

CHAPTER 2

FISH OIL POTENTIATES HIGH FAT DIET-INDUCED PERIPHERAL INSULIN RESISTANCE IN MICE

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ABSTRACT

Conflicting data have been reported on the effect of fish oil on insulin resistance and type 2 diabetes. Fish oil suppresses fat synthesis while promoting fat oxidation. We evaluated the metabolic consequences of fish oil with regard to substrate utilization and related this to alterations in glucose metabolism in diet-induced insulin resistant mice.

C57Bl/6J mice were fed high fat diets containing beef tallow or tallow/fish oil and compared to animals receiving low-fat chow. Whole-body substrate utilization and energy expenditure were determined by indirect calorimetry. Glucose metabolism was studied by stable isotope infusion. All experiments were performed in conscious, unrestrained animals.

Fish oil decreased the respiratory exchange ration (RER) in mice fed a high fat diet while energy expenditure remained unaffected. Furthermore, fish oil impaired glucose clearance. In contrast, fish oil decreased basal hepatic glucose production and normalized clamped hepatic glucose production.

In conclusion, fish oil increases the fat to glucose oxidation ratio without a concomitant rise in energy expenditure. This is associated with a deterioration of high fat diet-induced peripheral insulin resistance, while hepatic insulin sensitivity is restored. These data indicate that an increase in fat oxidation alone is not sufficient to prevent high fat diet induced peripheral insulin resistance in mice.



INTRODUCTION

Intake of high fat diets is associated with dyslipidemia, increased cardiovascular risk and insulin resistance in humans [1]. Although fish consumption has beneficial effects on blood lipid profiles and cardiovascular risk [2,3], its consequences for the development of insulin resistance are not conclusive; both improvement and deterioration have been reported [4–10].

PUFA, the bioactive component of fish oil, alter the activity of several transcriptional regulators such as peroxisome proliferator activated receptors (PPARs), the liver X receptors (LXRs), the farnesoid X receptors (FXRs), and the sterol and carbohydrate response element binding proteins (SREBP and ChREBP) [11–13]. As a result, expression of genes encoding enzymes involved in fat synthesis is suppressed while that of enzymes facilitating fat oxidation is increased. However, the effect of fish oil on substrate utilization *in vivo* is currently unknown. This is particularly interesting considering recent animal studies which have shown that high fat oxidation rates are linked to insulin resistance [14–18] and that glucose disposal is increased when fat oxidation is suppressed [14,19–21]. Clearly, more insight into the metabolic adaptations in response to fish oil is needed to establish its therapeutic potential in the prevention of diet-induced insulin resistance.

We therefore determined the effect of fish oil on substrate utilization in mice fed a high fat diet in relation to alterations in glucose metabolism. C57Bl/6J mice were subjected to a 6-week dietary challenge of a diet rich in beef tallow or a similar diet in which part of the tallow was replaced by fish oil and compared them to mice that were fed standard low-fat laboratory chow. We assessed whole-body substrate utilization, energy expenditure and basal and hyperinsulinemic glucose metabolism *in vivo* by dedicated techniques and related outcome to biometric and biochemical parameters as well as gene expression patterns.

EXPERIMENTAL PROCEDURES

Animals and experimental design

Male C57Bl/6J mice (Charles River, L'Arbresle Cedex, France), three months of age, were housed in a light- and temperature-controlled facility (lights on 6:30 AM–6:30 PM, 21 °C). They were divided into groups and fed three different diets for six weeks. One group received laboratory chow (RMH-B), the second group received high fat diet (beef tallow, 60 energy% fat) and the third group received a diet in which part of the tallow was replaced by fish oil (tallow: 35, fish oil: 25 energy% fat). The two high fat diets were hypercaloric compared to laboratory chow (chow, 3.3; tallow, 5.5; tallow/fish oil, 5.5 kcal/g). All diets were obtained from Abdiets, Woerden, The Netherlands. For dietary fatty acid composition see Table 1. At the end of the dietary period, mice were either sacrificed for basal plasma and tissue collection, subjected

to indirect calorimetry or to in vivo measurements of glucose metabolism. Experimental procedures were approved by the Ethics Committees for Animal Experiments of the Universities of Groningen and Leiden.

Table 1: Fatty acid composition experimental diets. Values are given in g/kg.

	chow	tallow	tallow/fish oil
C14:0	0.5	12.2	16.1
C16:0	8.4	92.5	79.5
C16:1	0.7	11.5	18.0
C18:0	3.7	76.3	50.5
C18:1	13.7	133.2	101.0
C18:2	16.9	11.5	9.7
C18:3	1.9	2.9	15.2
C20-22	0.4	4.0	53.3

Plasma and tissue sampling and analysis

The mice were fasted from 6-10 AM. Blood concentrations were measured using an EuroFlash meter (Lifescan Benelux, Beerse, Belgium). Mice were subsequently sacrificed by cardiac puncture under isoflurane anesthesia. Livers and skeletal muscles were quickly removed, snap-frozen in liquid nitrogen and stored at -80 °C. For adipocyte histology, epididymal fat was fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Blood was centrifuged (4000 xg for 10 min at 4 °C) and plasma was stored at -20 °C. Plasma triglyceride (TG) concentrations were determined using a commercially available kit (Roche Diagnostics, Mannheim, Germany). Plasma insulin concentrations were determined using ELISA (Ultrasensitive Mouse Insulin kit; Mercodia, Uppsala, Sweden). Plasma leptin concentrations were determined by Luminex® Multiplex technology (Luminex Corporation, Austin, TX) using Multiplex Immunoassays (Mouse adipokine panel; Millipore, Amsterdam, The Netherlands). Hepatic fatty acid content was determined by gas chromatography after transmethylation [22]. Hepatic glycogen content was determined as previously described [23]. For adipocyte histology, 3 µm paraffin sections were stained with hematoxylin and eosin and analyzed at 10x magnification. Fat cell area of two representative sections per group was quantified using image analysis software (Qwin, Leica, Wetzlar, Germany). RNA was extracted from liver and skeletal muscle using Tri reagent (Sigma-Aldrich, St. Louis, MO, USA). RNA was converted into cDNA by a reverse transcription procedure using M-MLV (Sigma) and random primers

according to the manufacturer's protocol. For realtime PCR, cDNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) and the appropriate primers and probes. Primer and probe sequences of the following genes have been published (www.LabPediatricsRug.nl): 18S, 36B4, carnitine-acylcarnitine translocase (Cact), fatty acid transporter (CD36), carnitine palmitoyltransferase 1b/2 (Cpt1b/2), carnitine acyltransferase (Crat), glucose-6-phosphate hydrolase (G6ph), glucose-6-phosphate translocase (G6pt), Pepck and peroxisome proliferator activated receptor gamma co-activator 1 α (Pgc-1 α). For acyl-CoA synthase (Acs), the following primers/probe were used: sense, GGA GCT TCG CAG TGG CAT C; antisense, CCC AGG CTC GAC TGT ATC TTG T; probe, CAG AAA CAA CAG CCT GTG GGA TAA ACT CAT CTT (accession number NM_007981). For long-chain acetyl-Coenzyme A dehydrogenase (Lcad), the following primers/probe were used: sense, TAC GGC ACA AAA GAA CAG ATC G; antisense, CAG GCT CTG TCA TGG CTA TGG; probe, CAC TTG CCC GCC GTC ATC TGG (accession number NM_007381). All mRNA levels were calculated relative to the expression of 18S (liver) or 36B4 (skeletal muscle) and normalized for expression levels of chow-fed mice.

Indirect calorimetry

We assessed *in vivo* energy metabolism in tallow and tallow/fish oil-fed mice using a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, USA). Mice were housed individually to enable real time and continuous monitoring of metabolic gas exchange. Detectors measured O₂ and CO₂ sequentially across each chamber for 45 seconds at seven-minute intervals. RER was calculated as the ratio between the volume of CO₂ produced (VCO₂) and the volume of O₂ consumed (VO₂). RER values were compared to data obtained in mice receiving a low-fat diet (Research Diet Services, Wijk bij Duurstede, The Netherlands). Carbohydrate and fat oxidation rates were calculated from VO₂ and VCO₂ using the following formulas [24]:


$$\text{Carbohydrate oxidation (kcal/h)} = ((4.585 \times \text{VCO}_2) - (3.226 \times \text{VO}_2)) \times 4/1000$$

$$\text{Fat oxidation (kcal/h)} = ((1.695 \times \text{VO}_2) - (1.701 \times \text{VCO}_2)) \times 9/1000$$

With VO₂ and VCO₂ given in mL/h. Total energy expenditure was calculated from the sum of carbohydrate and fat oxidation.

In vivo glucose metabolism

Five days prior to the experiment, mice were equipped with a permanent catheter in the right atrium via the jugular vein [25]. The two-way entrance of the catheter was attached to the skull using acrylic glue. Food was withdrawn nine hours (from 11 PM-8 AM) prior to the start and during the experiment. The mice were kept in experimental cages and had free access to water. All *in vivo* infusion experiments lasted six hours and were performed in conscious, unrestrained mice, since the



cages allowed frequent collection of blood spots without the use of anesthesia. During the experiment, we measured blood glucose concentrations every 15 minutes in a small drop of blood that was taken from the tail vein using an EuroFlash glucose meter. Every 30 minutes, a bloodspot was collected on filter paper via tail bleeding for GC-MS measurements. Basal glucose metabolism was studied by infusion of [U-¹³C]-glucose at 7.5 μmol/h for 120 minutes. Subsequently, a hyperinsulinemic euglycemic clamp was performed for 240 minutes. During the clamp, mice were infused with two solutions. The first solution consisted of BSA(1% w/v, Sigma-Aldrich) containing somatostatin (40 μg/mL, UCB, Breda, The Netherlands), insulin (44 mU/mL, Actrapid; Novo Nordisk, Bagsvaerd, Denmark), glucose (1078 mmol/L) and [U-¹³C]-glucose (33 mmol/L, 99% ¹³C atom %excess; Cambridge Isotope Laboratories, Andover, MA, USA) and was infused at a constant rate of 0.135 mL/h. The second solution consisted of glucose (1078 mmol/L) and [U-¹³C]-glucose (33 mmol/L) and its infusion rate was adjusted according to the blood glucose concentration in order to maintain euglycemia. Prior to these experiments, dose-responsiveness of insulin-mediated suppression of hepatic glucose production and stimulation of glucose clearance was tested in separate groups of mice. We performed hyperinsulinemic euglycemic clamps using the protocol described earlier and applied different insulin doses (0-30 mU/hr) and determined at which insulin dose the half-maximal effect on peripheral glucose clearance and hepatic glucose production was reached. This dose (6 mU/hr) was used for the clamps performed on the animals fed the different diets. At the end of all in vivo infusion experiments, the mice were sacrificed under isoflurane anesthesia.

Analysis of in vivo glucose metabolism

Analytical procedures for extraction of glucose from blood spots, derivatization of the extracted compounds and GC-MS measurements of derivatives were performed according to van Dijk et al. [26]. Calculations were performed according to Grefhorst et al. [27]. Mean hepatic glucose production rates and metabolic clearance rates (MCR; a measure of glucose disposal) were calculated for the period of steady-state isotope dilution.

Statistics

All data are presented as means ± SEM. Statistical analysis was performed using SPSS for Windows software (SPSS 12.02, Chicago, IL, USA). Analysis of two groups (chow versus tallow, tallow versus tallow/fish oil) was assessed by Mann-Whitney U-test for biometric, plasma and tissue parameters or by ANOVA for repeated measurements for the infusion experiments. In all statistical tests performed, the null hypothesis was rejected at the 0.05 level of probability.

RESULTS

Fish oil induces additional weight gain and increases plasma adipokine levels in mice fed a tallow diet

Caloric intake was higher in mice fed the tallow and tallow/fish oil diets compared to chow-fed animals (Table 2). Despite similar caloric intake, body weight gain in mice fed the tallow/fish oil diet was more pronounced compared to mice fed the tallow diet. Adipose tissue mass was also increased in these animals (unpublished observations). Plasma leptin concentrations were increased in mice fed the tallow/fish oil diet compared to mice fed chow and tallow diet (Table 2). Average adipocyte size of mice fed the tallow/fish oil diet was increased compared to that of mice fed the tallow diet and chow (Figure 1A and B).

Increased fat to glucose oxidation in mice fed a tallow or tallow/fish oil diet

Fish oil is thought to lower plasma and tissue lipid levels by suppressing hepatic VLDL production and de novo lipogenesis while promoting lipid clearance and oxidation [3]. As expected, plasma TG concentrations (Table 2) and hepatic TG content (unpublished observations) were decreased in mice fed the tallow/fish oil diet. Total hepatic fatty acid content was increased in mice fed the tallow diet and normalized in mice fed the tallow/fish oil diet to levels observed in mice fed chow (Table 2). Blood glucose and plasma insulin levels were increased in mice fed the tallow and tallow/fish oil diets compared to chow-fed mice (Table 2).

Table 2: Metabolic parameters and metabolite levels in C57Bl/6J mice fed chow, tallow and tallow/fish oil diets for 6 weeks.

Mice were fasted from 6-10 AM. Glucose concentrations were measured in tail blood using an Euro-Flash meter. Plasma was obtained by centrifugation of blood from cardiac puncture. Leptin, insulin and triglyceride concentrations were determined using commercially available kits. Hepatic fatty acid content was determined by gas chromatography after transmethylation. Values represent means \pm SEM for $n=5-7$; * $p<0.05$ tallow vs. chow; # $p<0.05$ tallow/fish oil vs. tallow (Mann-Whitney U-test).

	chow	tallow	tallow/fish oil
Body weight gain (%)	11 \pm 2	17 \pm 2	27 \pm 4#
Caloric intake (kcal/24 h)	12.3 \pm 0.1	17.1 \pm 0.8*	16.8 \pm 1.0
Plasma leptin (ng/mL)	1.4 \pm 0.2	2.9 \pm 0.3	6.3 \pm 1.1#
Blood glucose (mmol/L)	8.7 \pm 0.9	9.5 \pm 0.6	12.9 \pm 0.8#
Plasma insulin (ng/mL)	0.2 \pm 0.0	0.7 \pm 0.2*	0.8 \pm 0.2
Plasma triglycerides (mmol/L)	0.6 \pm 0.1	0.6 \pm 0.1	0.4 \pm 0.1#
Hepatic fatty acids (μ mol/g)	123 \pm 8	158 \pm 6*	120 \pm 8#

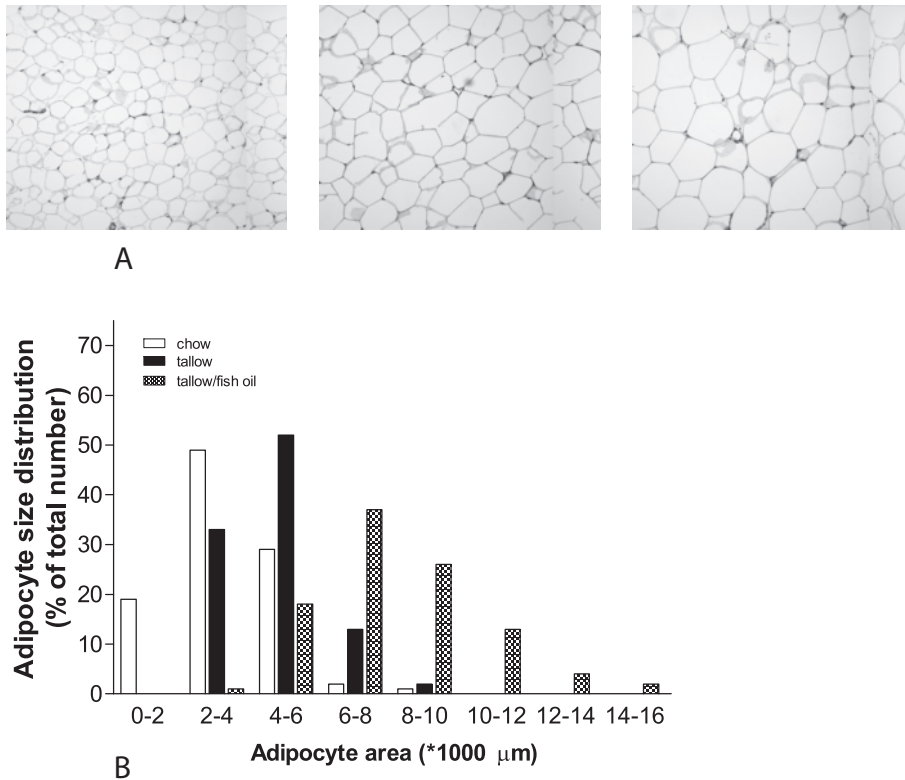
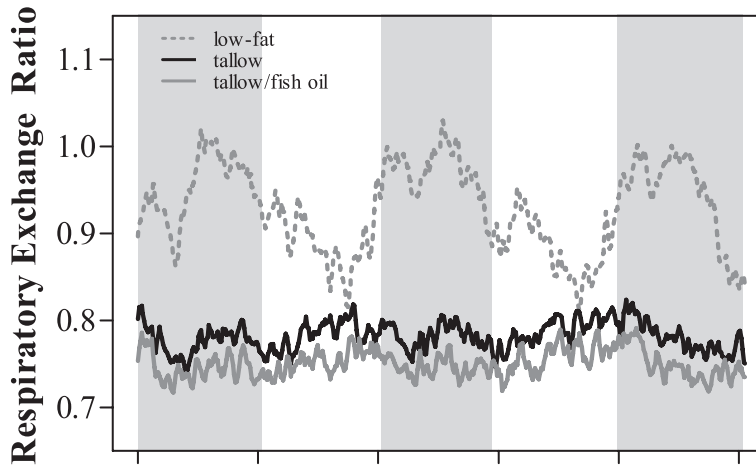
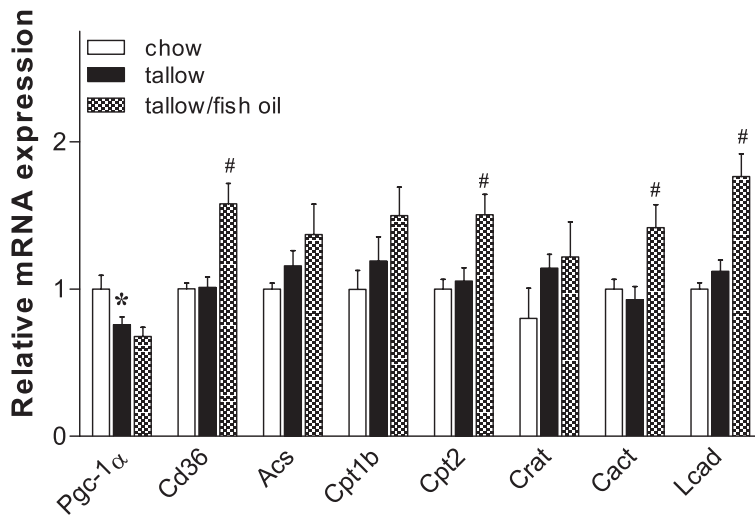


Figure 1: Adipocyte morphology in C57Bl/6J mice fed chow, tallow and tallow/fish oil diets for 6 weeks. Hematoxylin and eosin stained paraffin sections were analyzed and fat cell area was estimated using image analysis software. A: adipocyte morphology and B: adipocyte size distribution in representative samples of epididymal fat tissue. Open bars, chow diet; filled bars, tallow diet; dotted bars, tallow/fish oil diet.

Mice fed a low-fat diet exhibit a high RER during the dark phase, which decreases during the light phase (dark: 0.95 ± 0.01 , light: 0.90 ± 0.02 , $p < 0.05$ dark versus light, Figure 2A), indicating a switch from whole-body fat (dark: 0.12 ± 0.02 , light: 0.08 ± 0.02 kcal/h, $p < 0.05$ dark versus light) to carbohydrate (dark: 0.44 ± 0.02 , light: 0.33 ± 0.03 kcal/h, $p < 0.05$ dark versus light) oxidation. In contrast, tallow-fed mice exhibited a low RER during both the dark and light phase (dark: 0.79 ± 0.01 , light: 0.79 ± 0.01 , Figure 2A). This reduction in RER was even more pronounced in mice fed the tallow/fish oil diet (dark: 0.76 ± 0.01 , light: 0.76 ± 0.01 , Figure 2A). No significant differences in RER between dark the dark and light phase were observed in both tallow and tallow/fish oil-fed animals (tallow: $p = 0.17$, tallow/fish oil: $p = 0.18$). Direct comparison of the groups on high fat diets revealed a lower 24-h RER in tallow/fish oil-fed mice (tallow: 0.78 ± 0.01 , tallow/fish oil: 0.75 ± 0.01 , $p < 0.05$), indicating a higher



A



B

Figure 2: Substrate utilization in C57Bl/6J mice fed a low-fat diet, tallow and tallow/fish oil diets for 6 weeks. Whole-body substrate utilization was analyzed by indirect calorimetry using the Comprehensive Laboratory Animal Monitoring System (CLAMS). Gene expression levels in skeletal muscles were determined by qPCR. A: Respiratory Exchange Ratio (RER) and B: expression of genes involved in fat oxidation. Dashed line, chow diet; black line, tallow diet; grey line, tallow/fish oil diet. Open bars, chow diet; filled bars, tallow diet; dotted bars, tallow/fish oil diet. Average RER values represent means for n=7-8. Average qPCR values represent means \pm SEM for n=5-7; * p<0.05 tallow vs. chow; # p<0.05 tallow/fish oil vs. tallow (Mann-Whitney U-test).

fat to carbohydrate oxidation ratio. Absolute 24-h carbohydrate oxidation rates were significantly lower in animals on tallow-fish oil compared to mice fed tallow (tallow: 0.12 ± 0.01 , tallow/fish oil: 0.07 ± 0.01 kcal/h, $p < 0.05$) while absolute 24-h fat oxidation rates only tended to be increased in these mice (tallow: 0.28 ± 0.01 , tallow/fish oil: 0.32 ± 0.02 kcal/h, $p = 0.09$). Calculated 24-h whole-body energy expenditure was not different between mice fed the tallow and tallow/fish oil diets (tallow: 0.40 ± 0.01 , tallow/fish oil: 0.39 ± 0.01 kcal/h, $p = 0.15$) because of the increased contribution of fatty acid oxidation to total energy expenditure in tallow/fish oil-fed animals.

Consistent with this increase in fat to carbohydrate oxidation ratio, expression of genes involved in fatty acid uptake and oxidation was solely increased in skeletal muscles of mice fed the tallow/fish oil diet compared to mice fed chow and tallow diet (Figure 2B) while Pgc-1 α expression was decreased in both high fat diet groups (Figure 2B).

Fish oil aggravates impaired basal and insulin-stimulated glucose clearance in tallow-fed mice

Insulin resistance has been shown to be associated by elevated β -oxidation and impaired switching to carbohydrate utilization during the fasted-to-fed transition [14]. Therefore, we determined basal and insulin-stimulated glucose clearance in mice receiving chow, tallow and tallow/fish oil diets. Basal glucose clearance was slightly reduced in tallow-fed mice (chow: 19 ± 2 , tallow: 16 ± 1 mL/kg/min, $p = 0.09$),

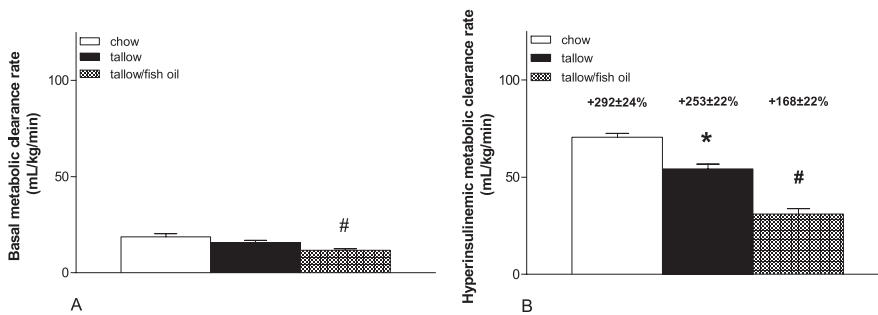


Figure 3: Peripheral glucose clearance in C57Bl/6 mice fed chow, tallow and tallow/fish oil diets for 6 weeks. Glucose disposal was quantified by infusing tracer amounts of [U-13C]glucose (basal period) or insulin (6 mU/h) and [U-13C]glucose (hyperinsulinemic euglycemic clamp) in conscious, unrestrained mice. Glucose kinetics were determined by GC-MS analysis of blood spots taken via tail bleeding on filter paper at regular time intervals. Blood glucose concentrations were measured in tail blood using a EuroFlash meter. Metabolic clearance rate was calculated as previously described [27]. A: metabolic clearance rate during the basal period and B: metabolic clearance rate during the hyperinsulinemic clamp. Open bars, chow diet; filled bars, tallow diet; dotted bars, tallow/fish oil diet. Inset, relative increase of metabolic clearance rate from basal to hyperinsulinemic period. Values represent means \pm SEM for $n = 5-9$ during stable isotope infusion ($t = 75-120$ min for basal period; $t = 270-360$ min for hyperinsulinemic clamp); # $p < 0.05$ tallow/fish oil vs. tallow (ANOVA for repeated measurements).

but significantly impaired in mice fed the tallow/fish oil diet (tallow/fish oil: 12 ± 1 mL/kg/min, $p < 0.05$ tallow/fish oil versus tallow, Figure 3A). Prolonged continuous insulin infusion (6 mU/h) increased glucose clearance in all mice compared to basal conditions. However, the hyperinsulinemic glucose clearance (chow: 71 ± 2 , tallow: 54 ± 2 , tallow/fish oil: 31 ± 3 mL/kg/min, $p < 0.05$ tallow versus chow and tallow/fish oil versus tallow, Figure 3B) as well as the stimulation of glucose clearance by insulin infusion was impaired in mice fed the tallow diet and even further deteriorated by fish oil ($p < 0.05$ tallow versus chow and tallow/fish oil versus tallow, Figure 3B), indicating peripheral insulin resistance in tallow and tallow/fish oil-fed animals. Blood glucose concentrations and the glucose infusion rates are given in Table 3. Glucose infusion rate under hyperinsulinemic conditions was lower in mice fed the tallow diet and further reduced by fish oil.

Table 3: Clamped blood glucose concentrations and glucose infusion rates required to maintain euglycemia under hyperinsulinemic conditions in C57Bl/6J mice fed chow, tallow and tallow/fish oil diets for 6 weeks. Glucose metabolism was assessed by infusing insulin (6 mU/h) and [U-13C]glucose in conscious, unrestrained mice. Glucose concentrations were measured in tail blood using an EuroFlash meter. Values represent means \pm SEM for $n=5-9$ during stable isotope infusion ($t=270-360$ min); * $p < 0.05$ tallow vs. chow; # $p < 0.05$ tallow/fish oil vs. tallow (ANOVA for repeated measurements).

	chow	tallow	tallow/fish oil
Clamped blood glucose concentration (mmol/L)	7.0 ± 0.2	7.1 ± 0.2	6.9 ± 0.2
Glucose infusion rate ($\mu\text{mol/kg/min}$)	443 ± 14	$304 \pm 14^*$	$145 \pm 10^\#$

Fish oil suppresses basal and insulin-stimulated hepatic glucose production in tallow-fed mice

Fish oil has recently been shown to protect against hepatic insulin resistance in mice fed a safflower oil diet [28]. mRNA expression of Pgc1 α , Pepck, G6ph and G6pt, genes involved in hepatic gluconeogenesis was decreased in mice fed the tallow/fish oil diet (Figure 4A). Furthermore, hepatic glycogen content was lower in these mice (chow: 334 ± 24 , tallow: 357 ± 37 , tallow/fish oil: 269 ± 26 $\mu\text{mol/g}$ $p < 0.05$ tallow/fish oil versus tallow). We determined hepatic glucose production under basal and hyperinsulinemic conditions in mice fed chow, tallow and tallow/fish oil diets. Tallow feeding did not affect basal endogenous production (chow: 128 ± 6 , tallow: 136 ± 5 $\mu\text{mol/kg/min}$, $p=0.42$, Figure 4B) while it was reduced in tallow/fish oil fed mice compared to mice fed chow or tallow (tallow/fish oil: 102 ± 5 $\mu\text{mol/kg/min}$, $p < 0.05$ tallow/fish oil versus tallow, Figure 4B). Prolonged continuous insulin infusion (6 mU/h) suppressed hepatic glucose production in all mice compared to basal conditions. Hepatic glucose production under hyperinsulinemic conditions was higher in mice fed the tallow diet compared to chow-fed mice, (chow: 43 ± 10 , tallow: 75 ± 8 $\mu\text{mol/kg/min}$, $p < 0.05$ tallow versus chow, Figure 4C) and insulin-mediated suppression of hepatic glucose production was blunted in these animals ($p < 0.05$ tallow

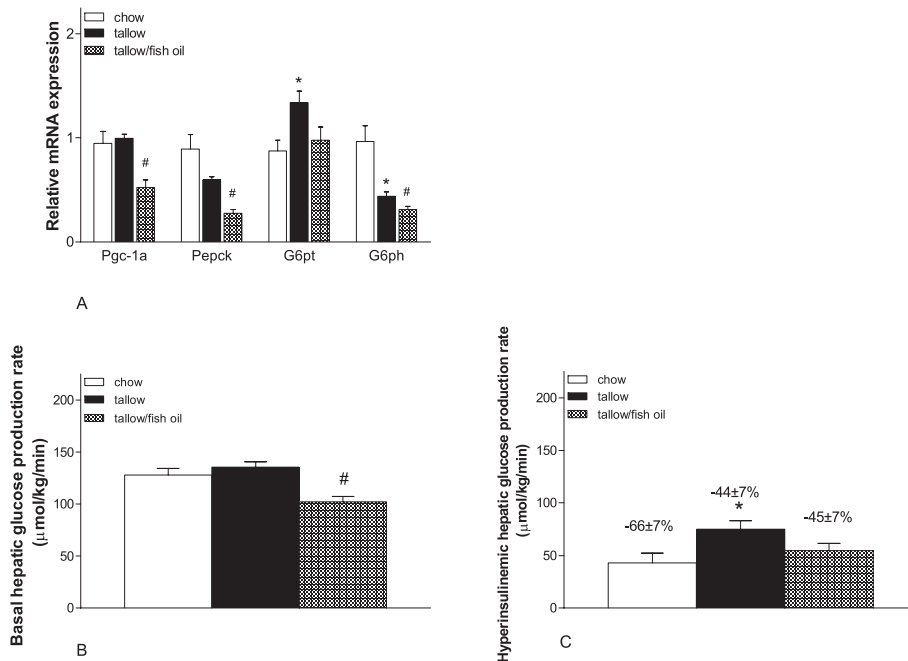


Figure 4: Hepatic glucose production in C57Bl/6J mice fed chow, tallow and tallow/fish oil diets for 6 weeks. Gene expression levels in livers were determined by qPCR. Glucose production was quantified by infusing tracer amounts of [U-13C]glucose (basal period) or insulin (6 mU/hr) and [U-13C]glucose (hyperinsulinemic euglycemic clamp) in conscious, unrestrained mice. Glucose kinetics were determined by GC-MS analysis of blood spots taken via tail bleeding on filter paper at regular time intervals. Hepatic glucose production was calculated as previously described [27]. A: expression of genes involved in glucose production. B: hepatic glucose metabolism during the basal period and C: hepatic glucose metabolism during the hyperinsulinemic clamp. Open bars, chow diet; filled bars, tallow diet; dotted bars, tallow/fish oil diet. Inset, relative decrease of hepatic glucose production from basal to hyperinsulinemic period. Values represent means \pm SEM for $n=5-9$ during stable isotope infusion ($t=75-120$ min for basal period; $t=270-360$ min for hyperinsulinemic clamp); * $p<0.05$ tallow vs. chow; # $p<0.05$ tallow/fish oil vs. tallow (Figures A and C, ANOVA for repeated measurements; Figure B, Mann-Whitney U-test).


versus chow, Figure 4C). Fish oil partially normalized hepatic glucose production to control values under hyperinsulinemic conditions (tallow/fish oil: $55 \pm 7 \mu\text{mol/kg/min}$, $p=0.15$ tallow/fish oil versus tallow, Figure 4C). However, insulin-mediated suppression of hepatic glucose production in these mice was similar to that observed in tallow-fed mice ($p=0.70$, Figure 4C).

DISCUSSION

High fat diets predispose to development of insulin resistance and type 2 diabetes [5]. So far, studies on the effect of fish oil supplementation on glucose control are inconclusive [4–10]. Moreover, the relationship between fatty acid oxidation and glucose disposal is unclear [14–21] and *in vivo* data on the concurrent alterations in substrate utilization and glucose metabolism are lacking. This prompted us to study the metabolic effects of fish oil in mice fed a high fat diet. C57Bl/6J mice were subjected to a 6-week dietary challenge by a diet rich in beef tallow or a similar diet in which part of the tallow was replaced by fish oil and outcome was compared to animals that were fed normal laboratory chow.

Mice fed the tallow/fish oil diet exhibited a larger body weight gain compared to mice fed the tallow diet and histological analysis revealed remarkable adipocyte enlargement in these animals. Accordingly, plasma levels of leptin were increased in tallow/fish oil-fed mice. A similar phenotype in high fat-fed mice receiving fish oil has recently been reported by Coenen et al. [29]. However, others have found a body weight-reducing effect of fish oil in obese diabetic mice [30–32], mice on high α -linoleic acid diets [33] and in case of long-term dietary intervention [34,35]. Taken together, these data illustrate that the metabolic state of the animal determines the effects of fish oil on adipose tissue development [36].

Tallow feeding clearly altered the circadian pattern of substrate utilization. Animals fed a chow diet switch from glucose oxidation during the dark phase to fat oxidation during the light phase. These day-night variations in substrate utilization correlate with those reported for plasma insulin levels [37]. Kohsaka et al. [38] have reported disturbed circadian rhythmicity of serum insulin concentrations (i.e. increased insulin concentrations during both day and light phases) upon high fat feeding in mice. In our study, mice fed a tallow diet did not switch from fat oxidation during the light phase toward carbohydrate oxidation during the dark phase. These mice displayed sustained high fat to carbohydrate oxidation ratios and, thus, insulin responsiveness of substrate utilization must have been disturbed. RER values in mice fed the tallow/fish oil diet were consistently lower compared to animals fed tallow and fat to carbohydrate oxidation ratio was further increased while fat oxidation was somewhat increased. However, total energy expenditure was similar in tallow and tallow/fish oil-fed mice and the increased fat oxidation therefore coincided with a reduced glucose oxidation. Impaired ability to switch between glucose and lipid oxidation has been reported in obese and/or diabetic subjects [39,40]. Moreover, metabolic inflexibility to glucose is related to impaired glucose clearance in type 2 diabetic subjects [41]. We therefore studied the consequences of the altered substrate utilization in more detail under basal conditions and during prolonged hyperinsulinemia. Basal glucose clearance was reduced in both tallow and tallow/fish oil-fed animals compared to mice receiving chow. Insulin-mediated stimulation of glucose clearance was impaired by tallow feeding, indicative for peripheral insulin resistance.



This effect was potentiated by fish oil. Thus, the reduced rates of carbohydrate oxidation in tallow and tallow/fish oil-fed mice during the light and dark phase were paralleled by and correlated to an impaired glucose clearance. This phenotype in which an increased whole body fat to carbohydrate oxidation ratio is associated with a deterioration of insulin sensitivity has also been observed in type 2 diabetic subjects receiving long-term fish oil supplementation [6]. Skeletal muscle is the major site for insulin-stimulated glucose clearance [42] and an increased fatty acid oxidation in skeletal muscle is associated with an impaired glucose metabolism [14–17] while inhibition of fatty acid uptake and oxidation enhances glucose disposal in vivo in normal and insulin-resistant mice [14,19–21]. It is therefore tempting to speculate that the peripheral insulin resistance in mice fed the tallow and tallow/fish oil diets mainly resulted from an impaired glucose clearance into the muscles [41]. However, the obese phenotypes may to some extent have contributed to an impaired glucose disposal into the adipose tissue of these animals.

Basal hepatic glucose production rates were comparable in chow and tallow-fed mice (Figure 4A), despite the fasting hyperinsulinemia observed in the animals receiving tallow. Consistent with this hepatic insulin insensitivity under basal conditions, hepatic glucose production was higher in tallow-fed mice under hyperinsulinemic conditions as compared to animals on chow. Basal hepatic glucose production was lower in tallow/fish oil-fed mice (-25% vs. tallow), indicating an improvement of hepatic insulin responsiveness under these conditions. In parallel, hepatic gluconeogenic gene expression levels were decreased and hepatic glycogen content was reduced in tallow/fish oil-fed animals. Although additional in vivo isotope studies are needed to quantify the actual gluconeogenic flux in fish oil fed-mice, these observations suggest a decreased gluconeogenic flux in these mice [43,44]. Consistent with the lower basal hepatic glucose output, fish oil partially normalized hepatic glucose production under hyperinsulinemic clamp conditions (-27% vs. tallow). However, as a result of the reduction of basal hepatic glucose production by fish oil, the relative suppression of hepatic glucose production from basal to hyperinsulinemic conditions was similarly impaired in tallow and tallow/fish-oil fed mice. One possibility for this similar relative suppression is that maximal insulin-responsiveness was reached in fish oil-fed mice under hyperinsulinemic conditions. Alternatively, the reduced basal glucose production in tallow/fish oil fed mice may therefore not just result from a restoration of hepatic insulin sensitivity. As yet unidentified mechanisms could be mediating the effect of fish oil on basal hepatic glucose production.

Interestingly, Neschen et al. [28] performed a similar study on fish oil replacement of a diet rich in vegetable oil in mice on a SV129 background. Major differences in the response to the dietary challenges were observed compared to our study. Strikingly, Neschen et al. did not observe peripheral insulin resistance in mice either fed safflower oil or a safflower/fish oil diet for 2 weeks. Moreover, these authors observed severe hepatic insulin resistance in safflower oil-fed animals, which was partially prevented by fish oil. In addition to differences in duration of dietary intervention,

fat sources and the genetic background of the mice [45], the experimental conditions under which in vivo glucose metabolism was studied were different from ours. This may be of importance in this respect [46]. We performed 6-h stable isotope infusion studies in awake, freely-moving mice. In this experimental setup, insulin dose-dependently increases glucose clearance while hepatic glucose production is suppressed (Figure 5). Tissue glucose demand is diminished if animals are anaesthetized or restrained (unpublished observations). Therefore, in our opinion, relevant outcome is best guaranteed using methods in which normal physiology is minimally disturbed.

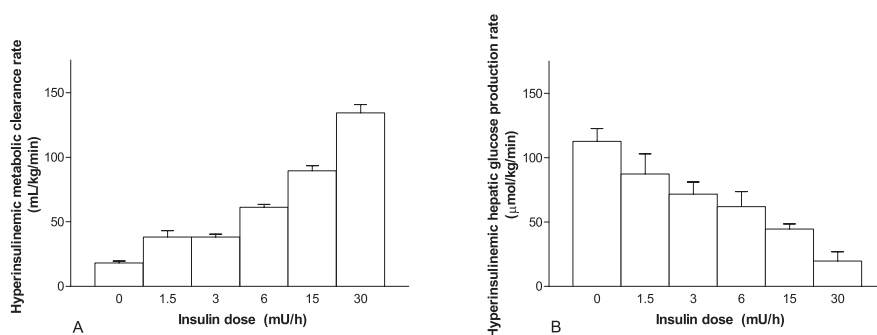


Figure 5: Dose-dependent effect of insulin on glucose disposal and glucose production under clamp conditions. Clamps were performed different doses of insulin (0-30 mU/h) and [U-13C]glucose in conscious, unrestrained mice. Glucose kinetics were determined by GC-MS analysis of blood spots taken via tail bleeding on filter paper at regular time intervals. Blood glucose concentrations were measured in tail blood using a EuroFlash meter. Metabolic clearance rate was calculated as previously described [33]. A: metabolic clearance rate and B: hepatic glucose production rate. Values represent means \pm SEM for n=4-6.

In summary, we have shown that fish oil alters substrate utilization by increasing the fat to glucose oxidation ratio. This is associated with a further deterioration of insulin-mediated glucose clearance in mice fed a high fat diet. Our data indicate that an increased fat to carbohydrate oxidation ratio per se does not improve obesity and peripheral insulin sensitivity and emphasizes the need for a change in energy balance to arrest diet-induced obesity and peripheral insulin resistance. These insights will allow us to define the metabolic conditions under which dietary approaches may be useful to prevent the development of insulin resistance and type 2 diabetes.

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