

Resolution of glucose intolerance in long-term high-fat, high-sucrose-fed mice

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Abstract

The high-fat, high-sucrose diet (HFSD)-fed C57Bl/6 mouse is a widely used model of prediabetes. However, studies typically implement a relatively short dietary intervention lasting between 4 and 16 weeks; as a result, little is known about how a long-term HFSD influences the metabolic profile of these mice. Therefore, the aim of this investigation was to examine the effects of consuming a HFSD for 42 weeks on the development of hyperinsulinaemia and glucose intolerance in male C57Bl/6 mice. Two cohorts of HFSD mice were studied at independent institutes and they underwent an oral glucose tolerance test (OGTT) with measures of plasma insulin and free fatty acids (FFA). Age-matched chow-fed control mice were also studied. The HFSD-fed mice were hyperinsulinaemic and grossly obese, being over 25 g heavier than chow-fed mice, which was due to a marked expansion of subcutaneous adipose tissue. This was associated with a 3-fold increase in liver lipid content. Glucose tolerance, however, was either the same or better than control mice due to the preservation of glucose disposal as revealed by a dynamic stable isotope-labelled OGTT. In addition, plasma FFAs were suppressed to lower levels in HFSD mice during the OGTT. In conclusion, we have made the paradoxical observation that long-term HFSD feeding results in the resolution of glucose intolerance in the C57Bl/6 mouse. Mechanistically, we propose that the gross expansion of subcutaneous adipose tissue increases the glucose disposal capacity of the HFSD-fed mouse, which overcomes the prevailing insulin resistance to improve glucose tolerance.

Key Words

- ▶ glucose tolerance
- ▶ insulin resistance
- ▶ diet-induced obesity
- ▶ high-fat
- ▶ high-sucrose diet

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Introduction

With the increasing prevalence of obesity and insulin resistance, the reliance on mouse models of metabolic disease has increased dramatically over the last decade (Kowalski & Bruce 2014). The high-fat, high-sucrose-fed C57Bl/6 mouse is one of, if not the most commonly used

model of metabolic disease and prediabetes as it rapidly and robustly develops obesity, insulin resistance and glucose intolerance (Surwit *et al.* 1988, 1991, Winzell & Ahren 2004, Turner *et al.* 2013, Attane *et al.* 2016). Indeed, this model has been at the forefront of preclinical

research for pharmacological testing and is extensively used as the background strain for genetically engineered mice (Surwit *et al.* 1988, 1991, Winzell & Ahren 2004, Attane *et al.* 2016, Fisher-Wellman *et al.* 2016).

The widespread use of the high-fat, high-sucrose-fed C57Bl/6 mouse has no doubt advanced our understanding of the mechanisms that regulate insulin action and glycaemic control (Winzell & Ahren 2004, Kowalski & Bruce 2014). However, it is important to highlight that unlike humans who may live with insulin resistance and impaired glycaemic control for decades, the majority of mouse studies using this model have been performed over relatively short time frames. Typical studies using the diet-induced model of obesity and prediabetes in this strain are conducted over a period of between 4 and 16 weeks (for example, see Ahren *et al.* 1997, Park *et al.* 2005, Winzell *et al.* 2007, Turner *et al.* 2013, Raichur *et al.* 2014, Wu *et al.* 2014, Jordy *et al.* 2015, Kowalski *et al.* 2015a,b,c, Kraakman *et al.* 2015, Meex *et al.* 2015, Selathurai *et al.* 2015, Williams *et al.* 2015, Attane *et al.* 2016, Chaurasia *et al.* 2016, Fisher-Wellman *et al.* 2016, Mottillo *et al.* 2016, Murphy *et al.* 2016, Nagarajan *et al.* 2016, Petersen *et al.* 2016). Given the basis of using mice is to largely model the human condition, it is therefore critical to conduct long-term high-fat, high-sucrose feeding studies to examine how this influences the metabolic profile of C57Bl/6 mice. Such studies are essential to increase the ability to translate the findings from preclinical studies to the human condition. Surprisingly, only a few studies have specifically examined the impact of long-term high-fat, high-sucrose feeding on the metabolic profile of C57Bl/6 mice (Ahren & Pacini 2002, Winzell & Ahren 2004, Sumiyoshi *et al.* 2006, Ahren *et al.* 2010, Agardh & Ahren 2012), and interestingly not all these studies assessed glucose tolerance (Winzell & Ahren 2004, Ahren *et al.* 2010).

With this in mind, we aimed to examine the effects of consuming a long-term (i.e. 42-weeks) high-fat, high-sucrose diet (HFSD) on the development of hyperinsulinaemia and glucose intolerance in C57Bl/6 mice. Paradoxically, despite the fact that mice maintained on the long-term HFSD exhibited marked obesity and hyperinsulinaemia, glucose tolerance was the same or better than that of chow-fed age-matched control mice.

Materials and methods

Animals

Male C57Bl/6J mice were used. Two independent cohorts of C57Bl/6J mice were studied; Cohort 1 was maintained

at Monash University (Melbourne, Australia), whereas Cohort 2 was studied at the Baker IDI Heart and Diabetes Institute (Melbourne, Australia). Mice for Cohort 1 were sourced from Monash University Animal Services (designated C57Bl/6JMARF) whereas mice for Cohort 2 were from the Alfred Medical Research Precinct Animal Services (C57Bl/6J). All experiments were approved by the Monash University Animal Research Platform Animal Ethics Committee or the Alfred Medical Research and Education Precinct Animal Ethics Committee and were conducted in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation. Mice were maintained at $22\pm 1^\circ\text{C}$ on a 12-h light/darkness cycle, with free access to food and water. At 8 weeks of age, mice were randomly assigned to one of two dietary conditions, either continuing to be maintained on a standard chow control diet or were switched to a HFSD (42% energy from fat, 20% by weight from sucrose, Specialty Feeds SF4-001, Glen Forrest, WA, Australia) for 42 weeks. The chow diet used for Cohort 1 contained 12.6 kJ/g with 5% energy from fat (Barastoc Rat and Mouse, Ridley AgriProducts, Melbourne, Australia), whereas the chow diet used for Cohort 2 contained 14 kJ/g with 4.8% energy from fat (Specialty Feeds, Glen Forrest, WA, Australia). The numbers of mice in each group were as follows: Cohort 1 chow ($N=7$); Cohort 1 HFSD ($N=17$); Cohort 2 chow ($N=6$) and Cohort 2 HFSD ($N=10$).

Body composition

Fat and lean mass were determined in all mice in Cohort 2 using the EchoMRI 4-in-1 (Echo Medical Systems, Houston, TX, USA).

Oral glucose tolerance test (OGTT)

For Cohort 1, OGTTs were conducted on conscious mice after 22 and 42 weeks of HFSD, whereas for Cohort 2, an OGTT was only performed after 42 weeks of HFSD. The week prior to any OGTT experiment, daily sham gavages were performed on every mouse, typically for 4–5 consecutive days. This involved scruffing mice and performing an oral gavage without any liquid in the syringe. The tails of the mice were also gently 'milked' to simulate what would occur during the blood collection period during the OGTT. This routine familiarised the mice to the OGTT procedure as well as to the handlers themselves, thus minimising the stress response. On the actual day of experimentation, following a 5-h fast (initiated at 07:00h),

mice were weighed and blood (~30 µL) was obtained from the tail vein in the conscious state for determination of fasting glucose, insulin and free fatty acids (FFAs). For Cohort 1, mice received a fixed dose of 50 mg of glucose, whereas mice in Cohort 2 underwent a stable isotope-labelled OGTT where a 1:1 mix of 6,6-[²H]glucose and 2-[²H]glucose was administered at a dose of 2 g/kg lean mass. The administration of isotopically labelled glucose provides the assessment of dynamic glucose disposal, the pattern of endogenous glucose production (EGP) and hepatic futile glucose cycling (Kowalski *et al.* 2015c, 2016). For all studies, glucose was administered via oral gavage, and blood samples were obtained at 15, 30, 45, 60, 90 and 120 min. Blood glucose was measured with a hand-held glucose metre (Accu-Check, Roche, NSW, Australia). For the 42-weeks OGTTs, blood was obtained for the measurement of plasma insulin and FFAs under fasting conditions and again at 15 min after glucose administration. In Cohort 2, additional blood samples were obtained prior to and at 15, 30, 60 and 120 min after glucose gavage for the determination of glucose tracer enrichment.

Plasma analysis

Plasma insulin was measured by ELISA (Millipore). Plasma FFAs were measured spectrophotometrically by an enzymatic colorimetric assay (NEFA C Kit; Wako Chemicals). The enrichment of glucose tracer in the plasma was determined by gas chromatography–mass spectrometry (GC–MS) according to our previously described methods (Kowalski *et al.* 2015c, 2016).

Tissue weights

Three days after the OGTT, mice in Cohort 1 were humanely killed (CO₂ asphyxiation) and tissues including the liver, heart, quadriceps muscle as well as the inguinal and epididymal fat pads were collected, weighed and frozen. Triglyceride (TAG) content was determined in liver, heart and muscle using a colorimetric assay

(Triglycerides GPOPAP; Roche Diagnostics) as described previously (Kowalski *et al.* 2015c, 2016). Liver and muscle glycogen content were measured in KOH digests subjected to enzymatic hydrolysis followed by the quantification of free glucosyl units via glucose oxidase assay.

Statistics

All data are reported as mean ± s.e.m. Data were analysed by independent *t*-test or two-way repeated-measures ANOVA where appropriate. For two-way repeated measures ANOVA, Holm-Sidak's multiple comparisons tests were used to determine the differences between groups. Statistical significance was set at *P* < 0.05. Incremental area under the curve (AUC) for the blood glucose response during the OGTT was calculated using the trapezoidal method.

Results

Cohort 1

After 22 weeks of dietary intervention, mice fed the HFSD were heavier and had impaired glucose tolerance compared with chow-fed mice (Fig. 1A, B and C). Long-term (42 weeks) HFSD caused a marked elevation in body mass, with mice fed the HFSD being ~25 g heavier than those maintained on the chow diet (41.1 ± 1.8 vs 67.2 ± 1.3 for chow and HFSD, respectively; *P* < 0.0001; Fig. 2A). The mass of the liver (+80%; *P* < 0.001), heart (+25%; *P* < 0.001) and inguinal subcutaneous fat pad (+300%, *P* < 0.0001) were increased in the HFSD mice, but somewhat surprisingly the mass of the epididymal fat pad was similar between the chow and HFSD mice (Fig. 2A, B, C, D and E). Fasting blood glucose and FFA levels were similar in the 42-weeks chow and HFSD mice, although fasting plasma insulin was elevated in those fed the HFSD (Table 1). In contrast to our observation at 22 weeks in which the HFSD mice were glucose intolerant, glucose levels during the OGTT as

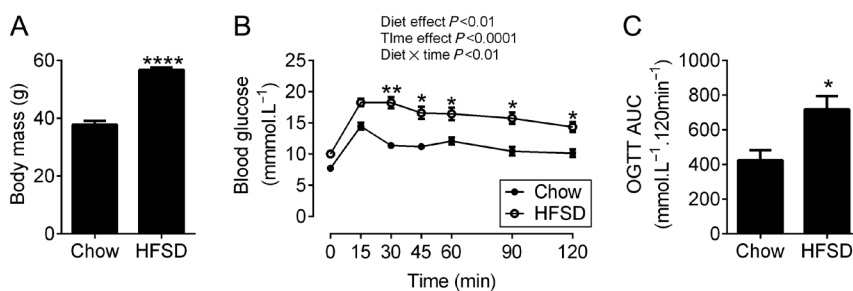
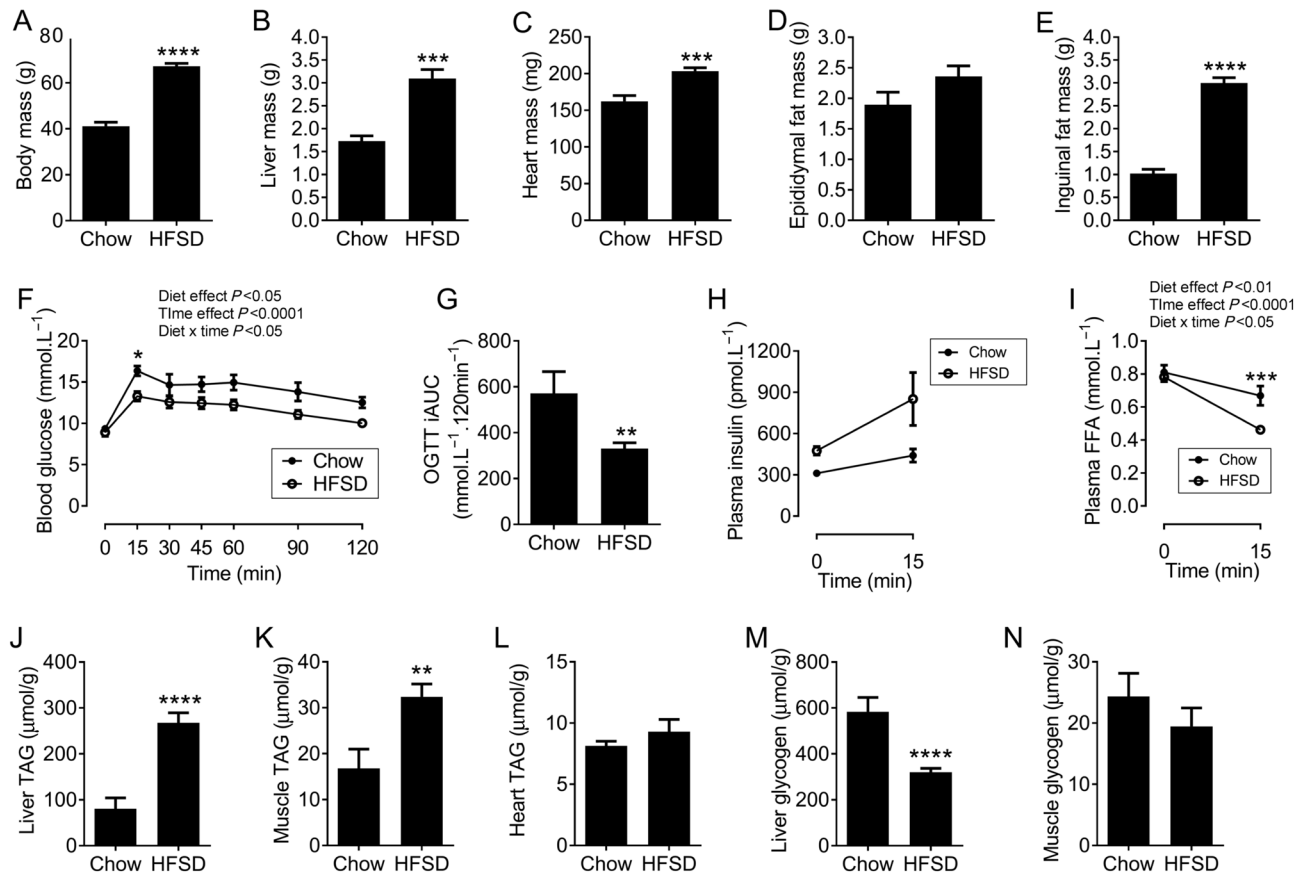


Figure 1 Body mass (A) and glucose tolerance (B and C) in mice fed either a chow or high-fat, high-sucrose diet (HFSD) for 22 weeks. Data are mean ± s.e.m. **P* < 0.05; ***P* < 0.01; *****P* < 0.0001 vs chow at a specified time point. *N* = 7 for chow; *N* = 17 for HFSD.

**Figure 2**

The effect of 42 weeks of high-fat, high-sucrose diet (HFSD) on body mass (A), liver mass (B), heart mass (C), epididymal fat pad (D) and inguinal fat pad (E) mass, glucose tolerance (F and G), plasma insulin (H) and FFA levels (I) during the OGTT, triglyceride content in the liver (J), muscle (K) and heart (L), and glycogen content in liver (M) and muscle (N). Data are mean \pm s.e.m. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ vs chow at specified time point. $N = 7$ for chow; $N = 17$ for HFSD.

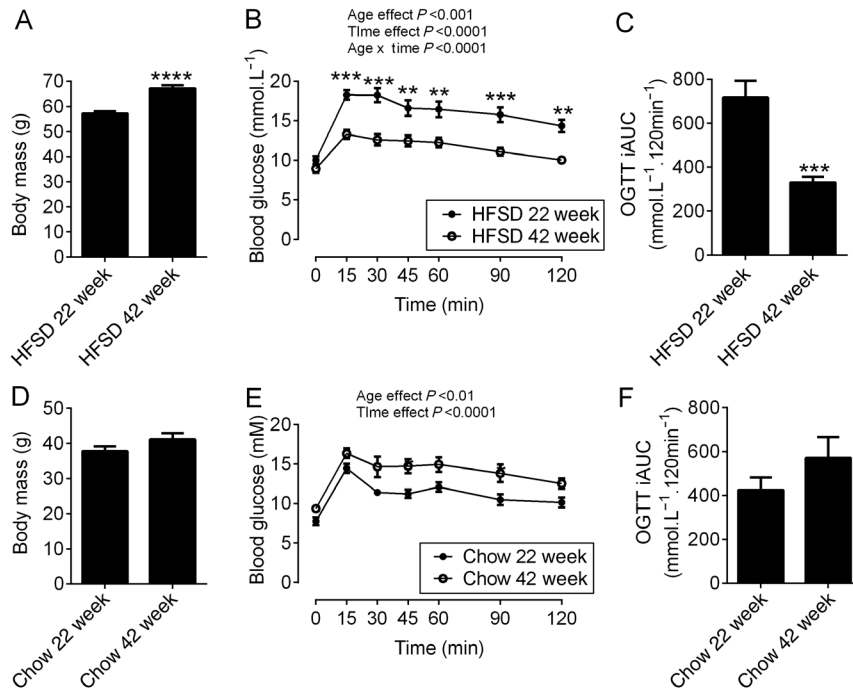
well as the glucose AUC were lower in the 42-weeks HFSD-fed mice (Fig. 2F and G). Plasma insulin levels during the OGTT (i.e. at 15 min) were not statistically different between the two groups (Fig. 2H); however, plasma FFAs were suppressed to a greater extent in the mice fed the HFSD (Fig. 2I). Liver and muscle TAG content were increased by >300% and 200%, respectively, in the long-term HFSD mice (Fig. 2J and K); yet, the TAG content of the heart was similar to that in age-matched chow-fed control mice (Fig. 2H). The

HFSD caused a 50% reduction liver glycogen content (Fig. 2M), whereas muscle glycogen was not different between chow and HFSD conditions (Fig. 2N). When comparing the metabolic parameters of the HFSD mice at 22 and 42 weeks, there was a clear improvement in glucose tolerance from weeks 22 to 42, despite the significant increase in body mass over time (Fig. 3A, B and C). In contrast, although body mass was similar (Fig. 3D), there was a modest impairment in glucose tolerance in chow-fed mice from 22 to 42 weeks

Table 1 Basal metabolic data of mice fed a HFSD for 42 weeks.

	Cohort 1		Cohort 2	
	Chow	HFSD	Chow	HFSD
Fasting blood glucose (mmol/L)	9.4 \pm 0.3	8.9 \pm 0.5	8.7 \pm 0.6	10.5 \pm 0.2*
Fasting plasma insulin (pmol/L)	312 \pm 26	475 \pm 31*	399 \pm 60	2295 \pm 270**
Fasting plasma FFA (mmol/L)	0.8 \pm 0.1	0.8 \pm 0.1	1.1 \pm 0.1	0.7 \pm 0.1***

Data are mean \pm s.e.m. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$ for the specified chow vs HFSD comparison within each cohort. Statistical analysis performed using an independent t-test.

**Figure 3**

Comparison of the effects of 22 vs 42 weeks of HFSD or chow feeding on body mass (A, D) and glucose tolerance (B, C, E and F). These data are also presented in Figs 1 and 2. Data are mean \pm s.e.m. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ at specified time point. $N = 7$ for chow; $N = 17$ for HFSD.

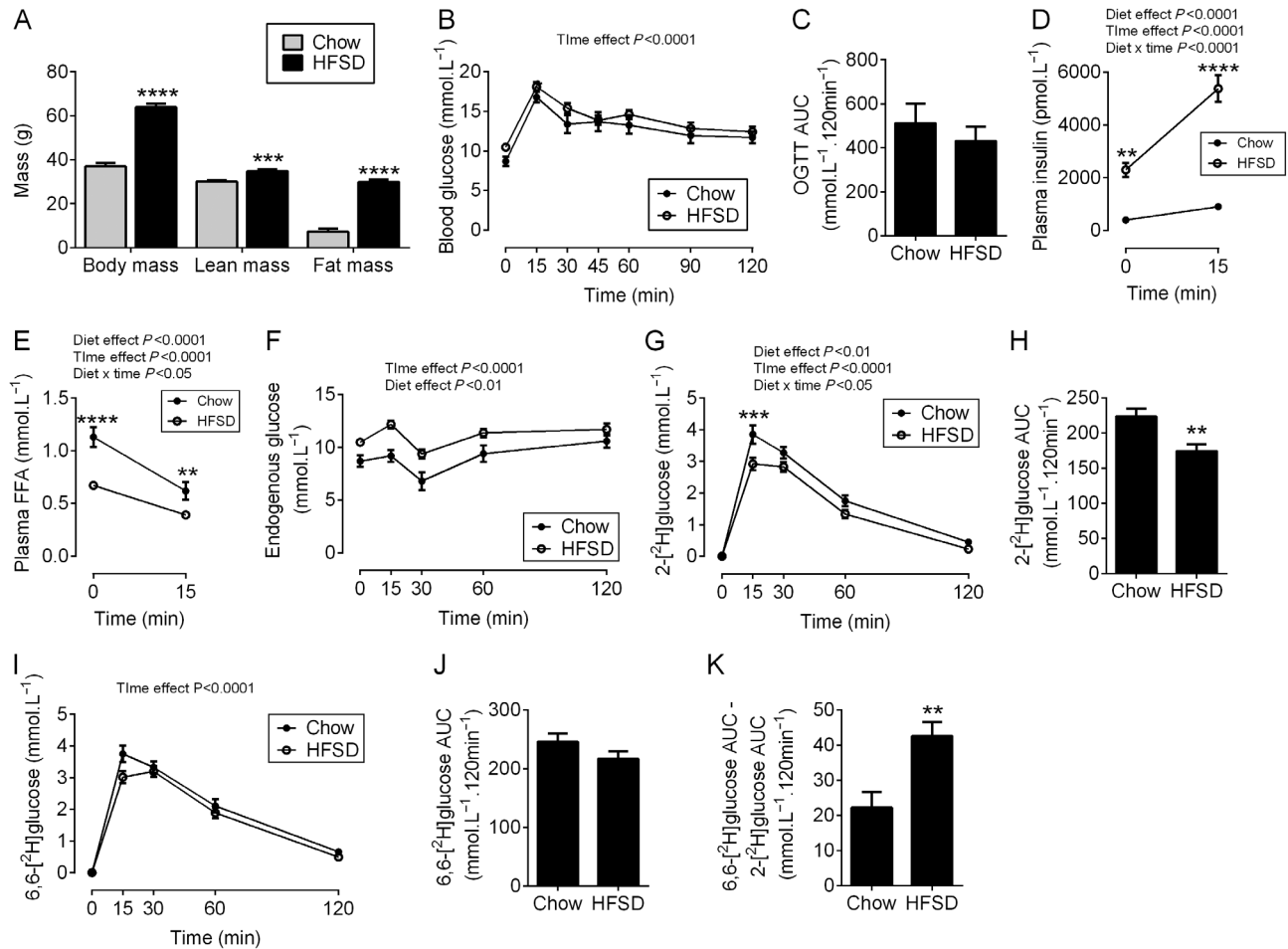
(main effect for age $P < 0.01$; Fig. 3E), although the AUC was not significantly different (Fig. 3F).

Cohort 2

Given the paradoxical finding that long-term HFSD feeding improved glucose tolerance, an additional cohort of mice fed a HFSD for 42 weeks was studied at another institute. Body composition of this chow and HFSD mouse cohort is presented in Fig. 4A. Similar to Cohort 1, mice fed the HFSD were ~ 27 g heavier than their chow-fed counterparts (37.0 ± 1.5 vs 63.8 ± 1.7 g for chow and HFSD, respectively; $P < 0.0001$; Fig. 4A). Although lean mass was increased by $\sim 16\%$ ($P < 0.001$; Fig. 4A) in the HFSD group, the increase in body mass was mostly attributed to the additional ~ 22 g of fat carried by the HFSD mice (7.4 ± 1.3 vs 29.9 ± 1.2 for chow and HFSD, respectively; $P < 0.0001$; Fig. 4C). In this cohort, the HFSD mice displayed moderately elevated fasting blood glucose levels and dramatic hyperinsulinaemia; however, fasting plasma FFAs were lower compared with chow-fed mice (Table 1). Despite the stark difference in adiposity, the HFSD mice exhibited the same glucose tolerance as chow-fed controls (Fig. 4B and C), whereas 15 min after gavage, insulin levels were markedly higher in the HFSD mice (Fig. 4D). As with Cohort 1, FFAs were also lower in the HFSD mice 15 min after glucose administration (Fig. 4E).

Given that we used stable isotope-labelled OGTT for Cohort 2, we were able to use mass spectrometry to

resolve the postprandial blood glucose concentrations into that derived from endogenous sources (liver and kidney) or that from the administered oral glucose load (exogenous) (Fig. 4F, G, H, I, J and K), thus permitting a readout of dynamic glucose turnover (Vaitheesvaran *et al.* 2010). Endogenous glucose concentrations were higher in the HFSD mice compared with chow controls (Fig. 4F). With the determination of load-derived glucose, as measured with two simultaneously administered complementary tracers, one being subjected to hepatic recycling (2-[2 H]glucose) while the other not (6,6-[2 H]glucose) (Efendic *et al.* 1985, Vaitheesvaran *et al.* 2010, Kowalski *et al.* 2015c, 2016), we were able to assess dynamic whole body glucose disposal and hepatic futile glucose cycling. Hepatic futile glucose cycling is an index of the efficiency of hepatic glucose uptake and is a function of the activity of hepatic glucokinase relative to that of glucose-6-phosphatase (hepatic glucose phosphorylation potential). Interestingly, the 2-[2 H]glucose excursion was lower in the HFSD mice, particularly at 15 min, which also resulted in a reduction in the 2-[2 H]glucose AUC, thus indicating a more rapid clearance of this tracer in the HFSD mice (Fig. 4G and H). However, the 6,6-[2 H]glucose concentrations and AUC did not significantly differ between HFSD and chow-fed mice (Fig. 4I and J), indicating that hepatic futile glucose cycling (difference in 6,6-[2 H] and 2-[2 H]glucose AUCs) was elevated in the HFSD-fed mice (Fig. 4K). Thus, during the OGTT, long-term HFSD fed mice maintained the same absolute whole

**Figure 4**

Body composition (A), blood glucose (B and C), plasma insulin (D), FFAs (E), endogenous glucose levels (F), disposal of 2-[²H]glucose (G and H) and 6,6-[²H]glucose (I and J) and hepatic futile glucose cycling (K) during the stable isotope-labelled OGTT. Data are mean \pm s.e.m. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ vs chow at a specified time point. $N = 6$ for chow; $N = 10$ for HFSD.

body glucose disposal rates as chow controls in the face of modestly diminished hepatic glucose uptake (elevated futile glucose cycling).

Discussion

The high-fat, high-sucrose-fed C57Bl/6 mouse is the most widely used rodent model of metabolic disease and prediabetes as it is highly susceptible to weight gain and develops insulin resistance and glucose intolerance within days to weeks (Park *et al.* 2005, Turner *et al.* 2013). Despite its widespread use, relatively little attention has been devoted to characterising the long-term metabolic effects of high-fat, high-sucrose feeding in the C57Bl/6 mouse. Here, in two cohorts of mice studied at independent institutes, we investigated the metabolic

adaptations induced by 42 weeks of high-fat, high-sucrose feeding in male C57Bl/6 mice. Paradoxically, despite the fact that mice maintained on the long-term HFSD were grossly obese, hyperinsulinaemic and had >3-fold higher hepatic TAG content, glucose tolerance was either the same or better than that of age-matched chow-fed mice. These unexpected results reveal unique insight into the metabolic adaptations that occur in response to the consumption of a long-term HFSD in C57Bl/6 mice.

The most intriguing finding of the current study was the resolution of glucose intolerance between 22 and 42 weeks of high-fat, high-sucrose feeding in our first cohort of mice. In fact, in this cohort, the 42-week HFSD fed mice exhibited glucose tolerance that was significantly better than that of the chow-fed control mice. In contrast, there was a modest impairment in glucose tolerance in chow-fed mice over time, suggesting some unique event

caused by long-term HFSD feeding led to the resolution of glucose intolerance. Given the unexpected nature of this result, we studied an additional cohort of mice at an independent institute to either confirm or refute this finding. Similar to our first cohort, the second cohort of mice fed the HFSD for 42 weeks had glucose tolerance that was identical to that of the chow control group. Although an OGTT was not performed in this second cohort at 22 weeks, as in Cohort 1, to confirm the presence of glucose intolerance, we and others have previously shown that mice fed the HFSD for between 12 and 20 weeks in this facility consistently developed glucose intolerance (Kowalski *et al.* 2011, Nicholls *et al.* 2011, Turner *et al.* 2013, Lancaster *et al.* 2016). We therefore have good reason to believe that the HFSD fed mice in this second cohort were indeed glucose intolerant at earlier time points and, as in Cohort 1, this resolved by 42 weeks. Together, these findings from two independent cohorts, provide a clear demonstration that long-term high-fat, high-sucrose feeding is not necessarily accompanied by glucose intolerance.

Despite the resolution of glucose intolerance in the HFSD mice, some defects in glucose homeostasis were still evident. The HFSD in Cohort 2 exhibited a mild fasting hyperglycaemia, whereas both cohorts of HFSD mice displayed fasting hyperinsulinaemia, which is indicative of insulin resistance. In addition, the HFSD mice in Cohort 2 were markedly hyperinsulinaemic during the OGTT. The use of a stable isotope-labelled OGTT allowed blood glucose to be resolved into that derived from endogenous or exogenous (administered oral glucose load) sources. Although the pattern of endogenous glucose concentrations throughout the OGTT was relatively similar between groups, the concentrations were indeed elevated in the HFSD-fed mice, indicating a slightly expanded whole body glucose pool size. Interestingly, during the OGTT, the long-term HFSD-fed mice maintained the same absolute whole body glucose disposal (6,6-²H]glucose clearance) as chow controls despite the fact that net hepatic glucose uptake rates were modestly diminished, indicating elevated rates of futile glucose cycling. Specifically, the fact that the 2-²H] glucose tracer was cleared more rapidly than the 6,6-²H glucose tracer (despite being given in equimolar amounts) in HFSD mice indicates that a greater portion of the administered glucose was taken up by hepatocytes, phosphorylated by glucokinase and then rather than entering downstream metabolism was dephosphorylated by glucose-6-phosphatase and released back out into the circulation. This 'tug of war' between the most proximal and distal steps of hepatic glucose

metabolism, respectively, is termed futile glucose cycling as the initial phosphorylation and 'trapping' of glucose involves an investment of 1 ATP, with the subsequent dephosphorylation prior to entry into glycolysis (ATP generation) results in a net loss of 1 ATP. Enhanced rates of hepatic futile glucose cycling are a common feature of type 2 diabetes in humans (Efendic *et al.* 1985). Our previous studies in 8-week HFSD-fed glucose-intolerant C57BL/6 mice have also demonstrated elevated rates of hepatic futile glucose cycling (Kowalski *et al.* 2016). Thus, it seems that the long-term HFSD-fed mice still maintain this metabolic feature; however, this defect is not large enough to impair whole body glucose disposal, indicating whole body insulin resistance is adequately compensated for in these mice.

Although a few studies have examined the effects of long-term high-fat diets on glucose tolerance in C57BL/6 mice, the results are conflicting. In contrast to our results, studies by Ahrén and coworkers have shown that C57BL/6 mice fed a high-fat diet for either 10 (Ahren & Pacini 2002) or 19 months (Agardh & Ahren 2012) were glucose intolerant. Similar to our findings here, Sumiyoshi and coworkers reported that the glucose tolerance observed after 10 weeks of HFSD feeding resolved when mice were studied after 30 and 55 weeks on the diet (Sumiyoshi *et al.* 2006). It is important to highlight some key differences in how glucose tolerance was assessed in these studies as they could account for these divergent findings. Ahrén and coworkers used either an intravenous (Ahren & Pacini 2002) or intraperitoneal (Agardh & Ahren 2012) glucose tolerance test, whereas our study and that of Sumiyoshi and coworkers (Sumiyoshi *et al.* 2006) employed an OGTT. Although the route of glucose administration can elicit different glucose and insulin responses (Kowalski & Bruce 2014), we believe that the most likely source of variation is the different glucose dosing strategies used. We employed two different dosing protocols; a fixed dose of 50 mg of glucose for Cohort 1 and a dose of 2 g/kg lean body mass for Cohort 2, equating to ~60 mg of glucose for chow mice and ~70 mg the HFSD group. Similar to our approach in Cohort 1, Sumiyoshi and coworkers (Sumiyoshi *et al.* 2006) administered a fixed dose of 556 µmol (or 100 mg) of glucose per mouse. The studies from Ahrén's group, however, administered glucose at a dose of 1 g/kg total body mass. The implication of administering glucose based on total body mass is that it results in large differences in the absolute amount of glucose given to normal weight and obese animals (McGuinness *et al.* 2009, Omar *et al.* 2014). Indeed, as the high-fat-fed mice were roughly double the weight of the chow-fed animals in the studies

from Ahren's laboratory (Ahren & Pacini 2002, Agardh & Ahren 2012), the amount of glucose administered to those animals was around double that given to chow mice. As lean tissue is the major site of glucose disposal and the HFSD-induced increase in lean mass is dramatically less than the increase in fat mass (Fig. 4A), dosing relative to body weight disproportionately increases the glucose dose in relation to the major glucose-utilising tissues, which biases the obese mice toward a phenotype of impaired glucose tolerance (McGuinness *et al.* 2009). Together with the fact the standard OGTT used for clinical diagnostic purposes in humans involves the ingestion of fixed dose of 75 g of glucose, we support the recent suggestion that the use of the fixed dosing regime is the most appropriate approach with the greatest relevance to the human condition (Omar *et al.* 2014).

Although the glucose intolerance was resolved with long-term HFSD feeding, it is important to highlight that these mice still exhibited a number of significant metabolic abnormalities. Specifically, the long-term HFSD fed mice were grossly obese, hyperinsulinaemic, which is strongly suggestive of insulin resistance, had fatty liver and perhaps most significantly exhibited a marked increase in heart size. Thus, the long-term HFSD-fed mice should not necessarily be considered metabolically healthy.

Our findings also reveal interesting insight into the ability of specific adipose tissue depots to expand in response to a HFSD. Despite the fact that the HFSD fed mice had over 20 g of additional fat, contrasting observations were made with respect to the mass of the epididymal (i.e. intra-abdominal) and inguinal (i.e. subcutaneous) fat depots. Although the inguinal fat pad weight was elevated by ~3-fold in the high-fat, high-sucrose-fed mice, there was no difference in epididymal fat pad weight. This suggests the intra-abdominal epididymal fat pad has a finite expansion capacity and when this capacity is reached, lipid deposition is achieved by expanding the subcutaneous depots. In support of this, whilst dissecting the HFSD mice, there were noticeable subcutaneous fat depots in areas of the body not generally associated with fat storage including around the ankles, behind the knees and around the neck and head. In addition to adipocyte hypertrophy, these observations would support the notion that substantial adipogenesis occurs in subcutaneous adipose tissue in response to long-term high-fat, high-sucrose feeding (Joe *et al.* 2009).

The expansion of subcutaneous adipose tissue could mechanistically play an important role in resolving the glucose tolerance in the long-term HFSD-fed mice. Our observations suggest that in mice, regardless of whether

fed a chow or HFSD, glucose uptake into subcutaneous adipose tissue is around 4- to 5-fold higher than that of epididymal fat during a euglycaemic hyperinsulinaemic clamp (Kowalski and Bruce, unpublished observations). Even though subcutaneous adipose tissue would be expected to exhibit some degree of insulin resistance with respect to glucose uptake, this would be overcome by the marked expansion of subcutaneous fat depot in the long-term HFSD mice, thereby increasing the glucose disposal capacity of the animal (i.e., there is an increased glucose sink). Similar findings have been reported in obese humans who have reduced rates of glucose uptake but because of increased fat mass, the absolute amount of glucose taken up by adipose is not reduced (Virtanen *et al.* 2002).

In agreement with our previous findings (Kowalski *et al.* 2015c, 2016, Lancaster *et al.* 2016), we show that plasma FFA levels are not elevated in obese HFSD-fed mice. In fact in Cohort 2, fasting FFA levels were lower than the chow-fed controls, which is consistent with our previous findings (Kowalski *et al.* 2015c, 2016, Lancaster *et al.* 2016) and that of others in mice (Park *et al.* 2005) and rats (Chalkley *et al.* 2002, Hegarty *et al.* 2002). In addition, plasma FFA concentrations were lower during the OGTT in both cohorts of HFSD mice. Given circulating FFAs are largely supplied by subcutaneous fat depots (Jensen 1995) and are controlled by lipolytic and re-esterification mechanisms (Reshef *et al.* 2003), the fact that the long-term HFSD fed mice had a dramatic expansion of subcutaneous fat mass and lower FFA concentrations during the OGTT could support the concept of the expanded subcutaneous fat depots being the site(s) of additional glucose uptake. Given that adipose tissue lacks glycerol kinase activity (Reshef *et al.* 2003, Tan *et al.* 2003), which requires the generation of glycerol-3-phosphate from glycolysis or glyceroneogenesis (Reshef *et al.* 2003, Bederman *et al.* 2009) to esterify (trap) fatty acids inside of adipocytes, increased subcutaneous glucose uptake during the OGTT could allow glucose tolerance to be enhanced (Cohort 1) or remain intact (Cohort 2) while providing the substrate to generate adipocyte glycerol-3-phosphate (Bederman *et al.* 2009), thus driving FFA esterification and lowering plasma FFA levels.

It is worthy to highlight some notable differences between cohorts. Although the glucose tolerance in the HFSD-fed mice was actually better than that of the chow controls in Cohort 1 (fixed dose), glucose tolerance was not different in Cohort 2 (lean mass dosing). In addition, the two cohorts exhibited differences in baseline fasting plasma parameters (Table 1). The fasting blood glucose

concentrations were similar between the chow and HFSD mice in Cohort 1, whereas in Cohort 2, the HFSD mice displayed a modest increase in blood glucose. Furthermore, the hyperinsulinaemia in the HFSD mice in Cohort 2 was markedly greater than that observed in the HFSD mice of Cohort 1 (475 vs 2295 pmol/L). There are a number of potential reasons to account for these observations and include the fact the mice were sourced from independent lines of C57Bl/6J mice and there is likely to be some genetic drift between these lines. Furthermore, mice were housed at different institutes. This supports recent reports highlighting that metabolic differences can exist between substrains of C57Bl/6J mice (Hull *et al.* 2017).

In conclusion, we have identified the paradoxical observation that long-term HFSD feeding results in the resolution of diet-induced glucose intolerance. This occurred despite the fact the HFSD-fed mice were grossly obese, insulin resistant and had elevated hepatic lipid content. We propose that the gross expansion of subcutaneous adipose tissue increases the glucose disposal capacity of the HFSD-fed mouse, which is substantial enough to improve glucose tolerance by compensating for any defect in insulin action.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

G M K, M J K, A J M and C R B conceptualised, designed and conducted the studies. G M K, S A M and C R B were involved in the data analysis, interpretation and drafting of the manuscript. M J K and A J M critically revised the manuscript. All authors approved the final version.

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