Figure 1

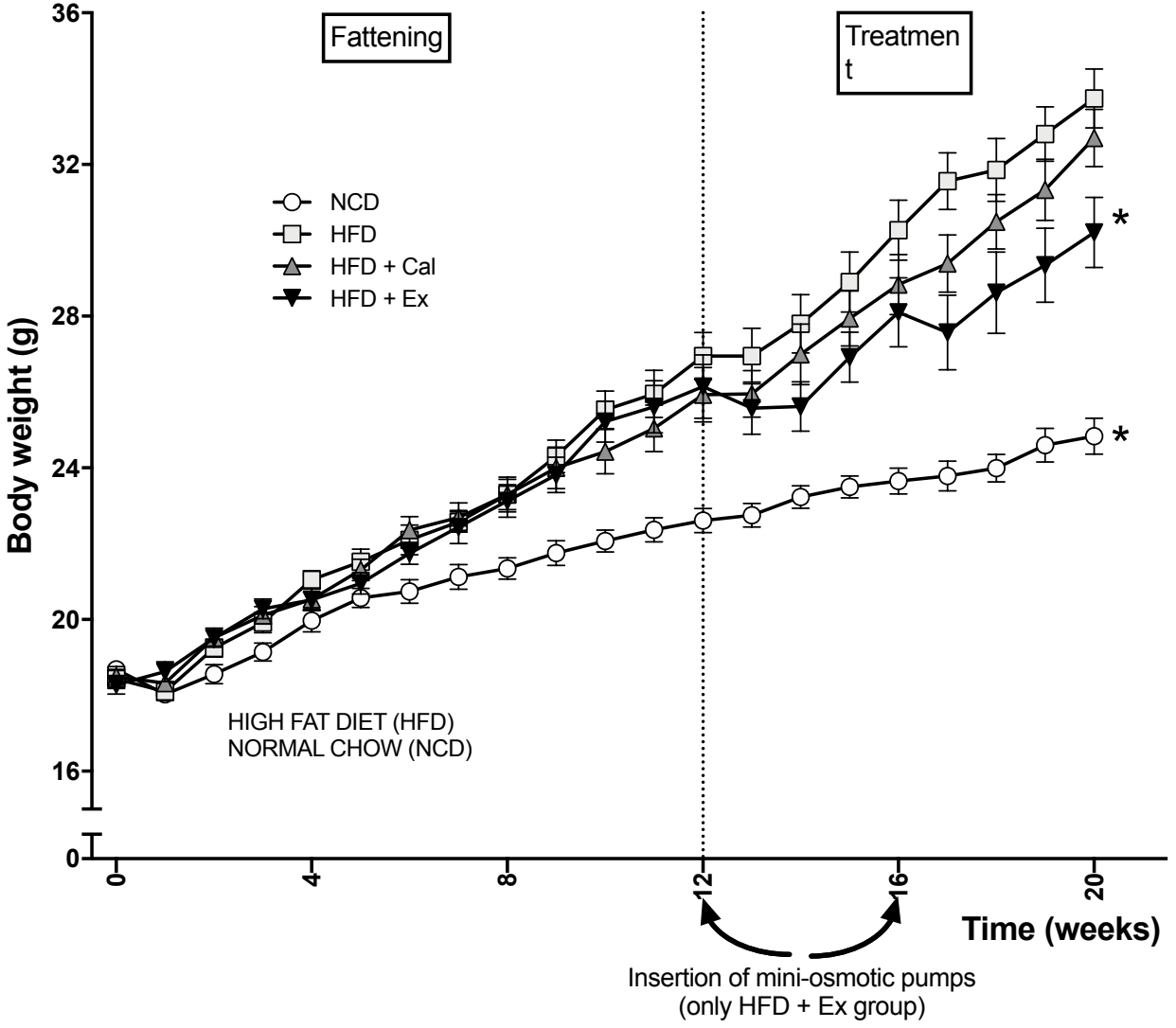
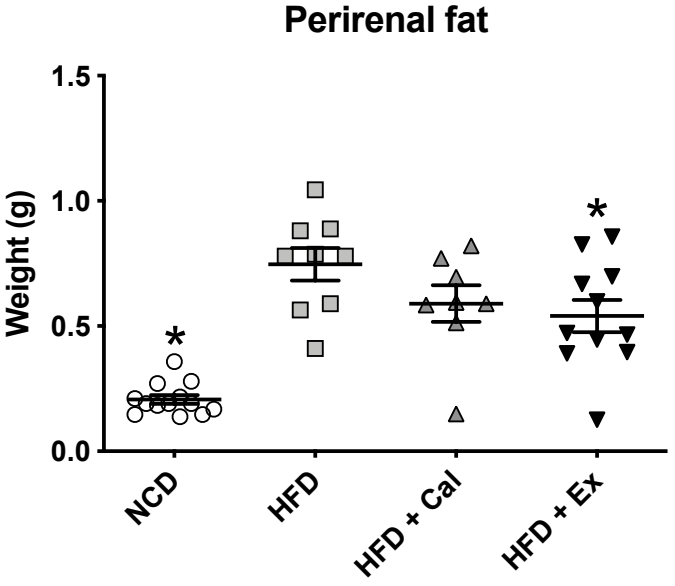
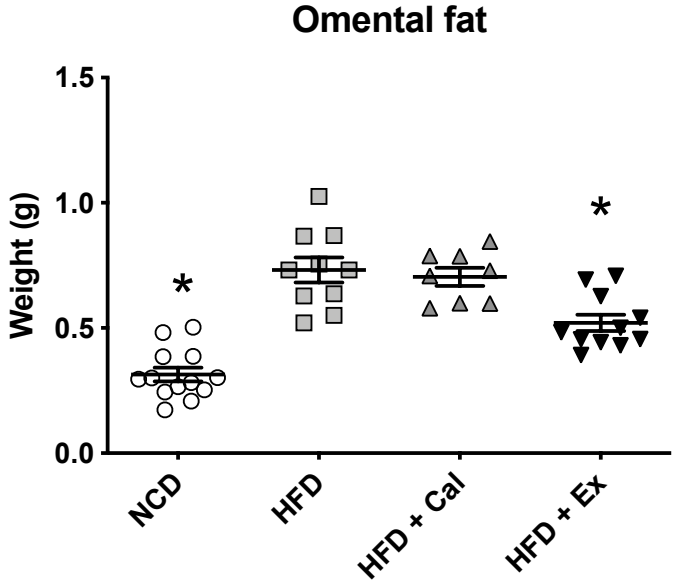
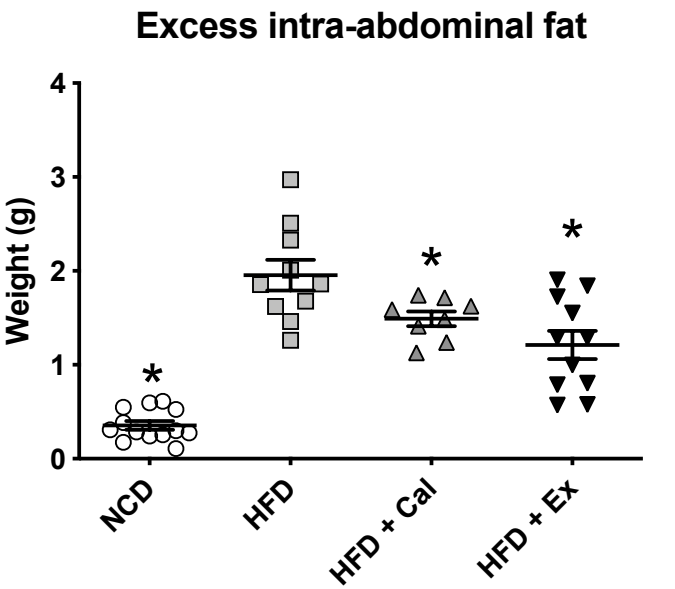

Fattening
Treatmen
t
NCD
HFD
HFD + Cal
HFD + Ex
Body weight (g)
36
32
28
24
20
16
0
0
4
8
12
16
20
Time (weeks)
*
*
HIGH FAT DIET (HFD)
NORMAL CHOW (NCD)
Insertion of mini-osmotic pumps
(only HFD + Ex group)

Figure 2


Perirenal fat
Weight (g)
1.5
1.0
0.5
0.0
*
*
NCD
HFD
HFD + Cal
HFD + Ex


Omental fat
Weight (g)
1.5
1.0
0.5
0.0
*
*
NCD
HFD
HFD + Cal
HFD + Ex


Excess intra-abdominal fat
Weight (g)
4
3
2
1
0
*
*
*
NCD
HFD
HFD + Cal
HFD + Ex

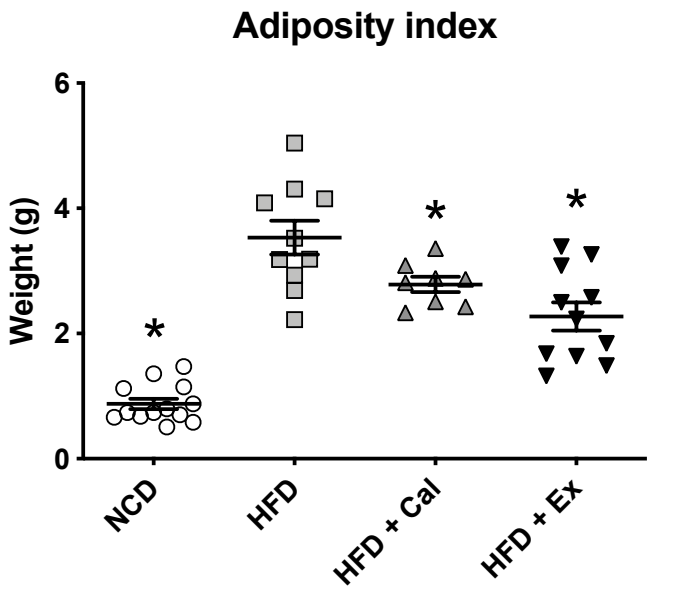
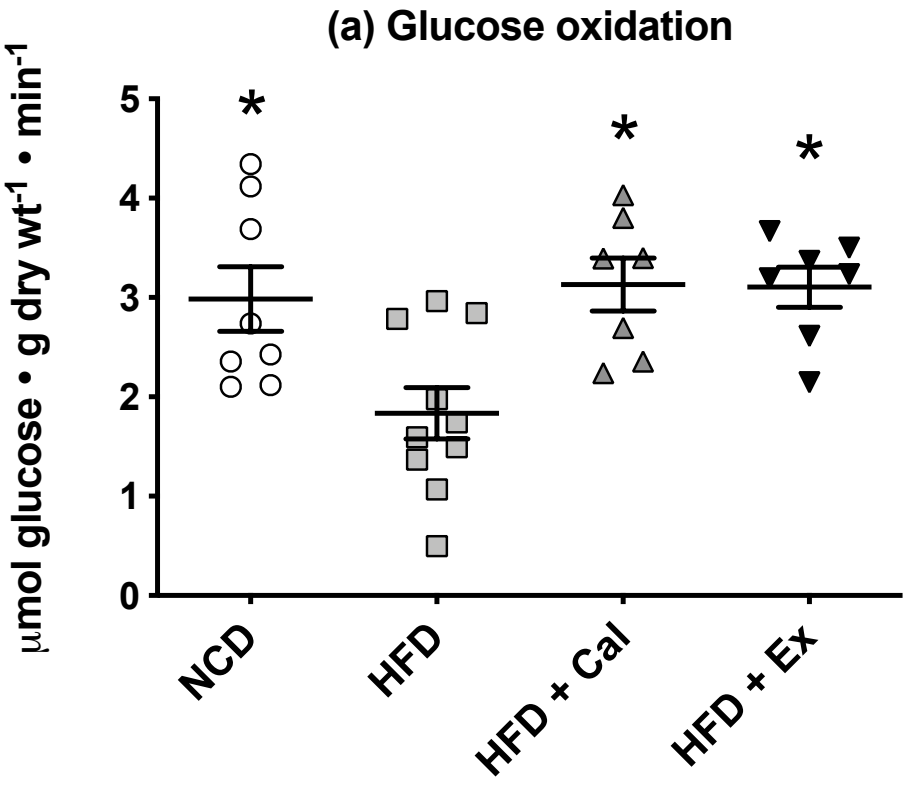

Adiposity index
Weight (g)
6
4
2
0
*
*
*
NCD
HFD
HFD + Cal
HFD + Ex

Figure 3


(a) Glucose oxidation
μmol glucose • g dry wt-1 • min-1
0
1
2
3
4
5
*
*
*
NCD
HFD
HFD + Cal
HFD + Ex

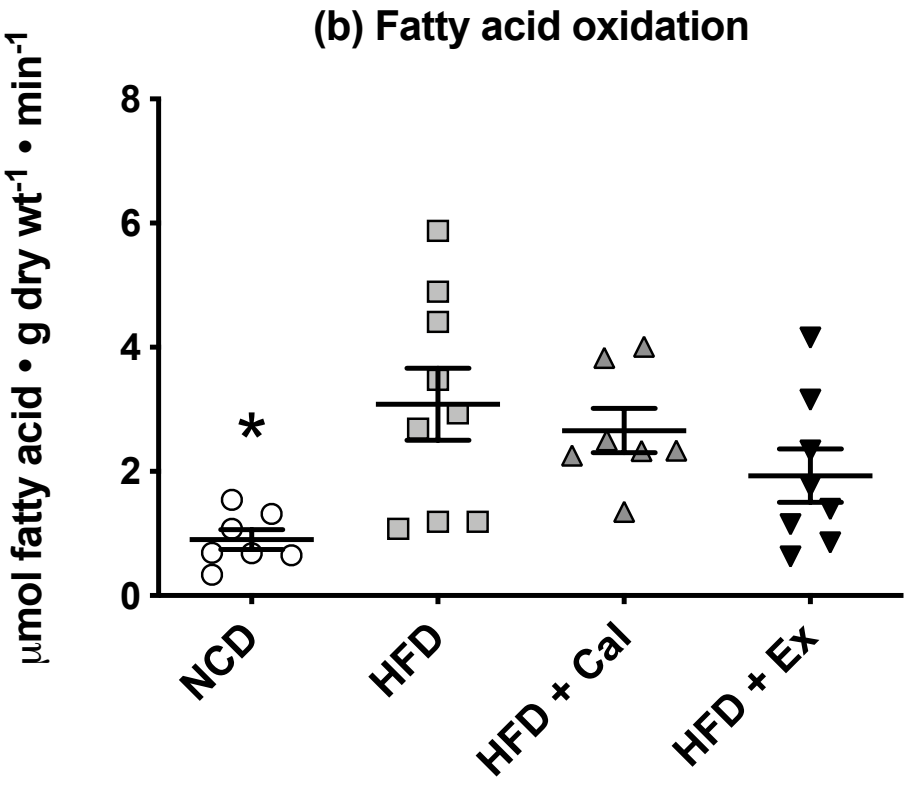

(b) Fatty acid oxidation
μmol fatty acid • g dry wt-1 • min-1
0
2
4
6
8
*
NCD
HFD
HFD + Cal
HFD + Ex

Figure 4

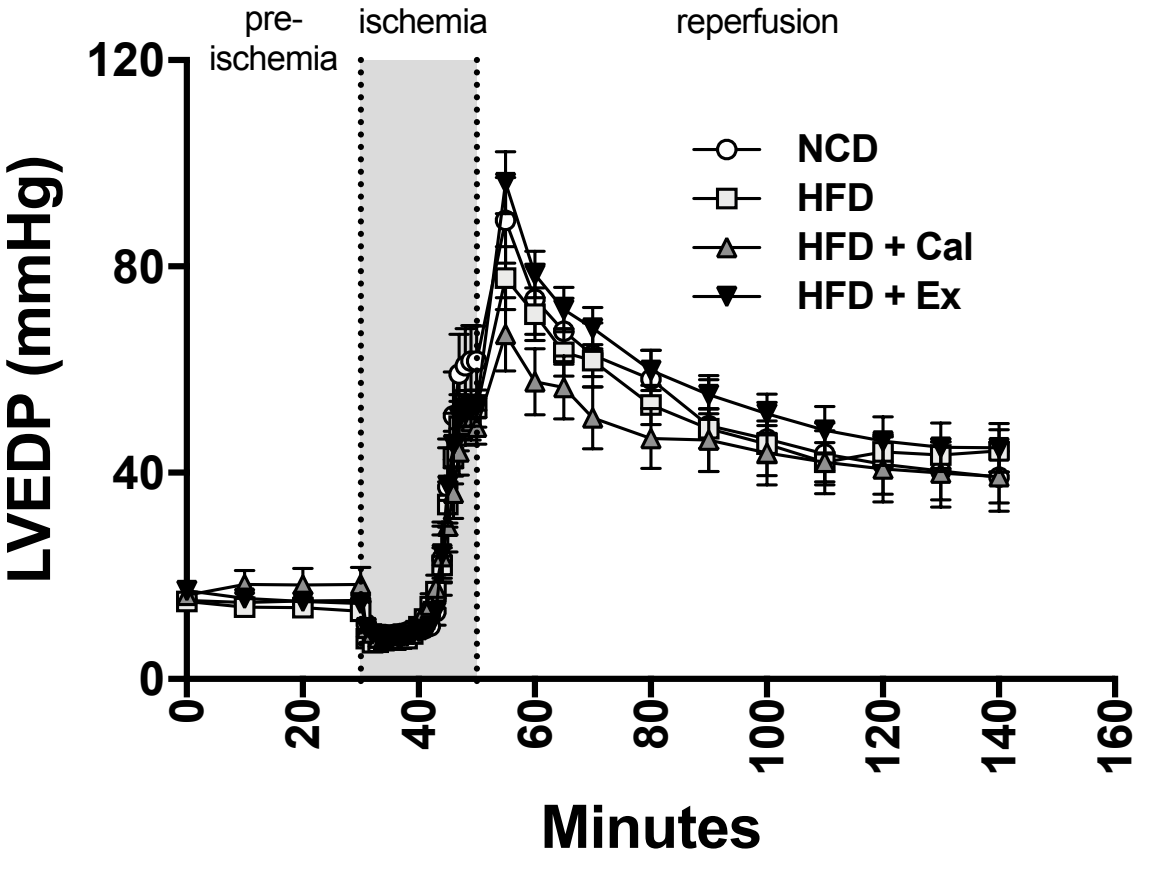

pre-
ischemia
ischemia
reperfusion
120
80
40
0
LVEDP (mmHg)
NCD
HFD
HFD + Cal
HFD + Ex
0
20
40
60
80
100
120
140
160
Minutes

Figure 5

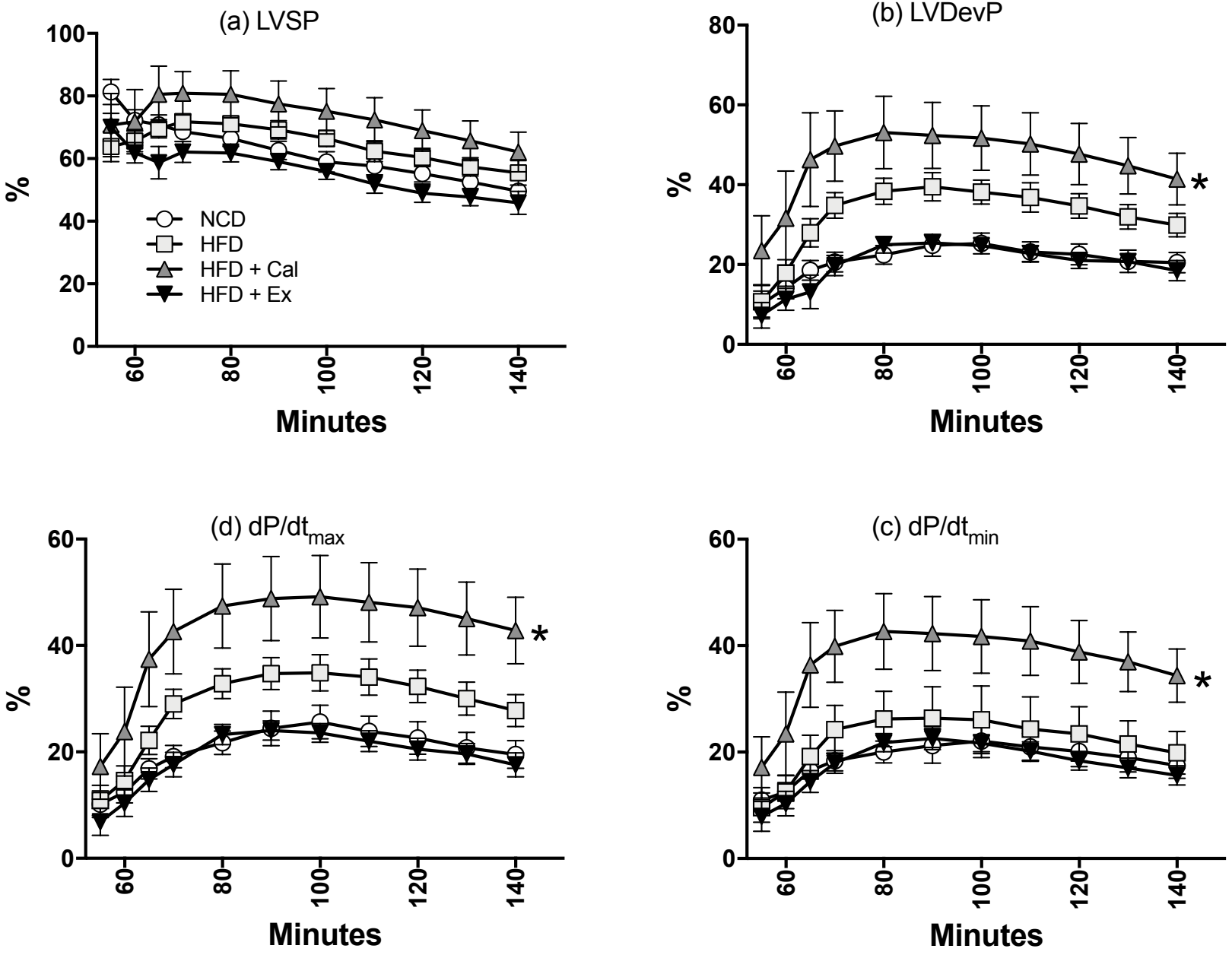
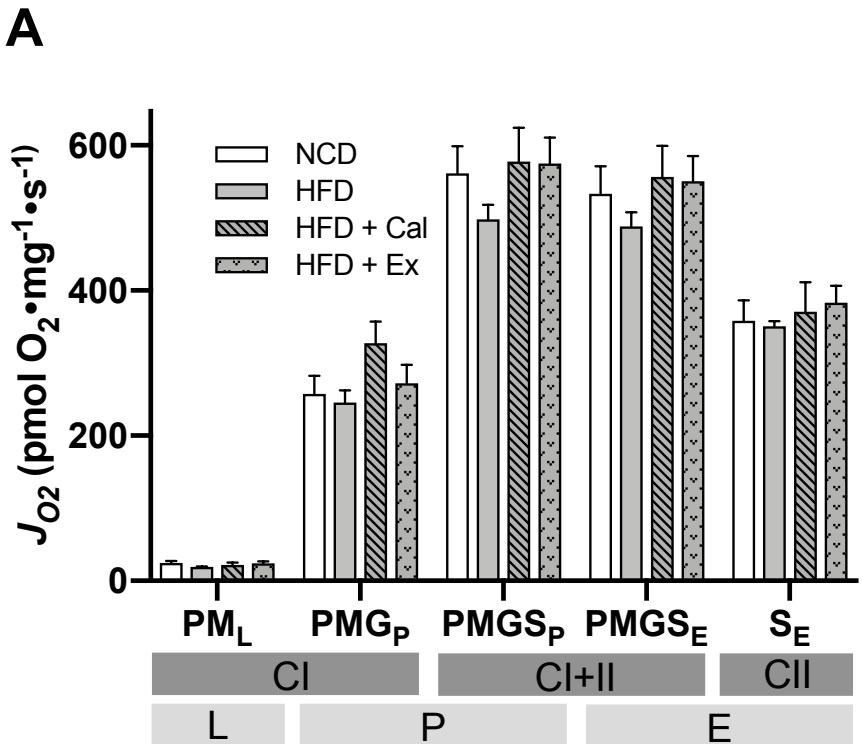
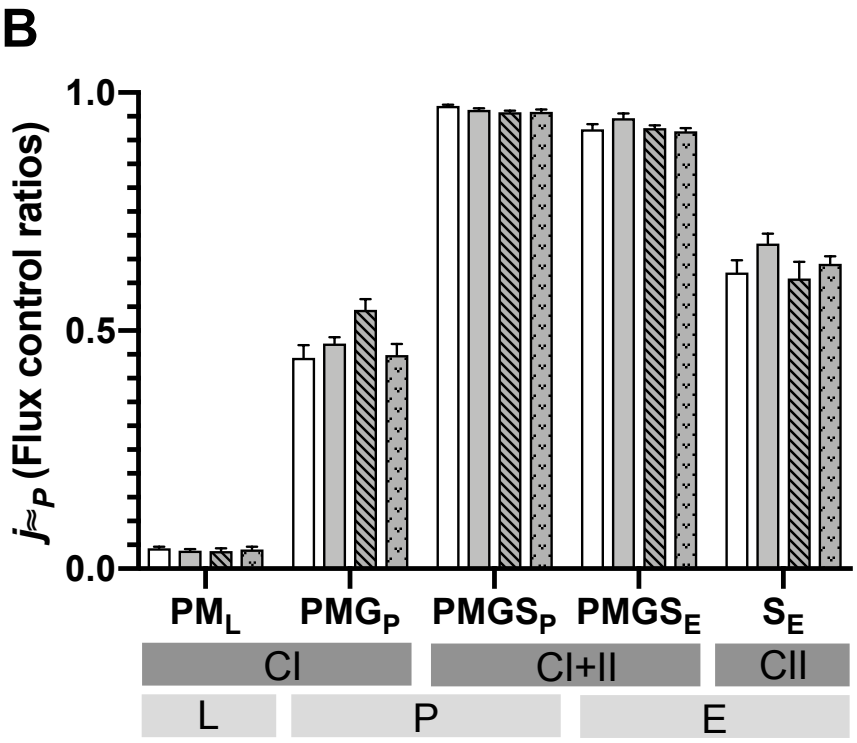

(a) LVSP
100
80
60
40
20
0
%
NCD
HFD
HFD + Cal
HFD + Ex
60
80
100
120
140
Minutes
(b) LVDevP
80
60
40
20
0
%
*
60
80
100
120
140
Minutes
(d) dP/dtmax
60
40
20
0
%
*
60
80
100
120
140
Minutes
(c) dP/dtmin
60
40
20
0
%
*
60
80
100
120
140
Minutes

Figure 6


A
NCD
HFD
HFD + Cal
HFD + Ex
J_{O2} (pmol O_2•mg^{-1}•s^{-1})
600
400
200
0
PM_L
PMG_P
$PMGS_P$
$PMGS_E$
S_E
CI
CI+II
CII
L
P
E


B
$j_{\approx P}$ (Flux control ratios)
1.0
0.5
0.0
PM_L
PMG_P
$PMGS_P$
$PMGS_E$
S_E
CI
CI+II
CII
L
P
E

Dietary Calanus oil recovers metabolic flexibility and rescues post-ischemic cardiac function in obese female mice

Short title: Energy metabolism and cardiac function

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Abstract

The aim of this study was to find out if dietary supplementation with Calanus oil (a novel marine oil) or infusion of exenatide (an incretin mimetic) can counteract obesity-induced alterations in myocardial metabolism and improve post-ischemic recovery of left ventricular (LV) function. Female C57bl/6J mice received high-fat diet (HFD, 45% energy from fat) for 12 weeks, followed by 8 weeks feeding with non-supplemented HFD, HFD supplemented with 2% Calanus oil, or HFD plus exenatide infusion (10 μg/kg/day). A lean control group was included, receiving normal chow throughout the whole period. Fatty acid and glucose oxidation was measured in *ex vivo* perfused hearts during baseline conditions, while LV function was assessed by an intra-ventricular fluid-filled balloon before and after 20 min global ischemia. HFD-fed mice receiving Calanus oil or exenatide showed less intra-abdominal fat deposition, compared to mice receiving non-supplemented HFD. Both treatments prevented the HFD-induced decline in myocardial glucose oxidation. Somewhat surprising, recovery of LV function was apparently better in hearts from mice fed non-supplemented HFD, relative to hearts from mice fed normal chow. More importantly however, post-ischemic recovery of hearts from mice receiving HFD with Calanus oil was superior to that of mice receiving non-supplemented HFD and mice receiving HFD with exenatide, as expressed by better pressure development, contractility and relaxation properties. In summary, dietary Calanus oil and administration of exenatide counteracted obesity-induced derangements of myocardial metabolism. Calanus oil also protected the heart towards ischemia, which could have implications for the prevention of obesity-related cardiac disease.

Key words: Ischemia-reperfusion; Myocardial fatty acid oxidation; Myocardial glucose oxidation; Obesity; Ventricular function

Introduction

Diabetes and diet-induced obesity are characterized by elevated supply and uptake of fatty acids to the heart, leading to a shift in myocardial energy metabolism towards fatty acid oxidation at the expense of glucose (1, 4, 23, 34). The rate of fatty acid uptake, however, may exceed the capacity of fatty acid oxidation, resulting in accumulation of lipid intermediates (diacylglycerol, ceramides, reactive oxygen species) which, over time, will create a lipotoxic state and impair myocardial metabolism as well as ventricular function (41).

Recent reports suggest that dysregulation of adipose tissue metabolism, in particular of the intra-abdominal fat depots, play a central role in linking obesity to impairment of cardiac metabolism and function (7). A key finding is that adipocyte hypertrophy in response to nutrient stress creates a local low-grade inflammatory response with production of pro-inflammatory cytokines (TNFα, IL-6 and IL-1β) and chemokines (14, 19-21). Adipose tissue inflammation can also result in elevated serum levels of inflammatory cytokines (13), which in turn leads to activation of IKKβ/NFκB and JNK pathways and dysregulation of insulin action in peripheral tissues, such as liver and skeletal muscle. The central role of abdominal fat in this process is probably due to its high lipolytic activity and direct drainage of inflammatory molecules and fatty acids to the liver via the portal vein (18). In fact, abdominal obesity has been regarded as the most serious new risk factor for cardiovascular and metabolic complications.

Of particular interest for this paper, Park et al. (32) reported that chronic high-fat feeding and obesity in mice impairs myocardial glucose metabolism, which was associated with ventricular hypertrophy and cardiac dysfunction. The same group reported that diet-induced obesity in mice increased macrophage and cytokine levels in heart, which was associated with significant reductions in AMPK phosphorylation and downregulation of glucose metabolism (25). The inflammatory response in obese adipose tissue is believed to be triggered by local hypoxia and activation of HIF-1α(39), because as the adipocytes expand and become hypertrophic, the distance between the blood bearing vessels increases and diffusion of oxygen becomes limited. Over time, this condition leads to local fibrosis and necrosis of adipocytes (39), which ultimately lead to inflammation and metabolic dysfunction, including increased mobilization of the fat stores (3, 24, 26). Therefore, the

obvious solution to prevent adipose tissue inflammation and the accompanying metabolic and cardiovascular complications is to apply strategies for targeted reduction of this particular fat store in obese subjects. We have previously reported that dietary supplementation with a small amount of oil from the marine crustacean, *Calanus finmarchicus*, reduces both intra-abdominal and hepatic fat deposition, while at the same time exerting a strong anti-inflammatory action in adipose tissue during high-fat feeding in male C57bl/6J mice. The main purpose of this paper is to find out if these beneficial effects of Calanus oil also translate into improved myocardial metabolism and cardiac function in diet-induced obese mice. For comparative reasons, we also tested the effect on these parameters of the GLP-1 receptor agonist, exenatide (marketed as Byretta), which is reported to increase insulin resistance from the pancreas and lower plasma glucose in diabetic patients (30).

Materials and methods

Animals and study design

The experiments were approved by the local authority of the National Animal Research Authority in Norway (FOTS id 8430), and the mice were treated according to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes (Directive 2010/63/EU). The animals were housed at 23°C (three animals per cage) under a reversed light/dark cycle (12-h dark/12-h light) with *ad libitum* access to food and drinking water. Body weight and food intake were recorded once every week.

Seven week-old C57Bl/6J female mice (Charles River, Sulzfeld, Germany) were randomly divided in four groups (16 mice each), one receiving normal chow diet (NCD) containing 10% energy from fat (#58Y2, Test Diet, IPS Ltd, Notts, UK), whereas the other three groups received a lard-based high-fat diet (HFD) containing 45% energy from fat (#58V8, Test Diet, IPS Ltd, Notts, UK, https://figshare.com/s/12580e0361db69d5cf5e). After an initial 12 weeks feeding period the diet was replaced by HFD supplemented with 2% Calanus oil for one of the high fat-fed groups (HFD + Cal, https://figshare.com/s/7fdffdb52f0ceafb4948), while another (HFD + Ex) received 10 μg/kg/day of the incretin mimetic, exenatide (Polypeptide

Laboratories Pvt Limited, Ambernath India), via mini-osmotic pumps (Alzet Micro-Osmotic Pump Model 1004, DURECT Corporation, ALZET Osmotic Pumps, Cupertino, CA, USA). This feeding regimen continued for another 8 weeks, so that the total feeding period lasted for 20 weeks.

The first 3-4 days after surgery and insertion of mini-osmotic pumps, the mice were single-housed in order to secure healing of the operation wound. This was also the reason why we used female mice, which are less aggressive than male mice. Also, mice who did not undergo surgery were subjected to single housing. Temgesic analgesia (0.1 mg/kg) was given 8 and 20 h postoperatively. A few mice were classified as *low responders* to the high fat diet (mice that did not increase their body weights above that of the lean controls) or *high responders* (mice whose body weight exceeded 40 g). These mice were excluded from the study. In addition, a few hearts were lost during perfusion, due to technical problems.

Heart perfusion and recording, substrate oxidation and ventricular function

The mice were anaesthetized with pentobarbital (100 mg/kg, 300 µL i.p.) mixed with heparin (100 U). Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit bicarbonate buffer (KHB), containing (in mmol/L): NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.25, $NaHCO_3$ 25.0 and glucose 11.1. The aorta was immediately cannulated, and the hearts were retrogradely perfused with KHB (gassed with 95% O_2 and 5% CO_2, pH 7.4, 37 ^oC) under a pressure of 73.5 mmHg. A small fluid-filled ballon connected to a pressure transducer (Transpac® IV; Abbott Laboratories, North Chicago, IL, USA) was inserted into the left ventricle via the mitral valve, and pressure signals were amplified and recorded by locally designed software (LabVIEW based). Thereafter, the hearts were connected to a buffer reservoir containing KHB supplied with 0.2 mmol/L palmitate bound to 3% BSA (fatty acid free, Europa Bioproducts Ltd., Cambridge UK) and perfused in recirculating mode.

After a 10 min stabilization period, the perfusion system was closed, and rates of glucose and fatty acid oxidation were determined simultaneously during the next 30 min by measuring $^{14}CO_2$ released from oxidation of [U-^{14}C] glucose and ^3H_2O released from oxidation of [9,10-^{3}H] palmitate, respectively, as described previously (2, 4, 5, 31). During this period, we also recorded pre-ischemic values of left ventricular function.

The hearts were next subjected to 20 min no-flow ischemia, followed by 90 min reperfusion. Left ventricular end-diastolic pressure was measured both during ischemia and reperfusion, and is given in mmHg. Recovery of other functional parameters was recorded and expressed as % of the corresponding pre-ischemic values. At the end of reperfusion, hearts were frozen at – 20 ^{o}C and cut in slices of 1 mm thickness and stained by 1% 2,3,5-triphenyl-2H-tetrazolium chloride solution. Infarct size was calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

Blood and tissue samples

Blood was collected (prior to and after excision of the heart) by puncture of the saphenous vein, while tissue samples and organs were taken immediately after excision of the heart, for later analyses of blood lipids and mRNA expression.

Quantitative real-time PCR

RNA isolation was performed using quantitative reversed real-time PCR (qPCR). Perirenal white adipose tissue samples were immersed in Allprotect Tissue Reagent (Qiagen) overnight at 4 °C. 90-110 mg tissue was used for RNA extraction in accordance to the RNeasy Lipid Tissue kit Protocol (Qiagen). RNA concentrations were measured by use of Nanodrop and stored at -80 °C before cDNA was prepared. cDNA was subsequently made according to High Capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific, Walthman, Massachusetts US). cDNA was stored at -20 °C until qPCR was performed in a Roche LightCycler 96, using a 1:5 dilution of the cDNA and the fast start essential DNA green master (Roche, Basel Swiss). Five house-keeping genes were analyzed to normalize the expression of the target genes to the geometric mean of the two best house-keeping genes, which were selected on the basis of the average expression stability values determined with geNorm. For quantification of the gene expression in the perirenal white adipose tissue, we used HMBS (hydroxymethylbilane synthase) and Cyclo. Forward and reverse primers of the target genes analyzed in the perirenal WAT are shown in Supplementary Table S1 (https://figshare.com/s/57449b263aac9bf7de86).

High resolution respirometry

Mitochondrial function was assessed by high-resolution respirometry (Oroboros Oxygraph-2k; Oroboros instruments, Innsbruck, Austria). All respirometry experiments were performed on fresh heart tissue. Following excision of the heart, a piece cardiac tissue was cut out of the left ventricle, washed and stored in relaxing and biopsy preservation solution (BIOPS, containing in mmol/L: Ca_2K_2EGTA 2.8, K_2 EGTA 7.2, ATP 5.8, $MgCl_2$ 6.6, taurine 20, Na_2 phosphocreatine 15, imidazole 20, dithiothreitol 0.5, and MES 50), pH 7.1. The tissue was thereafter homogenized using a PBI shredder SG3 (Pressure BioSciences Inc., MA, USA) to a final concentration of 0.8 mg/mL in mitochondrial respiration media (MiR05; containing in mmol/L: EGTA 0.5, $MgCl_2$ 3, K-lactobionate 60, KH_2PO_4 10, HEPES 20, and sucrose 110), pH 7.1. Mitochondria respiration was measured in the presence of several substrates, as previous described by Carles Cantó and Pablo M. Garcia-Roves (6) The O_2 flux that was left after adding antimycin A (residual oxygen consumption) was subtracted for the values of each step. Normalized flux ratios were calculated by dividing each value by the maximum flux.

Fatty acid composition of red blood cell membranes

Fatty acid composition of red blood cell membranes was determined after methylation as described by Hahn and Christie (17). The fatty acid methyl esters (FAMEs) were analyzed by capillary GLC using a Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph with a 50 m × 0.25 mm Chrompack CP-Sil 88 CB capillary column (Varian Inc., Palo Alto, CA, USA). The content of the individual fatty acids in the samples was expressed in percent of the total fatty acid content.

Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM). Graphs and statistical analyses were done in GraphPad prism (GraphPad Software, San Diego, CF USA). Significant differences between treatment groups were assessed by one-way ANOVA, followed by Dunnett's post-hoc test. A p-value < 0.05 was considered statistically significant.

Results

Administration of exenatide (but not dietary supplementation with Calanus oil) resulted in lower weight gain in mice on a high-fat diet (HFD), compared to that of mice on HFD alone

We have previously reported that feeding young male mice a high-fat diet (HFD) supplemented with 2% Calanus oil over an 8 weeks period resulted in a slightly reduced body weight gain, compared to that of mice given HFD alone (36). In the current study, we used adult female mice, which were made obese through an initial 12 weeks feeding period on HFD. In this case, dietary Calanus oil had no effect on body weight gain. On the other hand, administration of exenatide resulted in a near 30% lower weight gain ($p<0.05$) relative to the untreated HFD group (Fig. 1). In this group we also noted a small temporary drop in body weight during the first week of treatment, which we assume was due to the surgery, since a similar drop in body weight was recorded in a few mice receiving saline-filled pumps (data not shown). There was no differences between the high-fat diet groups with respect to organ weights (heart, liver, kidney and spleen, supplementary Fig. 1; https://figshare.com/s/57449b263aac9bf7de86), and food intake (supplementary Fig. 2; https://figshare.com/s/57449b263aac9bf7de86) was similar for all HFD groups.

The reduced body weight gain in exenatide-treaded mice was reflected in reduced deposition of intra-abdominal fat, equivalent to 35% ($p<0.05$) reduction of the adiposity index. In the Calanus oil group, the adiposity index was reduced by 22% ($p<0.05$), mainly due to reductions of perirenal and excess intra-abdominal fat (Fig. 2).

Dietary Calanus oil, as well as exenatide administration, prevented the obesity-induced alterations in myocardial substrate oxidation

Following sacrifice at the end of the 8 weeks treatment period, hearts were excised and perfused during baseline, normoxic conditions for measurement of myocardial substrate oxidation. In line with previous results (16), fatty acid oxidation was significantly increased ($p<0.05$) in HFD mice at the expense of glucose oxidation (Fig. 3). Both dietary Calanus oil and exenatide administration, however, counteracted the obesity-induced switch in myocardial metabolism, leading to full recovery of the capacity for glucose oxidation,

without having any clear effect of fatty acid oxidation. (Fig. 3). There was no difference between the two treatments regarding their impact on myocardial substrate oxidation.

Dietary Calanus oil, but not exenatide administration, rescued myocardial ischemia-reperfusion injury

To test whether the improvements in myocardial energy metabolism had any cardio-protective correlate, hearts from the various groups were subjected to ischemia-reperfusion (20 min no-flow ischemia followed by 120 min reperfusion). Pre-ischemic functional parameters are given in Table 1, indicating slightly better pressure development, as well as inotropic (dP/dt_{max}) and lusitropic (dP/dt_{min}) states in hearts from all HFD groups, relative to the lean controls. None of these differences were, however, statistically significant.

The ischemic insult produced a marked increase in the intraventricular pressure, plateauing at values around 50 mmHg, again with no differences between the groups (Fig. 4). A post-ischemic peak in the pressure (LVEDP) was recorded 5 min after start of reperfusion, but again there were no differences in the peak values or rate of decline of LVEDP throughout the reperfusion period.

In contrast to the prevailing view, we did not observe any negative impact of high-fat feeding on post-ischemic recovery of the other parameters of ventricular function (LVdevP, dP/dt_{max} and dP/dt_{min}) (Fig. 5). The important finding, however, was that post-ischemic recovery of these functional parameters in the HFD group receiving Calanus oil was superior to that of the non-treated HFD group, as well as the HFD + Ex and lean control groups ($p<0.05$). We also measured infarct size. However, the values were similar for all groups (including the lean control group), ranging between 47-58% (supplementary Fig. 3; https://figshare.com/s/57449b263aac9bf7de86).

Effect of Calanus oil and exenatide on cardiac mitochondrial function

Differences in cardiac mitochondrial respiration between the various groups were studied in freshly dissected tissue from the left ventricle, using high-resolution respirometry. No statistically significant differences were observed between the groups for any of the respiratory states, except for a slightly higher (non-significant) oxygen flux in the Calanus-oil group in the presence of complex I substrates relative to the other groups (Fig. 6 A). This difference was also evident when calculating the flux control ratios for the different respiratory states (i.e. the relative contribution of each respiratory state to the maximum flux (Fig. 6B). Maximum respiration in the coupled state (following addition of glutamate and succinate), with electron input through both complex I and II (C I+II) was not different between groups. Furthermore, oxygen flux was essentially unaltered following addition of the exogenous uncoupler FCCP, reflecting the efficiency of the phosphorylation system (adenine nucleotide translocase, phosphate transporter, and ATP synthase) in matching the potential of the electron transfer system in mouse cardiac muscle.

Fatty acid composition of red blood cell membranes

Gas chromatography analysis revealed significantly higher content of poly-unsaturated omega-3 fatty acids in red blood cell membranes of mice receiving Calanus oil-supplemented HFD, compared to that of the other HFD groups, as well as the lean control (NCD) group. This resulted in a marked increase in the omega-3 index and the n-3/n-6 ratio (Table 2).

Gene expression

In order to find out whether the observed alterations in metabolism were reflected at the gene level, we examined mRNA expression of genes involved in metabolic regulation in adipose tissue. Accumulation of intra-abdominal fat in the HFD groups was associated with increased mRNA expression of CD36 in perirenal adipose tissue relative to that of normal chow-fed mice, in line with a high fatty acid uptake in the adipocytes (Fig. 4, supplementary data; https://figshare.com/s/57449b263aac9bf7de86). This response was not influenced, however, by Calanus oil supplementation or administration of exenatide. Expression of GLUT4 was somewhat lower in mice receiving Calanus oil, while the expression of PDK4 was reduced both in the Calanus oil and exenatide group, which might be a compensatory

mechanism to maintain the flux through the PDH complex despite of reduced glucose uptake.

Low-grade inflammation and release of pro-inflammatory adipokines in obese adipose tissue are suggested to cause insulin resistance in peripheral tissues. We found, however, that mRNA expression of pro-inflammatory genes like IL-6 and TNFα was extremely low expressed in perirenal adipose tissue (data not included), but other indicators of inflammation (MCP1 and EMR1) was significantly increased in the HFD group, relative to normal chow-fed mice. All three HFD groups showed increased expression of GPR120, and a very unexpected finding was that adiponectin was significantly increased in the HDF groups, relative to the lean control group.

Discussion

Main findings

Obesity induced by obesogenic diets is characterized by a shift in myocardial energy metabolism towards increased fatty acid oxidation at the expense of carbohydrates. In the present study we show, however, that dietary supplementation with Calanus oil, as well as administration of the GLP-1 receptor agonist, exenatide (incretin mimetic), were able to prevent the obesity-induced decline in myocardial glucose utilization, while fatty acid utilization was not significantly affected. In contrast to the notion that obesity impairs recovery of cardiac function after an ischemic insult, we observed that the post-ischemic recovery of ventricular function in *ex vivo* perfused hearts from high fat-fed mice was not impaired relative to hearts from mice receiving normal chow. More importantly, post-ischemic recovery of hearts from mice receiving high-fat diet with Calanus oil exhibited significantly better recovery than hearts from mice on non-supplemented high-fat diet, indicating obesity-dependent cardio-protective properties of the Calanus oil.

Anti-obesogenic effect of Calanus oil and exenatide

High-fat feeding resulted in increased deposition of intra-abdominal fat (supported by increased mRNA expression of CD36 and GPR120). However, the results confirmed previous reports demonstrating that both dietary Calanus oil and administration of exenatide (30) have anti-obesogenic effects, although less pronounced in the female mice used in the present study than that previously reported for male mice (19, 20). The mechanism behind the anti-obesogenic effect is so far unknown, and both current and previous results (19, 20) exclude the possibility that it is due to reduced energy intake. Mack et al. (30) reported, however, decreased food intake and a drop in body weight gain in diet-induced obese (DIO) mice during the first week following administration of exenatide and claimed that this could be due to discomfort of the animals, since both emesis and nausea have been reported with clinical use of the drug. Food intake dropped temporarily following infusion of exenatide also in the current experiment - both after the first and second insertion of mini-osmotic pumps. However, we believe that this response was due to the discomfort associated with the surgical procedure, since insertion of saline-filled mini-osmotic pumps (in a few mice) showed a similar drop in body weight (not shown).

Effect of Calanus oil and exenatide on adipose tissue metabolism

Obesity is associated with increased adipose tissue lipolysis and increased release of fatty acids to the circulation, due to increased size of the adipocytes as well as insulin resistance (3, 11, 26). Moreover, obesity is tightly associated with the development of a local low-grade inflammation in adipose tissue. Thus, expansion of adipocytes results in elevated production of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 in obese individuals (21), which may negatively influence insulin action in adipocytes and hepatocytes via activation of IKKβ/NFκB and JNK pathways(37). In contrast to previous results in male DIO mice(19, 20), mRNA expression of TNF-α and IL-6 was hardly detectable in the current study on HFD-fed female mice (data not shown); the only evidence of obesity-induced inflammation was an apparent increase in the expression of MCP1 and EMR1, which was not influenced by Calanus oil or exenatide treatment. The low inflammatory status could probably be explained by the finding that high-fat diet induced only a relative mild degree of adiposity, so that the signal for adipokine secretion(39) was missing. In addition, it has been reported that genes involved in inflammation are more highly upregulated in males than in

females (15). Also, the present observation of increased mRNA expression of *adiponectin* in adipose tissue in response to HFD is in line with previous reports (10). Still, dietary Calanus oil or infusion of exenatide resulted in reduced deposition of intra-abdominal fat, compared to that of untreated HFD mice. The underlying mechanism is not clear, but increased adipose tissue lipolysis and/or decreased lipogenesis could be involved. In addition, increased hepatic uptake of fatty acids could drain fatty acids from the abdominal fat stores. However, these possibilities need to be further investigated.

Effect of Calanus oil and exenatide on myocardial metabolism

The high energy demand of the heart is covered to a large extent by oxidation of fatty acids. Obesity, however, leads to an imbalance between fatty acid uptake and oxidation, where the myocardial fatty acid supply exceeds the fatty acid oxidation capacity of the heart. The obesity-induced changes in myocardial substrate oxidation were confirmed in the female hearts used in the current experiments. Of note, however, dietary Calanus oil supplementation, as well as exenatide administration, were able to restore the ability of the heart to oxidize glucose, but did not significantly suppress the myocardial over-reliance of fatty acid oxidation. Thus, one gets the impression that the two treatments led to an increase in total myocardial substrate oxidation, but calculating the sum of ATP-production from fatty acids and glucose (38 ATP/mole glucose and 131 ATP/mole fatty acid) showed that the total ATP production was similar in the three high fat-fed groups. Having said that, we have no information of any contribution from other substrates, e.g. ketone bodies).

Intuitively, one would expect that the reduction in fat mass (and probably also in hepatic fat content) in response to these treatments be reflected in reduced fatty acid delivery from adipose tissue (and tri-acylglycerol from the liver) to the blood. Thus, the observed improvement in myocardial metabolism following these treatments could be explained in terms of the Randle effect (23, 34), where lower levels of circulating lipid substrates (FA and TAG) render the heart less fatty acid–dependent for energy production, while allowing recovery of the myocardial capacity for glucose oxidation. Measurements of plasma fatty acid and tri-acylglycerol concentrations revealed, however, no difference between the

groups for any of these lipids (supplementary Fig. 5; https://figshare.com/s/57449b263aac9bf7de86). Still, one should bear in mind that the observed concentrations of these lipid substrates represent merely spot measurements, which do not necessarily reflect the long-term supply of lipids to the heart. Also, it is reason to believe that suppression of adipose tissue inflammation and reduced release of adipose tissue-derived pro-inflammatory cytokines (19) play a role, because these substances can negatively influence metabolic pathways and insulin signaling in the heart (27).

Effect of Calanus oil and exenatide on post-ischemic recovery of ventricular function

Normally, one would expect that high-fat feeding leads to accumulation of myocardial TG, and that mobilization of these TG stores (catalyzed by the adipose triglyceride lipase, ATGL) should release fatty acids, which together with the external fatty acid supply, could lead to lipid overload and accumulation of toxic lipid metabolites. Thus, previous studies on rodent models (1, 28) have reported that obesity-induced shifts in myocardial fuel selection in favor of fatty acids are associated with accumulation of toxic lipid intermediates and contractile abnormalities (16, 22, 42). Moreover, Peterson (33) reported reductions in both systolic myocardial velocity and early diastolic myocardial velocity with increasing BMI in young healthy obese women. In the present study, using *ex vivo* perfused female mouse hearts, long-term feeding with high-fat diet did not impair ventricular function. If anything, the functional parameters obtained during baseline normoxic conditions were indicative of improved performance of hearts from the high fat-fed groups, although the effects were not statistically significant (Table 1). Furthermore, high-fat feeding did not compromise post-ischemic functional recovery, since average pressure development and contractility were not impaired, relative to hearts from lean controls. If anything, HFD hearts recovered better than hearts from the lean controls. At a first glance, and in light of several reports in the literature (29, 40, 44, 45), we were surprised with these observations, but a deeper analysis of the literature revealed that increased resistance to ischemic heart injury has been reported previously both in rats fed a high-fat diet (35, 43), as well as in high fat-consuming humans (the “obesity paradox”) (8). Furthermore, a recent study by Edland et al. (9) showed that long-term consumption of an obesogenic diet in mice increased the tolerance to ischemia-reperfusion injury by reducing infarct size in *ex vivo* perfused hearts from these mice. Of

note, the study by Edland et al., as well as the present study, used female mice, and it would be of interest to find out if there are gender differences that might influence the outcome of an ischemic insult in obesity.

The explanation why post-ischemic recovery of hearts from mice fed with Calanus oil-supplemented HFD was superior to that of the other groups is not clear. In particular do we need an explanation why hearts from mice treated with exenatide did not recover LV function to the same degree as hearts from Calanus oil-treated mice. The metabolic pattern prior to ischemia revealed that both treatments abrogated the obesity-induced suppression of glucose oxidation. Such an improvement in cardiac metabolism is expected to result in increased cardiac efficiency (22) and less accumulation of lipotoxic metabolites (12), which in turn would prime the hearts to better tolerate the ischemic insult and the subsequent stress during reperfusion. Therefore, the finding that hearts from Calanus oil-treated mice showed significantly better recovery of LV function than those from exenatide-treated mice (as well as the other groups), appears to be unrelated to the improvement in glucometabolic control. Probably, eight weeks of Calanus oil treatment might have led to neurohumoral and/or hemodynamic alterations which have the potential to change the intrinsic properties of the heart, which persist in ex vivo perfusions. Moreover, obesity influences more than just glucose and fatty acid oxidation, leaving the possibility that Calanus oil–derived omega-3 fatty acids might have influenced additional aspects of myocardial metabolism, e.g. reduction of oxidative stress (38).

In an attempt to find out whether dietary Calanus oil supplementation was associated with improved myocardial energy production, we measured mitochondrial respiration in cardiac fibers, using a standard protocol. However, we were not able to detect any differences between the groups for any of the respiratory states, except for a slightly (non-significant) elevated oxygen flux in the presence of complex I substrates. Alternatively, one might speculate whether Calanus oil (or its metabolites) has a direct effect on the contractile apparatus due to incorporation of omega-3 fatty acids into the sarcolemma, thereby modifying the membrane fluidity and improving calcium transport in the cardiomyocytes. In

support of this hypothesis, we measured a significantly higher omega-3 index (as well as n-3/n-6 ratio) in red blood cell membranes from the Calanus oil-treated group (Table 2). Further studies are needed, however, to explain the beneficial impact of Calanus oil on post-ischemic contractile function in hearts from obese mice.

Conclusion

Obesity induced by high-fat feeding shifts myocardial substrate metabolism towards almost exclusively fatty acid oxidation at the expense of glucose. Both dietary Calanus oil and exenatide treatment counteracted these metabolic derangements. Calanus oil supplementation of the high-fat diet provided, in addition, protection from ischemia-reperfusion damage, apparently unrelated to the concomitant improvement in myocardial metabolism.

Limitations

One limitation of this study is the lack of any assessment of insulin sensitivity or insulin signaling - both in response to high-fat feeding and following treatment with Calanus oil and exenatide. The study would also have benefitted from assessments of inflammatory markers in plasma and/or cardiac tissue in order to suggest causality between the reported parameters. Finally, inclusion of fatty acids as respiratory substrate would have added additional information regarding the mitochondrial function in response to the treatments.

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Conflict of interest

Conflict of interest: Prof. Terje Larsen (senior author) has a small position as scientific advisor in Calanus AS.

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Figure legends

Figure 1: Body weight development in HFD and NCD-fed mice during the initial 12 weeks feeding period, as well as during the subsequent 8 weeks treatment period. The HFD and NCD groups stayed on their diet for the full 20 weeks and served as lean and obese controls, respectively. After the 12 week fattening period, two groups were treated with either Calanus oil (HFD + Cal) or exenatide (HFD + Ex) for another 8 weeks. Calanus oil was mixed into the pellets by the manufacturer (TestDiet), while exenatide was administered via mini-osmotic pumps.
*, $p<0.05$ vs HFD (n = 10-15). Significant differences between treatment groups were assessed by two-way repeated measures ANOVA.

Figure 2: Weight of different intra-abdominal fat depots at sacrifice of the various groups of mice. Adiposity index was calculated as the sum of the individual fat depots. *, $p<0.05$ vs HFD (n = 8-13). Significant differences between treatment groups were assessed by one-way ANOVA, followed by Dunnett's post-hoc test.

Figure 3: Myocardial glucose (a) and fatty acid (b) oxidation in the various groups of mice during baseline normoxic perfusion. *, $p<0.05$ vs HFD (n = 7-10). Significant differences between treatment groups were assessed by one-way ANOVA, followed by Dunnett's post-hoc test.

Figure 4: Development of cardiac contracture during no-flow ischemia, as well as post-ischemic rise and decline in left ventricular end-diastolic pressure (LVEDP) in hearts from the various groups of mice. LVEDP during the normoxic, pre-ischemic perfusion was set to 15 mmHg. No statistically significant differences were observed between treatment groups as tested by two-way repeated measures ANOVA.

Figure 5: Post-ischemic recovery of left ventricular function in *ex vivo* perfused hearts from the various groups of mice. (a) LVSP, left ventricular systolic pressure; (b) LVDevP, left ventricular developed pressure; (c) dP/dt_{max}, maximum rate of pressure change during isovolumic contraction; (d) dP/dt_{min}, maximum rate of pressure change during isovolumic relaxation. Heart rate recovered to approximately 80% of the pre-ischemic values in all groups (not shown). * $p< 0.05$ vs HFD (n = 7-11 in each group). Area under the curve was calculated for each heart in the various groups. Significant differences between treatment groups were assessed by one-way ANOVA, followed by Dunnett's post-hoc test.

Figure 6: Mitochondrial respiration in cardiac muscle at the end of the experimental period for the same group of mice as described in Fig. 1. Oxygen flux (J_{O2}) was measured with an Oroboros-2k oxygraph. A: First, pyruvate and malate (PM) were added for assessment of oxygen flux in the LEAK state (L). Thereafter, ADP, cytochrome C and glutamate (G) were added to measure oxygen flux in the OXPHOS state (P) with electron flow from complex I (CI). Maximum coupled respiration with electron flow from both complex I and II (CI+II) was obtained following addition of succinate (S). The electron transfer system (E) capacity was measured after addition of FCCP, followed by rotenone (complex I inhibitor) to determine the specific contribution from complex II (CII). Finally, antimycin A was added to inhibit complex III, and the remaining oxygen flux (residual oxygen consumption) was

625 subtracted from each of the previous respiratory states. B: Flux control ratios (j≈P), i.e. oxygen flux
626 rates in the various respiratory states normalized to maximum flux rate. n=8, 7, 7 and 10 for NCD,
627 HFD, HFD+Cal and HFD + Ex, respectively.

628

Table 1: Pre-ischemic left ventricular function during baseline, normoxic conditions

	NCD (n=9)	HFD (n=11)	HFD + Cal (n=7)	HFD + Ex (n=9)
LVSP	121 ± 12	145 ± 6	135 ± 8	150 ± 6
LVDevP	109 ± 12	134 ± 6	124 ± 8	139 ± 7
LVEDP	15 ± 1	14 ± 1	15 ± 1	15 ± 1
dP/dt_{max}	4493 ± 500	5818 ± 263	5340 ± 376	5762 ± 281
dP/dt_{min}	-3133 ± 391	-4219 ± 263	-3698 ± 364	-3988 ± 224
BPM	279 ± 11	289 ± 12	279 ± 20	291 ± 15

Table 2: Fatty acid composition of red blood cell membranes (RBC)

Fatty acid	NCD Mean ± SD	HFD Mean ± SD	HFD + Cal Mean ± SD	HFD + Ex Mean ± SD
16:0	25.58 ± 0.33	23.87 ± 0.38	25.36 ± 0.36	23.98 ± 0.29
16:1n-7	1.24 ± 0.44	0.83 ± 0.23	0.87 ± 0.13	0.81 ± 0.11
18:0	13.66 ± 0.60	16.71 ± 0.93	16.26 ± 0.54	17.03 ± 0.76
18:1n-9	14.86 ± 0.46	12.95 ± 0.31	12.99 ± 0.25	13.08 ± 0.39
18:1n-7	2.41 ± 0.21	1.40 ± 0.11	1.31 ± 0.06	1.42 ± 0.11
18:2n-6	6.75 ± 0.47	9.41 ± 0.55	9.50 ± 0.20	8.17 ± 3.06
18:4n-3	n.d.	n.d.	0.55 ± 0.02	n.d.
20:4n-6	22.84 ± 0.62	22.92 ± 0.71	17.02 ± 0.28*	22.53 ± 0.50
20:5n-3	0.84 ± 0.12	0.71 ± 0.15	2.77 ± 0.38*	0.64 ± 0.08
22:4n-6	1.98 ± 0.09	2.45 ± 0.09	1.12 ± 0.07*	2.47 ± 0.09
24:1n-9	1.31 ± 0.07	1.21 ± 0.14	n.d.	1.17 ± 0.09
22:5n-3	0.55 ± 0.04	0.64 ± 0.06	1.66 ± 0.07*	0.61 ± 0.04
22:6n-3	7.96 ± 0.48	6.91 ± 0.49	10.72 ± 0.40*	7.09 ± 0.34
Omega-3 index	10.1 ± 0.4	9.0 ± 0.7	18.5 ± 1.0*	9.2 ± 0.6
n-3:n-6 ratio	29.5 ± 1.6	23.7 ± 1.5	56.4 ± 2.6*	25.4 ± 3.2

Table legends

Table 1: Ventricular function was assessed using a fluid-filled balloon in the left ventricle. LVSP, left ventricular systolic pressure; LVDevP, left ventricular developed pressure; dP/dtmax, maximum rate of pressure change during isovolumic contraction; dP/dtmin, maximum rate of pressure change during isovolumic relaxation. Values (mean ± SEM) are based on the three last recordings before ischemia.

Table 2: Relative percent of various fatty acids in red blood cell membranes from the various groups of mice. Note significantly higher values of n-3 PUFAs (and lower content of n-6 PUFAs) in RBC from HFD + Cal mice, resulting in a significantly higher omega-3 index and n-3:n-6 ratio for this group. Also, stearidonic acid (18:4n-3) was detected only in RBC from the HFD + Cal group. * $p<0.05$ versus all other groups (n = 7-9)