High-fat diet results in postprandial insulin resistance that involves parasympathetic dysfunction

Ricardo A. Afonso[1,2], W. Wayne Lautt[3], Josh Schafer[3], Dallas J. Legare[3], Antonio G. Oliveira[4] and M. Paula Macedo[2,5]*

[1]*Department of Biochemistry, Faculdade de Ciências Médicas (FCM), Universidade Nova de Lisboa (UNL), Campo Martires da Patria, 130, 1169-056 Lisboa, Portugal*

[2]*Department of Physiology, Faculdade de Ciências Médicas (FCM), Universidade Nova de Lisboa (UNL), Campo Martires da Patria, 130, 1169-056 Lisboa, Portugal*

[3]*Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, A210-753 McDermot Avenue, Winnipeg, MB, Canada R3E 0T6*

[4]*Department of Biostatistics and Informatics, Faculdade de Ciências Médicas (FCM), Universidade Nova de Lisboa (UNL), Campo Martires da Patria, 130, 1169-056 Lisboa, Portugal*

[5]*Portuguese Diabetes Association (APDP), R. do Salitre 118-120, 1250-203 Lisboa, Portugal*

Different diets have distinct impacts on glucose homoeostasis, for which insulin sensitivity (IS) after a meal (postprandial IS) is highly relevant. Postprandial IS depends upon hepatic parasympathetic activation and glutathione content elevation. We tested the hypothesis that postprandial IS is compromised in high-fat diet (HFD)-induced obesity. Sprague–Dawley rats were fed a standard diet (STD, *n* 10), 1-week HFD (*n* 9) or 4-week HFD (*n* 8). IS was tested in postprandial state using the rapid IS test (RIST) before and after the blockade of the parasympathetic nerves (atropine, 1 mg/kg); parasympathetic-dependent IS was obtained from the difference between control and post-atropine RIST. Fasting IS was also assessed in the STD-fed rats (*n* 4) and 4-week HFD-fed rats (*n* 3) using the RIST. Whole-body fat and regional fat pads were heavier in the 1-week HFD-fed rats (79·8 (SE 7·9) and 23·7 (SE 1·0) g, respectively) or 4-week HFD-fed rats (106·5 (SE 6·1) and 30·1 (SE 1·4) g, respectively) than in the STD-fed rats (32·5 (SE 3·7) and 13·7 (SE 1·0) g, respectively; *P* < 0·001). Fasted-state IS was similar between the groups studied. Postprandial IS was higher in the STD-fed rats (185·8 (SE 5·6) mg glucose/kg body weight (bw)) than in both the 1-week HFD-fed rats (108·8 (SE 2·9) mg glucose/kg bw; *P* < 0·001) and 4-week HFD-fed rats (69·3 (SE 2·6) mg glucose/kg bw; *P* < 0·001). Parasympathetic-dependent IS was impaired in both HFD-fed groups (STD, 108·9 (SE 3·9) mg glucose/kg bw; 1-week HFD, 38·6 (SE 4·2) mg glucose/kg bw; 4-week HFD, 5·4 (SE 1·7) mg glucose/kg bw; *P* < 0·001). Total (postprandial) and parasympathetic-dependent IS correlated negatively with whole-body fat (R^2 0·81 and 0·87) and regional adiposity (R^2 0·85 and 0·79). In conclusion, fat accumulation induced by HFD is associated with postprandial insulin resistance, but not with fasting insulin resistance. HFD-associated postprandial insulin resistance is largely mediated by impairment of parasympathetic-dependent insulin action, which correlates with adiposity.

Obesity: High-fat diet: Insulin resistance: Parasympathetic nerves

Alterations in glucose homoeostasis seem to occur in the postprandial state long before they appear in the fasted state[1–5]. Under physiological conditions, following a meal, insulin released by the pancreas stimulates glucose uptake by peripheral tissues, but this effect is mediated to a large extent by concomitant activation of the hepatic parasympathetic nerves[6–8], as demonstrated by the observations that hepatic parasympathetic denervation[9,10] causes a major reduction (up to 60 %) in postprandial insulin sensitivity (IS)[9], with the same effect being observed after atropine administration[9,11]. In fact, atropine administration does not produce any additional impairment of IS in previously hepatic parasympathetic denervated rats[9,12]. Atropine's effects are dose dependent, and the same effect on IS can be achieved by intraportal and intravenous (i.v.) atropine administrations, although with different median effective doses (ED_{50}), but without any changes in plasma concentrations of glucagon or insulin[13,14]. Both hepatic denervation and atropine-induced insulin resistance can be reversed by intraportal acetylcholine administration[10,15], suggesting that both procedures interfere with the same pathway and that the activation of hepatic parasympathetic nerves leads to acetylcholine release in the

liver[8,15,16]. Acetylcholine stimulates muscarinic receptors[15] and results in hepatic NO production[16,17]. As with acetylcholine[10,15], administration of a NO donor to a denervated liver[17] also restores whole-body postprandial IS.

This hepatic parasympathetic-dependent NO mechanism is additionally dependent upon the availability of hepatic glutathione, which is increased after a meal, when hepatic pathway activation is also maximal[18]. This hepatic-dependent mechanism seems to result in the hepatic release of a humoral factor, referred to as hepatic insulin-sensitising substance (HISS), which acts mainly in the skeletal muscle[9] and appears to be responsible for 50–60 % of whole-body insulin action in the postprandial state[6,9,11,18,19]. This mechanism accounts for the increment in IS from the fasted to the fed state[20].

The HISS-dependent contribution to overall postprandial insulin action can be calculated from the difference in postprandial IS before and after the inhibition of the HISS pathway, which can be achieved by either fasting[6,11], parasympathetic denervation[9,15], blockade with atropine[15] or hepatic NO synthase inhibition (e.g. using NG-monomethyl-L-arginine or NG-nitro arginine methyl ester)[17,18,21]. Similar impairment of insulin action can be obtained by hepatic glutathione depletion[18]. Autonomic dysfunction is observed in obesity[22–24], suggesting that decreased parasympathetic activity is associated with increased body fat[7,22,24].

Earlier experiments that were performed by our group, in which only IS was assessed, suggest that short-term high-fat diet (HFD, 1 week) induces partial impairment of postprandial IS[25]; however, to what extent and the time course during which HFD induces the deterioration of postprandial IS are not known, and neither the relationship between adiposity and postprandial IS or hepatic glutathione content is known. In the present study, we aimed to evaluate the effects of different HFD durations on fat deposition and obesity, as well as to relate them with the postprandial IS, which depends on HISS action. For the first time, we have aimed to study the relationship between abdominal and whole-body adiposities and HISS action.

Thus, in the present work, we have hypothesised that a HFD induces postprandial insulin resistance, which is accounted for by the decrease of HISS-dependent insulin action and is proportional to the degree of obesity. We tested this by measuring the effect of muscarinic nerve blockade on IS in rats fed a standard diet (STD), short-term (1 week) HFD or long-term (4 weeks) HFD. Fat mass deposition and its correlation with HISS-dependent and HISS-independent insulin resistance were evaluated. The present work reveals further pathways interconnecting the impact of diets on insulin resistance.

Experimental methods

Animals

Male Sprague–Dawley rats (Charles River Laboratories, Saint Constant, QC, Canada) were used. The rats reached our facilities at the age of 4 weeks, and were housed under climate-controlled conditions and a 12 h light–dark cycle (07.00–19.00 hours). During the conditioning period, rats were fed *ad libitum* with a standard laboratory chow (Prolab RMH 3000 5P00; Labdiet, St Louis, MO, USA), and they had free access to tap water.

At the end of the experiment, rats were killed by i.v. administration of an overdose of anaesthetic (sodium pentobarbital).

All applicable institutional and national guidelines for the care and use of animals were followed, and all the experimental procedures involving animals were approved by the University of Manitoba Ethics Committee on Animal Care. These studies were compliant with the NIH Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985), the Canadian Council on Animal Care guidelines and the Laboratory Animal Care Guidelines of the European Union (86/609/CEE).

Diets and feeding protocols

The control diet that was used was the standard laboratory chow (STD), which consisted of 26 % protein, 60 % carbohydrates and 14 % fat, whereas the HFD (DIO Series Diets D12492; Research Diet, Inc., New Brunswick, NJ, USA) consisted of 20 % protein, 20 % carbohydrates and 60 % fat (percentage of total energy) as described previously[26]; the fat content was mostly provided by soyabean oil and lard (5·5 and 54·4 % of the total energy, respectively). After 1 week of conditioning, the rats were randomly divided into three groups according to the diet they were fed: the control group (*n* 14), which was fed the standard laboratory diet for the whole period of the study; the HFD-1 group (*n* 9), which was fed HFD for 1 week, between 8 and 9 weeks of age; and the HFD-4 group (HFD-4, *n* 10), which was fed HFD for 4 weeks, between 5 and 9 weeks of age.

All the rats were tested at the age of 9 weeks. They were given free access to food and water. On the day before the experiment, rats were fasted for 8 h, starting from 22.00 hours. Free access to water was maintained. At 06.00 hours on the next day, rats were allowed access to food for 2 h (HFD for the HFD-1 (*n* 9) and HFD-4 (*n* 7) groups; STD for the STD group (*n* 10)) in order to ensure that they had eaten by the time the experiment was started (postprandial studies).

A small group of STD-fed (*n* 4) and HFD-4-fed (*n* 3) rats were subjected to a 24 h fast, which was started at 08.00 hours on the day before the experiment in order to assess IS in the fasted state.

Surgical procedure

The rats were anaesthetised using sodium pentobarbital (65 mg/kg, intraperitoneal). Body temperature was maintained at $37·0 \pm 0·5°C$ throughout the experiment using a Homeothermic Blanket Control Unit (Harvard Apparatus, Holliston, MA, USA).

A tracheotomy was performed to allow spontaneous respiration. Anaesthesia was maintained by continuous infusion of sodium pentobarbital (10 mg/h per kg, i.v.) through a left jugular vein catheter (polyethylene tubing PE50, Intramedic; Becton Dickinson, Franklin Lakes, NJ, USA), which was also used to infuse glucose.

The right femoral artery and vein were also catheterised. A femoral arterial-venous shunt was created by connecting the catheters through a silicone sleeve, which allows blood circulation[27]. The arterial-venous shunt was primed with

sodium heparin (200 IU/ml) to prevent blood clotting. Mean arterial pressure was obtained by clamping the venous side of the shunt, and was monitored using the Lab View Software (National Instruments, Austin, TX, USA).

Multiple arterial blood samples were collected by puncturing into the arterial side, and i.v. drug infusions were given through the venous side of the shunt. After the surgery, a 30 min period (minimum) was allowed for the measurement of blood glucose and blood pressure before further procedures were undertaken.

Insulin sensitivity assessment

The rapid IS test (RIST) was used to assess IS as described previously[27]. The RIST is a modified euglycaemic clamp procedure, which is suitable for the rapid and reproducible assessment of IS. Since there is no carry-over effect between RIST, it is possible to repeat the RIST up to four times in the same animal on the same day[27], providing a suitable index for IS both in anaesthetised and conscious animals[11,28]. The RIST also allows for the assessment of IS in both the fasted and the fed states[6].

Before each RIST, basal arterial glycaemia (baseline) was determined. The RIST was started with the administration of an i.v. bolus of insulin (50 mU/kg, 5 min; $t = 0$ min), and 1 min later ($t = 1$ min), glucose infusion (i.v.) was given (at a rate of 3 mg/kg per min). Then, at 2-min intervals, arterial glycaemia was measured, and the glucose infusion rate was adjusted (every 2 min) accordingly (using a 'Genie' infusion pump; Kent Scientific Corporation, Torrington, CT, USA) in order to maintain euglycaemia. The RIST was considered complete when no further glucose infusion was required to maintain euglycaemia. The total amount of glucose infused during the RIST is referred to as the RIST index (mg glucose per kg body weight, bw), and it is the parameter used to evaluate IS.

Glycaemia was determined by the glucose oxidase method using a glucose analyser (1500 Sport Analyser; Yellow Springs Instruments, Yellow Springs, OH, USA).

Insulinaemia quantification

Before and after each RIST, arterial blood samples (40 μl) were collected and centrifuged (3 min, 16 755 *g*; Corning Costar Corporation centrifuge, Cambridge, MA, USA) to determine plasma insulin levels, which was done using an ELISA technique (Rat Ultrasensitive Insulin ELISA; Mercodi, Inc., Winston Salem, NC, USA).

Hepatic glutathione quantification

At the end of the experiment, before the rats were killed, liver samples were collected and immediately frozen ($-70°C$) for spectrophotometric quantification of glutathione using the Bioxytech GSH-420 kit (from Oxisresearch, Portland, OR, USA).

Fat mass assessment

Fat mass assessment was done by measuring both whole-body fat mass and the weights of specific fat pads. Whole-body fat mass was estimated by tetrapolar bioelectrical impedance analysis using a Bioelectrical Body Composition Analyser (Quantum II; RJL Systems, Clinton Township, MI, USA) as described previously[29].

At the end of the experiment (fed rats), a laparotomy was performed in order to harvest the abdominal fat pads, i.e. the fat pads surrounding the kidneys (perinephric), the testis (epididymal) and the intestine (perienteric). Total abdominal fat pad mass was the sum of the individual weights of perinephric, epididymal and perienteric fat pads.

Experimental protocols

An initial postprandial stable arterial glycaemic baseline (control baseline) was determined, and a control RIST was performed. Atropine (1 mg/kg, 5 min, i.v.) was then infused to block the HISS pathway. A 30 min period was then allowed for stabilisation before a new glycaemic baseline (post-atropine baseline) was determined, and another RIST (post-atropine RIST) was performed. The HISS-dependent component of insulin action was quantified by subtracting the post-atropine RIST index from the control RIST index.

In the STD-fed and HFD-fed rats that were to be tested in the fasted state (24 h fast), a single RIST was performed, following the determination of fasted glycaemic baseline.

Before and after each RIST (protocols followed in the fed state), arterial blood samples were collected for the determination of plasma insulin levels as described previously. At the end of the experiment, liver samples were collected for glutathione quantification (see above).

Statistical analysis

Data are presented as means with their standard errors. The significance of the differences was calculated through two-tailed Student's *t* tests and one-way ANOVA followed by the Tukey–Kramer multiple comparison tests, repeated-measures ANOVA or through ANCOVA, as applicable. Differences were accepted as statistically significant at $P < 0.05$.

Regression analysis (linear and polynomial) was used to assess the relationship between insulin action (total, HISS-dependent and HISS-independent) and fat mass, both whole body and regional (perinephric, epididymal and perienteric pads), using Stata version 9.2 (StataCorp, College Station, TX, USA).

Drugs

Sodium pentobarbital (Somnotol) was obtained from Biomeda-MTC Animal Healt, Inc., Cambridge, ON, Canada; sodium heparin was obtained from Leo Pharma, Thornhill, ON, Canada; insulin (Novolin) was obtained from Novo Nordisk, Mississauga, ON, Canada; and D-glucose and atropine were supplied by Sigma-Aldrich, Oakville, ON, Canada. All solutions were prepared in saline (Baxter Corporation, Toronto, ON, Canada).

Results

Body weight and blood pressure

A relationship between bw and HFD duration was observed: bw of the STD-fed rats (STD, 371·6 (SE 7·3) g, n 10) was lower than those of the 1-week HFD-fed rats (HFD-1, 401·4 (SE 5·2) g, n 9; P < 0·001) and the 4-week HFD-fed rats (HFD-4, 420·5 (SE 4·7) g, n 7; P < 0·001).

Basal mean arterial pressure was similar in all the groups (STD, 116·6 (SE 5·4) mmHg; HFD-1, 115·0 (SE 5·6) mmHg; HFD-4, 115·0 (SE 5·8) mmHg), and was unaltered throughout the experiment (post-atropine: STD, 118·0 (SE 6·6) mmHg; HFD-1, 110·7 (SE 5·2) mmHg; HFD-4, 113·8 (SE 11·4) mmHg).

Glycaemia

There was no difference in the basal postprandial arterial glucose levels between the HFD-1-fed (7·1 (SE 0·4) mmol/l, n 9), HFD-4-fed (7·6 (SE 0·8) mmol/l, n 7) and STD-fed (6·5 (SE 0·1) mmol/l, n 10) rats, although the glucose levels tended to be higher in the HFD-fed rats. These glycaemic levels were not significantly affected either by the control RIST (STD, 6·5 (SE 0·1) mmol/l; HFD-1, 7·0 (SE 0·4) mmol/l; HFD-4, 7·8 (SE 0·8) mmol/l) or by atropine administration (STD, 6·5 (SE 0·1) mmol/l; HFD-1, 7·1 (SE 0·3) mmol/l; HFD-4, 7·8 (SE 1·0) mmol/l).

The fasting (24 h fast) arterial glycaemia was similar between the STD-fed (5·2 (SE 0·2) mmol/l, n 4) and HFD-4-fed (5·7 (SE 0·2) mmol/l, n 3) rats, despite it showing a tendency to increase with the HFD feeding.

Insulinaemia

Basal postprandial insulinaemia was not statistically different between the STD-fed (2·3 (SE 0·4) μg/l) and HFD-1-fed (4·2 (SE 0·5) μg/l) rats, but it was higher in the HFD-4-fed rats (4·6 (SE 0·7) μg/l; P < 0·05 v. STD). After the control RIST, insulin levels remained at baseline (STD, 2·4 (SE 0·4) μg/l; HFD-1, 4·6 (SE 0·3) μg/l; HFD-4, 6·1 (SE 1·2) μg/l). Atropine administration did not affect insulinaemia (STD, 1·9 (SE 0·4) μg/l; HFD-1, 6·0 (SE 0·9) μg/l; HFD-4, 4·9 (SE 0·7) μg/l), and the post-atropine RIST insulin levels were also similar to the baseline levels (STD, 2·3 (SE 0·4) μg/l; HFD-1, 5·4 (SE 1·2) μg/l; HFD-4, 4·9 (SE 0·8) μg/l).

Hepatic glutathione levels

Hepatic glutathione levels were different between the STD-fed (6·2 (SE 0·1) μmol/g, n 10) and HFD-fed (HFD-1, 5·1 (SE 0·2) μmol/g, n 8; P < 0·01 v. STD; HFD-4, 5·4 (SE 0·3) μmol/g, n 7; P < 0·05 v. STD) rats, although there was no difference between the HFD-1-fed and HFD-4-fed rats.

Insulin sensitivity assessment

Fasting IS, measured after a 24 h fast, in which HISS contribution is absent, was not different between STD-fed (80·2 (SE 5·4) mg glucose/kg bw, n 4) and HFD-4-fed (79·0 (SE 6·0) mg glucose/kg bw, n 3) groups, suggesting that the HFD does not induce changes in the HISS-independent component of insulin action (insulin action per se).

Postprandial insulin action comprises both the HISS-dependent and HISS-independent components. As indicated by the control RIST index (Fig. 1(a)), postprandial IS was lower in the HFD-4-fed rats (69·3 (SE 2·6) mg glucose/kg bw, n 7) than in both the HFD-1-fed (108·8 (SE 2·9) mg glucose/kg bw, n 9; P < 0·001) and STD-fed (185·8 (SE 5·6) mg glucose/kg bw, n 10; P < 0·001) rats, suggesting that insulin resistance increases with HFD duration. The difference in insulin action between the HFD-1-fed and STD-fed rats was also statistically significant (P < 0·001).

The insulin action observed after the blockade of the hepatic parasympathetic nerves (post-atropine RIST index), i.e. after completely abolishing the HISS component, corresponded to the HISS-independent insulin action (Fig. 1(a)). The HISS-independent (post-atropine) insulin action was similar between the STD-fed (76·9 (SE 3·8) mg glucose/kg
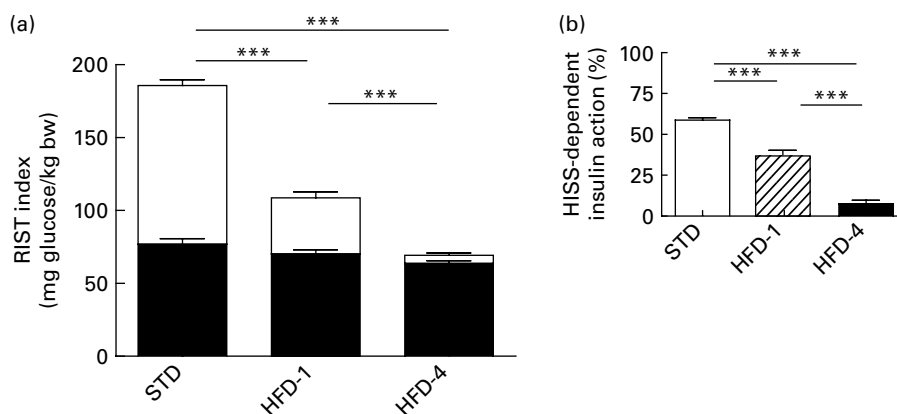
Fig. 1. Postprandial insulin sensitivity, assessed by the rapid insulin sensitivity test (RIST), was significantly lower in both the 1-week high-fat diet-fed (HFD-1, n 9) and 4-week HFD-fed (HFD-4, n 7) rats than in the control rats (standard diet (STD), n 10), an effect which seems to be primarily due to the impairment of the hepatic insulin-sensitising substance (HISS) pathway-dependent component. (a) RIST index for the HISS-dependent (□) and HISS-independent (post-atropine, ■) components of insulin action. The HISS-independent component represents the direct insulin action. The sum of both components represents the control RIST index (postprandial insulin action) for each group. (b) Relative contribution (%) of the HISS-dependent component to the overall postprandial insulin action in the STD-fed (□), HFD-1-fed (▨) and HFD-4-fed (■) rats. Data are means with their standard errors. *** Mean values were significantly different (P < 0·001). bw, Body weight.

bw, n 10) and HFD-1-fed (70·2 (SE 2·9) mg glucose/kg bw, n 9) rats, but it was slightly lower in the HFD-4-fed rats (63·8 (SE 1·8) mg glucose/kg bw, n 7; $P < 0.05$ $v.$ STD only).

In contrast, the HISS-dependent component of insulin action (Fig. 1(a)), obtained by subtraction of the post-atropine RIST index from the control RIST index, decreased significantly with HFD duration: STD, 108·9 (SE 3·9) mg glucose/kg bw; HFD-1, 38·6 (SE 4·2) mg glucose/kg bw ($P < 0.001$ $v.$ STD); HFD-4, 5·4 (SE 1·7) mg glucose/kg bw ($P < 0.001$ $v.$ STD and HFD-1).

HISS pathway contribution to overall insulin action (Fig. 1(b)) was reduced in the HFD-1-fed rats (37·1 (SE 3·4)%), and became relatively insignificant in the HFD-4-fed rats (7·5 (SE 2·2)%; $P < 0.001$ $v.$ HFD-1) than in the STD-fed rats (58·7 (SE 1·5)%; $P < 0.001$ $v.$ HFD-4 and HFD-1).

HISS-dependent insulin action was decreased by 64·6% in the HFD-1-fed rats than in the STD-fed rats, increasing to 95% suppression after 4 weeks of HFD feeding. In contrast, the HISS-independent component was less affected, since the fall was only significant in the HFD-4-fed rats, in which the HISS-independent insulin action was 17% lower than that in the STD-fed rats. HFD seems to affect the HISS pathway first, and that too to a greater extent than the HISS-independent component.

Fat mass assessment

Whole-body fat mass, determined by tetrapolar bioelectrical impedance, was significantly higher in the HFD-4-fed rats (n 7) than in the HFD-1-fed ($P < 0.05$) and STD-fed (n 9; $P < 0.001$ $v.$ HFD-1 and HFD-4) rats (Table 1). The non-fat mass was similar between the HFD-4-fed (314·1 (SE 8·0) g) and HFD-1-fed (323·0 (SE 6·9) g) rats, but it was higher in the STD-fed rats (347·4 (SE 7·5) g; $P < 0.05$ $v.$ HFD-4). In terms of bw percentage, whole-body fat mass was 25·5 (SE 1·5)% in the HFD-4-fed rats, 19·7 (SE 1·8)% in the HFD-1-fed rats ($P < 0.05$) and 8·5 (SE 0·9)% in the STD rats ($P < 0.001$ $v.$ HFD-1; $P < 0.001$ $v.$ HFD-4).

There was a negative polynomial (square) correlation between whole-body fat mass, measured by bioimpedance,

Fig. 2. There is a negative polynomial correlation between whole-body fat mass, measured by bioelectrical impedance, and insulin action. (a) Whole-body fat mass correlates (negative polynomial correlation) with total insulin action (R^2 0·81; $P < 0.05$). (b) There is also a negative polynomial correlation between hepatic insulin-sensitising substance (HISS) pathway-dependent insulin sensitivity and whole-body fat mass (●, R^2 0·87; $P < 0.01$); however, there is no correlation between whole-body fat mass and HISS-independent insulin action (▲; $P = 0.73$). bw, Body weight.

and overall postprandial insulin action, given by the control RIST index (R^2 0·81, n 16; $P < 0.05$) (Fig. 2). There was also a similar negative polynomial correlation between whole-body fat mass and HISS-dependent insulin action (R^2 0·87; $P < 0.01$) (Fig. 2). In contrast, there was no correlation between the HISS-independent insulin action (post-atropine RIST) and whole-body fat mass, either polynomial (R^2 0·20; $P = 0.73$) or linear (R^2 0·19; $P = 0.09$) (Fig. 2). These results suggest that the increase in whole-body adiposity affects the HISS-dependent component of insulin action (or vice versa) in a fast and pronounced way, whereas it is not associated with significant changes in the HISS-independent insulin action.

The individual weights of the abdominal fat depots collected (perinephric, epididymal and perienteric) increased significantly with HFD duration (Table 1). Total regional fat pad content, given by the sum of the individual fat pad weights, was also higher in the HFD-4-fed rats than in the HFD-1-fed rats ($P < 0.01$), which had higher content than the STD-fed rats ($P < 0.001$ $v.$ HFD-1 and HFD-4) as shown in Table 1.

The weight of each of the abdominal fat depots collected (perirenal, perienteric and epididymal) was also negatively correlated with IS as shown in Table 2. Indeed, a negative linear correlation was observed between each of the individual fat pad masses and overall insulin action (control RIST index), HISS-dependent and HISS-independent insulin action

Table 1. Whole-body fat, individual abdominal fat pads (perinephric, epididymal and perienteric) and total regional fat pad masses of rats fed the standard chow diet (STD) and of rats fed the high-fat diet (HFD) for 1 week (HFD-1) and 4 weeks (HFD-4)§
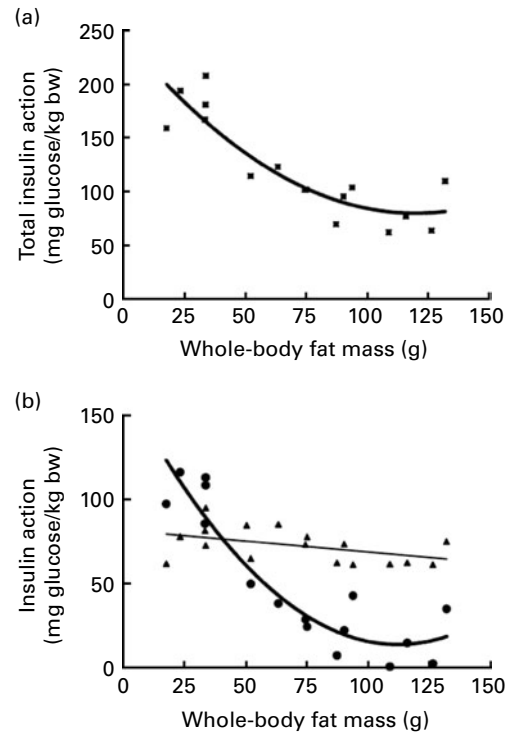
(Mean values with their standard errors)

	STD		HFD-1		HFD-4	
	Mean	SE	Mean	SE	Mean	SE
Whole-body fat mass (g)	32·5	3·7	79·8*	7·9	106·5*†	6·1
Perinephric fat mass (g)	4·8	0·5	9·6*	0·4	12·3*‡	0·7
Perienteric fat mass (g)	4·5	0·4	6·9*	0·3	8·2*	0·3
Epididymal fat mass (g)	4·4	0·3	7·1*	0·4	9·6*‡	0·7
Total fat pad mass (g)	13·7	1·0	23·7*	1·0	30·1*‡	1·4

* Mean values were significantly different compared with STD ($P < 0.001$).
† Mean values were significantly different compared with HFD-1 ($P < 0.05$).
‡ Mean values were significantly different compared with HFD-1 ($P < 0.01$).
§ Total fat pad mass was determined by the sum of perinephric, epididymal and perienteric fat pads masses.

Table 2. Correlation of both the components of whole-body insulin action (hepatic insulin-sensitising substance (HISS)-dependent and HISS-independent) with perinephric, perienteric and epididymal fat masses

(Mean values with their standard errors and R^2 values)

|	Total insulin action (mg glucose/kg bw)			HISS-dependent insulin action (mg glucose/kg bw)			HISS-independent insulin action (mg glucose/kg bw)		
	R^2	Mean slope	SE	R^2	Mean slope	SE	R^2	Mean slope	SE
Perinephric fat mass (g)	0·88	− 11·7*	1·2	0·85	− 10·1*§	1·1	0·37	− 1·6†	0·6
Perienteric fat mass (g)	0·73	− 20·7*	3·4	0·67	− 17·2*§	3·3	0·43	− 3·4‡	1·1
Epididymal fat mass (g)	0·73	− 16·8*	2·6	0·69	− 13·9*§	2·5	0·51	− 3·0‡	0·8

bw, Body weight; R^2, squared R of the linear regressions.
* Mean values were significantly different from zero ($P < 0.001$).
† Mean values were significantly different from zero ($P < 0.05$).
‡ Mean values were significantly different from zero ($P < 0.01$).
§ Mean values were significantly different from HISS-independent action ($P < 0.001$).

(Table 2). Similarly, there was also a negative linear correlation between the sum of the individual abdominal fat pad masses (total regional fat) and total insulin action (R^2 0·85; $P < 0.001$), HISS-dependent insulin action (R^2 0·79; $P < 0.001$) and HISS-independent insulin action (R^2 0·45; $P < 0.01$) (Table 2 and Fig. 3). However, this correlation was stronger for the HISS-dependent component (adjusted R^2, 0·78) than for the HISS-independent component (adjusted R^2, 0·41; $P < 0.001$) of insulin action (Fig. 3). In Fig. 3, the slopes of the linear regressions for total (− 5·4 (SE 0·6)) and HISS-dependent insulin action (− 4·6 (SE 0·6)) are more pronounced than the slopes of the linear regressions for the HISS-independent insulin action (− 0·9 (SE 0·3)), similar to that observed for the individual abdominal fat depots (Table 2), suggesting that the change in regional adiposity has a more pronounced impact on the HISS-dependent component than on the HISS-independent component.
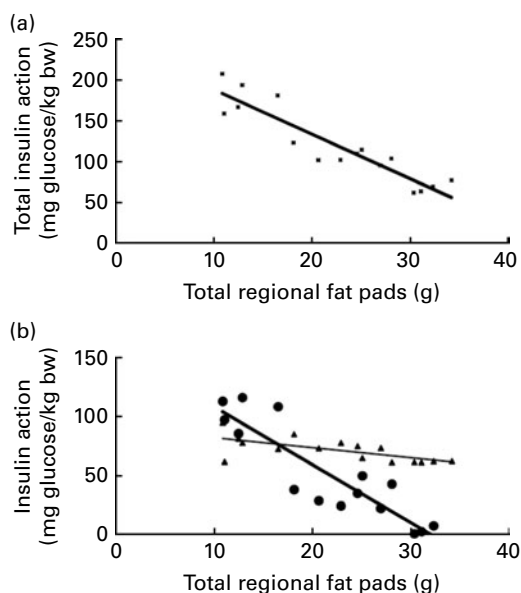
Fig. 3. Total regional fat pad mass and insulin action present a negative linear correlation. (a) Total regional fat pad mass v. overall insulin action (R^2 0·85, adjusted R^2 0·84; $P < 0.001$). (b) Regional fat mass v. hepatic insulin-sensitising substance (HISS)-dependent (●, R^2 0·79, adjusted R^2 0·78; $P < 0.001$) and HISS-independent (▲, R^2 0·45, adjusted R^2 0·41; $P < 0.01$) insulin action. The total regional fat pad mass is given by the sum of the individual weights of the perinephric, perienteric (abdominal) and epididymal fat depots.

Discussion

In the present study, we observed that increasing HFD durations lead to increasing insulin resistance, specifically in the postprandial state, with less impact on fasting IS. Our data suggest that the major contribution to the HFD-induced postprandial insulin resistance derives from the significant impairment of HISS action, which correlates with adiposity.

Methodological considerations

Most methods used to assess IS are based solely on the steady-state glucose and insulin concentrations observed in the fasted state, disregarding postprandial insulin action. We used the transient euglycaemic clamp, RIST, to assess IS, since this is the most appropriate test for the present study, which requires more than one evaluation of IS in the postprandial state. Anaesthesia does not alter the outcome of the RIST[11], and insulin administered as a bolus does not induce vagal impairment, as observed for the methods that require insulin infusions that are to be given for a longer duration resulting in hyperinsulinaemic states[30]. The RIST is reproducible within the same experiment (up to four consecutive RIST) while retaining high sensitivity[21,27], and it has been used in several animal models[11,21,27,31,32] and in human subjects[33], with results comparable to those obtained using the insulin tolerance test[14].

The use of atropine to specifically block the HISS-dependent component of insulin action has been validated already by numerous studies performed in several different models, showing no additional effects on insulin action per se or insulinaemia[6,11,17,21,32,34,35]. Moreover, the impact on postprandial IS by the use of atropine equals that observed after selective hepatic parasympathetic surgical ablation[11,15]. The fasted-state experiments further provided an estimation of the efficacy of the blockade of parasympathetic nerves by atropine.

Our aim was to compare a regular STD with a diet that was clearly rich in fat content (HFD). We used a 60 % HFD (energetic contribution of fat to the diet) in order to detect changes induced by dietary fat in a short period of time. This was done to avoid having ageing as a confounding factor for the present study, since we reported previously that ageing affects HISS-dependent insulin action[34].

The effect of high-fat diet on hepatic glutathione

Reports from several groups suggest that HFD induces oxidative stress[36–38] in a process that may involve glutathione depletion[37]. Whether the oxidative stress caused by an HFD-induced depletion of glutathione is directly linked to the impairment of IS remains unclear. We determined glutathione content in the liver, since a decrease in hepatic glutathione could lead not only to a state of reduced free radical quenching, but also to a compromise in postprandial insulin action as glutathione is required for HISS-dependent insulin action[18].

The hepatic glutathione levels were higher in the STD-fed rats than in the HFD-fed rats, but there was no difference between the HFD-1- and HFD-4-fed rats, although postprandial insulin resistance was aggravated during these periods. These results suggest that glutathione impairment might not be directly involved in the progressive increase of insulin resistance with HFD duration, although it might play a role in the initiation of the process.

The effect of high-fat diet on insulin sensitivity

The HFD induced postprandial insulin resistance, which was already present after 1 week (HFD-1: approximately 59 % of the IS in the STD-fed rats) and was further aggravated by 4 weeks on HFD feeding (approximately 37 % of the IS observed in the STD-fed rats), in accordance with the previous observations[39–41].

Postprandial insulin action depends not only on insulin *per se*, but also on a hepatic mechanism that involves both parasympathetic nerves[15] and glutathione[18] – the HISS-dependent component of insulin action[20].

HISS-independent insulin action was unaltered during the HFD-1 feeding, and it was only slightly decreased in the HFD-4-fed group. This observation possibly explains the fact that not all authors observe differences in insulin-induced glucose utilisation between HFD-fed and control animals[42], although a majority do[39–41]. Such differences could arise from variations in diet composition, diet duration and the methodology used to assess IS. HFD feeding that is carried out for a period longer than 4 weeks may also lead to the deterioration of the HISS-independent component of insulin action, and result in fasting hyperglycaemia or frank diabetes.

The reduction in HISS-dependent insulin action paralleled the reduction in total postprandial IS: an impairment of about 65 % was observed after 1 week of HFD feeding, and by 4 weeks of HFD feeding, HISS action was completely absent. Since 1 week of HFD feeding was not sufficient to affect HISS-independent insulin action, HISS impairment seems to be primarily responsible for the early postprandial insulin resistance associated with HFD.

Because there were no differences in hepatic glutathione levels between the HFD-1-fed and HFD-4-fed rats, the progressive decrease of HISS action appears to be solely due to the impairment of hepatic parasympathetic nerves.

The postprandial insulin resistance described for the HFD-induced obesity model studied herein is mechanistically different from that observed in other animal models of obesity, such as the genetically obese Zucker (fa/fa) rats, in which the two components of insulin action were impaired in the same proportion[21], probably due to post-insulin receptor abnormalities at the level of the skeletal muscle[43,44]. This does not seem to be the case with HFD-induced model, in which insulin resistance was observed only in the postprandial state for the period studied, which was caused by impairment of HISS action.

High-fat diet-induced obesity, parasympathetic dysfunction and postprandial insulin resistance

The importance of hepatic parasympathetic nerves in insulin-induced glucose homoeostasis has been addressed previously[6,11,15,45,46]. Work done by others has shown functional decrease of intra-hepatic muscarinic nerves in livers of streptozotocin-treated rats, suggesting that impairment of hepatic parasympathetics can deprive the chronically diabetic patient of an adequate response to postprandial hyperglycaemia[46]. More recently, studies performed by Li *et al.*[47] in mice suggest that the hepatic muscarinic acetylcholine receptors are not essential to ensure adequate IS in the fasted state. These findings are in agreement with our previous data, showing that although hepatic parasympathetic nerves are not critical for fasting IS, they are required for proper postprandial IS as we reported previously[11]. Furthermore, Li *et al.*[47] did not observe any effect of overexpression of hepatic muscarinic receptors in the fasted state, which in the light of the HISS hypothesis should be expected, since besides parasympathetics, hepatic glutathione content must also increase, which is achieved by feeding[18].

Several studies have addressed the association between obesity and parasympathetic dysfunction[7,22,24], and although no tissue specificity of such parasympathetic dysfunctions has been reported, the liver seems to be affected[7]. A hepatic parasympathetic dysfunction associated with obesity could result in the impairment of the HISS pathway and, consequently, of the postprandial insulin action.

Obesity has been associated with an imbalance between sympathetic and parasympathetic nervous systems caused by increased sympathetic tone[24,48], therefore leading to the impairment of parasympathetic activity[24,49]. The existence of an association between the parasympathetic nervous system, visceral fat and liver, proposed by Fliers *et al.*[50], together with the observations suggesting that attenuation of parasympathetic activity promotes insulin resistance[10,11,15,21,32,50–53], provides further support for our conclusions. In addition to hepatic parasympathetic nerves, acetylcholine and NO are also intermediates in the HISS pathway, and functional impairment of either can result in HISS-dependent insulin resistance[15–18,21]. Therefore, recent reports of impaired acetylcholine-induced activation of NO synthase[54] and NO-related abnormalities in HFD-induced obese models[55,56] are also consistent with our data.

Fat mass and insulin resistance

The decrease in postprandial IS observed in the HFD-fed rats was associated with the increase in adipose mass as in previous reports[38,57–59]. The present results add to this by showing that the effect of adiposity is predominantly mediated by the abrupt decrease in the HISS-dependent component of insulin action. Indeed, there was a significant polynomial

correlation between whole-body fat mass and HISS-dependent insulin action, which was similar to the correlation between whole-body fat mass and total insulin action, whereas the HISS-independent insulin action did not correlate with the whole-body fat mass (Fig. 2). On the other hand, the regional abdominal fat mass presents a linear correlation with both components of insulin action, although this correlation was stronger for the total and for HISS-dependent insulin action than for the HISS-independent insulin action, as given by the adjusted R^2 and linear regression slopes (Table 2 and Fig. 3). This suggests either that both the HISS-dependent and HISS-independent components of insulin action decrease with adiposity, or that the fat accumulation results from the observed insulin resistance. Nevertheless, we also showed that the HISS-independent insulin action (insulin action *per se*) tends to decrease at a slower rate than the HISS-dependent action.

In the present study, we evaluated fat content by measuring both whole-body fat and specific regional fat pad masses. The measurements of whole-body fat and specific abdominal fat depots, which provided us with an assessment of the fat accumulation in adipose tissue in response to the HFD, were shown for the first time to be associated with pronounced insulin resistance due to HISS pathway impairment. These results are in agreement with the previous reports associating high adiposity with severe insulin resistance, both in human subjects[24] and in animal models[41,58–60]. Despite this strong correlation, we cannot differentiate cause and effect, i.e. whether the inadequate HISS-dependent insulin action causes the high-fat storage and concomitant adiposity, or vice versa. However, the HISS hypothesis proposes that the absence of HISS action shifts the balance of nutrient storage away from glycogen in skeletal muscle towards fat deposition.

Studies performed by other groups support the involvement of visceral and/or abdominal fat in the process of obesity-related insulin resistance[24,57], and report that visceral fat removal improves IS in animal models of obesity[57,58]. However, the increase in regional abdominal fat pad mass and that in whole-body fat mass were correlated, suggesting that HFD does not induce fat accumulation in any specific site, but rather induces a general increase in adiposity, i.e. whole-body fat, which is similar to the observations reported by others[59,60]. Therefore, the development of insulin resistance due to an inadequate HISS pathway action does not seem to be associated with fat deposition in any particular depot.

In conclusion, HISS pathway is essential for postprandial insulin action. The present study shows that impairment of HISS action is the major contributor for HFD-induced insulin resistance. It was observed for the first time that animals fed an HFD present increasing disturbances of glucose homoeostasis observed primarily and more severely in the postprandial state than in the fasted state: HFD resulted in insulin resistance, higher insulinaemia and slightly higher (NS) glycaemia in the postprandial state readily after 1 week of HFD feeding. Furthermore, the HISS impairment correlated with adiposity, as it did with the overall postprandial insulin resistance. In the HFD-4-fed group, fasted rats did not show significant alterations in insulin responsiveness.

Thus, our data suggest that HFD-induced obesity leads to a progressive decline in HISS-dependent IS, resulting in postprandial insulin resistance and predisposing to frank diabetes.

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