

Long-term characterization of the diet-induced obese and diet-resistant rat model: a polygenetic rat model mimicking the human obesity syndrome

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Abstract

The availability of useful animal models reflecting the human obesity syndrome is crucial in the search for novel compounds for the pharmacological treatment of obesity. In the current study, we have performed an extensive characterization of the obesity syndrome in a polygenetic animal model, namely the selectively bred diet-induced obese (DIO) and diet-resistant (DR) rat strains. We show that they constitute useful models of the human obesity syndrome. DIO and DR rats were fed either a high-energy (HE) or a standard chow (Chow) diet from weaning to 9 months of age. Metabolic characterization including blood biochemistry and glucose homeostasis was examined at 2, 3, 6, and 9 months of age. Furthermore, in 6-month-old HE-fed DIO rats, the anti-obesity effects of liraglutide and sibutramine were examined in a 28-day study. Only HE-fed

DIO rats developed visceral obesity, hyperleptinemia, hyperinsulinemia, and dyslipidemia, and showed a worsening of glucose tolerance over time. In line with the hyperlipidemic profile, a severe hepatic fat infiltration was observed in DIO rats at 6 months of age. The effects of liraglutide and sibutramine were tested in 6-month-old DIO rats. Both compounds effectively reduced food intake and body weight in DIO rats. Liraglutide furthermore improved glucose tolerance when compared with sibutramine. Our data highlights the usefulness of a polygenetic animal model for screening of compounds affecting food intake, body weight, and glucose homeostasis. Furthermore, the results underscore the effectiveness of GLP-1 mimetics both as anti-diabetes and anti-obesity agents.

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Introduction

The global epidemic of obesity is rapidly evolving as one of the major global health issues as it is frequently associated with a number of diseases with high mortality and morbidity such as diabetes, cancer, arthritis, hypertension, stroke, and myocardial infarction (Reaven 1988, Anderson *et al.* 2001, Haslam & James 2005). It is generally accepted that the tremendous rise in the obesity prevalence across the globe is driven primarily by a combination of increased calorie intake and decreased physical activity (Bandini *et al.* 1999), and strongly influenced by our genetic background (Mutch & Clement 2006).

Although several single gene mutations causing obesity in humans have been identified (e.g. the proopiomelanocortin gene (Leibel *et al.* 1997), the melanin-concentrating hormone

receptor 1 (Hebebrand *et al.* 2003), leptin (Montague *et al.* 1997), and the leptin receptor (Clement *et al.* 1998)), the occurrence of single gene mutations is rare, and it is generally believed that the individual sensitivity to the modern obesogenic environment is determined by a complex polygenetic background (for review, see Bell *et al.* (2005) and Ridderstrale & Groop (2009)). Therefore, to investigate the pathophysiology of the human obesity syndrome, and in the search of novel anti-obesity agents, polygenetic animal models closely mimicking the human obesity syndrome are crucial.

One such polygenetic model is the diet-induced obese (DIO) and diet-resistant (DR) out-bred rat models (Levin *et al.* 1997). While some short-term studies have been performed to characterize the development of obesity and to address the presence of some of the associated metabolic

disturbances (Levin *et al.* 1997, Levin & Govek 1998, Ricci & Levin 2003, Tkacs & Levin 2004, Gorski *et al.* 2006), a thorough and long-term metabolic and pharmacological characterization of these models has not previously been performed. Thus, the aim of the present study was to track the development of obesity and metabolic alterations in the DIO and DR rats fed either a high-fat diet or normal chow diet for 9 months. In addition, we examined the body weight reduction and metabolic effects after administration of sibutramine and the novel human GLP-1 analog liraglutide for 28 days.

Materials and Methods

Animals

All experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals, and were approved by the Danish committee for animal research. Studies were carried out in male DIO and DR rats (Rheoscience breeding colony; Rheoscience, Rødovre, Denmark). In 2001, Rheoscience A/S received breeding pairs of selectively bred DIO rats and DR rats originated from a Sprague–Dawley background from Prof. Barry Levin's breeding facility (Newark, NJ, USA). At Rheoscience A/S, the breeding of DIO rats and DR rats was continued with the main focus on lowering the genetic drift of the two strains. This was done by minimizing the replacement of breeding animals, and no first-degree or second-degree relatives were mated. Replacement of breeding pairs, mainly breeding mothers, has been kept to a minimum.

All animals were kept on a 12 h light:12 h darkness cycle (lights on at 0600 h) in a temperature-controlled environment (22–24 °C) with free access to food and water unless otherwise stated.

Metabolic characterization study of the DIO and DR rats

Feeding paradigm After weaning, 48 DIO and 48 DR male rats were housed individually, and stratified according to body weight into two groups, and were offered *ad libitum* water and either normal chow diet (chow; 2.85 kcal/g – energy%: carbohydrate 60.7%, fat 12.6%, and protein 26.7%; diet #1324 Altromin, Brogård, Denmark) or high-fat diet (high-energy (HE); 4.41 kcal/g – energy%: carbohydrate 51.4%, fat 31.8%, and protein 16.8%; diet #12266B; Research Diets, NJ, USA).

Oral glucose tolerance test

The oral glucose tolerance tests (OGTTs) were carried out at 2, 3, 6, and 9 months of age. Each OGTT started at 0800 h, and was performed on eight DIO and eight DR rats that were offered HE diet, and eight DIO and eight DR rats that were offered chow diet. The OGTT was performed in semi-fasted rats, and hence, on the day before the test, animals were

offered 50% of their daily energy requirements (average of the previous week). Blood samples (100 µl/time point) were taken from the tip of the tail at time points –15, 0, 15, 30, 60, 90, 120, 180, and 240 min after oral administration of 2 g/kg glucose (using 500 mg glucose/ml distilled H₂O). To minimize the blood loss, the OGTT performed in 2-month-old rats was terminated after 180 min. The oral glucose load was given as gavage via a gastric tube connected to a syringe to ensure accurate dosing. Plasma glucose was measured at all time points; baseline triacylglycerol (TAG) and total cholesterol were measured at time point –15 min (DT60II model, Orthoclinical Diagnostics, Johnson and Johnson, Nordic AB, Sollentuna, Sweden). Plasma insulin was measured in duplicate at all time points using a commercially available insulin ELISA assay (Diamyd Diagnostics, Stockholm, Sweden).

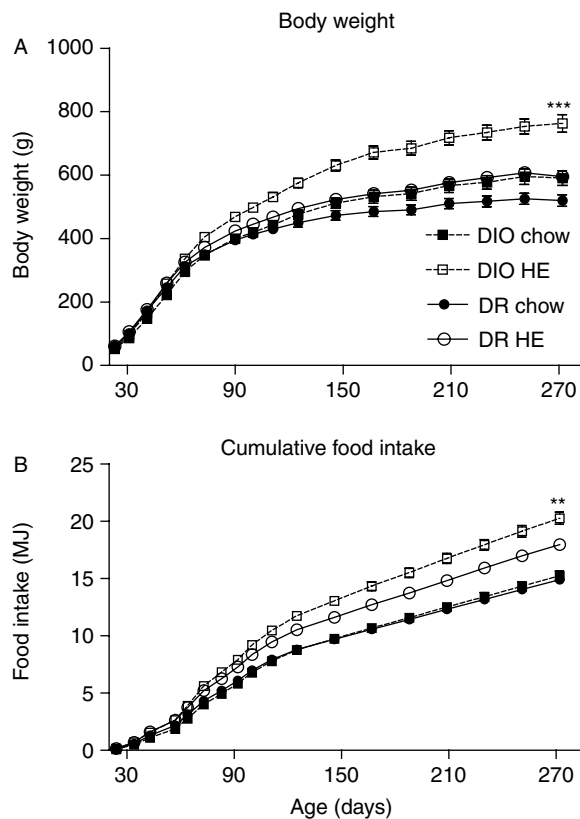


Figure 1 (A) Body weight and (B) cumulative food intake measured weekly for DIO and DR rats on normal chow diet (DIO chow and DR chow) or high-fat diet (DIO HE and DR HE) for the characterization period of 9 months. Dashed lines and squares represent DIO rats, and lines and circles represent DR rats. Open symbols (squares and circles) represent HE-fed diets, whereas closed symbols represent chow-fed diets. For body weight and food intake, there was an interaction between genotype, diet, and time (BW, genotype \times diet \times time: $F=23.9$, $P<0.0001$; food intake: genotype \times diet \times time: $F=13.5$, $P<0.001$). Asterisks indicate significant differences (** $P<0.001$ and *** $P<0.0001$). Data are means \pm S.E.M. of $n=10$.

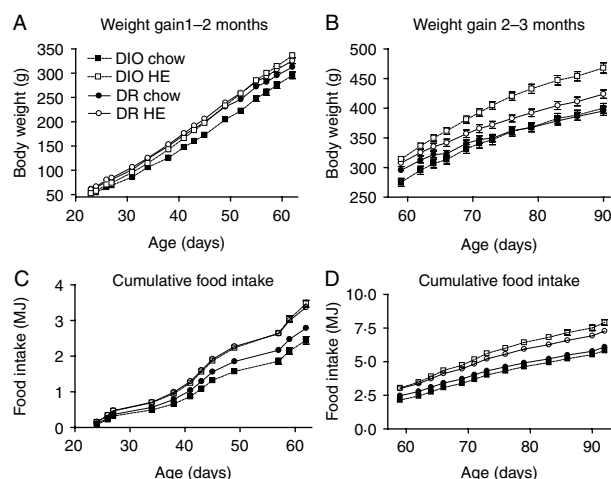


Figure 2 (A) Body weight at 1–2 months of age, (B) body weight at 2–3 months of age, (C) cumulative food intake at 1–2 months of age, and (D) cumulative food intake at 2–3 months of age for DIO and DR rats on HE diet or chow diet. Symbols are same as in Fig. 1 (Altromin standard # 1324, Energy: 11.90 kJ/g). Data are means \pm S.E.M. of $n=8$.

Circadian rhythm hormone profile

Measurements of the circadian rhythms of leptin and insulin were performed in rats aged 3, 6, and 9 months (eight DIO and eight DR rats on HE diet, and eight DIO and eight DR rats on chow diet). Blood samples were collected every 4th hour for 20 h starting at 0800 h. Rats had *ad libitum* access to food during the entire sampling period. Blood samples (100 μ l/time point) were drawn from the tip of the tail. The samples were collected in EDTA tubes for measurements of insulin and leptin. Insulin and leptin were analyzed simultaneously, and were measured in duplicate at all time points using a commercially available rat endocrine Linco-plex kit (Rendo-85K, Linco Research, St Charles, MO, USA) and a Luminex 100 reader (Luminex 100 analyzer, Austin, TX, USA).

Table 1 Fat pads weighed at 3 and 6 months of age. The weight (gram) of the epididymal (E), inguinal (I), mesenteric (M), and retroperitoneal (R) is shown. All measures were compared by two-way ANOVA for main effect of diet and genotype as well as a diet by genotype interaction (F value is shown in Table). Data are expressed as mean \pm S.E.M. Individual groups are compared using one-way ANOVA followed by Fisher's *post-hoc* analysis

	DIO chow (a)	DIO HE (b)	DR chow (c)	DR HE (d)	Geno	Diet	Geno \times diet
Fat depot							
E 3	4.7 \pm 0.3 ^{b†}	12.9 \pm 1.0	5.3 \pm 0.7 ^{d†}	7.3 \pm 0.7	$F=12^\dagger$	$F=51^\ddagger$	$F=18^\ddagger$
E 6	9.9 \pm 0.9 ^{b*,c*}	18.2 \pm 0.8 ^{d†}	6.3 \pm 0.6 ^{d†}	12.4 \pm 1.5	$F=22^\ddagger$	$F=51^\ddagger$	$F=1$
I 3	1.7 \pm 0.2 ^{b†}	4.4 \pm 0.6 ^{d†}	1.3 \pm 0.1	1.5 \pm 0.2	$F=31^\ddagger$	$F=24^\ddagger$	$F=16^\ddagger$
I 6	3.6 \pm 0.5 ^{b,c†}	6.0 \pm 0.7 ^{d†}	1.2 \pm 0.2	2.4 \pm 0.2	$F=47^\ddagger$	$F=17^\ddagger$	$F=2$
M 3	6.4 \pm 0.3 ^{b*,c*}	13.0 \pm 0.8 ^{d†}	4.2 \pm 0.3 ^{d*}	5.5 \pm 0.3	$F=112^\ddagger$	$F=74^\ddagger$	$F=33^\ddagger$
M 6	10.3 \pm 0.9 ^{b*,c*}	19.0 \pm 0.9 ^{d†}	3.6 \pm 0.2 ^{d*}	6.1 \pm 0.7	$F=177^\ddagger$	$F=58^\ddagger$	$F=18^\ddagger$
R 3	3.3 \pm 0.3 ^{b†}	8.4 \pm 0.3 ^{d†}	2.7 \pm 0.4 ^{d†}	4.2 \pm 0.5	$F=40^\ddagger$	$F=76^\ddagger$	$F=21^\ddagger$
R 6	7.8 \pm 0.4 ^{b*,c†}	16.6 \pm 0.4 ^{d†}	3.3 \pm 0.4 ^{d†}	7.5 \pm 0.9	$F=104^\ddagger$	$F=96^\ddagger$	$F=11^\ddagger$

* $P<0.05$, $^\dagger P<0.01$, and $^\ddagger P<0.001$. Data with different letters differ from each other by $P>0.5$. Geno, genotype; sum of fat = $2 \times (I+R+E)+M$.

Termination

Rats were killed by CO_2/O_2 anesthesia and decapitation. Thereafter, from 3- and 6-month-old animals, inguinal (I), retroperitoneal (R), epididymal (E), and mesenteric (M) fat depots were removed and weighed. In addition, from 6-month-old rats, a piece of the right liver lobe was removed from three representative animals/group (with respect to BW). The liver was frozen on dry ice and stored at -80°C for subsequent oil red O staining to determine the degree of fat infiltration.

Oil red O staining Liver sections were cut into Superfrost Plus slides on a freezing microtome (12 μm thick sections). The sections were allowed to air dry for 30 min, and then fixed in ice-cold 10% formalin for 5 min, rinsed three times in distilled water, and placed in absolute propylene glycol for 5 min. Sections were next stained at 60°C for 8 min in an oil red O solution, rinsed in 85% propylene glycol for 5 min, and subsequently rinsed in distilled water. Counterstaining was performed in Gill's hematoxylin solution. The sections were mounted with an aqueous mounting medium and examined using a Nikon E1000M microscope fitted with a DT1200 digital camera. Images were adjusted for brightness and contrast in Adobe Photoshop.

28-day chronic study in HE-fed DIO rats

A total of 30 DIO animals were included in the study. The DIO rats were fed HE diet from the time of weaning and included in the study at the age of 19 weeks. The animals were stratified according to body weight into three groups of $n=10$: group 1, vehicle A + vehicle B; group 2, vehicle A + liraglutide; group 3, sibutramine + vehicle B. Vehicle A (Natrosol (0.5% w/v)) or sibutramine dissolved in vehicle A (5 mg/kg) was administered per orally (PO) once daily (QD) for 28 days in the morning between 0700 and 0800 h. Vehicle B (PBS) or liraglutide dissolved in vehicle B (200 $\mu\text{g}/\text{kg}$) was administered s.c. twice daily (BID) for 28 days in the morning between 0700 and 0800 h, and in the afternoon between 0300 and 0400 h.

The experiment was preceded by a 3-day run-in period with mock gavages. From day 0 and onwards, food and water intakes were measured daily, while body weight was measured biweekly. At the day of termination (day 28), the rats were subjected to an OGTT (performed as described above except for time points: -30, 0, 15, 30, 60, 90, 120 and 180 min). The animals were killed by CO₂/O₂ anesthesia and cervical dislocation. White adipose tissue compartments were removed and weighed (inguinal, retroperitoneal, epididymal, and mesenteric), and plasma levels of glucose, insulin, cholesterol, and TAG were measured as described above.

Statistical analysis

Results from body weight and food intake measurements for DIO and DR rats on either chow or HE diet were compared by two-way ANOVA for repeated measurements (genotype × diet (HE and chow) × time). Results from fat pads weight, plasma lipids, and hormone measurements were compared using two-way ANOVA (genotype and diet). Pairwise comparison of groups was performed using one-way ANOVA followed by Fisher's *post-hoc* analysis. Not all groups were compared with each other. For example, chow-fed DIO rats were compared with chow-fed DR rats and HE-fed DIO rats, but not to HE-fed DR rats (differential genotype and diet). Data from the test of anorectic compounds were compared with the vehicle group using one-way ANOVA followed by Fisher's *post-hoc* analysis. Values (hormone concentrations, arbitrary units, and body weight) are expressed as means ± s.e.m. $P < 0.05$ was considered statistically significant.

Results

DIO rats prone to obesity on an HE diet

Figure 1 shows body weight and cumulative food intake in the DIO and DR rats fed either chow or HE diet from weaning to 9 months of age. Figure 2 depicts the interesting first 3 months after weaning in more details: the DR rats were heavier than the DIO rats at weaning. However, when HE-fed DIO rats gained weight more rapidly than the corresponding group of HE-fed DR rats, and at 2 months of age, their body weight had surpassed that of the HE-fed DR rats (Fig. 2B). The increases in body weight did not differ between groups during the first month after weaning. Then, during the second and third months after weaning, the weight gain was higher in HE-fed DIO rats compared to all other groups (Fig. 2B).

While the gravimetric intake of diet did not differ between HE-fed and chow-fed groups (data not shown), as seen in Fig. 2, energy intake was higher in both DIO and DR rats on HE diet during the first 2 months following weaning (Fig. 2C and D), but despite a nearly similar energy intake, weight gain in HE-fed DR rats was much slower than in HE-fed DIO rats (Fig. 2B).

Body composition analysis, liver histology, and plasma lipids

HE-fed DIO rats had significantly larger fat stores compared with all other groups both at 3 and 6 months of age (Table 1). Especially, the mesenteric fat compartment (the visceral) was expanded, being three times larger in DIO rats on HE diet compared to DR rats on HE diet (Table 1 and Fig. 3E). In line with the vastly expanded visceral fat depot – and hence presumably increased hepatoportal lipid flux – the HE-fed DIO rats displayed a high degree of fat infiltration in the liver at 6 months of age (Fig. 3B). Histological examination using oil red O staining revealed large droplets of fat in hepatocytes

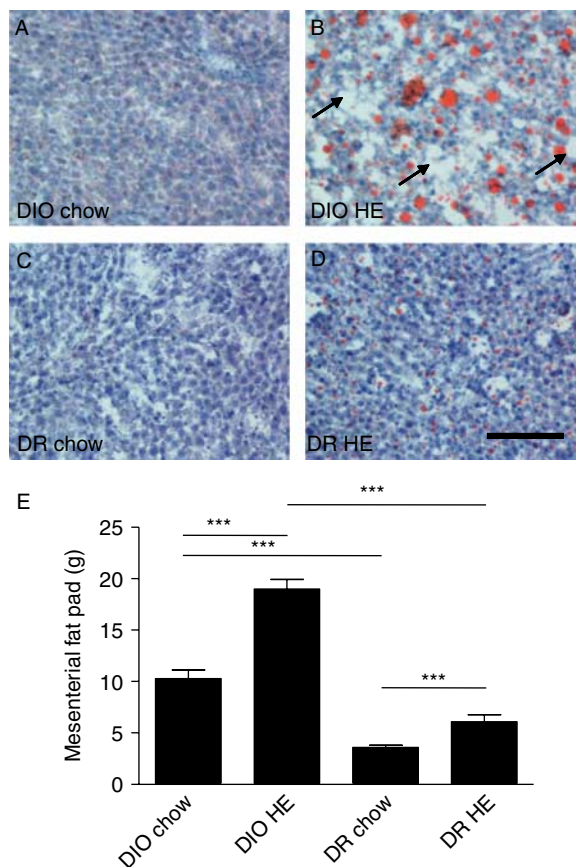


Figure 3 Microphotographs of oil red O-stained liver tissue from 6-month-old (A) DIO chow-fed, (B) DIO HE-fed, (C) DR chow-fed, and (D) DR HE-fed rats. Only HE-fed DIO rats display a marked fat infiltration (red color). The fat droplets in liver of the HE-fed DIO rats are large, and have been displaced (due to their size) during the staining procedure leaving as seen by the empty holes in the hepatocytes (arrows). Scale bar = 100 μ m. (E) The degree of visceral obesity in the HE-fed DIO rat at 6 months of age. There was a main effect of diet and genotype as well as a diet by genotype interaction (diet: $F=57.56$, $P<0.0001$; genotype: $F=176.93$, $P<0.0001$; genotype × diet: $F=17.94$, $P=0.0002$). Data are expressed as mean ± s.e.m. Individual groups are compared using one-way ANOVA followed by Fisher's *post-hoc* analysis. $P < 0.05$ is considered significant. Asterisks indicate significant differences (** $P < 0.0001$). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0004>.

in HE-fed DIO rats. While fat droplets were virtually absent in livers from chow-fed animals of either genotype (Fig. 3A and C), small fat droplets were observed in HE-fed DR rats (Fig. 3D).

Cholesterol plasma levels were significantly higher for the DR rats at weaning compared to the DIO rats (Table 2). Similar to that observed for body weight, this relationship reversed, and at both 6 and 9 months of age, the HE-fed DIO rats had a significantly higher cholesterol level compared to all other groups (Table 2). Likewise, the plasma TAG levels increased gradually throughout the experiment, and were significantly higher in HE-fed DIO rats at 3 and 6 months of age compared to the other groups (Table 2).

Oral glucose tolerance test

All animals developed and exhibited an impaired glucose tolerance up to 6 months of age. At 2, 3, and 6 months of age, HE-fed DIO rats had significantly impaired glucose tolerance compared with all other groups (Fig. 4 and Table 3). Plasma insulin levels were measured throughout the OGTT, and the glucose-induced insulin secretion curves are shown in Fig. 4. The HE-fed DIO rats had higher peak insulin levels than all other groups at 3 and 6 months of age. At 9 months of age, the insulin secretion peak for the HE-fed DIO rats deteriorated along with a worsened glucose tolerance (Fig. 4) as evidenced by increased area under the curve (AUC) of both the glucose and insulin curves following oral gavage with glucose (Table 3). Although glucose intolerance clearly deteriorated with age, HE-fed DIO rats never developed frank diabetes. No differences were found in HbA1c levels between the groups at 6 and 9 months of age (data not shown).

Circadian blood hormone profile

Non-fasting HE-fed DIO rats had elevated basal levels of leptin and insulin throughout the 24-h period of measurement at 3, 6, and 9 months of age (Fig. 5). However,

chow-fed DIO rats almost reached the same insulin secretion levels as HE-fed DIO rats at 6 months of age. The elevated leptin levels correspond well to the degree of obesity at the time points for the hormone profile. The elevated insulin secretion observed for HE-fed DIO rats was in accordance with the elevated fasting insulin levels in the HE-fed DIO rats already observed at 2 months of age.

Effects of 28 days of anorectic treatment in HE-fed DIO rats

As seen in Fig. 6A, both sibutramine and liraglutide administration gave rise to a significant drop in body weight (expressed as % of day 0). During the dosing study, food and water intake was measured daily. Significance was tested on days 14 and 28. Both liraglutide and sibutramine reduced food intake especially over the first 7 days of the study. However, daily food intake was also significantly lower at days 14 and 28 in liraglutide-treated rats ($P < 0.01$ on both days 14 and 28, Fig. 6B). Cumulative food intake was significantly lower at day 14 as well as at day 28 ($P < 0.001$; Fig. 6C and Table 4). There was no difference in cumulated water intake (data not shown). The OGTT performed after 28 days of treatment showed that liraglutide-treated animals exclusively had a significantly lower glucose AUC compared to vehicle ($P < 0.05$; Fig. 7A and Table 4). For insulin, the AUC was reduced following all treatments compared to vehicle (Fig. 7B and Table 4). On the final day of the experiment, plasma levels of glucose, insulin, and TAG were measured in mildly fasted animals. The plasma glucose, plasma insulin, and HbA1c levels were similar in all groups (data not shown). Compared with vehicle-treated animals, plasma levels of TAG were significantly reduced in both treatment groups (Table 4). Analysis of the fat depots at termination showed a reduction in the subcutaneous inguinal, the retroperitoneal, the epididymal, and the mesenteric fat depot in animals treated with liraglutide and sibutramine compared with vehicle-treated animals (Table 4).

Table 2 Blood lipids in fasted animals. The plasma levels of lipids, triacylglycerol from 1 to 9 months of age (TAG 1–9), and cholesterol from 1 to 9 months of age (Chol 1–9) are shown. All measures were compared by two-way ANOVA for main effect of diet and genotype as well as a diet by genotype interaction (F value is shown in Table). Data are expressed as mean \pm s.e.m. Individual groups were compared using one-way ANOVA followed by Fisher's *post-hoc* analysis

	DIO chow (a)	DIO HE (b)	DR chow (c)	DR HE (d)	Geno	Diet	Geno \times diet
Lipids (mmol/l)							
TAG 1	1.1 \pm 0.2 ^{bt}	2.8 \pm 0.3	0.8 \pm 0.7 ^{dt}	0.6 \pm 0.1	$F = 43^{\ddagger}$	$F = 16^{\ddagger}$	$F = 23^{\ddagger}$
TAG 2	0.9 \pm 0.1 ^{bt,c*}	1.1 \pm 0.1 ^{dt}	0.6 \pm 0.0 ^{dt}	0.7 \pm 0.1	$F = 19^{\ddagger}$	$F = 6^*$	$F = 1$
TAG 3	1.3 \pm 0.1 ^{bt}	2.2 \pm 0.2 ^{dt}	0.9 \pm 0.1	1.2 \pm 0.2	$F = 17^{\ddagger}$	$F = 15^{\ddagger}$	$F = 2$
TAG 6	1.5 \pm 0.2 ^{bt,ct}	2.8 \pm 0.2 ^{dt}	0.8 \pm 0.1	0.9 \pm 0.1	$F = 87^{\ddagger}$	$F = 25^{\ddagger}$	$F = 21^{\ddagger}$
TAG 9	2.4 \pm 0.3 ^{bt,c*}	3.0 \pm 0.3 ^{dt}	1.0 \pm 0.1 ^{d*}	1.9 \pm 0.3	$F = 20^{\ddagger}$	$F = 6^*$	$F = 0.4$
Chol 1	1.7 \pm 0.1 ^{bt,ct}	1.9 \pm 0.1 ^{dt}	1.9 \pm 0.1 ^{d*}	1.7 \pm 0.1	$F = 11^{\ddagger}$	$F = 1$	$F = 0.4$
Chol 2	1.3 \pm 0.1 ^{bt}	1.4 \pm 0.1 ^{dt}	1.4 \pm 0.1 ^{dt}	1.5 \pm 0.1	$F = 4$	$F = 3$	$F = 0$
Chol 3	1.3 \pm 0.1 ^{bt,ct}	1.8 \pm 0.1 ^{dt}	1.4 \pm 0.1 ^{dt}	1.7 \pm 0.1	$F = 0.1$	$F = 38$	$F = 0.4$
Chol 6	1.3 \pm 0.1 ^{bt}	2.6 \pm 0.2 ^{dt}	1.3 \pm 0.1 ^{d*}	1.6 \pm 0.1	$F = 22^{\ddagger}$	$F = 60^{\ddagger}$	$F = 25^{\ddagger}$
Chol 9	1.7 \pm 0.1 ^{bt,ct}	4.6 \pm 0.4 ^{dt}	1.5 \pm 0.1 ^{dt}	2.6 \pm 0.1	$F = 23^{\ddagger}$	$F = 79^{\ddagger}$	$F = 15^{\ddagger}$

* $P < 0.05$, $^{\ddagger}P < 0.01$, and $^{\ddagger\ddagger}P < 0.001$. Data with differing letters differ from each other by $P < 0.05$. Geno, genotype.

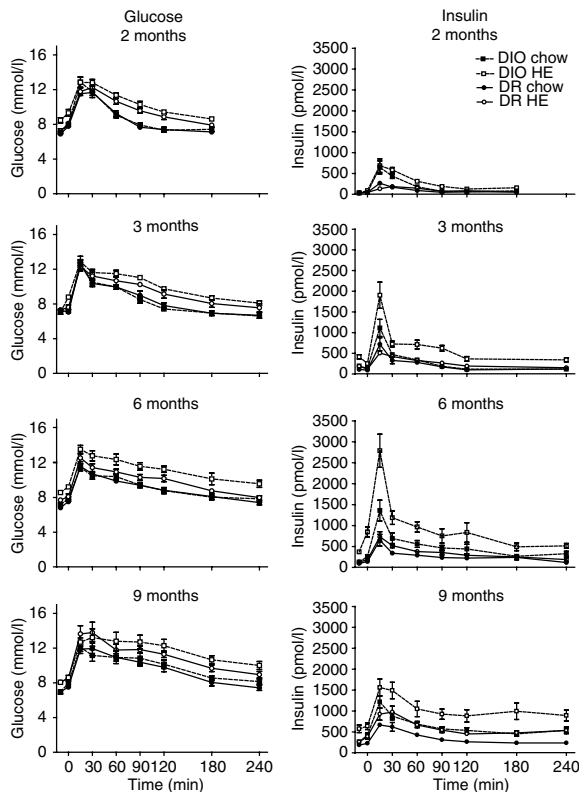


Figure 4 Blood plasma glucose and insulin levels during OGTTs after weaning, and at 2, 3, 6, and 9 months of age for DIO and DR rats on either HE diet or chow diet. Animals were semi-fasted 24 h before the OGTT (2 g/kg). Symbols are same as in Fig. 1. Data are means \pm S.E.M. of $n=8$.

Discussion

Human obesity is often a lifelong phenomenon that may originate from childhood obesity (Birch & Ventura 2009, Sinha & Kling 2009), and therefore it is important to describe and understand long-term metabolic characteristics, as the

obesity syndrome develops and progresses. The polygenetic and inheritable background of the DIO trait as well as the homogeneous development of obesity in the selectively bred DIO rats on HE diet (Levin *et al.* 1997, 2005) makes it an appropriate animal model for the study of human obesity (Bouchard & Perusse 1993, Bell *et al.* 2005), and here we present the effects after 9 months of either a high-fat or normal chow challenge on DIO prone or DR rat models. Furthermore, these DIO rats develop obesity when fed a diet with 31% energy from fat, a diet very similar in terms of fat content of the human diet in most developed countries.

As previously described (Levin *et al.* 1997, 2003, 2004, Ricci & Levin 2003), we observed that the DIO rats were profoundly affected by the HE diet, whereas the DR rats on HE diet were capable of controlling and defending their body weight to a much higher degree than the DIO rats. Thus, the HE-fed DIO rats developed pronounced obesity and obesity-associated hyperleptinemia. In fact, following 3 months of HE diet, we observed much higher body weight in DIO rats versus DR rats, an increase that can be primarily ascribed to a massive increase of fat mass (Table 1). Interestingly, a relatively high proportion of the fat accumulated as mesenteric (or central) fat, which has been described as a hallmark of the metabolic syndrome often associated with human obesity (Despres & Lemieux 2006). This feature of the HE-fed DIO rat makes the model relevant for the study of factors or compounds that affect fat distribution, such as the PPAR γ agonists (Larsen *et al.* 2003). The HE-fed DIO rats compared with the control groups developed dyslipidemia in the form of elevated plasma TAG and cholesterol levels, and do not spontaneously develop frank diabetes but further develop hyperinsulinemia and glucose intolerance.

Another observation from the present study pointing to a role of the visceral fat in the development of insulin resistance in the HE-fed DIO rats is the pronounced intracellular fat infiltration observed in the hepatocytes at 6 months of age. Liver exposure to high levels of free fatty acids (delivered from the visceral fat via the portal vein) is known to affect liver

Table 3 AUC values for oral glucose tolerance testing in DIO-DR rats. Data are mean \pm S.E.M.

	DIO chow	DIO HE	DR chow	DR HE
AUC glucose OGTT				
2 months	1656 ^b \pm 13	1973 ^a \pm 11	1621 ^c \pm 6	1832 ^c \pm 12
3 months	2056 ^b \pm 14	2476 ^a \pm 9	2082 ^b \pm 21	2309 ^c \pm 26
6 months	2249 ^b \pm 19	2770 ^a \pm 34	2229 ^b \pm 13	2442 ^c \pm 13
9 months	2432 ^{b,c} \pm 33	2891 ^a \pm 52	2361 ^c \pm 31	2726 ^{a,b} \pm 37
AUC insulin OGTT				
2 months	33 078 ^b \pm 1163	50 673 ^a \pm 1223	17 430 ^c \pm 554	19 398 ^c \pm 693
3 months	61 316 ^b \pm 1922	137 879 ^a \pm 3908	48 143 ^b \pm 2647	60 583 ^b \pm 2013
6 months	115 174 ^b \pm 6239	217 229 ^a \pm 10 761	63 118 ^b \pm 2357	81 501 ^b \pm 2437
9 months	149 708 ^b \pm 4988	253 240 ^a \pm 12 560	83 047 ^c \pm 2879	143 348 ^{b,c} \pm 5259

Data with differing letters differ from each other by $P \leq 0.05$. One-way ANOVA, followed by Fisher's PLSD. AUC, area under the curve (glucose, mmol/l \times min; insulin, pmol/l \times min).

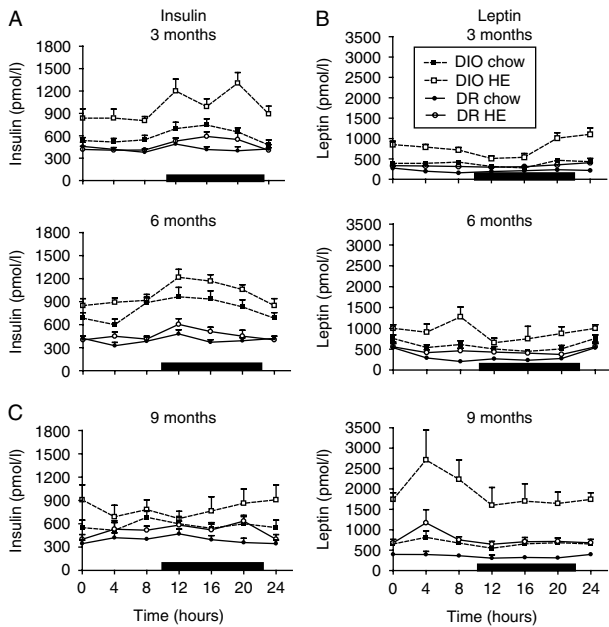


Figure 5 Circadian rhythm of insulin and leptin at 3, 6, and 9 months of age in free-feeding animals. Blood plasma samples were harvested every 4 h for a 24-h period. The 12-h dark period is illustrated with a black line. Symbols are same as in Fig. 1. Values are means \pm S.E.M. of $n=8$.

glucose metabolism and insulin resistance (Jensen 2008). Thus, taken together, the HE-fed DIO rats share a number of important characteristics with obese and pre-diabetic humans (Bell *et al.* 2005).

Elevated circadian rhythm of insulin and an elevated insulin response to a glucose challenge reflecting insulin resistance were evident in HE-fed DIO rats at 3, 6, and 9 months of age. Notably, this effect appeared to be genotype specific, as also the chow-fed DIO rats showed a deterioration in insulin sensitivity compared with the DR rats. Importantly, at 9 months of age, the insulin secretion peak for the HE-fed DIO rats deteriorated along with a worsened glucose tolerance. This development in the insulin response to a glucose load could be a consequence of a lowering effect in the secretory capacity of the insulin-producing β -cells in pancreas with age, and accordingly the circadian profile of plasma insulin was lower in 9- vs 6-month-old DIO rats, which is similar to the situation in humans as early states of the metabolic syndrome progress towards a diabetic situation (Karaca *et al.* 2009). It should be noted that we did not measure pancreatic function directly in the present study, and therefore cannot exclude that the observation should be ascribed to a difference in the level of insulin clearance. Also, in this study during the 9 months of observation period, we did not observe the development of frank diabetes in the HE-fed DIO rats as compared with the controls, i.e. there were no differences in glucose or HbA1c levels at 6 and 9 months of age. As a matter of fact, we have never observed frank diabetes in HE-fed DIO rats for up to 2 years (unpublished data). The absence of

diabetes in the HE-fed DIO rats is in accordance with previous reports of long-term fat feeding of Wistar rats (Chakley *et al.* 2002).

In this study, we observed an elevation in blood lipids in DIO rats compared to DR rats on HE diet at an age of 2 months, before the onset of insulin resistance. This observation could indicate that the hyperlipidemia associated

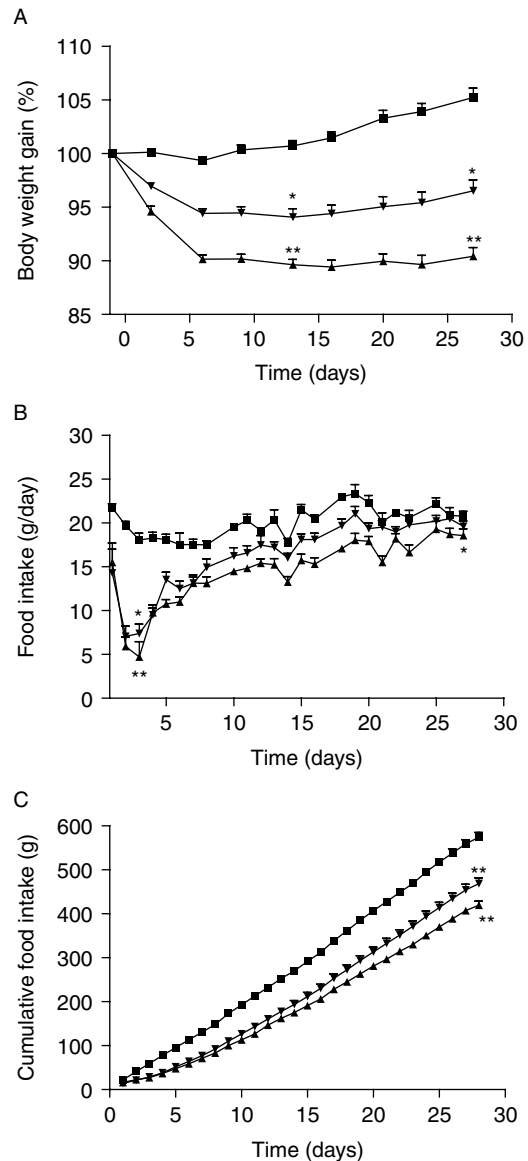


Figure 6 Body weight gain, food intake, and cumulative food intake for HE-fed DIO rats weighed biweekly from day -2 to day 28. The rats were dosed with liraglutide (200 μ g/kg), sibutramine (5 mg/kg), or vehicle. Squares represent vehicle, triangles represent sibutramine, and diamonds represent liraglutide. Statistics: liraglutide and sibutramine groups are compared with vehicle using one-way ANOVA followed by Fisher's *post-hoc* analysis. $P<0.05$ is considered significant. Asterisks indicate significant differences (* $P<0.001$ and ** $P<0.0001$). Data are means \pm S.E.M. of $n=10$.

Table 4 Effects of 28 days of anorectic treatment in high-energy-fed diet-induced obese rats. Body weight, plasma triacylglycerol (TAG), fat pads weight, and area under the curve for glucose and insulin are shown. All measures were compared by two-way ANOVA for main effect of diet and genotype as well as a diet by genotype interaction (*F* value is shown in Table). Data are expressed as mean \pm s.e.m. Individual groups are compared using one-way ANOVA followed by Fisher's *post-hoc* analysis

Parameter	Vehicle	Liraglutide	Sibutramine
BW day -1 (g)	559.1 \pm 13.6	562.1 \pm 11.7	563.7 \pm 11.7
BW day 14 (%)	100.7 \pm 0.5	89.6 \pm 0.1 [†]	94.1 \pm 0.1 [†]
BW day 28 (%)	105.2 \pm 0.9	90.4 \pm 0.8 [†]	96.5 \pm 1.0 [†]
TAG (mmol/l)	1.3 \pm 0.1	0.8 \pm 0.1 [†]	1.0 \pm 0.1 [†]
Mes. fat (g)	12.5 \pm 0.2	6.8 \pm 0.4 [†]	8.5 \pm 0.7 [†]
Peri. fat (g)	25.1 \pm 1.0	14.9 \pm 1.2 [†]	10.0 \pm 0.1 [†]
Ing. fat (g)	11.5 \pm 0.6	7.0 \pm 0.6 [†]	8.2 \pm 0.6 [†]
Epi. fat (g)	12.9 \pm 0.8	9.4 \pm 0.8 [†]	10.6 \pm 0.7 [†]
AUC glucose (10 ³ nmol/l \times min)	800.5 \pm 50.2	633.0 \pm 22.7*	750.2 \pm 70.7
AUC insulin (10 ³ nmol/l \times min)	94.11 \pm 6.4	63.15 \pm 4.52*	76.06 \pm 6.76*

Asterisks indicate significant differences (**P*<0.05, [†]*P*<0.01, and ^{††}*P*<0.001).

with high-fat feeding may be causative in the development of insulin resistance, possibly via increased fat accumulation in non-adipose tissue such as muscle and liver or reduced muscle fatty acid oxidation (Kraegen *et al.* 2001). Accordingly, following 6 months on either chow or HE diet, clearly hepatic level of TAG was elevated in HE-fed DIO rats as compared with controls (Fig. 3). The factors leading to this intra-organ accumulation are not clear, but it could derive from elevated circulating free fatty acids, basal or post-prandial TAG, or reduced muscle fatty acid oxidation (Kraegen *et al.* 2001).

In order to validate the HE-fed DIO rat as a useful animal model of obesity, we examined the body weight lowering effect of the well-known anti-obesity agent sibutramine and the once-daily human GLP-1 analog liraglutide. The findings of a transient (7 day) effect on food intake and a approximate 10% weight loss following 28 days of treatment with sibutramine is in agreement with data reported previously from other rodent DIO models (Boozar *et al.* 2001, Bush *et al.* 2006, Mashiko *et al.* 2008) as well as in a previous study in the DIO rat (Levin & Dunn-Meynell 2000). The body weight loss of \sim 10% seen in rodent models of obesity translates into an approximate 5% sustained weight loss in humans (Bray *et al.* 1999, James *et al.* 2000).

Interestingly, when compared with sibutramine, the once-daily human GLP-1 analog liraglutide caused a significantly greater weight loss in addition to additional beneficial effects on glucose homeostasis when administered to HE-fed DIO rats for 28 days. The anorectic and body weight lowering effects of liraglutide are in general agreement with previously published data from chronic animal studies, including in normal and obese monosodium glutamate-treated Wistar rats

(Larsen *et al.* 2001), in candy-fed obese Sprague-Dawley rats (Raun *et al.* 2007a), and in severely obese minipigs (Raun *et al.* 2007b). Liraglutide was recently approved by the EMEA for the treatment of type 2 diabetes, but in agreement with the rodent and pig studies, anti-obesity effects in humans have also been reported in the LEAD-3 and LEAD-4 studies (Garber *et al.* 2009, Zinman *et al.* 2009). Also, Astrup *et al.* (2009) just published data from a phase II study comparing the body weight lowering effects of liraglutide with that of orlistat. This study showed that liraglutide given to obese subjects subjected to an energy-deficit diet and exercise program led to a sustained and clinically relevant dose-dependent weight loss demonstrating the potential of liraglutide as a pharmacological treatment for the obese pre-diabetic patients (Astrup *et al.* 2009).

In conclusion, the current long-term metabolic characterization study has demonstrated that HE-fed DIO rats develop profound visceral and general obesity, insulin resistance, dyslipidemia, hepatic steatosis, and eventually impaired insulin secretion. Although the HE-fed DIO rat never

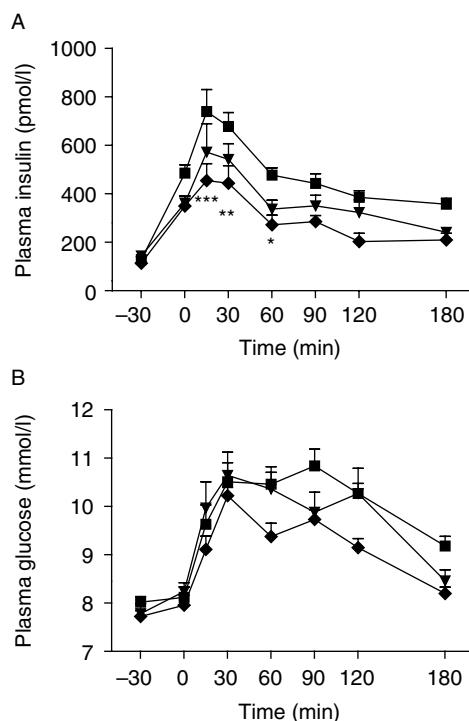


Figure 7 Blood plasma glucose and insulin levels during an OGTT for DIO rats on HE diet after 28 days of dosing with liraglutide (200 μ g/kg), sibutramine (5 mg/kg), or vehicle. Animals were semi-fasted 24 h before the OGTT and received 2 g/kg BW glucose. Squares represent vehicle, triangles represent sibutramine, and diamonds represent liraglutide. Statistics: liraglutide and sibutramine groups are compared to vehicle using two-way ANOVA followed by Fisher's *post-hoc* analysis. *P*<0.05 is considered significant. Asterisks indicate significant differences (**P*<0.05, ***P*<0.01, and ****P*<0.001). Data are means \pm s.e.m. of *n*=10.

develops frank diabetes, the 28-day chronic dosing study with sibutramine and the novel GLP-1 analog liraglutide demonstrates the versatility of the HE-fed DIO rat as a polygenetic model of human obesity and the associated metabolic alterations.

Declaration of interest

LBK is currently employed by Novo Nordisk A/S, the producer of liraglutide.

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