes in white adipose tissue (42, 52). Accordingly, it is interesting to study the effect of exogenously administrated ghrelin in this obesity-prone animal model. Further, several studies have demonstrated that obese animal models such as DIO rodents and db/db mice are resistant or have a blunted response to ghrelin’s orexigenic effects, although results are discrepant as to whether it is caused by the high fat diet per se or the resultant obesity (5, 14, 23, 42, 43). Interestingly, the ghrelin resistance seems to affect only the orexigenic and not lipogenic actions of ghrelin (42).

The aim of the present study was to examine the molecular and physiological backgrounds for increased fat accumulation by performing an integrated and simultaneous analysis of food intake, energy expenditure, substrate utilization and
activity levels under un-stressed home-cage conditions in free-fed and fasted male OP and OR rats using an indirect calorimetry system. This was done after feeding a Western diet for 76 days where obesity was fully established, in order to study the translational value of this model for human obesity. In addition, juvenile OP and OR rats were also examined before severe obesity had developed in the indirect calorimetry system in order to establish if the phenotype observed in the adult obese rats were primary or secondary to obesity. Adult OP and OR rats were also given a single dose of peripherally administered ghrelin in order to assess potential differences in ghrelin sensitivity between OP and OR rats.

Materials and methods

Animals and indirect calorimetry

Two cohorts of selectively bred male OP and OR rats (Taclevin DS and DR rats, Taconic, Hudson, NY) were used in these studies. All rats were single housed from four weeks of age with ad libitum access to water and a Western diet (31.8% fat, 16.8% protein and 51.4% carbohydrate (25.7% sucrose), #12266B, Research Diets, Brogaarden, Lyng, Denmark) or a chow diet (13% fat, 27% protein and 60% carbohydrate, altromin #1310, Brogaarden) under a 12-hour light/dark cycle (lights off at 1800 h). The first cohort of rats (twelve OP and twelve OR rats) was placed on the Western diet for 76 days. During this time period, food intake and body weight were monitored every three-four days and body composition determined every two weeks by quantitative magnetic resonance imaging (MRI) using EchoMRI (Echo Medical Systems, Houston, TX) in unanesthetized rats. At 15 or 17 weeks of age (balanced between OP and OR rats), rats were single-housed in an indirect
calorimetry system (Phenomaster; TSE systems, Bad Homburg, Germany), where food intake, energy expenditure, respiratory exchange ratio (RER: VCO₂/VO₂) and activity levels were recorded. Rats were acclimatized to the system for three days followed by three days of baseline measurements. Next, rats were fasted for 24 hours followed by 24 hours of re-feeding and a recovery period, after which ghrelin (2 mg/kg in 2.5 ml/kg s.c, PolyPeptide, Limhamn, Sweden) or saline was administered just before the onset of the dark phase in a cross-over design. The ghrelin dose was chosen based on previous publication (1, 5) and further optimized in our laboratory - in lean rats, this dose increases the active ghrelin concentration about five fold from 903±82 pg/ml to 4393± 162 pg/ml in plasma after 40 minutes (unpublished observation).

The second cohort of rats (eight OP and eight OR) was immediately placed in the indirect calorimetry system upon arrival with access to standard chow. The rats were acclimatized for one week before measurements were started at an age of five weeks. Data were collected for one week and then the rats were switched to the Western diet and data collected for another week. Body composition was determined before and after measurements. The indirect calorimetry system was calibrated before each study and for each study period the indirect calorimetry system measured each cage every 20 minutes for 1 minute and 10 seconds and the reference air every 20 minutes.

DIO mice and lean controls (C57BL/6) were purchased from Taconic after they had been fed a high-fat diet (60% fat, D12492, Research diets) or control diet for 10 weeks from six weeks of age (17). DIO rats and lean controls (Sprague Dawley, Taconic, Ry, Denmark) were started on a high-fat/high sucrose diet (45% fat, 12451, Research diets) or a low fat control diet (10% fat, D12450B, Research diets) at a body weight of 200 g and maintained on diets for 15 weeks. DIO mice were euthanized at
17 weeks of age and DIO rats at 22 weeks of age. Tissue was collected as described for OP and OR rats. At euthanasia, DIO mice had a body weight of 38.2 ± 0.6 g versus chow-fed animals 27.5 ± 0.6 g (17). DIO rats had a body weight of 665.2 ± 39.3 g versus control rats 470.6 ± 18.0 g. All animal experiments were approved by the Animal Experiments Inspectorate (Copenhagen, Denmark) and conducted in accordance with institutional guidelines.

Tissue collection

Adult obese rats were returned to baseline maintenance conditions after calorimetry studies for at least one week before tissue collection at an age of 20 weeks. Rats were food deprived from the early light phase and euthanized three-five hours into the light phase by decapitation after brief CO₂ anesthesia. The hypothalamus was dissected by making a cut posterior to the optic chiasm and anterior to the mammillary body and then it was scooped out along its lateral margins in an approximate depth of two mm and snap frozen in liquid nitrogen. Epididymal and subcutaneous white adipose tissue and soleus and extensor digitorum longus (EDL) muscle were removed and snap frozen in liquid nitrogen. Trunk blood was collected and plasma separated. All samples were stored at -80°C until further processed.

Quantitative real-time PCR

RNA (hypothalamus and adipose tissue) was extracted and DNase treated using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany) or trizol (muscle) (Invitrogen, Carlsbad, CA). cDNA was synthesized by reverse transcription using ImProm-II™ reverse transcriptase (Promega, Madison, WI). Relative mRNA levels were determined in duplicates using a Lightcycler 480 II (Roche Applied Science,
Penzberg, Germany) and SYBRPremix Ex Taq (Takara, Otsu, Japan). The relative gene expression level was determined using the ΔΔCT method normalizing to TATA-binding protein (TBP). All primers used (Table 1) were initially tested for specificity and efficiency by running standard curves.

Western blot
Western blotting was performed as previously described (44). The following antibodies were used: Total Acetyl-CoA carboxylase #3662, phosphorylated Acetyl-CoA carboxylase #3661 (Cell Signaling, Boston, MA) diluted 1:1000.

Plasma assays
Insulin and leptin were analyzed using the Mouse/Rat insulin kit and the Rat Leptin Kit (MesoScale Discovery, Rockville, MD).

Statistical analyses
Statistical analyses were performed using Prism version 6.0a (Graphpad Software, San Diego, CA). Data were analyzed using two-way ANOVA with Sidak post-test, two-way ANOVA repeated measures with Sidak post-test or two-tailed t-tests. Energy expenditure data was analyzed by one-way or two-way ANCOVA as recommended (54) with lean mass as covariate using SAS version 9.2 (SAS software, Cary, North Carolina). All data represent means ± SEM. Values of p≤0.05 were considered significant.

Results
Food intake, body weight, and body composition
The body weight of OP and OR rats was similar for the initial part of the feeding period, but during the final 20 days a difference in body weight developed between OP and OR rats (541 ± 9 g versus 500 ± 12 g, p<0.01) (Figure 1A). No difference in food intake was detected between OR and OP rats assessed by weighing of remaining food pellets (Figure 1B). OR and OP rats initially had similar body composition but already after two weeks on the Western diet, OP rats had significantly higher fat percentage and fat mass (Figure 1C and E) and lower lean percentage compared to OR rats, while the absolute lean mass was similar between OP and OR rats (Figure 1D and F). The difference in body composition between OP and OR rats continued to increase throughout the study and after 76 days the difference in fat percentage was 19.7 ± 0.6% in OP rats versus 14.2 ± 0.4% in OR rats (p<0.0001).

**Indirect calorimetry**

During the 72 hours of baseline measurements, OP rats had higher RER values (Figure 2A and B) and lower activity levels (Figure 2C and D) compared to OR rats, while OP and OR rats had similar cumulative food intake (Figure 2H) and energy expenditure (Figure 2E-G). In order to determine if the differences observed in the adult obese rats were primary and contributing or secondary to the obese phenotype we examined juvenile rats in the indirect calorimetry system before and during the introduction of the Western diet. Juvenile OP rats on chow showed increased RER values during the dark phase (Figure 2J) and during the light phase after the diet was switched to the Western diet (Figure 2L). No differences in activity levels were observed between juvenile OP and OR rats (Figure 2K and M). The juvenile OP and OR rats had similar body weight before the calorimetry study, while OP rats were
heavier afterwards (Figure 2I and N). This was likely caused by the higher food intake observed in juvenile OP rats (data not shown).

We also fasted the adult obese OP and OR rats for 24 hours and monitored their ability to shift from a mixed oxidation of carbohydrate and fat to an almost complete fat oxidation (Table 2). During the first three hours of fasting the OP rats had significantly higher RER, but during more extreme situations such as longer-lasting fasting the OP rats obtained the same capacity for fat oxidation as observed for the OR rats. During re-feeding OP rats likewise had similar RER values to OR rats. Further, OR rats had higher energy expenditure during dark phase re-feeding.

**Ghrelin administration**

Subcutaneous administration of ghrelin in the adult obese rats (2 mg/kg) induced a trend towards increased cumulative food intake (p=0.07) in OR rats and (p=0.12) in OP rats with a significant main effect of ghrelin treatment (p=0.006) (Figure 3A). Ghrelin decreased activity levels in OR rats (p=0.002) and energy expenditure in both OR (p<0.0001) and OP (p=0.0025) rats at 40 minutes, whereas no difference was observed after 120 min. (Figure 3B and C). Ghrelin induced a robust increase in RER values at both time points representative of a shift away from fat oxidation towards carbohydrate metabolism in both the OP and OR rats (Figure 3D).

**Adipose tissue and muscle gene expression**

In order to examine possible molecular backgrounds of the observed higher fat accumulation and higher RER values in OP rats, the expression of genes involved in lipogenesis and fatty acid metabolism were examined in the adult obese rats. In the intra-abdominal fat compartment, epididymal fat, OP rats had lower mRNA levels of
carnitine palmitoyl transferase 1b (CPT1b) and both isoforms of acetyl-CoA carboxylase (ACC) (Figure 4A). CPT1b mediates the transport of fatty acids across the mitochondrial membrane, which may be responsible for the impaired capacity of fat oxidation within this adipose compartment for OP rats. ACC mediates the synthesis of two pools of malonyl CoA, of which malonyl CoA produced by ACC1 is used in the synthesis of triglycerides, while malonyl CoA produced by ACC2 inhibits CPT1b and thus the β-oxidation pathway (55). As ACC is active only in the non-phosphorylated state, western blotting was used to determine the degree of phosphorylation. No difference was observed in total or phosphorylated ACC levels between OP and OR rats (Figure 4B) and thus the activity of ACC did not differ between OP and OR rats. In order to examine if low CPT1b levels in intra-abdominal fat is generally observed in obese animals, the CPT1b expression levels were also examined in outbred DIO mice and rats, obtained by simple high-fat diet feeding, hence not predisposed to obesity. In contrast to the OP rats, DIO mice had higher CPT1b levels compared to chow-fed controls (Figure 4C), while DIO rats had similar CPT1b levels to lean control rats (Figure 4D). High levels of CPT1b correspond well with earlier described low RER levels in outbred DIO rodents (15, 49).

In subcutaneous fat, OP rats had higher mRNA levels of stearoyl-CoA desaturase 1 (SCD1), the rate-limiting enzyme in unsaturated fatty acid synthesis (Figure 4E). No differences in mRNA levels in muscle were observed between OP and OR rats, except a trend towards higher expression of SCD1 in both soleus (oxidative) and EDL (mixed oxidative and glycolytic) in OP rats was observed (Figure 4F-G).

Hypothalamic gene expression
In the hypothalamus, the adult obese OP rats had lower expression of AgRP, uncoupling protein 2 (UCP2) and the ghrelin receptor (Figure 4H). As mentioned above, low expression of the ghrelin receptor is associated with obesity (5), which is consistent with these results. AgRP mRNA in ARC is upregulated when ghrelin activates the NPY/AgRP neurons and this is also the neuronal population described to be responsible for the ghrelin resistance associated with obesity. Further, hypothalamic UCP2 mRNA is also upregulated by ghrelin and UCP2 is required for a complete food intake response of ghrelin, as it mediates ghrelin’s activation of NPY/AgRP neurons by lowering free radicals generated by hypothalamic fatty acid oxidation (2). Thus, low mRNA levels of the ghrelin receptor in OP rats could decrease ghrelin mediated gene expression of AgRP and UCP2, which in turn likely contribute to the observed attenuated ghrelin sensitivity in OP rats.

Plasma hormones

Plasma levels of leptin and insulin were assessed to determine basal differences in metabolic blood markers in adult obese rats. OP rats had higher leptin and insulin levels than OR rats (Table 3). High leptin and insulin levels are often observed in obese animals as leptin mirrors adiposity, while high insulin could be indicative of insulin resistance in OP rats.

Discussion

In the present study we demonstrated that the selectively bred OP rats fed a moderate high-fat diet have an impaired capacity to use fat as an energy source demonstrated by increased RER value during normal physiological baseline conditions. We suggest that this could be one of the causative factor responsible for the severe increase in fat
accumulation in the OP compared to OR rats, since the increased RER level was observed both before and after severe obesity developed in the OP rats. For the first time we also dissect an important molecular pathway that may explain the decreased capacity to oxidize fat in the intra-abdominal fat compartment. Here we observed decreased expression of CPT1b, which is responsible for the transfer of free fatty acids into the mitochondrial matrix suggesting lower fat oxidation. In the subcutaneous fat compartment, the rate limiting enzyme in unsaturated fatty acid synthesis, SCD1, was upregulated. Also, in the skeletal muscles a trend towards upregulation of this enzyme was observed. The regulation of these markers could contribute to the impaired fat oxidation and explain the increased propensity for fat accumulation observed in OP rats. Interestingly, increased fat accumulation is observed prior to the increase in body weight, and this redistribution likely contributes to the following increase in body weight. Finally, we have shown that this strain of obese rats does not develop full ghrelin resistance with respect to food intake. However, the ghrelin-induced decrease in activity and energy expenditure were attenuated in the OP rats compared to the OR rats, while OR and OP rats responded similarly to ghrelin induced increase in the RER values.

Valid animal models that reflect the complicated interplay between environmental and genetic factors are required in the attempts to develop novel and efficient pharmaceutical treatments of obesity. In addition, such animal models may also be important for the molecular understanding of the pathophysiology responsible for the development of obesity. The OP rat model that we have studied may constitute such a model system. The OP rats in our study developed a strong degree of fat accumulation, which is a cornerstone in the pathophysiology of obesity and important for the accompanied diseases (10). Thus, it is important to characterize the metabolic
parameters under unstressed “home-cage” conditions for longer periods of times to
determine mechanisms of this severe fat accumulation, which the indirect calorimetry
system accommodates. In the ad libitum fed state, OP rats had higher RER values
indicating a preferential usage of carbohydrate over fat as their energy substrate,
despite the higher fat mass of OP rats. This difference was not caused by higher food
intake in the OP rats since this study, in contrast to previous studies (12, 25, 26, 31,
33, 36, 45) did not demonstrate increased food intake in OP rats compared to OR rats.
Juvenile OP rats likewise had higher RER values suggesting that impaired fat
oxidation is primary and causative and not secondary to obesity in this strain. On the
contrary, lower activity levels in OP rats were only observed in adult rats after the
obese phenotype was established. While it is well established that low activity levels
can maintain and exacerbate an obese phenotype (4), the contribution of low activity
levels as the primary underlying factor for obesity is more controversial. Rodent
studies show conflicting results as some find activity levels predictive of future
weight gain (4, 51), while others do not (7, 30, 41). In this particular strain of OP rats
most studies found no pre-existing differences in activity between OP and OR rats
(25, 39) while one study found activity differences prior to the establishment of
obesity (51). Thus, the causal relationship between activity and obesity is complex
and incompletely understood. This study suggests that low activity is secondary to
excessive fat accumulation and not one of the underlying factors contributing to the
OP and OR phenotypes of this strain.
A previous study (25) working on the same strain of rats found that selectively bred
OP rats preferentially oxidize carbohydrate over fat, but the study by Jackman et al.
(2010) only measured the energy expenditure and RER value just before and after
short (five days) exposure to high-fat diet, before obesity was fully established (25).
Impaired fat oxidative capacities of the OP rats is also supported by tracer studies after intra-gastric administration of $^{14}$C-labeled palmitate, where OP rats oxidized less dietary fat and had higher disposal of tracer to the adipose tissue than OR rats (24, 26). However, it has never previously been shown that OP rats oxidize less fat than the OR rats after long-term high-fat feeding, where severe fat accumulation in the OP rats has been obtained. The observation that OP rats display decreased fat oxidation both under lean and fat conditions indicate that this may be one of the primary causative factor for the excessive fat accumulation. Further our study suggests that the molecular responsible factor is a lower level of CPT1b in the fat tissue. This is in contrast to other obese animal models such as DIO rodents where diet alone is responsible for the obesity, as these have lower RER value compared to chow-fed controls (15, 49). The DIO mice and rats examined in the present study had higher or similar CPT1b levels compared to low-fat fed controls, which is probably an adaptive mechanism to their higher fat intake and the consequent high fat accumulation. This indicates that obesity or high adiposity per se is not associated with high RER values and low CPT1b levels, substantiating that the high RER and low CPT1b levels observed in the OP rats could be one of the genetic underlying and molecular factors responsible for the high level of fat accumulation in this obese phenotype. Interestingly, human studies suggest that impaired fat oxidative capacities may be an important contributor to obesity as obese individuals oxidize less fat than lean individuals (3) and fat oxidation capacities have been shown to predict body weight (13, 16, 37, 46, 58). Increased desaturase activities and SCD1 expression have been described in skeletal muscle of obese humans (20, 56), while decreased CPT1 expression has been observed (48). This is in line with our results from the OP and
OR rats and emphasizes the high translational value of this animal model when studying obesity.

We also examined the sensitivity of the OP and OR rats to the orexigenic hormone ghrelin. As described in lean animal models, we observed a trend towards increased food intake, a shift in the substrate use towards carbohydrate, and decreased activity and energy expenditure in OR rats, despite their high-fat diet. OP rats also showed a trend towards increased food intake and an attenuated decrease in activity levels and energy expenditure compared to OR rats. Only with respect to increased RER values a comparable ghrelin response was observed between OP and OR rats. Several obese and diabetic rodent models are resistant to ghrelin’s orexigenic effects (5, 14, 23, 43).

One study showed that feeding a high-fat diet overnight prior to central ghrelin administration was sufficient to blunt ghrelin’s orexigenic effect, suggesting that the diet interferes with ghrelin signaling (42). However, others find that longer time periods and increased body weight are necessary before ghrelin resistance occurs (6). Thus, the underlying cause e.g. the diet or the resultant obesity is not fully understood.

A recent study suggest that hyperleptinemia associated with obesity is important for ghrelin resistance (6). Interestingly, obese humans are not ghrelin resistant as increased food intake and hunger ratings were observed after infusion of 5 pmol/kg/minute ghrelin for 75 minutes in both obese and lean subjects (11). Also, very obese subjects (mean BMI 51.4) responded similarly to ghrelin infusions with respect to food intake as lean subjects (19). We did not observe full ghrelin resistance in the OR rats despite long-term Western diet feeding supporting that diet per se does not cause ghrelin resistance. The OP rats also showed a trend towards increased food intake after ghrelin administration, but with attenuated response compared to OR rats. Thus the OP and OR rats may also in this respect, constitute a better and more
predictable model for human obesity compared to other rodent models. An attenuated ghrelin response of OP rats compared to OR rats was also observed in ghrelin’s suppression of activity levels and energy expenditure, overall suggesting that adiposity or downstream factors affect ghrelin sensitivity. In contrast, OP and OR rats responded similarly to the ghrelin-induced shift in substrate use towards carbohydrate oxidation, in line with previous publications demonstrating that ghrelin’s adipogenic effects are not blunted in the obese state (42). Thus, the increased fat accumulation and decreased oxidative capacity observed for the OP rats is not caused by an increased sensitivity to ghrelin.

In addition to ghrelin, other studies have shown that the OR rats are more sensitive to leptin (21, 22, 32, 33), insulin (8, 21) and glp-1 agonists (12) compared to OP rats. Thus, it seems that NPY/AgRP neurons loose their ability to correctly integrate and respond to both orexigenic and anorexigenic hormonal signals in obese animals independently of the high-fat diet.

**Perspectives and significance**

In conclusion, the present study suggests that increased fat accumulation in the selectively bred OP rats may be caused by impaired capacity to oxidize fat. Impaired fat oxidative capacities were demonstrated both in adult obese and juvenile OP rats prior to the development of obesity, indicating that this defect could be causative. The low capacity to metabolize fat could be mediated via decreased expression of CPT1b. This, in combination with the observed high expression of SCD1, may constitute the molecular explanation for the increased fat accumulation in OP rats. The impaired fat oxidative capacity combined with increased fat accumulative ability has also been shown to characterize human obesity. Furthermore, ghrelin did not seem to contribute
to the high fat accumulation of OP rats, although OP rats were less resistant to ghrelin in terms of food intake compared to other rodent models of obesity. This also points to a high translational value for human obesity. Overall, we suggest that the OP and OR rat model constitute a suitable model for future studies on human obesity.

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Conflict of interest

KR is employed at Novo Nordisk A/S and owns shares in Novo Nordisk A/S.

Author contributions

BH, CR, ANM and GMK designed the study. CR, ANM, KSP, LJS, OHM, KR and LVK carried out experiments and analyzed data. CR drafted the manuscript. All authors reviewed and approved the manuscript.


**Figure 1. In vivo data during the 76 days of Western diet feeding in OP and OR rats.** (A) Body weight (genotype main effect p=0.013), (B) average weekly food intake, (C) fat % (genotype main effect p<0.0001), (D) lean % (genotype main effect p<0.0001), (E) fat mass (genotype main effect p<0.0001), and (F) lean mass. Data tested with two-way ANOVA repeated measures with Sidak post-test (A (12 comparisons made), C-F (six comparisons made)) and two-tailed t-tests (B). N=12 per genotype. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Data are means ± SEM.

**Figure 2. Indirect calorimetry for adult obese and juvenile OP and OR rats.** (A) 72-hour RER values (genotype main effect p=0.0043), (B) average RER values during light and dark phase, (C) 72-hour ambulatory activity (genotype main effect p=0.0023), (D) average ambulatory activity during light and dark phase, (E-G) average energy expenditure adjusted for lean mass during light and dark phase (H) cumulative food intake during light and dark phase, (I) body weight of juvenile rats before calorimetry, (J) average RER values of juvenile rats on chow during light and dark phase, (K) average ambulatory activity of juvenile rats on chow during light and dark phase, (L) average RER values of juvenile rats on Western diet during light and dark phase, (M) average ambulatory activity of juvenile rats on Western diet during light and dark phase, (N) body weight of juvenile rats after calorimetry. Shaded areas indicate dark phase. Activity data represent average beam breaks measured during 20-minute periods. Data tested with two-way ANOVA repeated measures (A and C),
two-way ANOVA with Sidak post-test (B,D,G,H and J-M (two comparisons made)),
two-tailed t-test (I and N) and ANCOVA (E-G). N=12 per genotype (A-H) and N=8
per genotype (I-M). *p<0.05, **p<0.01, ***p<0.001. Data are means ± SEM.

Figure 3. Sensitivity of OP and OR rats to a single dose of ghrelin (2 mg/kg s.c.).
Ghrelin administered just before the onset of the dark phase and data measured 40 and
120 minutes after administration. (A) Cumulative food intake (40 minutes: treatment
main effect p=0.0062), (B) energy expenditure (40 minutes: treatment main effect
p<0.0001), (C) ambulatory activity represented as average number of beam breaks
during 20 minute intervals (40 minutes: treatment main effect p=0.0011), and (D)
average RER values (40 minutes: treatment main effect p<0.0001, genotype main
effect p=0.018, 120 minutes: treatment main effect p<0.0001, genotype main effect
p=0.0018). Each time point tested with two-way ANOVA with Sidak post-test (two
comparisons made) except (B) where two-way ANCOVA was used. N=12 per
genotype. **p<0.01,***p<0.001 ****p<0.0001. Data are means ± SEM.

Figure 4. mRNA and protein levels of genes involved in fatty acid synthesis and
metabolism in epididymal fat, subcutaneous fat and skeletal muscle and
hypothalamic appetite regulation. (A) mRNA levels in epididymal fat of adipogenic
genesis (left), fat synthesis genes (middle) and fat breakdown genes (right), (B) protein
levels of ACC and pACC in epididymal fat (pictured western blot lanes represent
ACC (top), pACC (middle) and β-Actin (bottom), all proteins determined on the same
blot), (C) CPT1b mRNA levels in epididymal fat of DIO mice, (D) CPT1b mRNA
levels in epididymal fat of DIO rats, (E) mRNA levels in subcutaneous fat of
adipogenic genes (left), fat synthesis genes (middle) and fat breakdown genes (right),
(F) mRNA levels in Soleus, (G) mRNA levels in EDL, and (H) mRNA levels in hypothalamus. N=12 per genotype for OP and OR rats, N=14-15 for DIO mice and N=7 for DIO rats. *p<0.05. Data tested with two-tailed t-test. Data are means ± SEM.
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<td>Acacb</td>
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<td>CCGGCTCAAAGGTGGCGTAA</td>
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<tr>
<td>Agrp</td>
<td>GCTGCAAGAGCAGAAGC</td>
<td>GACTCGTGCAGCCTACACA</td>
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<td>Cnr1</td>
<td>TGCTTGCGATCATGGTGTAT</td>
<td>TGCTCAGCTCGTCCTCTC</td>
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<tr>
<td>Cpt1b (rat)</td>
<td>GCCTGTCGATCCACAGCCCT</td>
<td>AGTCCACAGCGCTTTGTCGCG</td>
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<td>Cpt1b (mouse)</td>
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<td>CGGGGCCTAGAGGACAG</td>
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<td>ACTCCACAGTGGAACAAAG</td>
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<tr>
<td>Ghsr</td>
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<td>AAAGGACACCAGGTTGCAGT</td>
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<tr>
<td>Lipe</td>
<td>CGCTCCCTGACAGCTCTCTC</td>
<td>CCAGCAAACTGGGTCTAT</td>
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<tr>
<td>Mc4r</td>
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<td>TCAGGGAAGCCCCAGTGGT</td>
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<tr>
<td>Npy</td>
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<td>TCTCAGGGCTTGATCTCTTGCA</td>
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<tr>
<td>Npy1r</td>
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<td>CGTTGATCCCTGGTGCTCA</td>
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<td>GCCAAGTTGTGAAATGCGC</td>
</tr>
<tr>
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<td>GCAGAGGAGCCACAGCAGG</td>
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<td>CCAGACATTGAGAAGGACCTG</td>
<td>CAGCAACCATGGGTCAG</td>
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<td>Ppargc1a</td>
<td>CCGAGAATTCATGGAACAT</td>
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<td>Scd1</td>
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<td>ACACCACATCCCTCAAATGTCAG</td>
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<tr>
<td>Sirt1</td>
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<td>TGGATTTCTCTGAAAGTGAACCA</td>
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<td>Tbp</td>
<td>TCAACACCCAGAAATTGTTCCTC</td>
<td>GGTAGATGGTTTCAATGCTTCA</td>
</tr>
<tr>
<td>Ucp2</td>
<td>CCTCAAGACCATGTGGCAGA</td>
<td>TGTCATGAGGGTGCTTTCA</td>
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</table>
Table 2. RER, Energy expenditure, activity, and food intake during 24 hours fasting followed by 24 hours re-feeding in obesity-prone and obesity resistant rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dark phase</th>
<th>Light phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P-value</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>OR</td>
</tr>
<tr>
<td>RER</td>
<td>0.766±0.003</td>
<td>0.760±0.002</td>
</tr>
<tr>
<td>Fasting 3 h</td>
<td>0.830±0.009</td>
<td>0.809±0.005</td>
</tr>
<tr>
<td>Energy expenditure (Kcal/h)</td>
<td>3.12±0.06</td>
<td>3.20±0.06</td>
</tr>
<tr>
<td>Activity (beam breaks)</td>
<td>225±13</td>
<td>245±17</td>
</tr>
<tr>
<td>Re-feeding 3 h</td>
<td>467±29</td>
<td>482±43</td>
</tr>
<tr>
<td>RER</td>
<td>0.887±0.003</td>
<td>0.886±0.003</td>
</tr>
<tr>
<td>Energy expenditure (Kcal/h)</td>
<td>3.47±0.06</td>
<td>3.64±0.06</td>
</tr>
<tr>
<td>Activity (beam breaks)</td>
<td>296±31</td>
<td>354±22</td>
</tr>
<tr>
<td>Cumulative food intake (g)</td>
<td>17.9±0.9</td>
<td>19.2±0.7</td>
</tr>
</tbody>
</table>

Energy expenditure represents average values adjusted for lean mass using ANCOVA. All other data tested with two-tailed t-test. N= 12 per genotype. Data are means ± SEM. RER: respiratory exchange ratio, OP: obesity-prone, OR: obesity-resistant.
Table 3. Plasma levels of leptin and insulin in obesity-prone and obesity-resistant rats.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>OP</th>
<th>OR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin (ng/ml)</td>
<td>19.6 ± 1.7</td>
<td>15.5 ± 0.9</td>
<td>0.047</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>4.4 ± 0.4</td>
<td>3.2 ± 0.4</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Data tested with two-tailed t-test. N= 12 per genotype. Data are means ± SEM. OP: obesity-prone, OR: obesity-resistant.