Glucose metabolism, diet composition, and the brain

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Chapter 3

Short-term fasting restores free-choice high-fat, high sugar diet-induced insulin resistance in rats

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Submitted

Abstract

We previously showed that rats fed a free-choice high-fat, high-sucrose (fcHFHS) diet for one week become obese and glucose intolerant. In the present study we investigated whether the observed glucose intolerance in rats on a fcHFHS diet is explained by lower insulin sensitivity. Since fasting has been shown to ameliorate glucose metabolism in humans with diabetes, we additionally studied whether short-term fasting reverses diet-induced insulin resistance.

Male Wistar rats received a fcHFHS diet or chow diet for one week. At day 8, rats were subcutaneously injected with an insulin bolus or saline after either an overnight fast or 15 grams of chow consumption. Stomach content and systemic concentrations of glucose and gluocregulatory hormones and free fatty acids (FFA) were assessed.

Insulin failed to suppress plasma glucose concentrations in overnight-fed fcHFHS rats. Interestingly, after an overnight fast, insulin's ability to reduce plasma glucose concentrations in fcHFHS rats was similar to fed or overnight-fasted chow diet rats. These findings could not be explained by differences in circulating glucoregulatory hormones or FFA.

These results show that glucose intolerance in short term fcHFHS rats is explained by insulin resistance and that short-term fasting restores insulin sensitivity in rats on a fcHFHS diet but does not affect glucose metabolism in lean, chow-fed rats.

Introduction

The prevalence of obesity and type 2 diabetes mellitus (T2DM), is reaching epidemic proportions. The molecular pathways underlying obesity-induced insulin resistance are divers and involve among others increased concentrations of fatty acids and their intermediates, inflammatory changes, adipose tissue dysfunction, oxidative and ER-stress as well as mitochondrial dysfunction (Bergman & Ader, 2000; Reaven, 2002b; Samuel *et al*., 2010). Besides these mechanisms, direct effects of the diet itself might contribute to insulin resistance (Storlien *et al*., 1988). We recently showed that rats consuming a choice diet consisting of saturated fat, 30% sucrose water, standard pellet chow and tap water (a free-choice high fat, high sugar (fcHFHS) diet) become obese within a week. This is associated with persistent hyperphagia, with increased meal frequency (la Fleur et al., 2014b) and increased food motivation (la Fleur *et al*., 2007). Furthermore, rats fed a fcHFHS diet show glucose intolerance, while rats on a free-choice high fat (fcHF) diet become equally obese but remain glucose tolerant (la Fleur *et al*., 2011). These data show that, in a hypercaloric setting, dietary composition independently affects glucose metabolism. Glucose intolerance occurs when either insulin sensitivity and/ or insulin secretion is reduced. Previous studies have shown that short-term feeding of hypercaloric cafeteria (Davidson & Garvey, 1993) or HF diets (Cruciani-Guglielmacci *et al*., 2005; Kraegen *et al*., 1991a) induce insulin resistance in rats. Since short-term (one week) fcHFHS-fed rats showed glucose intolerance not explained by reduced insulin secretion (la Fleur *et al*., 2011) our first aim was to study whole body insulin sensitivity in rats following one week of fcHFHS feeding.

Insulin resistance and hyperglycemia can be reversed upon weight loss induced by hypocaloric feeding (Henry & Gumbiner, 1991) and in diabetic subjects, fasting reduces hyperglycemia which is mainly explained by a reduction in endogenous glucose production (Andrikopoulos *et al*., 2008; Bowe *et al*., 2014; Fery & Balasse, 1994; Glauber *et al*., 1987; Jackson *et al*., 1971). In contrast, peripheral insulin sensitivity has been shown to be reduced by short-term fasting in lean humans (Newman and Brodows, 1983; Soeters *et al*., 2008) (48h and 62h respectively). Interestingly, in mice insulin sensitivity improves upon fasting (Ayala *et al*., 2006) but remains unaffected in rats (Youn & Buchanan, 1993). The difference in effects of fasting on glucose metabolism seems to be species-specific, in addition to different methods used to assess glucose metabolism across studies. Most of the rodent studies have been performed in mice, which have a much higher metabolic rate compared to humans (Andrikopoulos *et al*., 2008; Bowe *et al*., 2014). Of note the metabolic rate of rats is intermediate to that of mice and humans. However, rat data reporting the effect of short-term fasting on insulin sensitivity following a short-term hypercaloric diet are very limited and whether short-term hypercaloric diet-induced insulin resistance is reversible upon fasting in rats is unknown. Therefore our second aim was to investigate whether we could reverse the effects of a short-term fcHFHS diet on insulin sensitivity with an overnight fast in rats. To address these aims, we subjected rats to a fcHFHS diet or a chow (control) diet for one week. To measure insulin sensitivity in the

fed state all rats received chow overnight. To measure insulin sensitivity under fasting conditions, all rats were fasted overnight. The following day, rats were injected with an insulin bolus or saline (control) and killed. We assessed stomach content and systemic concentrations of glucose, glucoregulatory hormones and FFA.

Materials and Methods

Animals

Male Wistar rats (250-280g) (Charles River, Germany) were housed in plexiglass cages in groups of four to six per cage in a temperature (20±2°C), humidity (60±2%) and light controlled room with a 12/12h light-dark schedule (lights on at 7:00h). All rats had *ad libitum* access to standard laboratory chow (special diet service (SDS), England) and tap water. The experiment was approved by the Committee for Animal Experimentation of the Academic Medical Centre, the University of Amsterdam, the Netherlands.

Experimental Design

Rats (*n*=32, 280-300g) were subjected to a free choice, high-fat, high-sucrose (fcHFHS) diet *i.e., ad libitum* access to a dish of saturated fat (beef tallow (Ossewit/Blanc de Boeuf), Vandermoortele, Belgium) and a bottle of 30% sugar water (1.0M sucrose mixed from commercial grade sugar and tap water), in addition to their standard pellet chow and tap water bottle. The other rats (*n*=31, 280-300g) remained on *ad libitum* access to standard pellet chow and tap water. Body weights were matched between groups at the start of the diet. After seven days of diet, food was removed at the end of the light period (ZT12) and rats received either 0 (overnight fast, chow *n*=16, fcHFHS *n*=17) or 15 grams of chow (overnight fed) (chow *n*=15, fcHFHS *n*=15) to ensure similar intake between the chow and the fcHFHS diet group. The next day, at ZT5, each rat of the half of each group received a sc. injection of either saline (NaCl 0.9% B. Braun, Melsungen, Germany) or 0.06U Actrapid (Novo Nordisk, Alphen aan de Rijn, the Netherlands). Thirty minutes after injection rats were anaesthetized by CO_2/O_2 (6:4) followed by CO_2 only and killed by decapitation.

Data analysis

Body weight was measured daily upon arrival of the rats, at the evening prior to the experiment and at the experimental day prior to insulin injection. From the start of the diets, food intake was measured until the end of the experiment. From each rat total white adipose tissue weight was measured. Stomach content was calculated by subtracting empty stomach weight from full stomach weight.

Analytical methods

Trunk blood was collected and immediately chilled on ice in 10 ml greiner tubes, filled with 100 µL heparin (LEO Pharma B.V., Breda, the Netherlands and centrifuged at 4 °C

(15 min, 3000 rpm). Plasma was stored at -20°C until further analysis. Plasma glucose concentrations were measured by the glucose oxidation method using a Biosen glucose analyser (EKF-diagnostic GmbH, Barleben, Germany). Plasma concentrations of insulin, glucagon and corticosterone were measured using radioimmunoassay kits (Millipore, St Charles, MO, USA and Biochemicals, Costa Mesa, CA, respectively). The amounts of sample, standards, label, antibody and precipitating reagent, described in the manufacture's protocol, were divided by four. The coefficient of variation of the immunoassays was <10%. The FFA concentrations were determined with an enzymatic colorimetric method (NEFA-HR(2) test kit, Wako Chemicals GmbH, Neuss, Germany).

Statistics

All data are presented as means±SEM. Statistical analysis was performed using 3-way analysis of variance (ANOVA) (SPSS Inc, Chicago, USA) to test for effects of *Diet* (chow or fcHFHS), *Treatment* (*i.e.,* subcutaneous saline or insulin injection), *Feeding state* (*i.e.,* 0 or 15 grams of chow) and *Diet*Treatment, Diet* Feeding state, Treatment* Feeding state* or *Diet*Treatment* Feeding state* interaction. Individual group differences were detected using 1-way ANOVA when the 3-way ANOVA detected a significant effect. Data were tested on outliers with the Grubbs'outlier test (GraphPad Sofware, Inc, La Jolla, USA).

Results

Food intake, body weight and adiposity

Rats exposed to the fcHFHS-diet consumed significantly more calories compared to the chow-fed rats ($p<0.001$); Figure 1A). Intake of fcHFHS-fed rats consisted of 50.1±2.3% chow, 26.5±3.3% fat and 23.4±1.8% sucrose (Figure 1B). After one week of diet, body weight (BW) of chow-fed rats (330.2±2.7g) and fcHFHS-fed rats (338.8±3.2g) was not significantly different. Irrespective of whether or not food was given overnight, all rats lost body weight, but rats subjected to an overnight fast lost significantly more body weight than when given 15 grams of chow ($F(3,55)=25.74$, $p<0.001$); Figure 1C). Adiposity was significantly increased in fcHFHS-fed rats compared to chow-fed rats ($F(1,59)=56.404$, $p<0.001$). In contrast, adiposity was not affected by feeding status (*Gram:* $F(1.59)=2.090$, $p=0.154$) or insulin injection (*Injection*: $F(1,59)=0.262$, $p=0.611$) in either diet group (Figure 1D).

Stomach content

Stomach content was not different between rats on the fcHFHS or chow diet (*Diet* $F(1,59)=0.001$, $p=0.976$) and was independent of feeding status (*Gram*: $F(1,59)=1.296$, $p=0.260$). Three-way ANOVA detected an effect for injection ($F(1,59)=10.808$, $p=0.002$) (Figure 2), however, this was not due to one specific group as one-way ANOVA did not detect individual group difference. No significant interaction effects were detected.

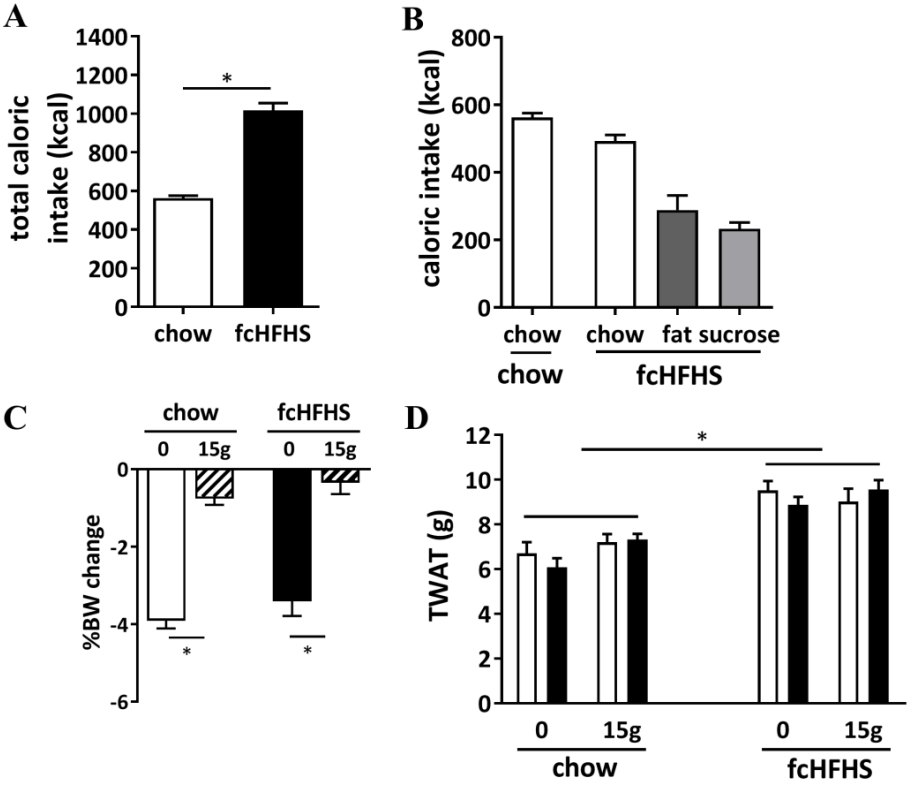

Figure 1. (A) Total caloric intake over 1 week was higher in fcHFHS-fed rats (black bar) compared to chow-fed rats (white bars). (B) Consumed calories from chow, saturated fat and 30% sucrose solution in rats on fcHFHS diet over 1 week. (C) Percentage body weight (BW) change was significantly higher in fasted compared to overnight-fed rats in both diet groups ($p<0.001$ after ANOVA detected a significant effect of feeding state ($p<0.001$) (white bar: chow diet, overnight-fasted rats; white striped bar: chow diet, overnight fed rats; black bar: fcHFHS diet, overnight-fasted rats; black striped bar: fcHFHS diet, overnight-fed rats. (D) Total white adipose fat stores in chow and fcHFHS-fed rats following an overnight fast or 15 grams of chow and a subcutaneous saline (white bars) or insulin injection (black bars). Total white adipose tissue was significantly increased in fcHFHS-fed rats compared to chow-fed rats and not affected by an overnight fast or sc. insulin injection in either diet group. TWAT was not corrected for BW considering difference in overnight food intake. Data are expressed as mean±SEM, *$p<0.05$.

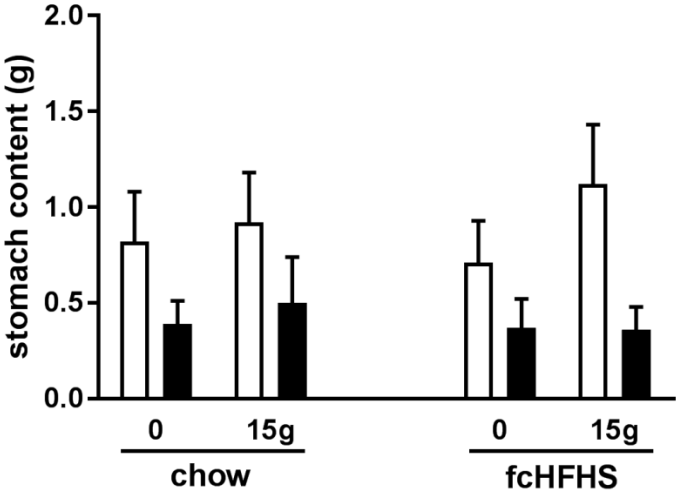

Figure 2. Stomach content (g) in chow- and fcHFHS-fed rats following an overnight fast or 15 grams of chow and a subcutaneous saline (white bars) or insulin injection (black bars). Stomach content was not different between fcHFHS-fed or chow-fed or between fasted and fed rats but decreased following a subcutaneous. insulin injection. Data are expressed as mean±SEM

Glucose and glucoregulatory hormones

Plasma glucose concentrations

Plasma glucose concentrations were not different between chow or fcHFHS fed rats (*Diet:* $F(1,59)=1.497$, $p=0.227$). However, three-way ANOVA did detect significant effects for injection (*Injection:* ($F(1,59)=44.642$, $p<0.001$) and feeding status (*Feeding state:* $F(1,59)=65.836$, $p<0.001$). The insulin injections significantly decreased plasma glucose concentrations in chow overnight-fasted rats ($p=0.015$) and chow overnight fed rats ($p=0.011$). The insulin-induced decrease of plasma glucose concentrations was reduced ($p<0.001$) in the fcHFHS overnight fed rats compared to the fcHFHS overnight-fasted rats. An overnight fast normalized the glucose response to insulin as the fcHFHS overnight-fasted, insulin-injected rats had lower plasma glucose concentrations ($p<0.001$) compared to their own saline-injected controls. Statistical analysis detected a significant interaction effect for *Feeding state*Injection* ($F(1,59)=4.757$, $p=0.034$ and *Diet*Feeding state*Injection* ($F(1,59)=5.213$, $p=0.027$) interaction. The latter can be explained by the equal plasma glucose concentrations in the fcHFHS, insulin-injected, overnight-fed rats compared to the fcHFHS, saline injected, overnight fed rats ($p=0.993$) (Figure 3A).

The effect of feeding status was due to significantly lower ($p=0.027$) plasma glucose concentrations in chow-fed, saline-injected, fasted rats compared to the chow-fed, saline-injected rats which received 15 grams of chow overnight.

Plasma glucagon concentrations

Three-way ANOVA detected an effect of diet for plasma glucagon concentrations (*Diet*: F(1,59)=7.790, p=0.007), but *Post hoc* analysis did not detect significant differences between the groups. A significant injection effect was detected (*Injection*: F(1,59)=15.664, p<0.001), which was due to higher plasma glucagon concentrations in the fcHFHS, insulin-injected, overnight-fasted rats compared to their saline controls (p=0.008). A trend for higher plasma glucagon concentrations was detected for the chow, insulin-injected, overnight-fasted, rats compared to their saline-injected controls (p=0.083). In contrast, feeding status did not affect plasma glucagon concentrations (*Feeding state*: (F(1,59)=1.088, p=0.302). In addition, a significant interaction effect was detected for *Feeding state*Injection* (F(1,59)=7.831, p=0.007) and a trend for *Diet*Feeding state* (F(1,59)=3.919, p=0.053) and *Diet*Feeding state*Injection* (F(1,59)=2.963, p=0.091).

Plasma corticosterone concentrations

Plasma corticosterone concentrations were not different between rats on a chow or fcHFHS diet (*Diet:* (F(1,59)=0.325, p=0.571). The insulin injection caused a significant increase in plasma corticosterone concentrations in all 4 treatment groups (*Injection*: (F(1,59)=41.980, p <0.001).

A significant effect was also detected for feeding status (*Feeding status*: (F(1,59)=6.277, p=0.015), because of higher corticosterone responses to insulin in the overnight-fasted rats. A trend was detected for the *Feeding state*Injection* interaction (F(1,59)=3.405; p=0.071) (Figure 3C).

Plasma insulin concentrations

Three-way ANOVA detected a significant difference in plasma insulin concentrations between fcHFHS- and chow-fed rats (*Diet*: F(1,57)=6.077, p=0.017). As expected the insulin injection significantly increased plasma insulin concentrations (*Injection:* (F(1,57)=57,256, p<0.001). Feeding status significantly affected plasma insulin concentrations (*Feeding state*: (F(1,57)=6.546; p=0.014). No significant *interaction* effects were detected (Figure 3D). Separate statistical analysis, between chow and fcHFHS-fed, saline-injected rats detected higher insulin concentrations in fcHFHS- compared to chow-fed rats (saline, overnight-fasted p=0.04; saline overnight-fed p=0.008).

Plasma free fatty acid concentrations

Plasma FFA concentrations were significantly increased in the fcHFHS-fed rats compared to the chow-fed rats (*Diet*: F(1,59)=8.186, p=0.006). The insulin injection significantly decreased plasma FFA concentrations (*Injection*: F(1,59)=56.745, p<0.001). *Post hoc* analysis revealed significantly lower plasma FFA concentrations in insulin-injected chow fasted (p=0.007), fcHFHS fasted (p<0.001) and fcHFHS overnight-fed (p=0.032) rats compared to their own saline controls. Feeding status did not significantly affect plasma

FFA concentrations in either diet group (*Feeding state*: F(1,59)=0.478, p=0,492). No significant *interaction* effects were detected (Figure 3E).

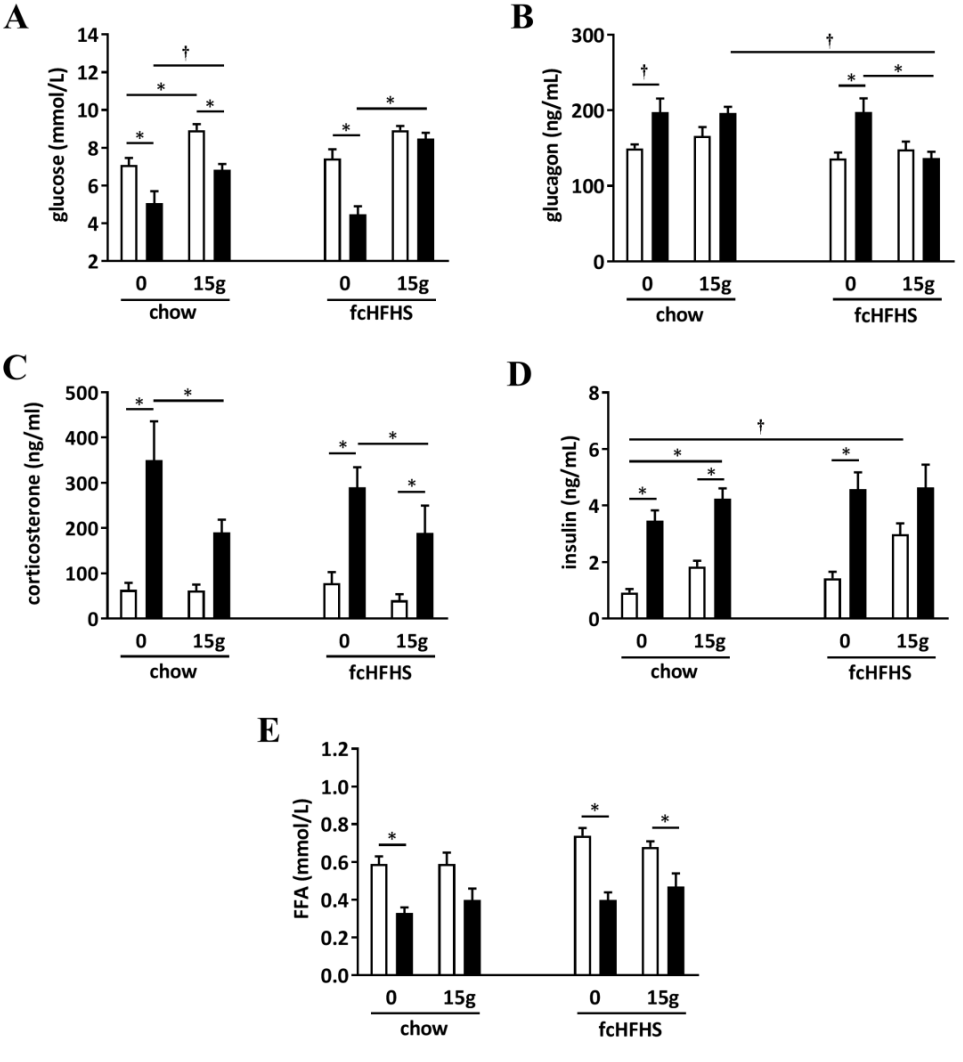

Figure 3. (A) plasma concentrations of glucose, (B) glucagon, (C) corticosterone, (D) insulin and (E) free fatty acids (FFA) in chow and fcHFHS-fed rats following an overnight fast or 15 grams of chow and a subcutaneous saline (white bars) or subcutaneous insulin injection (black bars). Data are expressed as mean±SEM, *p<0.05, $^{\dagger}p$<0.10.

Discussion

We previously showed that one week of consuming a fcHFHS diet induces obesity and glucose intolerance in rats. Here we extend these findings showing that the insulin-mediated decrease in plasma glucose concentrations is hampered in those rats, indicating a state of insulin resistance. Interestingly, fasting restores insulin action as it normalizes the insulin-induced glucose decrease in rats on the fcHFHS diet while it remains unchanged in chow-fed rats. These data show that: 1) a short-term high-fat, high-sucrose choice diet induces glucose intolerance through insulin resistance, 2) fasting restored the negative effect of a short-term high-fat, high-sucrose choice diet on insulin sensitivity and 3) insulin sensitivity in rats on a fcHFHS diet depends on feeding status.

In contrast to the rats on the fcHFHS diet, rats on the chow diet did not respond differently to the insulin injection depending on an overnight-fasted or -fed state, suggesting that an overnight fast does not alter insulin sensitivity in metabolically healthy rats. In line with our observations, Youn *et al*. (1993) observed no difference in hepatic insulin sensitivity assessed by a hyperinsulinaemic euglycaemic clamp, between overnight-fasted or -fed rats which received a normal chow diet. Contradicting these results, earlier studies have reported that fasting induces glucose intolerance (Cahill *et al*., 1966; Tzagournis & Skillman, 1970) and reduced peripheral insulin sensitivity in lean individuals (48h, (DeFronzo *et al*., 1978; Newman & Brodows, 1983) and metabolically healthy rats (84h, (Penicaud *et al*., 1985). However, all these studies included longer periods of fasting as compared to our study, and thus glucose metabolism in metabolically healthy humans and rats is probably affected after a prolonged period of fasting rather than overnight food deprivation. This is supported by the finding that hepatic insulin sensitivity is not affected in normal chow-fed rats following 48h food deprivation (Youn & Buchanan, 1993).
After an overnight fast, insulin injection increased plasma glucagon concentrations in the chow-fed rats. The increased plasma glucagon concentrations in the insulin-injected rats point to a physiological counter-regulatory response to the insulin-induced decrease in plasma glucose concentrations. Previous studies have demonstrated an increase in glucagon secretion after an insulin-induced decrease in glucose concentrations of approximately 4 mmol/L, but might also be apparent after smaller glucose changes (Havel *et al*., 1994; McCrimmon *et al*., 2002) and increased plasma glucagon concentrations were observed 10-30 minutes after an intravenous insulin bolus (Kalsbeek *et al*., 2006). This is further supported by the lack in glucagon increase in the fcHFHS, overnight fed rats in which insulin substantially failed to reduce plasma glucose concentrations. Besides an increase in plasma glucagon concentrations, plasma corticosterone concentrations were increased in the insulin-injected rats. Early studies have shown insulin-induced rises in plasma corticosterone concentrations (Arner *et al*., 1962; Bliss *et al*., 1954; Gershberg & Long, 1948; Gordon, 1950). This response has been explained as the counter regulatory response to the insulin-induced decrease in plasma glucose concentrations as co-administration of glucose prevented an increase in corticosterone (Gershberg & Long,

1948; Vogt, 1951). However, also on the fcHFHS diet fasting increased corticosterone significantly while glucose concentrations did not decrease, making it unlikely that the glucose decline is the only factor inducing corticosterone responses when injecting insulin. It could well be that changes in insulin receptor signalling in the hypothalamus due to the fcHFHS diet alter the complex cross-talk between energy status and the stress reactivity, as disruption of hypothalamic insulin receptors has been shown to alter stress responsivity (Chong *et al*., 2015). It remains, however, to be determined whether fcHFHS diet alter hypothalamic insulin signalling.

The reversal of insulin resistance by an overnight fast in fcHFHS fed rats is in line with human studies showing reduced glycaemia in diabetic subjects following fasting (Fery & Balasse, 1994; Glauber *et al*., 1987; Jackson *et al*., 1971) and reduced endogenous glucose production in lean subjects. However, there was no difference in fasting glucose suggesting that a fasting-induced reduction in EGP cannot account for our findings and thus indicates that insulin action either on hepatic glucose production or peripheral glucose uptake or both are affected.

Increased concentrations of circulating FFA are associated with insulin resistance and reduced insulin-mediated suppression of lipolysis and thus high plasma FFA is a hallmark of insulin resistant states. In our study, the suppression of FFA by insulin injections was equal between groups and therefore cannot explain the amelioration in insulin sensitivity in the fcHFHS, overnight-fasted rats nor the lack of an effect of insulin in the fcHFHS fed rats.

Taken together, fcHFHS overnight-fed rats showed reduced insulin sensitivity compared to chow-fed rats, which could be reversed after an overnight fast. The reversion of reduced insulin sensitivity could not be explained by a change in glucoregulatory hormones or FFA concentrations and must be due to difference in feeding status in combination with dietary history. This stresses the importance of timing in metabolic experiments with diet-induced insulin resistance.

In all rats residual food in the stomach was observed, irrespective of the amount of food given. This indicates that, although rats were food deprived overnight, food residues in the stomach might still be a source of glucose. However, as the amounts were not significantly different between chow fed and fcHFHS fed rats this will most likely not have affected our outcome. This is in line with an earlier study showing that the same oral glucose load was equally emptied in 24h and 6h food deprived rats (McCann & Stricker, 1986) indicating that duration of food deprivation does not affect gastric emptying time. In addition, our data showed that insulin injections reduced stomach content, independent of diet or overnight feeding state. This might be due to the insulin-mediated decrease in plasma glucose concentrations, which increases gastric emptying (McCann & Stricker, 1986), but again the effects were similar in rats on the fcHFHS diet, fed overnight, which did not have reduced plasma glucose concentrations following sc. insulin injection.

In line with previous rodent studies on fasting, we observed a decrease in BW following an overnight fast (Dallman *et al*., 1999; Strubbe *et al*., 1988; Youn & Buchanan,

1993) with no difference between groups. In contrast, there was no decrease in TWAT mass, indicating that overnight-fasted rats lost more body water and/or lean mass than overnight fed rats.

In summary, we here show that in rats subjected to a fcHFHS diet for one week, insulin did not suppress plasma glucose concentrations, pointing towards insulin resistance. Overnight fasting reversed insulin resistance in the fcHFHS-fed rats, while it did not affect insulin action in chow-fed rats suggesting that short-term (one week) diet-induced insulin resistance depends on feeding status. These findings could not be explained by differences in circulating glucoregulatory hormones or FFA. Whether the improvement in insulin action in the fcHFHS rats is caused by improved suppression of hepatic glucose production or stimulation of glucose uptake remains to be established.

Finally, our results stress the importance of feeding status *i.e.* fed, food deprived or fasted conditions, on metabolic responses and should be taken into account when designing future diet intervention studies.