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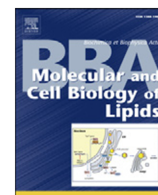

Cardiac lipoprotein lipase activity in the hypertrophied heart may be regulated by fatty acid flux

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ABSTRACT

Cardiac hypertrophy is characterised by an imbalance between lipid uptake and fatty acid β-oxidation leading to an accumulation of lipids, particularly triacylglycerol (TAG). It is unclear whether uptake mechanisms such as lipoprotein lipase (LPL) can be attenuated to diminish this uptake. Rats were cold acclimated to induce cardiac hypertrophy and increase cardiac LPL. Lipid uptake and metabolism were altered by feeding a 'Western-style' high fat diet (WSD) or feeding oxfenicine (2 g/L) in the drinking water. Diastolic stiffness (increased volume change/unit pressure change) was induced in hypertrophied hearts for rats fed WSD ($P<0.05$) or WSD + oxfenicine ($P<0.01$), although absolute performance of cardiac muscle, estimated from stress–strain calculations was unchanged. Cold acclimation increased cardiac endothelial LPL ($P<0.05$) but this was diminished following oxfenicine. Following WSD LPL was further decreased below WSD-fed control hearts ($P<0.05$) with no further decrease by oxfenicine supplementation. A negative correlation was noted between plasma TAG and endothelial LPL (correlation coefficient = −0.654; $P<0.001$) but not cardiac TAG concentration. Transcript levels of angiopoietin-like protein-4 (ANGPTL4) were increased 6-fold by WSD ($P<0.05$) and increased 15-fold following WSD + oxfenicine ($P<0.001$). For CA-hearts fed WSD or WSD + oxfenicine ANGPTL4 mRNA levels were preserved at chow-fed levels. VLDLR protein levels were increased 10-fold ($P<0.01$) by CA. ANGPTL4 protein levels were increased 2-fold ($P<0.05$) by WSD, but restored following oxfenicine. For CA-hearts WSD increased ANGPTL4 protein levels 3-fold ($P<0.01$) with WSD + oxfenicine increasing ANGPTL4 protein 4-fold ($P<0.01$). These data suggest that endothelial LPL levels in the heart are altered to maintain FA flux and may exploit ANGPTL4.
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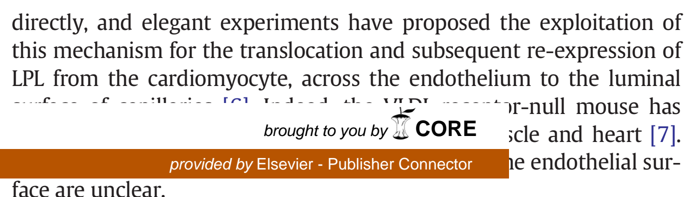
Introduction

The heart has a high and unremitting demand for energy and relies on metabolic flexibility to derive this energy from the prevailing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ly transported in the form of chylomicrons and must be assimilated into the myocardium through lipoprotein lipase, an endothelium-expressed enzyme that controls the transport of triacylglycerol-derived fatty acids (FA) into the cardiomyocyte across the endothelium. Chylomicrons may provide the majority of FA-derived energy as measured for the *in vitro* perfused myocardium [2] and in the whole animal [3]. LPL is intricately coupled to the very-low-density lipoprotein receptor (VLDL-receptor) and investigations have demonstrated the potency of the VLDL-receptor to bind lipoproteins and act as an anchor [4], facilitating the lipolysis of lipoprotein particles to release NEFA and thus generate a high local concentration at the endothelium. The VLDL-receptor may also facilitate uptake of core lipids from lipoproteins [5]. Further studies have also demonstrated the ability of VLDL-receptor to bind LPL directly, and elegant experiments have proposed the exploitation of this mechanism for the translocation and subsequent re-expression of LPL from the cardiomyocyte, across the endothelium to the luminal surface of capillaries [6]. Indeed, the VLDL-receptor-null mouse has ...scle and heart [7]. ...he endothelial surface are unclear.

We have recently demonstrated that chronic activation of AMPK with metformin increased the endothelial localisation of LPL [8]. Given the assertion that the hypertrophied heart is relatively energy-depleted and the subsequent activation of AMPK increases the uptake of substrates (both glucose and fatty acids) to offset this ATP shortfall, suggests that chronic activation of AMPK, as occurs in cardiac hypertrophy [9], and may result in accumulation of lipid in the myocardium through increased translocation of LPL to the capillary endothelium. This may be exacerbated further by the decline in fatty acid oxidation noted for the hypertrophed heart [10]. Intracellular lipid content depends on the balance of uptake (both NEFA and LPL-mediated TAG uptake) and the rate of FA oxidation. Lipid accumulation is believed to contribute to lipotoxicity and alters the sensitivity of the myocardium to catecholamine-mediated inotropy [11], altered insulin signalling [12] and triggers apoptosis [13]. However, experimental models have tended to rely upon constitutive over-expression of LPL at the luminal

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surface of the capillary. What is unclear is whether a mechanism for altering the transfer of LPL to the capillary endothelium is functional in the intact myocardium to prevent the ectopic accumulation of lipid in the cardiomyocyte. Multiple sites are potential targets including the synthesis of LPL, the translation of mRNA to functional enzyme or the transfer of functional LPL from the cardiomyocyte to the endothelial surface of capillaries.

We have exploited the cold-acclimated rat as a model for physiological cardiac hypertrophy that does not show impaired β-oxidation of fatty acids [14] to investigate for the first time the influence of hypertrophy on the expression of LPL and VLDL-receptor proteins and quantify the activity of cardiac lipoprotein lipase following changes to the lipid milieu in the intact rat. Lipid accumulation was initiated using either a Western-style high fat diet and/or the chemical inhibitor of carnitine palmitoyl-transferase 1 (CPT1), oxfenicine. Cardiac performance was estimated and the expression and activity of LPL enzyme quantified to determine whether altering substrate availability can change the presentation of LPL at the cardiac capillary surface.

Materials and methods

Materials

^{3}H-[9,10]-triolein were purchased from Amersham Biosciences (Chalfont, UK). Fatty acid-free bovine albumin and all buffer salts were purchased from Sigma (Poole, UK). All solvents were ANALAR grade and purchased from Fisher Scientific (Loughborough, UK). Kits for the measurement of plasma and tissue triacylglycerol and cholesterol were obtained from Randox (Crumlin, Antrim UK). Ventricular balloons were constructed 'in house' using Saran Wrap polythene film. RT-PCR reagents were obtained from Applied Biosystems (Carlsbad, CA, USA) (assay on demand VLDLR – Rn01498163_m1: LPL – Rn01446981_m1: ANGPTL4 – Rn01528817_m1: Internal reference GAPDH – 4308313).

Methods

Animals

Animals were maintained in accordance with the UK Home Office, Animal Scientific Procedures Act (1986) and the experiments were approved by the University of Birmingham Ethical Review Committee. Animals were housed at 22 °C 12 h light/12 h dark with *ad libitum* access to food and water.

Cold acclimation

Animals were acclimated to cold as previously outlined [14,15]. Briefly rats (60gm) were housed in pairs in an environmental chamber with minimal wood-chip cage bedding and ad libitum access to both food and water. The chamber was cooled from 21 °C to 4 °C over a period of 4 weeks, with day length reduced from 12 h light/dark to 1 h light/23 h dark over the same period. After this 4 week period rats were randomised into separate groups and maintained on either a chow diet or a Western-style high fat/sucrose diet (WSD-824503, Special Diet Services, Lilico Biotechnology, UK) for a further 2 weeks. These two groups were further sub-divided into groups receiving either tap water to drink or water supplemented with 4-hydroxy-phenyl-glycine (Oxfenicine — 2 g/L) [16], giving a total of 4-experimental groups. These groups were duplicated using control animals maintained under standard conditions of temperature and day length, but supplemented with either chow diet or WSD and water or oxfenicine solution (2 weeks–2 g/L oxfenicine).

Tissue isolation and heart perfusion

Animals were prepared surgically following 6 weeks of diet/temperature manipulation as outlined previously [17]. Briefly, anaesthesia was induced with isoflurane (~4% isoflurane in oxygen). Blood was collected in a heparinised syringe from inferior vena cava and following thoracotomy, hearts were excised. Hearts were perfused in retrograde fashion, as outlined previously [17]. A small flexible non-elastic balloon was inserted into the left atrium through the mitral valve and into the left ventricle. This fluid-filled balloon was attached to a fine plastic catheter and connected to a pressure transducer (MEMSCAP, Skoppum, Norway) and a graduated syringe (0–1000 μl: Hamilton, Nevada, USA). Hearts were maintained at 37 °C and perfused at a constant pressure (100 cm H_2O) with a Krebs–Henseleit crystalloid medium supplemented with glucose (10 mM) and $CaCl_2$ (1.3 mM) gassed with oxygen/CO_2 (95:5). Developed pressure was measured following isovolumic contraction of the fluid-filled balloon and recorded to computer using a digital interface (AD Instruments, Chalgrove, Oxford, UK).

Ventricular performance

Ventricular performance was estimated, as outlined previously [17]. Balloon volume was increased in incremental steps (50 μl) and developed pressure was recorded in real time. Pressures were allowed to stabilise until diastolic pressure remained constant before initiating further increases in balloon volume. Incremental increases in balloon volume were performed until the peak systolic pressure developed exceeded 200 mmHg. The balloon was then deflated and the process repeated. Coronary flow was estimated from timed collections of a known volume of perfusate and expressed as volume/unit time/unit mass of cardiac tissue. Ventricular performance was calculated off-line following the experiment using computer analysis software (Chart Version 5.0, AD Instruments, Chalgrove, Oxford, UK). Heart rate, systolic pressure and diastolic pressure were measured and hence developed pressure calculated. Rate of change of pressure (+dP/dt) was calculated from the maxima of first order derivative of pressure trace. Rate pressure product (RPP) was calculated at each balloon volume as the product of heart rate (bpm) x developed pressure (mmHg).

Contractile reserve

For selected hearts, ventricular balloon volume was adjusted to give a stable end-diastolic pressure (20 mmHg). Estimates of cardiac performance were made prior to, and after, addition of the sympathomimetic inotrope isoprenaline (final concentration 10 μM) to the perfusate. Increases in developed pressure and rate of pressure development were estimated once stable cardiac performance was achieved.

Cardiac stress–strain calculations

Cardiac stress–strain calculations were undertaken as detailed in Woodiwiss and Norton [18] with modifications [19]. Briefly, LVEDV was estimated as detailed previously for perfused hearts (Cheng and Hauton 2008). LV wall volume was calculated from LV wall wet mass x 0.943 [18]. Data was represented as gradients of linearised stress–strain relationships, following linear regression analysis, for animals within the same treatment group.

Total lipid extraction

Total cholesterol, phospholipid and triglycerides were also extracted from liver and heart tissue as described previously [15]. Briefly, aliquots (100 mg) of tissue powder were extracted with methanol:chloroform (1:2). Extracts were evaporated to dryness and resuspended in absolute ethanol. Hepatic and cardiac TAG, phospholipid and cholesterol were measured using commercial kits.

Lipid infiltration

Lipid infiltration was estimated from tissue sections stained with Oil Red 'O' [20]. Briefly, frozen sections (10 μm) were air-dried and fixed in formaldehyde solution (3.7%w/v). Sections were rinsed in distilled water and stained with Oil Red 'O' (300 mg/ml in 36% v/v triethyl phosphate in distilled water). Sections were de-stained in distilled water before mounting. Lipid droplets were visualised by fluorescence microscopy with Texas red filter (×200 magnification). 3 non-

consective sections were photographed (4-fields per section) using Zeiss Axioskop microscope and proprietary software. Images were analysed using Image J software (NIH). Images were split into primary colours, the red channel converted to binary image and smoothing function employed to remove individual 'stray' pixels. The remaining regions were quantified for mean particle size and coverage of section.

Lipoprotein lipase activity

Lipoprotein lipase (LPL) activity was measured as previously described [8]. Briefly, separate groups of hearts from chow and WSD-treated rats were perfused with Krebs–Hensleit medium containing glucose (10 mM) and $CaCl_2$ (1.3 mM) as outlined above. Perfusion was maintained initially in non-recirculating mode to wash out erythrocytes. Recirculating perfusion was established and maintained for 5 min, after which heparin(10U/ml final concentration) was added and recirculated for a further 2 min. Samples of perfusate were isolated and frozen in liquid nitrogen. Cardiac tissue was then snap-frozen in liquid nitrogen and cardiac mass noted. Aliquots of post-heparin perfusate and acetone-dried heart powders (10 mg) were reacted with triacylglycerol emulsion (final concentration 5.6 mM) pre-labelled with ^{3}H-[9,10]-triolein supplemented with human plasma (ratio plasma to final reaction volume 1:6) as a source of Apolipoprotein CII. Reactions were carried out in Tris.HCl buffer (0.1 M, pH = 8.0) supplemented with fatty acid free bovine albumin (final concentration 2.0%w/v). Incubations were carried out at 37 °C and activities were expressed per unit mass of cardiac tissue. Total cardiac LPL activity was estimated as the sum of tissue residual LPL and heparin-releasable LPL activity.

Estimation of tissue sphingomyelin

Tissue sphingomyelin contents were estimated by the method of Hojjati and Jiang [21] from ethanol extracts of powdered tissue. Briefly, samples and standards (0.5 mg/ml in ethanol) were incubated in reaction mixture comprising Tris.HCl buffer (50 mM, 0.66 mM $CaCl_2$, pH = 8.0); sphingomyelinase (0.5U/ml); alkaline phosphatase (10U/ml); choline oxidase (0.5U/ml); peroxidise (20U/ml); 4-aminoantipyrine (0.73 mM) and DAOS (0.73 mM). At termination absorbance was quantified (595 nm) and concentration calculated with reference to standard curve.

Serum lipids

Serum was collected for all groups and used to measure total cholesterol (Randox cholesterol reagent), triglycerides (Randox triglyceride reagent), glucose (Thermo-Electron glucose kit). All assays were quantified as outlined in kit instructions using a 96-well plate analyser.

Gene expression

Two-step RT-PCR was performed using 1 μg of RNA, random hexamers and Multiscribe reverse transcriptase enzyme (Applied Biosystems, UK). An ABI 7500 real time PCR machine was used to quantitate rat transcripts using specific primer pairs and probes (Applied Biosystems, USA). Real time expression assays specific for VLDLR, ANGPTL4 and GAPDH were purchased as 'assay on demand' from Applied Biosystems, UK. All samples were normalised to GAPDH transcript levels and data presented as arbitrary units (AU) calculated as $AU = 1000x2^{\Delta\Delta Ct}$.

Immunoblotting for proteins

Western blot analysis was carried out on frozen whole heart tissue following removal of adipose tissue and atria. Standard Western immunoblotting techniques were used for the detection and estimation of relative amounts of ANGPTL4 or VLDLR (20–100 μg protein). Briefly, cardiac tissue (50 mg) was powdered in liquid nitrogen and extracted with Radio-immunoassay Precipitation Assay (RIPA) buffer containing protease inhibitors, before centrifugation and recovery of the supernatant. The PVDF-membranes were probed with antibodies specific for VLDLR (Santa Cruz — dilution 1:2000), mouse monoclonal ANGPTL4 (1–2000 dilution). Appropriate HRP-linked secondary antibodies were used and membranes developed using enhanced chemiluminesence (ECL) detection (Roche). Densitometry of Western blots was estimated using ImageJ software (NIH). Protein expression was corrected for the expression of an internal control (tubulin).

Statistical analysis

Statistical analysis was carried out using 2-way ANOVA analysis where appropriate to compare the effect of diet or cold acclimation. Data represents mean ± standard deviation. Correlation coefficients were calculated using Spearmans rank method and 'best fit' was estimated assuming a linear relationship. Where appropriate statistical significance was calculated using Student's 't' test.

Results

Post mortem

Cold acclimation had no effect on the growth of rats and oxfenicine or WSD did not alter growth (Table 1). CA increased cardiac mass for chow-fed and WSD rats ($P<0.001$ for both; Table 1) yet supplementation of chow-fed CA rats with oxfenicine restored cardiac mass to control levels (NS; Fig. 1). By contrast, for CA-WSD rats, oxfenicine decreased cardiac mass, but did not restore it to WSD-fed control levels ($P<0.05$; Table 1).

Plasma glucose was preserved for all chow-fed rats, irrespective of cold acclimation or supplementation with oxfenicine (NS; Table 1). WSD significantly increased plasma glucose for untreated rats ($P<0.01$; Table 1) but this was restored to chow-fed levels by either CA or oxfenicine treatment (NS; Table 1).

Cold acclimation decreased plasma TAG for chow-fed animals ($P<0.05$; Table 1) however this was restored following supplementation with oxfenicine (NS; Table 1). WSD increased plasma TAG 2-fold for untreated and oxfenicine-supplemented rats ($P<0.01$ and $P<0.05$ respectively; Table 1). For CA-rats, WSD increased plasma TAG 6-fold ($P<0.001$; Table 1) and this was maintained following supplementation of CA-WSD rats with oxfenicine ($P<.001$, Table 1).

Analysis of hepatic lipids showed that oxfenicine decreased hepatic TAG content for chow-fed rats ($P<0.05$; Table 1). CA halved hepatic TAG content ($P<0.001$; Table 1) however supplementation with oxfenicine for CA-rats restored hepatic TAG (NS; Table 1). Animals maintained on WSD had increased hepatic TAG content, irrespective of treatment with CA, Oxfenicine or CA + oxfenicine in combination ($P<0.001$ for all; Table 1).

Cardiac performance

Estimate of cardiac performance with intraventricular balloon revealed that neither WSD nor WSD + oxfenicine had any effect of diastolic performance for hearts isolated from control animals (Supplemental Fig. 1A & 1B). However, for CA rats, WSD decreased the diastolic stiffness of the left ventricle (Supplemental Fig. 1 $P<0.05$ for CA; $P<0.01$ for CA + Oxfenicine) for CA rats fed WSD (Supplemental Fig. 1C) or WSD + oxfenicine (Supplemental Fig. 1D). For chow-fed rats stiffness was increased by oxfenicine and CA alone ($P<0.05$ for both, Fig. 1A). However WSD returned diastolic stiffness for CA hearts to control levels (NA, Fig. 1A). Investigation of the relationship between plasma TAG and diastolic stiffness from pooled data indicated a negative correlation between plasma TAG and LV diastolic stiffness (Fig. 1B, coefficient = −0.547; $P<0.001$). However estimation of LV stress–strain relationship, measured as the gradient of the linear regression of stress–strain relationship (Supplemental Fig. 2), showed that WSD did not alter the LV stress–strain relationship for control, oxfenicine-treated rats, CA rats or CA rats + Oxfenicine (Fig. 2). However, comparison of the effect of CA (Fig. 2) on stress–strain revealed that CA decreased the gradient of the stress–strain

Table 1
Post mortem data collected from control and cold-acclimated rats fed chow or a western-style high fat diet. Data represents MEAN ± SD (n = 6). Statistical significance represented as significantly different from untreated controls * P<0.05, ** P<0.01, *** P<0.001; effect of oxfenicine-treatment + P<0.05, ++ P<0.01, +++ P<0.001; Effect of Western-style diet †P<0.05, ††† P<0.001.

MEASUREMENT	Control — chow diet				Western diet			
	Untreated	Oxfenicine	Cold acclimated	Cold acclimated + Oxfenicine	Untreated	Oxfenicine	Cold acclimated	Cold acclimated + Oxfenicine
Body mass (g)	298 ± 8	370 ± 8	283 ± 18	290 ± 22	302 ± 16	322 ± 21	291 ± 22	302 ± 21
Heart mass (g)	1.29 ± 0.1	1.46 ± 0.2	$1.58 \pm 0.16^{***}$	1.32 ± 0.09	1.37 ± 0.08	1.40 ± 0.2	$1.74 \pm 0.10^{***}$	$1.52 \pm 0.08^{*} + ††$
Heart–body mass ratio (%)	0.43 ± 0.04	0.43 ± 0.03	$0.56 \pm 0.06^{**}$	$0.45 \pm 0.04^{++}$	0.45 ± 0.02	0.39 ± 0.03	$0.60 \pm 0.03^{***}$	$0.51 \pm 0.03^{+++\dagger}$
Plasma glucose (mM)	9.25 ± 0.56	8.06 ± 1.18	8.96 ± 1.56	9.13 ± 1.44	$11.46 \pm 1.49^{\dagger\dagger}$	7.60 ± 1.56	10.54 ± 1.73	$12.10 \pm 1.39^{\dagger\dagger}$
Plasma triacylglycerol (mM)	0.90 ± 0.23	1.36 ± 0.06	$0.62 \pm 0.13^{*}$	0.78 ± 0.39	$2.07 \pm 0.64^{\dagger\dagger}$	$2.80 \pm 1.20^{\dagger}$	$3.82 \pm 0.97^{**\dagger\dagger\dagger}$	$5.02 \pm 0.80^{***} + †††$
Hepatic TAG (μmoles/g)	10.2 ± 1.82	$7.15 \pm 0.93^{+}$	$5.69 \pm 1.71^{***}$	7.28 ± 3.73	$18.51 \pm 1.96^{\dagger\dagger\dagger}$	$20.23 \pm 3.04^{\dagger\dagger\dagger}$	$18.27 \pm 3.39^{\dagger\dagger\dagger}$	$18.31 \pm 2.70^{\dagger\dagger\dagger}$
Hepatic cholesterol (mg/g)	2.56 ± 0.22	$1.05 \pm 0.32^{+++}$	2.64 ± 0.30	2.16 ± 0.30	$4.08 \pm 0.67^{\dagger\dagger\dagger}$	$2.15 \pm 0.88^{\dagger+++}$	$3.49 \pm 0.54^{\dagger\dagger\dagger}$	$3.07 \pm 0.86^{\dagger\dagger\dagger}$

relationship when compared to corresponding untreated rats, and this was unaffected by WSD (P<0.01 for CA, P<0.01 for CA + WSD Fig. 2).

Isoprenaline-induce inotropy

Cardiac performance at fixed end-diastolic pressure (EDP) (estimated as developed pressure, rate-pressure product or + dP/dt) was unaffected by oxfenicine or CA for chow-fed rats, however combination of CA + oxfenicine increased developed pressure 25% (P<0.05), increased dP/dt 40% (P<0.01) and increased RPP 25% (P<0.01; Table 2). Addition of isoprenaline increased developed pressure (P<0.01), RPP (P<0.05) and dP/dt (P<0.01) for chow-fed hearts and this increase was preserved following oxfenicine treatment (P<0.01; Table 2). CA abolished the increase in cardiac work for chow-fed rats (NS; Table 2) but supplementation of CA-rats with isoprenaline augmented developed pressure 20% (P<0.05), RPP 30% (P<0.05) and dP/dt 50% (P<0.01; Table 2).

For untreated rats fed WSD isoprenaline also augmented cardiac work (P<0.01; Table2), however with addition of oxfenicine or CA no increase in cardiac work was noted following addition of isoprenaline (NS; Table 2). Combination of CA + oxfenicine for WSD-rats restored the response to isoprenaline, increased developed pressure 50% (P<0.001), RPP 100% (P<0.001) and dP/dt 2-fold (P<0.001) for corresponding CA-rats (Table 2).

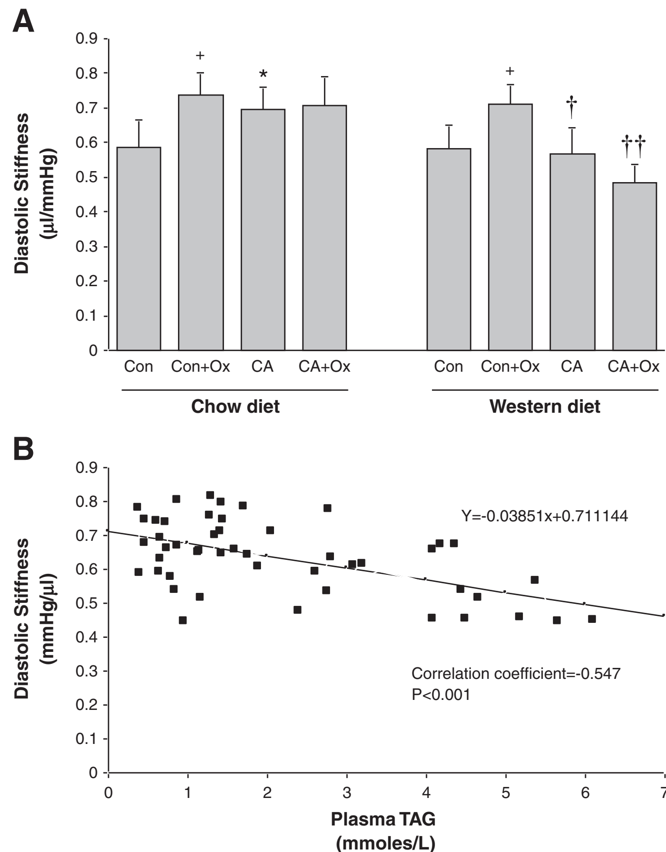

Fig. 1. Effects of western-style high fat diet or oxfenicine on diastolic stiffness for normal or hypertrophied rat hearts. A) Diastolic stiffness was estimated from the gradient of linear regression of diastolic performance curve for pressure values greater than zero at the point the regression line bisected the balloon volume at diastolic pressure = Zero. Hearts were perfused with Krebs–Hensleit buffer at fixed coronary perfusion pressure (100 cm H_2O) with pressure estimated using non-elastic balloon placed in the left ventricle. For further details see methods. B) Correlation between plasma triacylglycerol (TAG) and diastolic stiffness for perfused hearts.Plasma TAG concentrations were estimated from venous blood samples collected under terminal anaesthesia. Data represents MEAN ± SD (n = 6 hearts/group). Best fit was determined from linear regression of the data points giving the equation Y = − 0.03851x + 0.711144. Correlation was quantified using Spearman's rank test, with significance estimated by 't' test. Statistical significance is represented as: effects of oxfenicine + P<0.05; effect of cold acclimation * P<0.05; effect of western-style diet † P<0.05, †† P<0.01.

Cardiac lipid concentrations

Irrespective of treatment, cardiac phospholipid and sphingomyelin contents were unchanged by either CA, WSD or oxfenicine alone or in combination (Fig. 3). WSD alone had no effect on cardiac TAG content

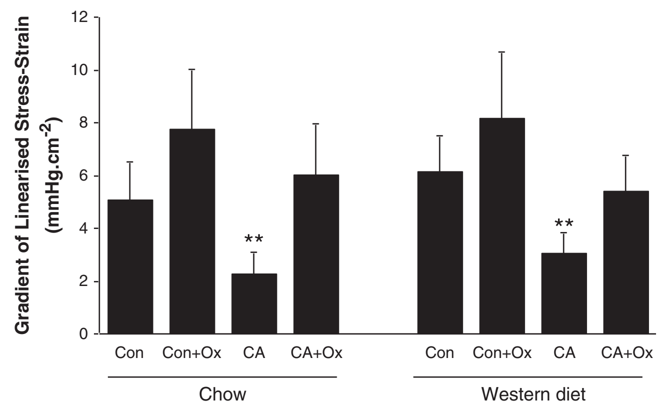

Fig. 2. Effect of cardiac hypertrophy and western diet on the intrinsic performance of rat left ventricle. Performance was estimated from the gradient of the linearised stress–strain relationship. Best fit was determined from linear regression of the data points and plotted as rend line through the appropriate points (see Supplemental Fig. 1). Cardiac stress and strain were calculated as outlined in the methods. Data represents MEAN ± SD (n = 6 hearts/group). Statistical significance is represented as: effect of cold acclimation ** P<0.01.Effect of cardiac hypertrophy and western diet on the intrinsic performance of rat left ventricle. Performance was estimated from the gradient of the linearised stress–strain relationship. Best fit was determined form linear regression of the data points and plotted as rend line through the appropriate points (see Supplemental Fig. 1). Cardiac stress and strain were calculated as outlined in the methods. Data represents MEAN ± SD (n = 6 hearts/group). Statistical significance is represented as: effect of cold acclimation ** P<0.01.

Table 2
Effects of Cold Acclimation and Isoprenaline on cardiac performance in Langendorff-perfused rat hearts. Hearts were perfused at constant pressure (100 cm H_2O) and performance measured with intraventricular balloon. Diastolic pressure was set at 20 mmHg. Data represents MEAN ± SD (n = 6). Statistical significance represented as: effects of isoprenaline * P<0.05; ** P>0.01: effects of oxfenicine + P<0.05; ++ P<0.01; +++P<0.001.

Group	Control			Isoprenaline (10 μM)		
	Developed pressure (mmHg)	+dP/dtmax (mmHg/s)	Rate-pressure product (mmHg/min)	Developed pressure (mmHg)	+dP/dtmax (mmHg/s)	Rate-pressure product (mmHg/min)
Chow diet						
Control	59 ± 12	1053 ± 216	16437 ± 3145	86 ± 6**	1903 ± 341**	25052 ± 4465*
Oxfenicine	86 ± 10	1323 ± 142	19166 ± 2086	103 ± 11*	1771 ± 199**	25723 ± 2015***
Cold-acclimated	59 ± 9	941 ± 157	14362 ± 2573	58 ± 8	1274 ± 226	18394 ± 3408
Cold Acclimated + Oxfenicine	75 ± 7+	1402 ± 241++	20330 ± 2822++	90 ± 11*+++	2106 ± 402**++	26247 ± 4834*+
Western Diet						
Control	60 ± 9	990 ± 185	14724 ± 3205	74 ± 3**	1389 ± 194**	18370 ± 1678**
Oxfenicine	82 ± 15	1313 ± 287	18604 ± 3183	92 ± 11	1563 ± 213	22525 ± 2214
Cold-acclimated	52 ± 11	867 ± 211	11344 ± 3208	56 ± 13	1089 ± 267	13370 ± 3820
Cold acclimated + oxfenicine	86 ± 8+++	1607 ± 286++	19766 ± 3847++	105 ± 11*+++	2424 ± 297**+++	27329 ± 2366**+++

for untreated rats (NS; Fig. 3). For chow-fed rats, oxfenicine increased cardiac TAG content 2-fold (P<0.05; Fig. 3). However when chow-fed CA rats were supplemented with oxfenicine, cardiac TAG levels were unchanged (NS; Fig. 3). For WSD rats, oxfenicine supplementation increased cardiac TAG 3-fold (P<0.01; Fig. 3) when compared to WSD-fed control rats. CA had no effect of cardiac TAG levels in either chow-fed or WSD-fed rats. For CA rats fed WSD, oxfenicine increased cardiac TAG levels 2-fold (P<0.05; Fig. 3).

Cardiac LPL activity

For chow-fed rats, CA increased heparin releasable LPL (hrLPL) 20% (P<0.05; Fig. 4A). Supplementation of diet with oxfenicine had no effect on hrLPL for control animals (NS; Fig. 4A) and restored hrLPL levels to those of control rats for CA rats supplemented with oxfenicine (NS; Fig. 4A). WSD had no effect on cardiac hrLPL levels for untreated or oxfenicine-treated rats when compared to control (NS, Fig. 4A).

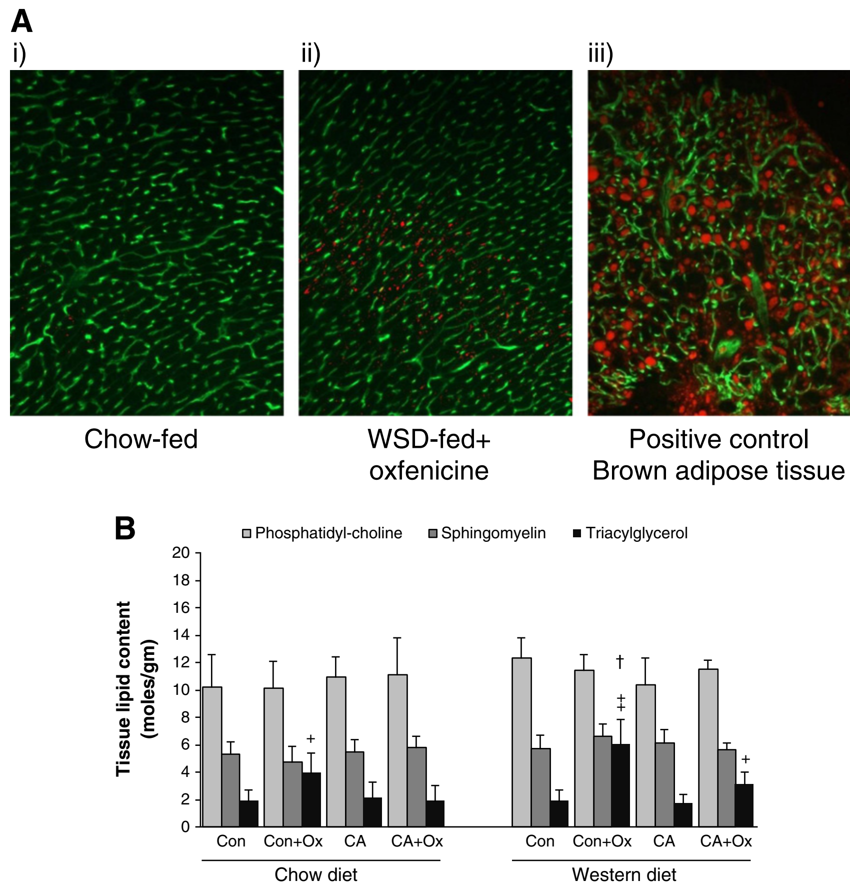

Fig. 3. Effects of hypertrophy, Western-style diet (WSD) and oxfenicine on lipid accumulation in the perfused heart. A) representative images of Oil Red 'O'-stained images of cardiac muscle from (i) chow-fed rats, (ii) WSD + oxfenicine fed rats (iii) positive control showing lipid staining in brown adipose tissue. Sections counterstained with FITC-labelled lectin to highlight capillary glycocalyx boundaries. B) Cardiac tissue lipid concentrations for isolated hearts. Phospholipid, triacylglycerol and sphingomyelin were estimated spectrophotometrically following solvent extraction of cardiac tissue. For further details, see methods. Data represents MEAN ± SD (n = 6 hearts/group). Statistical significance is represented as: effects of oxfenicine + P<0.05, ++ P<0.01; effect of western-style diet † P<0.05.

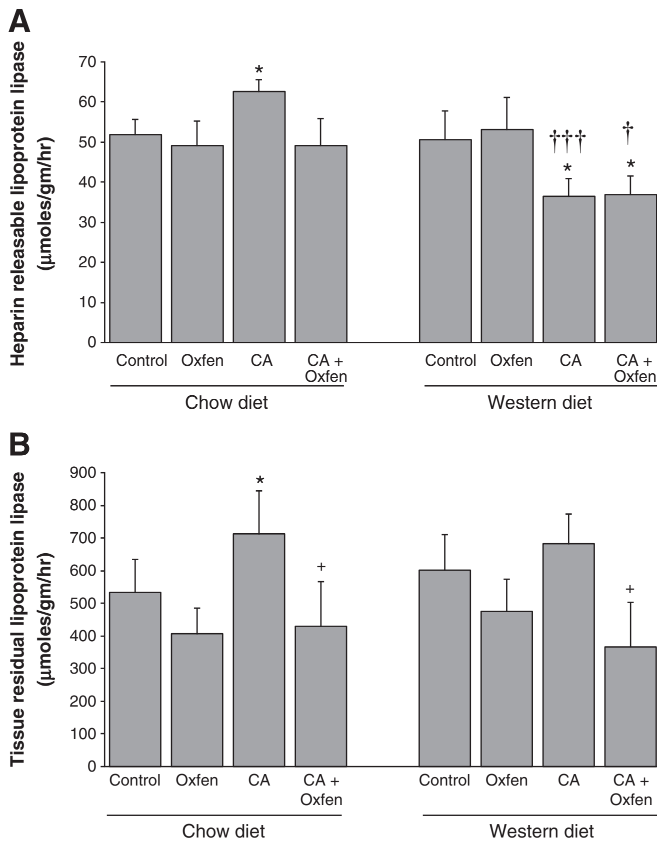

Fig. 4. Effect of hypertrophy, Western-diet and oxfenicine on cardiac lipoprotein lipase activity. A) Heparin-releasable lipoprotein lipase (hrLPL) activity. Heparin-releasable LPL was estimated by perfusion of hearts with Krebs–Henseleit buffer containing heparin (5 U/ml). B) Tissue-residual LPL activity. Tissue-residual LPL activity was estimated from acetone dried powders of cardiac tissue and activities corrected for cardiac wet-mass. For further details see methods. Data represents MEAN ± SD (n = 6 hearts/group). Statistical significance is represented as: effect of cold acclimation * P<0.05; effects of oxfenicine + P<0.05; effect of western-style diet † P<0.05, ††† P<0.001.

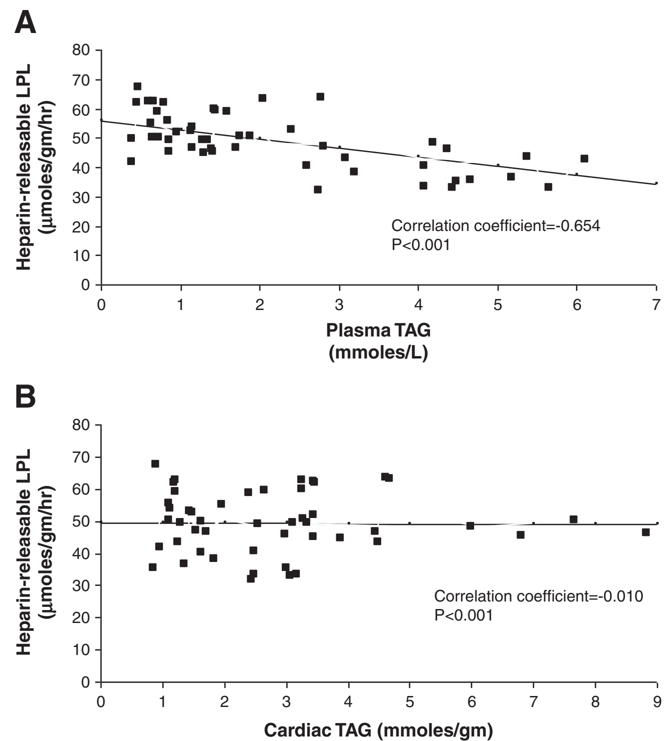

Fig. 5. Correlation of cardiac heparin-releasable lipoprotein lipase enzyme activity with A) plasma triacylglycerol concentration or B) cardiac tissue triacylglycerol concentration. Best fit was determined form linear regression of the data points and plotted to give trend line. Correlation was quantified using Spearman's rank test, with significance estimated by 't' test. Data represents N = 8 treatment groups or n = 6 hearts/group (total 48 rats).

However, following CA, WSD decreased hrLPL by one-third compared with chow-fed CA rats (P<0.001, Fig. 4A) and 20% lower that WSD-fed control rats (P<0.05, Fig. 4A). For CA rats fed WSD supplemented with oxfenicine hrLPL activity decreased 20% below WSD-fed control rats (P<0.05; Fig. 4A) and 20% below chow-fed CA + oxfenicine rats (P<0.05; Fig. 4A).

For chow-fed rats, CA increased tissue residual LPL (trLPL) 40% (P<0.05; Fig. 4B). Oxfenicine had no effect on trLPL for chow-fed rats, however for chow-fed CA rats, oxfenicine decreased trLPL 40% compared to corresponding CA rats (P<0.05; Fig. 4B). For untreated controls, WSD had no effect on trLPL (NS; Fig. 4B). Furthermore, for CA rats fed WSD, trLPL was also unchanged (NS; Fig. 4B). Treatment of CA-WSD rats with oxfenicine halved trLPL compared with CA-WSD rats (P<0.05; Fig. 4B). Investigation of the factors affecting the tissue levels of hrLPL revealed a negative correlation between plasma TAG and hrLPL for pooled data (correlation coefficient = −0.654; P<0.001; Fig. 5A) However, no correlation between cardiac tissue TAG levels and hrLPL (correlation coefficient = −0.01; P<0.001; Fig. 5B).

Transcript levels

All mRNA levels were expressed as fold change from untreated chow-fed rat hearts. Irrespective of treatment, whole heart levels of VLDLR transcript were unaltered by any treatment used (NS; Fig. 6A). For LPL, transcript levels were unchanged for hearts from chow-fed rats when supplemented with oxfenicine or cold acclimated, alone or in combination (NS for all; Fig. 6B). For WSD-fed rats, neither oxfenicine nor CA alone changed LPL mRNA levels (NS for both; Fig. 6B): however in combination LPL mRNA levels were halved (P<0.01; Fig. 6B). For chow fed rats, ANGPTL4 transcript levels were unchanged by oxfenicine or CA, alone or in combination (NS; Fig. 6C). WSD increased ANGPTL4 mRNA levels 6-fold compared to chow-fed controls (P<0.05; Fig. 6C). Supplementation of WSD-rats with oxfenicine increased ANGPTL4 mRNA levels 15-fold relative to chow-fed controls (P<0.001; Fig. 6C) and 2-fold over WSD-fed control rats (P<0.01; Fig. 6C). ANGPTL4 mRNA levels were unchanged for CA rats fed WSD or for CA-WSD rats receiving oxfenicine (NS for both; Fig. 6C).

Protein expression

All immunodetectable protein levels were expressed as fold change from untreated chow-fed rat hearts. Irrespective of treatment, CA increased VLDLR protein levels 8-fold (P<0.01; Fig. 7). This was unaffected by supplementation with oxfenicine or WSD (NS; Fig. 7). For chow-fed rats, ANGPTL4 protein levels were unchanged by oxfenicine or CA, alone or in combination (NS for both; Fig. 7). WSD increased ANGPTL4 levels 2-fold compared to chow-fed controls (P<0.05; Fig. 7). However supplementation of WSD-rats with oxfenicine restored ANGPTL4 to untreated levels (NS; Fig. 7). For WSD rats, CA increased ANGPTL4 3-fold compared to untreated control (P<0.05; Fig. 7) and supplementation of WSD-CA rats with oxfenicine increased ANGPTL4 protein 4-fold compared to untreated controls (P<0.05; Fig. 7).

Discussion

We investigated the effects of a variety of interventions designed to alter lipid metabolism and myocardial TAG handling and performance. We demonstrate that short-term feeding of WSD led to high plasma TAG concentration that was further increased following cold

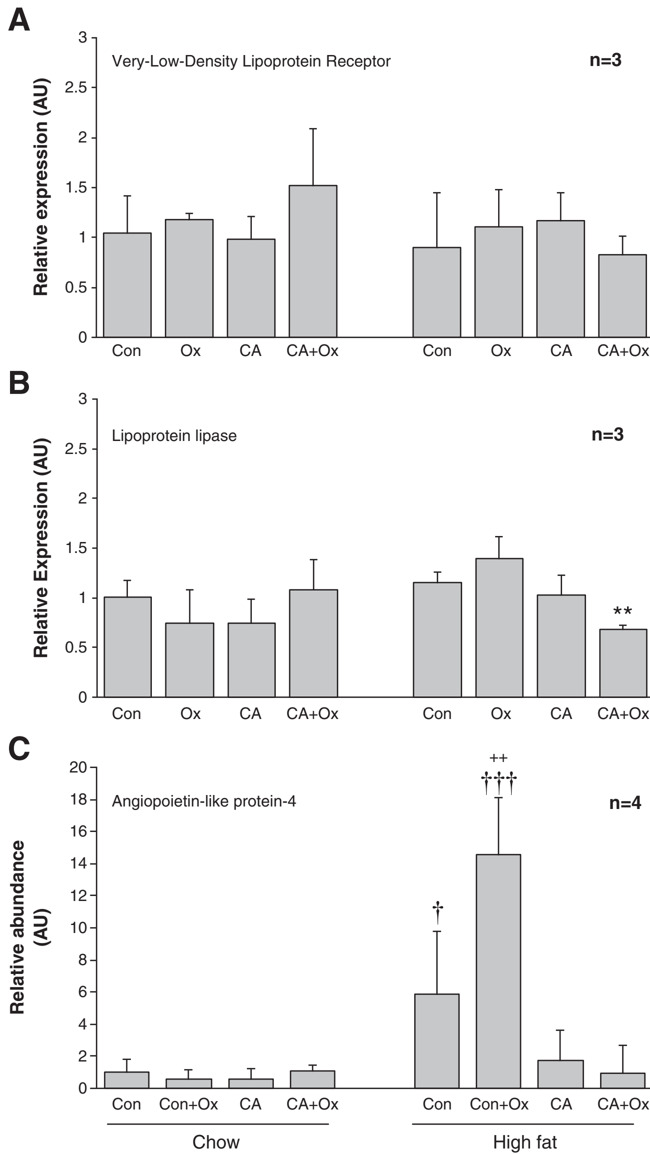

Fig. 6. RT-PCR gene transcript levels for cardiac A) very-low-density lipoprotein receptor, B) lipoprotein lipase and C) angiopoietin-like protein 4. RT-PCR was performed using 1 μg of RNA. Real time expression assays specific for VLDLR, ANGPTL4 and GAPDH were purchased as 'assay on demand' from Applied Biosystems, UK. All samples were normalised to GAPDH transcript levels and data presented as arbitrary units (AU) calculated as $AU=1000x2^{\Delta\Delta Ct}$. Data represents MEAN ± SD (n = 3–4 hearts/group). Statistical significance is represented as: effect of cold acclimation ** $P<0.01$; effects of oxfenicine ++ $P<0.01$; effect of western-style diet † $P<0.05$, ††† $P<0.001$.

acclimation, and contributed to cardiac dysfunction in the hypertrophied heart. This was characterised as a change in diastolic stiffness of the left ventricle that was not a result of changes to the intrinsic performance of the muscle (estimated as stress–strain). The correlation of diastolic stiffness with plasma TAG concentration was modest, however no correlation was noted with myocardial TAG content (data not shown). Furthermore, we show that inhibition of lipid oxidation with oxfenicine led to TAG accumulation in the myocardium. In addition, the activity of LPL for hypertrophied hearts was subject to regulation by components of a 'Western-style' high fat diet, activity decreased by the diet, possibly as a consequence of increased levels of ANGPTL4 protein leading to decreased synthesis of active enzyme protein. This down-regulation of hrLPL activity was so profound as to prevent the lipid accumulation anticipated and thus mitigate against further lipid-induced cardiac dysfunction. Interestingly, there appears to be a disconnection between ANGPTL4 mRNA levels and transcription of protein, indicative of a dynamic system 'sensing' the availability of lipid possibly involving PPAR [22].

Diastolic stiffness of the LV was maintained for animals fed a chow diet yet showed declines in performance for hypertrophied hearts when maintained on WSD. Investigation of the impact of plasma TAG indicates that a modest negative correlation between diastolic stiffness and plasma TAG, associating increased plasma lipid levels with decreased cardiac mechanical performance. However it was unclear whether this was a direct cause of altered performance of the muscle. Estimation of the stress–strain relationship for the LV indicated that for all hearts WSD had no effect on the stress–strain relationship although CA led to decreased mechanical performance. This is most likely a result of the poor performance coupled with the increased mass of the heart associated with CA previously noted [14] and is not a result of high fat feeding directly. Previous experiments illustrate that WSD may induce decreases in cardiac performance for control rats following long duration feeding [23]. Although, following hypertrophy changes may be faster — high fat diet inducing contractile dysfunction in hearts following aortic banding in as little as 7-days [24] and may be attributable to increased cardiac hrLPL, however this was not measured. This is at odds with longer duration experiments that suggest no exacerbation of cardiac dysfunction by high fat-feeding following pressure pathological overload hypertrophy [25] implying a temporal correction in lipid accumulation. Direct inhibition of CPT1 activity with oxfenicine did not itself induce cardiac hypertrophy or cardiac dysfunction for the control or worsen hypertrophy or performance in CA-heart, confirming previous observations for control rats [16].

Estimation of the lipid contents for cardiac tissue suggested that oxfenicine did inhibit the oxidation of fatty acids, resulting in the accumulation of lipids as TAG. This accumulation was modest, as evident from the Oil Red 'O' staining of myocardium. Moreover, this accumulation is not so severe as to alter tissue phospholipid or sphingomyelin contents which has been noted for agents that inhibit β-oxidation such as perhexiline and amiodarone [26]. Of interest was the lack of effect noted for CA hearts, suggesting that the mechanism for assimilation of lipids into the myocardium may have been under regulatory control. Given the central role of TAG in cardiac energetics [2] and that the majority of lipid present in plasma was TAG, the involvement of LPL in the uptake of TAG was explored.

The functional component of LPL for the assimilation of lipids is the endothelial compartment that is released following perfusion with heparin (hr) and this hrLPL is anticipated to increase lipid loading — particularly associated with decreased β-oxidation. As previously noted CA led to increased hrLPL in chow-fed rat hearts. This was subsequently blunted by oxfenicine. Given the drop in plasma TAG content noted for chow-fed CA rats this may reflect a need to preserve the influx rates of FA into the myocardium. Given that FA flux rates through LPL are governed by either concentration of substrate (in this case TAG) or the concentration of LPL enzyme, falling flux rates in this case were overcome through increased enzyme presentation at the endothelium. One possible origin for the increased rates of lipid assimilation in CA (hence diminished plasma TAG) is the increased burden of thermogenesis, previously noted for the CA rat [15]. For oxfenicine-treated CA rats the hrLPL levels were normalised as plasma TAG levels were restored, probably through a decrease in the reliance on TAG as a source of fuel for thermogenesis. For WSD-fed rats CA decreased hrLPL levels and the impact of oxfenicine was refractory to WSD, implying that lipid delivery levels to the myocardium were preserved. Given the increase in plasma TAG levels for CA rats fed WSD this may be a substrate concentration-mediated effect.

The strong negative correlation between plasma TAG and hrLPL levels implies that endothelial presentation of the enzyme may be dictated by the flux rates of FA entering the cardiomyocyte and thus ultimately related to the prevailing plasma milieu. Given the continual utilisation of LPL and the loss from the endothelial surface of capillaries with the recycling of LPL with remnant lipoprotein particles this

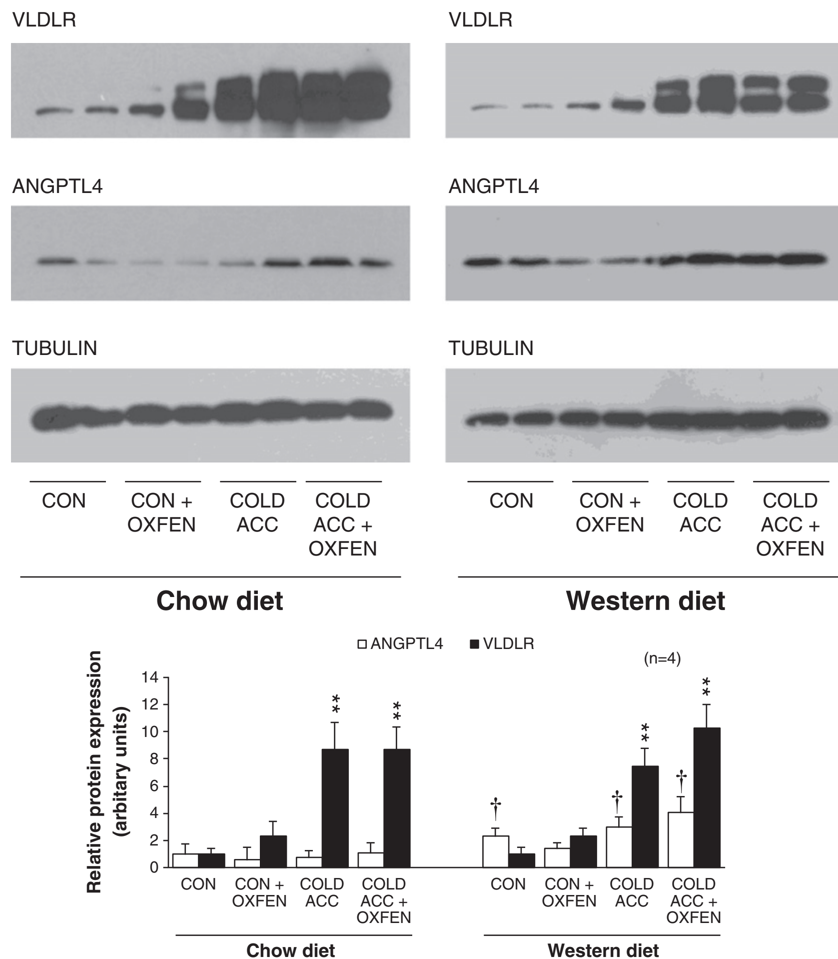

Fig. 7. Immunoblot analysis of cardiac levels of very-low-density lipoprotein receptor (VLDLR), angiopoietin-like protein 4 (ANGPTL4) and tubulin. Representative immunoblots for VLDLR, ANGPTL4 and tubulin for chow-fed and WSD-fed rats hearts. Cardiac tissue was powdered in liquid nitrogen and extracted with Radio-immunoassay Precipitation Assay (RIPA) buffer containing protease inhibitors. PVDF-membranes were probed with antibodies specific for VLDLR (Santa Cruz — dilution 1:2000), mouse monoclonal ANGPTL4 (1–2000 dilution). Densitometry of Western blots was estimated using ImageJ software (NIH). Protein expression was corrected for the expression of an internal control (tubulin). Data represents MEAN ± SD (n = 4 hearts/group). Statistical significance is represented as: effects of cold acclimation ** $P<0.01$; effect of western-style diet † $P<0.05$.

mechanism may show a degree of auto-regulation [27]. Both TAG and cholesterol accumulation in the liver following Western diet may be indicative of such remnant particle recovery [28]. However the determinants for the frequency of LPL replacement at the endothelium are unclear, one such trigger is the activation of AMPK by synthetic agents such as AICAR or perhexiline [29], metformin [8] or ischemia-reperfusion injury [30]. LPL mRNA expression was unchanged by high fat-feeding, confirming previous observations [31]. Investigation of cardiac TAG levels and the resulting hrLPL suggested that in this case cardiac TAG levels were not the determinant of hrLPL levels. However, given that the translocation of LPL to the surface of the endothelium requires multiple steps each with the potential for regulatory control of this process, we further investigated each step to elucidate the point(s) of control.

Neither LPL- nor VLDL-receptor mRNA levels were altered by treatment with WSD or cold acclimation, suggesting that any regulatory step must be post-translational, the advantage possibly being the rapidity with which changes could be initiated. Other pathological models of pressure-overload hypertrophy demonstrate similar increases in LPL activity yet this was also accompanied by an increase in LPL mRNA expression and no changes to VLDL-receptor mRNA [32]. It is unclear whether the changes to metabolism associated with such models of pressure overload, resulting in TAG accumulation [33], may initiate changes to VLDL-receptor or LPL expression through ligand-mediated activation of PPAR [34], although reporter-gene assays suggest that lipolysis of VLDL by LPL yields ligand activation of PPAR directly [35]. ANGPTL4 plays a critical role in the enzyme activity of LPL protein [36] with ANGPTL4-null mice showing decreased plasma TAG and elevated post-hpearin plasma LPL activity [37]. WSD led to large increases in ANGPTL4 transcript levels which were augmented by supplementation with oxfenicine and abolished following CA. This was unusual given the recent observations for adipose tissue illustrating an association between high levels of ANGPTL4 mRNA and low levels of LPL activity for adipocytes [38]. However, transcript levels do not necessarily dictate activity of the resulting protein and so immunoblot analysis was employed to explore changes to protein levels for both VLDL-receptor and ANGPTL4.

Cold acclimation increased the expression of VLDL-receptor protein, in contrast to our previous experiments [14]. VLDL-receptor protein may be closely-associated with the translocation of LPL to the luminal surface of the capillary endothelium [6], and this discrepancy may be a result of the longer exposure to chronic cold, our current experiment sustaining exposure to the lowest temperature (4 °C) for 14-days. However, direct comparison of this experiment and the previous [14] imply that levels of hrLPL are similar and thus increased translation of VLDL-receptor protein does not directly influence LPL presentation at the endothelium, merely facilitating the translocation of available LPL protein. Immunohistochemical analysis of the localisation of VLDL-receptor suggested this is indeed the case (Supplemental Fig. 3) with the majority of VLDL-receptor protein localised to apical/capillary regions of cardiomyocytes. We have previously illustrated the role of VLDLR in lipoprotein assimilation by the perfused heart, suggesting

uptake of lipoproteins in the absence of functional LPL [5]. Mice devoid of the VLDL-receptor gene show mild hypertriacylglycerolemia and decreased endothelial presentation of LPL in heart and skeletal muscle [7] but the direct contribution of VLDLR to this route of uptake may be small in comparison to LPL-mediated assimilation [7]. Furthermore, despite noting a 10-fold increase in VLDLR protein levels in these experiments we do not demonstrate a corresponding increase in cardiac TAG concentration supporting a limited role for VLDLR in direct assimilation of cardiac lipids for intact heart. Culture of isolated cardiomyocytes suggested more uniform staining of VLDL-receptor throughout the cell [39] possibly through a loss of cell polarity, however orientation of cells within cardiac tissue may be important for the localisaton of VLDL-receptor.

ANGPTL4 protein levels were increased by WSD-feeding, and further augmented by CA. High prevailing plasma TAG concentrations increased the flux of FA into the cardiomyocyte to fuel β-oxidation, and was blunted following oxfenicine treatment. Yet for CA plasma TAG levels remained high and thus to maintain the influx of FA hrLPL levels were attenuated through increased ANGPTL4 protein levels. Taken together, these observations for both mRNA and protein implied that upon feeding WSD mRNA transcript levels rose in response to WSD in the absence of translation to protein suggesting strong feedback control possibly as a consequence of continual removal of the assimilated lipid to fuel β-oxidation. However, with further rises in plasma TAG the ANGPTL4 protein was transcribed decreasing hrLPL, and thus diminished the flux of FA into the cardiomyocyte. Therefore the stimulus for increases gene transcription is blunted.

Previous experiments document that oxfenicine itself does not alter cardiac performance but does decrease oxygen consumption for a fixed cardiac workload [40]. Interestingly, cardiac mitochondria are more sensitive to the inhibition of CPT1 by oxfenicine than mitochondria from liver [41]. Dietary supplementation with oxfenicine at levels used in this experiment increased cardiac lipids in the absence of increased hepatic accumulation of lipid, even whilst maintenance on WSD (evidenced by hepatic TAG levels). Agents such as etomoxir that also inhibit CPT1 led to a decrease in CPT1 activity, despite increasing CPT1 protein expression, and without altering cardiac TAG content [42] implying an excess of CPT1 activity in myocardium. Interestingly, oxfenicine did restore cardiac performance to CA-rat hearts to control levels, implying that the dysfunction associated with CA was metabolic in origin. We have previously noted no change in glucose or fatty acid oxidation rates for the CA rat heart [14] and so improving efficiency of oxygen use (with respect to ATP production) is unlikely to result in this increased performance. The impact of the high dietary sucrose present in WSD also cannot be overlooked and this may reduce rates of glycolysis and increase rates of β-oxidation [43]. Indeed, sucrose feeding led to both echocardiographic changes and altered calcium uptake into sarcoplasmic reticulum, but over longer duration than those used here [44]. WSD alone decreased cardiac performance whilst increasing oxygen consumption thus contributing to decreased cardiac efficiency [33]. Other possible candidates for impairment of the cardiac performance may be the lipids ceramide or diacylglycerol, high levels of constitutively-active LPL led to accumulation of both lipids in the heart that was ameliorated in part by feeding myriocin, an inhibitor of *de novo* ceramide synthesis, and this restored cardiac efficiency [45].

Concluding remarks

Our data does not support the accumulation of TAG as a major contributor to the aetiology of lipotoxic cardiac dysfunction. In addition, we suggest that prevailing plasma TAG rather than tissue TAG concentration may alter the level of translocation of LPL to the surface of the capillary endothelium to preserve fatty acid flux into the heart to fuel contraction. This is achieved through multiple mechanisms, including altering the expression of ANGPTL4 protein rather than VLDL-receptor or LPL mRNA. Build-up of lipids as TAG in the cardiomyocytes is reduced by effective reductions in hrLPL suggesting that feedback autoregulation may prevent lipotoxicity in physiological hypertrophy. It is unclear whether lipid accumulation following pathological hypertrophy occurs through disruption of this process or whether mechanisms such as AMPK activation can override this mechanism.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbalip.2011.12.004.

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