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ARTICLE

S.-Y. Li · X. Yang · A. F. Ceylan-Isik · M. Du · N. Sreejayan · J. Ren

Cardiac contractile dysfunction in *Lep*/*Lep* obesity is accompanied by NADPH oxidase activation, oxidative modification of sarco(endo)plasmic reticulum Ca^{2+}-ATPase and myosin heavy chain isozyme switch

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Abstract *Aims/hypothesis:* Obesity is an independent risk factor for heart diseases but the underlying mechanism is not clear. This study examined cardiac contraction, oxidative stress, oxidative modification of sarco(endo) plasmic reticulum Ca^{2+}-ATPase (SERCA) and the myosin heavy chain (MHC) isoform switch in obese mice. *Methods:* Mechanical properties were evaluated in ventricular myocytes from C57BL/6J lean and *Lep*/*Lep* obese mice (formerly known as *ob*/*ob* mice), including peak shortening (PS), time to 50 or 90% PS, time to 50 or 90% relengthening (TR_{50}, TR_{90}), maximal velocity of shortening/relengthening (±dL/dt), intracellular Ca^{2+} and its decay (τ). Oxidative stress, lipid peroxidation, protein damage and SERCA activity were assessed by glutathione/glutathione disulfide, malondialdehyde, protein carbonyl and $^{45}Ca^{2+}$ uptake, respectively. NADPH oxidase was determined by immunoblotting. *Results:* Myocytes from *Lep*/*Lep* mice displayed depressed PS and ± dL/dt, prolonged TR_{50}, TR_{90}, elevated resting $[Ca^{2+}]_i$, prolonged τ, reduced contractile capacity at high stimulus frequencies and diminished responsiveness to extracellular Ca^{2+} compared with lean controls. Cardiac glutathione/glutathione disulfide was decreased whereas malondialdehyde, protein carbonyl, membrane $p47^{phox}$ and membrane $gp91^{phox}$ were increased in the *Lep*/*Lep* group. SERCA isoenzyme 2a was markedly modified by oxidation in *Lep*/*Lep* hearts and associated with decreased $^{45}Ca^{2+}$ uptake. The MHC isozyme displayed a shift from the α to the β isoform in *Lep*/*Lep* hearts. Short-term incubation of angiotensin II with myocytes mimicked the mechanical defects, SERCA oxidation and $^{45}Ca^{2+}$ uptake seen in *Lep*/*Lep* myocytes. Incubation of the NADPH oxidase inhibitor apocynin with *Lep*/*Lep* myocytes alleviated contractile defects without reversing SERCA oxidation or activity. *Conclusions/interpretation:* These data indicate that obesity-related cardiac defects may be related to NADPH oxidase activation, oxidative damage to SERCA and the MHC isozyme switch.

Keywords Cardiac myocytes · Contraction · MHC isozymes · NADPH oxidase · Obesity · SERCA

Abbreviations ±dL/dt: maximal velocity of shortening/relengthening · GSH: glutathione · GSSG: glutathione disulfide · MHC: myosin heavy chain · PS: peak shortening · ROS: reactive oxygen species · SERCA: sarco(endo)plasmic reticulum Ca^{2+}-ATPase · SERCA2a: sarco(endo)plasmic reticulum Ca^{2+}-ATPase isozyme 2a · TPS_{50}: time to 50% peak shortening · TPS_{50}: time to 90% peak shortening · TR_{50}: time to 50% relengthening · TR_{90}: time to 90% relengthening · τ: intracellular Ca^{2+} decay rate

Introduction

An emerging theme in obesity is the presence of compromised ventricular function [1, 2]. Cardiovascular regulation of the obese gene product leptin has attracted much attention because its deficiency or resistance leads to obesity and cardiovascular problems [3–6]. Leptin receptors exist in cardiomyocytes and are coupled to the signalling pathways regulating myocardial contractility [6, 7] and cellular growth [5, 8]. Mice lacking leptin (*Lep*/*Lep*, formerly known as *ob*/*ob*) or its receptor (*Lepr*/*Lepr*, formerly known as *db*/*db*) develop cardiac hypertrophy and contractile dysfunction, consolidating a role for leptin in

S.-Y. Li · X. Yang · A. F. Ceylan-Isik · N. Sreejayan · J. Ren (✉)
Division of Pharmaceutical Sciences and Center for Cardiovascular Research and Alternative Medicine, University of Wyoming,
1000 E. University Avenue,
Laramie, WY 82071, USA
e-mail: jren@uwyo.edu
Tel.: +1-307-7666131
Fax: +1-307-7662953

M. Du
Department of Animal Science, University of Wyoming,
Laramie, WY 82071, USA

the maintenance of cardiac architecture and function [5, 9, 10].

Oxidative stress is a major risk factor for ventricular hypertrophy and endothelial dysfunction [3, 11, 12]. Although intracellular reactive oxygen species (ROS) are essential for optimal insulin sensitisation [13], excessive oxidative stress initiates an NADPH oxidase-mediated reduction in nitric oxide bioavailability and endothelial dysfunction in obesity [14, 15]. Defects in oxidative capacity alter cardiac function through decreased sarco (endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) isozyme 2a (SERCA2a) activity and the redistribution of myosin heavy chain (MHC) isozymes [16–18]. Alterations in MHC isozymes have been reported in obesity-related respiratory complications, which may be attenuated by leptin repletion [19, 20]. Although leptin is considered an essential player in obesity and diabetes [4, 11], the cellular mechanism(s) responsible for cardiac dysfunction in leptin-deficient obesity have not been elucidated. The aim of our study was to evaluate cardiomyocyte function, oxidative stress, lipid peroxidation, NADPH oxidase, oxidative modification of the key Ca^{2+} regulating protein SERCA and MHC isozyme distribution in leptin-deficient *Lep*/*Lep* obese mice.

Materials and methods

Experimental animals and intraperitoneal glucose tolerance test

All procedures described here were approved by our institutional animal care and use committee. Male homozygous B6.V-lep<*Lep*>/J *Lep*/*Lep* mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 3 weeks of age and were housed within our animal facility until 9 weeks of age. Age-matched wild-type C57BL/6J mice were used as controls. Mice were allowed free access to water and lab chow. A glucose tolerance test was conducted in mice fasted for 12 h by intraperitoneal injection of glucose (2 g/kg body weight). Glucose levels were determined immediately before challenge and 15, 30, 60 and 120 min thereafter [21].

Cell isolation, short-term culture, cell shortening and intracellular Ca^{2+} measurement

Cardiomyocytes were isolated enzymatically as described [22]. Cohorts of cardiomyocytes from lean or *Lep*/*Lep* mice were incubated with angiotensin II (100 nmol/l), apocynin (100 μmol/l) or both for 4 h. Mechanical and intracellular Ca^{2+} properties were assessed using edge detection and fura-2 (0.5 μmol/l). Cell shortening and relengthening were assessed using the following indices: peak shortening (PS), times to 50 and 90% PS (TPS_{50} and TPS_{90}), times to 50 and 90% relengthening (TR_{50} and TR_{90}), and maximal velocity of shortening/relengthening (±dL/dt). Qualitative changes in intracellular Ca^{2+} were inferred from the ratio of fura-2 fluorescence intensities at two wavelengths (360 and 380 nm). Fluorescence decay time (τ) was measured to indicate the intracellular Ca^{2+} clearance rate [2, 22].

Glutathione and glutathione disulfide assay

Glutathione levels were determined as an indicator of oxidative stress [22]. Samples were homogenised in four volumes (w/v) of 1% picric acid and centrifuged at 16,000 ×*g* (30 min). Supernatant fractions were assayed for total glutathione (GSH) and glutathione disulfide (GSSG) by the standard recycling method. GSH was determined using a standard curve and GSSG was measured with 4-vinyl pyridine. The GSSG (as GSH ×2) was then subtracted from the total GSH to determine the actual GSH level.

Measurement of lipid peroxidation

Hearts were homogenised in ice-cold phosphate-buffered saline (20 mmol/l) containing protease inhibitor cocktail. The homogenate was centrifuged (3,000 ×*g*, 10 min at 4 °C) and the supernatant was used for assay according to a Bioxytech LPO-586 kit (Oxis, Portland, OR, USA) [23].

Quantification of protein carbonyl

Protein was precipitated by adding an equal volume of 20% trichloroacetic acid and centrifuged at 11,000 ×*g* for 1 min. The sample was resuspended in 10 mmol/l 2,4-dinitrophenylhydrazine (2,4-DNPH) solution for 15–30 min at room temperature before 20% trichloroacetic acid was added and samples were centrifuged (11,000 ×*g*) for 3 min. The precipitate was resuspended in 6 mol/l guanidine solution. The maximum absorbance (360–390 nm) was read against appropriate blanks (2 mol/l HCl) and carbonyl content was calculated using the formula: absorption at 360 nm×45.45 nmol/protein content (mg) [22].

MHC isoform analysis by gel electrophoresis

Briefly, homogenised heart tissue in sample buffer (1:30) was heated for 2 min at 95 °C and chilled on ice for 5 min before being centrifuged. Three microlitres of 1:10 diluted supernatant was loaded for electrophoresis [24]. The methods for gels and the running conditions were identical to those described by Reiser and Kline [25]. After running, gels were fixed for a minimum of 2 h in 5% glutaraldehyde before being silver-stained and scanned with a calibrated densitometer (GS-800; Bio-Rad, Hercules, CA, USA) to determine the amounts of MHC-α and MHC-β.

Western blot analysis of NADPH oxidase subunit expression and distribution

Subcellular fractions of myocytes were prepared using the Cell Compartment Kit Fractionation Procedure (Qiagen, Valencia, CA, USA). Western blot analysis of NADPH oxidase subunits was performed on cytosolic and membrane protein fractions. Samples were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk and were incubated with β-actin and antibodies to NADPH oxidase subunits $gp91^{phox}$ and $p47^{phox}$. The film was scanned and the intensity of immunoblot bands was detected with a calibrated densitometer (Bio-Rad) [22].

SERCA isoenzyme 2a immunoprecipitation and protein carbonyl immunoprobing

Cardiomyocytes were sonicated and solubilised in a buffer containing 0.5% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; 1 mg CHAPS/100 μg protein), 10 mmol/l Tris–HCl (pH 7.4), 50 mmol/l dithiothreitol, 0.3 mol/l sucrose, with protease inhibitors at 4 °C. After centrifugation (6,000 ×*g*, 10 min), antibody to sarco(endo)plasmic reticulum Ca^{2+}-ATPase isozyme 2a (SERCA2a) (Affinity BioReagent, Denver, CO, USA) was added to the supernatant and incubated overnight at 4 °C. An IgG–agarose slurry was added and rotary-mixed at 4 °C for 2 h. Oxidised SERCA2a was probed immunochemically after derivatisation with dinitrophenylhydrazine [26]. Total SERCA2a expression after immunoprecipitation was quantified and was used to normalise protein loading.

SERCA activity measured by $^{45}Ca^{2+}$ uptake

Cardiomyocytes were sonicated and solubilised in a Tris–sucrose homogenisation buffer consisting of 30 mmol/l Tris–HCl, 8% sucrose, 1 mmol/l phenylmethylsulfonyl-fluoride and 2 mmol/l dithiothreitol, pH 7.1. To determine SERCA-dependent Ca^{2+} uptake, samples were treated with and without 10 μmol/l of the SERCA inhibitor thapsigargin for 15 min. The difference between the two readings was deemed the thapsigargin-sensitive uptake through SERCA. Uptake was initiated by the addition of an aliquot of supernatant to a solution consisting of (mmol/l) 100 KCl, 5 NaN_3, 6 $MgCl_2$, 0.15 EGTA, 0.12 $CaCl_2$, 30 Tris–HCl pH 7.0, 10 oxalate, 2 ATP and 1 μCi $^{45}CaCl_2$ at 37 °C. Aliquots of samples were injected onto glass filters on a suction manifold and washed three times. Filters were then removed from the manifold, placed in scintillation fluid and counted. SERCA activity was expressed as cpm/mg protein [27].

Statistical analysis

Data are expressed as mean ± SEM. Differences were assessed using ANOVA followed by the Newman–Keuls post hoc test. A p value less than 0.05 was considered statistically significant.

Results

General features of lean control and obese mice

The effects of obesity on body, organ weight, tibia length, glucose tolerance and blood pressure are shown in Table 1 and Fig. 1. Obesity increased the weights of the body and organs without affecting tibia length or systolic and diastolic blood pressures. The ratios of heart weight to tibia length and liver weight to tibia length were significantly higher in *Lep*/*Lep* mice. The ratio of kidney weight to tibia length was not different between lean and obese mice. Obesity moderately but significantly elevated the fasting blood glucose levels in conjunction with glucose intolerance.

Mechanical properties of cardiomyocytes

Cardiomyocytes from obese mice exhibited significantly enlarged cross-sectional area and reduced contractile capacity, indicated by decreased PS and ±dL/dt compared with lean controls. Durations of relengthening (TR_{50} and TR_{90}) were significantly prolonged and associated with normal duration of shortening (TPS_{50} and TPS_{90}) in *Lep*/*Lep* myocytes (Fig. 2). Obesity led to elevated resting and peak $[Ca^{2+}]_i$, slowed intracellular Ca^{2+} decay (prolonged τ) and increased area underneath the fluorescence curve, an indication of compromised Ca^{2+} clearing. The electrically

Table 1 General characteristiscs of lean control and *Lep*/*Lep* obese

	Mouse group	
	Lean (n=12 mice)	*Lep*/*Lep* (n=10 mice)
Body weight (g)	25.7±1.2	54.2±1.7[a]
Heart weight (mg)	221±19	288±20[a]
Heart weight/tibia (mg/cm)	124±10	154±11[a]
Liver weight (mg)	1424±108	3267±240[a]
Liver weight/tibia (mg/cm)	800±61	1742±128[a]
Kidney weight (mg)	368±25	434±22
Kidney weight/tibia (mg/mm)	20.7±1.4	23.1±1.2[a]
Tibia length (mm)	17.8±0.3	18.7±0.6
Systolic pressure (mmHg)	109.0±1.8	111.9±3.4
Diastolic pressure (mmHg)	80.0±2.8	79.9±3.7
Fasting blood glucose (mmol/l)	89.7±9.8	7.17±1.14[a]

Mean±SEM
[a]p<0.05 vs lean control

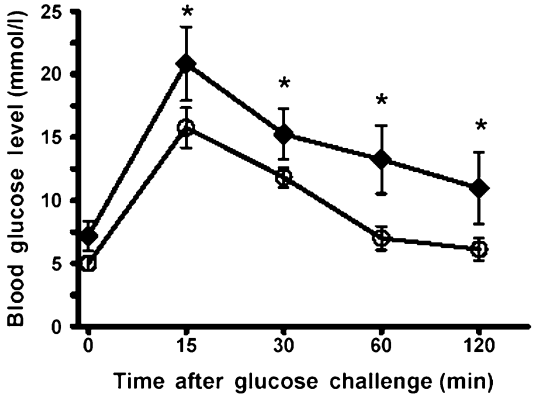

Fig. 1 Intraperitoneal glucose tolerance test. Blood glucose levels in response to intraperitoneal glucose challenge (2 g/kg) in *Lep*/*Lep* (*filled symbols*) and lean (*open symbols*) control mice. Mice were fasted for 12 h before the tests. Mean±SEM, n=6 mice, *p<0.05 vs lean control

stimulated rise in $[Ca^{2+}]_i$ was similar between the two groups (Fig. 3).

Effect of extracellular Ca^{2+} and stimulus frequency on myocyte shortening

To examine the influence of extracellular Ca^{2+} levels on PS, myocytes were placed in a contractile buffer with 0.5 mmol/l $CaCl_2$ before increasing extracellular Ca^{2+} levels to 1.0, 2.0 and 3.0 mmol/l, with a 5-min interval in between. Figure 4a shows that PS was not significantly different between lean and obese myocytes under 0.5 mmol/l Ca^{2+}. Stepwise increases in extracellular Ca^{2+} resulted in increased PS in lean control myocytes. However, such a rise in PS was significantly dampened in *Lep*/*Lep* myocytes. The maximal PS (at 3 mmol/l Ca^{2+}) was significantly less in *Lep*/*Lep* myocytes than in those of lean controls. These results suggest reduced myocyte responsiveness to extracellular Ca^{2+} and/or a diminished sarcoplasmic reticulum Ca^{2+} load under obesity. To evaluate the impact of obesity on cardiac function at higher pacing rates, cells were initially stimulated to contract at 0.5 Hz for 5 min to ensure a steady state before commencing the frequency response study. All recordings were normalised to PS at 0.1 Hz of the same myocyte. Figure 4b shows a steeper decline of PS in *Lep*/*Lep* myocytes with increasing stimulus frequency compared with lean control myocytes (with the exception of 3.0 Hz). These data indicate that *Lep*/*Lep* myocytes displayed reduced intracellular Ca^{2+} cycling or stress tolerance.

Effect of obesity on cardiac oxidative stress, lipid peroxidation level, protein carbonyl content and expression of MHC isozymes

Obesity is often associated with enhanced oxidative stress, leading to irreversible damage of proteins or lipids [14]. Figure 5a demonstrates that the GSH/GSSG ratio was significantly decreased, by ~40%, in *Lep*/*Lep* heart. Similarly, lipid peroxidation and protein carbonyl formation were both significantly elevated in *Lep*/*Lep* hearts (Fig. 5b,c). It has been reported that expression of MHC isozymes is associated with cardiac function, especially in myocardial remodelling and cardiomyopathies [17, 28–31]. We examined the pattern of MHC isozyme distribution in *Lep*/*Lep* heart. The result shown in Fig. 6 revealed an MHC isozyme switch from α to β in *Lep*/*Lep* hearts. While MHC-α was the predominant isoform in lean hearts, the MHC-β content was significantly elevated in *Lep*/*Lep* hearts compared with the lean group (Fig. 6).

Role of NADPH oxidase in obesity-associated cardiac mechanical dysfunction

NADPH oxidase is a predominant source of ROS [32, 33]. Our earlier study revealed that angiotensin receptor-mediated activation of NADPH oxidase plays a role in hyperglycaemia-induced cardiac dysfunction [34]. To test whether the angiotensin II-NADPH oxidase cascade plays a role in obesity-induced cardiac dysfunction, lean and *Lep*/*Lep* myocytes were incubated with angiotensin II (100 nmol/l) or apocynin (100 μmol/l) for 4 h before the cell mechanics were evaluated. Data shown in Fig. 7 indicate that neither angiotensin II nor apocynin affected cell cross-sectional area in lean and *Lep*/*Lep* myocytes. Interestingly, apocynin blunted the obesity-induced reduction in PS, ±dL/dt and prolongation of TR_{90} in *Lep*/*Lep* myocytes without affecting cell mechanics in lean cells. On the contrary, angiotensin II mimicked the cardiomyocyte contractile phenotype of *Lep*/*Lep* myocytes to a great extent, an effect which was abolished by apocynin. TPS_{90} was not affected by either angiotensin II or apocynin. Surprisingly, co-incubation with both agents significantly shortened TPS_{90} in lean control myocytes, although the mechanism remains unknown. Apocynin and angiotensin II exhibited effects on TPS_{50} and TR_{50} similar to those of TPS_{90} and TR_{90} in lean and *Lep*/*Lep* myocytes (data not shown). These data suggest that angiotensin II and NADPH oxidase activation play important roles in the cardiac dysfunction observed in *Lep*/*Lep* obesity. To further elucidate the role of NADPH oxidase in obesity-associated cardiac dysfunction, levels of two major subunits of NADPH oxidase, $gp91^{phox}$ and $p47^{phox}$, were examined in lean and *Lep*/*Lep* cardiomyocytes. Redistribution of NADPH oxidase subunits from cytosol onto membrane is considered a hallmark of NADPH oxidase activation [32]. Consistent with oxidative stress in *Lep*/*Lep* mouse hearts, the levels of $p47^{phox}$ and $gp91^{phox}$ were elevated in the membrane but not the cytosolic fraction of *Lep*/*Lep* myocytes or lean cells treated with angiotensin II. Apocynin attenuated elevated $pg91^{phox}$ distribution but not $p47^{phox}$ in *Lep*/*Lep* myocytes. Similarly, apocynin effectively antagonised angiotensin II-induced upregulation of NADPH oxidase subunits. Cytosolic NADPH oxidase was not affected by obesity, angiotensin II or apocynin (Fig. 8).

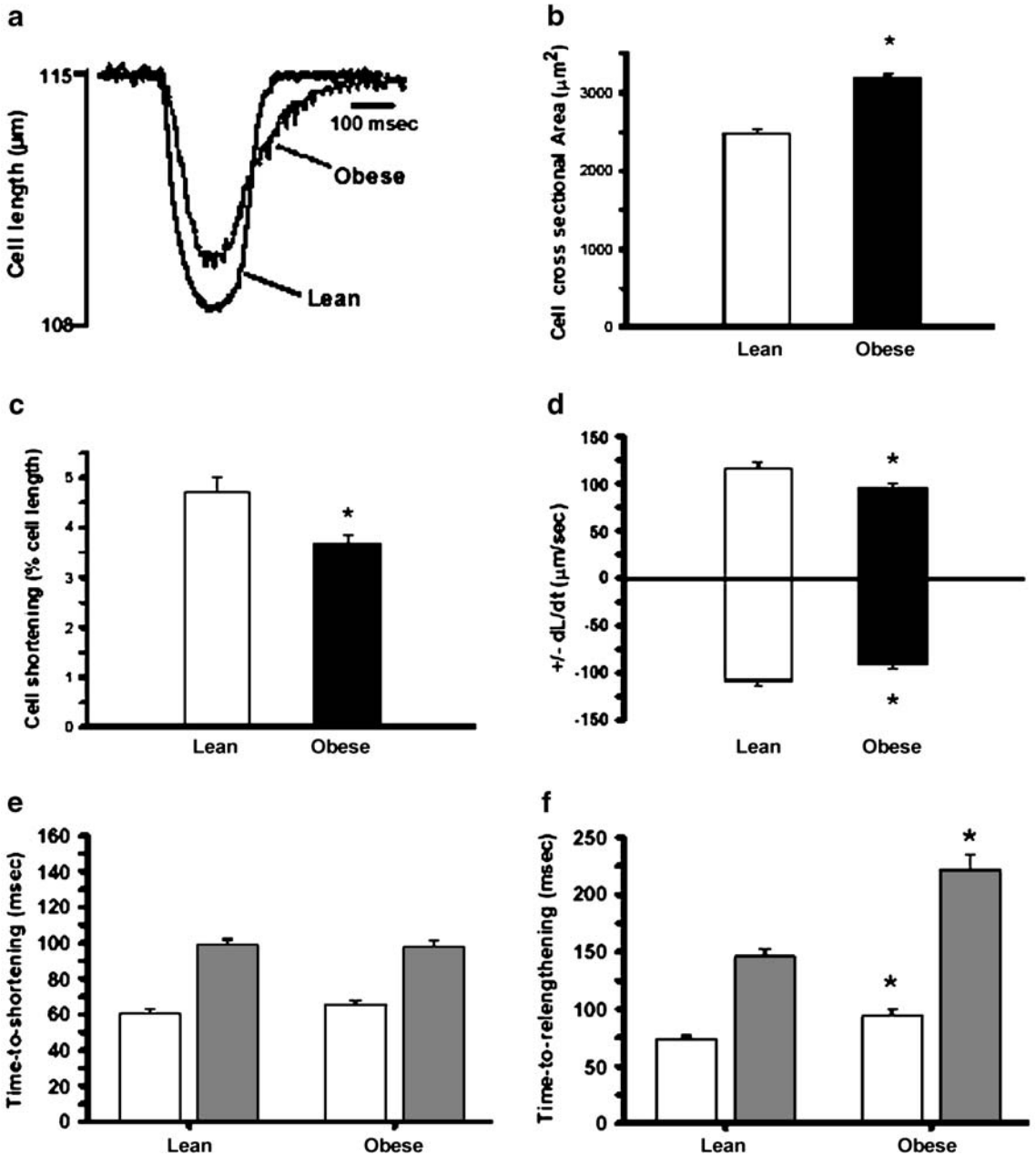

Fig. 2 Contractile properties of ventricular myocytes from lean and *Lep*/*Lep* obese mouse hearts. **a** Representative cell shortening traces in lean control and *Lep*/*Lep* mouse myocytes. **b** Cross-sectional area. **c** Peak shortening normalised to resting cell length. **d** Maximal velocity of shortening/relengthening (±dL/dt). **e** Times to 50 and 90% peak shortening (TPS_{50} and TPS_{90}, *open and shaded bars*, respectively). **f** Times to 50 and 90% relengthening (TR_{50} and TR_{90}, *open and shaded bars*, respectively). Cardiomyocytes were electrically stimulated to contract at a frequency of 0.5 Hz with an extracellular Ca^{2+} level of 1.0 mmol/l. Mean±SEM, n=146 cells evenly distributed from six mice, *p<0.05 vs lean control

Effect of obesity on SERCA2a oxidation and SERCA activity

Persistent oxidative stress leads to oxidative damage to proteins such as SERCA [27, 35, 36]. Given that protein carbonyl formation, a fundamental measure of oxidative damage in cardiac proteins [22, 37], is elevated in *Lep*/*Lep* heart, we evaluated oxidative damage to SERCA2a by quantitative immunoprecipitation of SERCA2a from cell lysates using specific SERCA2a antibody followed by immunoprobe of the protein carbonyl content. The total expression of immunoprecipitated SERCA2a proteins did not differ among lean myocytes, *Lep*/*Lep* myocytes and cells treated with apocynin or angiotensin. Interestingly, the levels of oxidised SERCA2a (both absolute abundance and the level normalised to total SERCA2a) were significantly increased in *Lep*/*Lep* myocytes and lean control myocytes treated with angiotensin II. Apocynin did not affect the oxidative modification SERCA2a in either the lean or the *Lep*/*Lep* group. Interestingly, the angiotensin

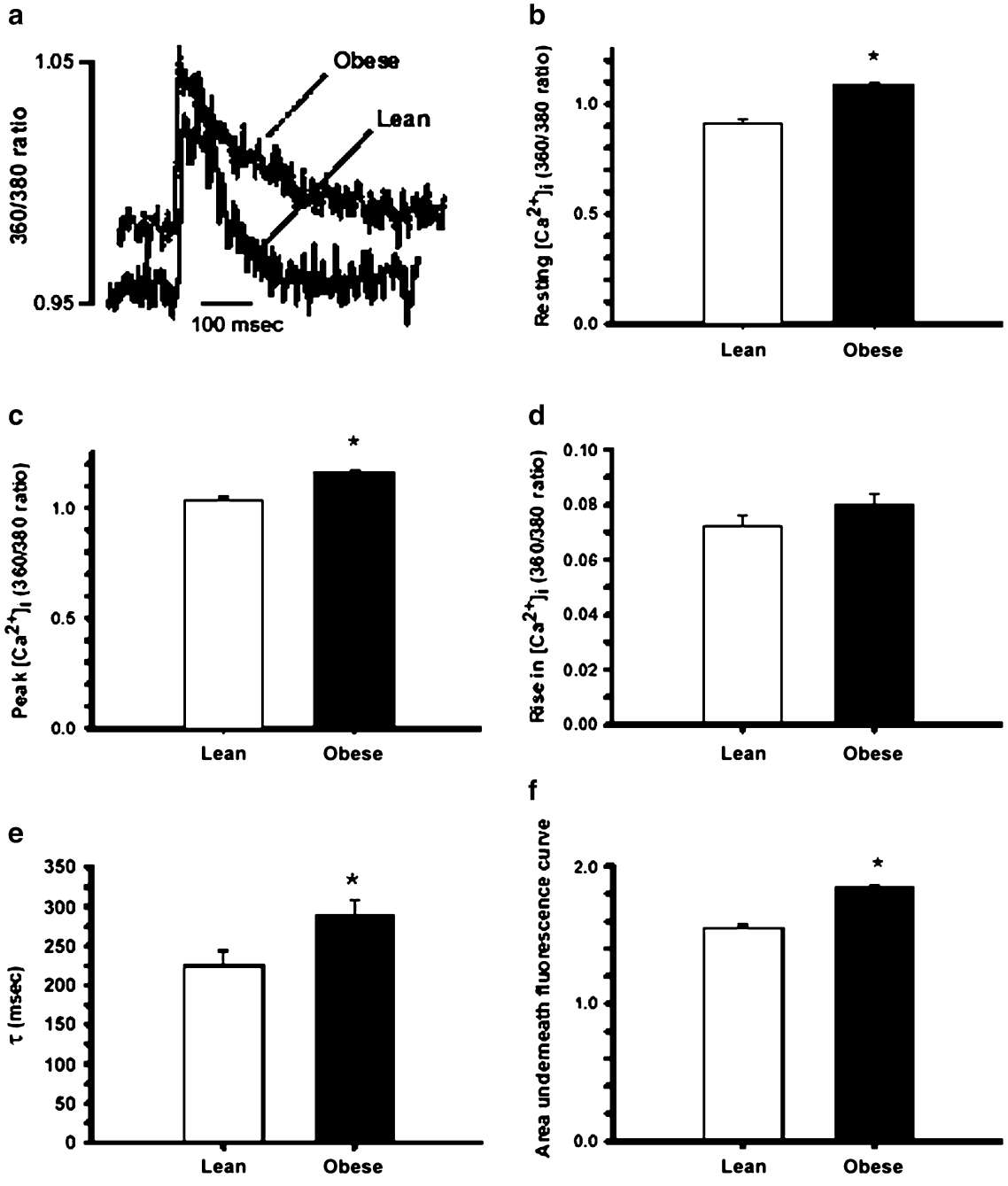

Fig. 3 Intracellular Ca^{2+} transient properties of ventricular myocytes from lean and *Lep*/*Lep* obese mouse hearts. **a** Representative intracellular Ca^{2+} transients in lean and *Lep*/*Lep* mouse myocytes. **b** Resting intracellular Ca^{2+} levels. **c** Peak intracellular Ca^{2+} levels. **d** Electrically stimulated rise in intracellular Ca^{2+}. **e** Intracellular Ca^{2+} decay rate (τ). **f** Area integration underneath Ca^{2+} fluorescence curve. Cardiomyocytes were electrically stimulated to contract at a frequency of 0.5 Hz with an extracellular Ca^{2+} level of 1.0 mmol/l. Mean±SEM, n=56 cells evenly distributed from four mice, *p<0.05 vs lean control

II-elicited increase in SERCA2a oxidative modification was significantly attenuated by apocynin (Fig. 9). Consistent with enhanced SERCA2a oxidative modification in *Lep*/*Lep* myocytes, SERCA activity was significantly reduced in myocytes from *Lep*/*Lep* mice. Angiotensin II treatment suppressed SERCA activity in lean control myocytes to a similar extent. Somewhat surprisingly, apocynin did not exhibit any affect on the reduced SERCA activity in *Lep*/*Lep* myocytes whereas it significantly attenuated angiotensin II-induced reduction in SERCA activity (Fig. 10).

Discussion

This study demonstrated that *Lep*/*Lep* obesity is associated with cardiomyocyte dysfunction and intracellular Ca^{2+} mishandling, manifested as reduced contractile capacity

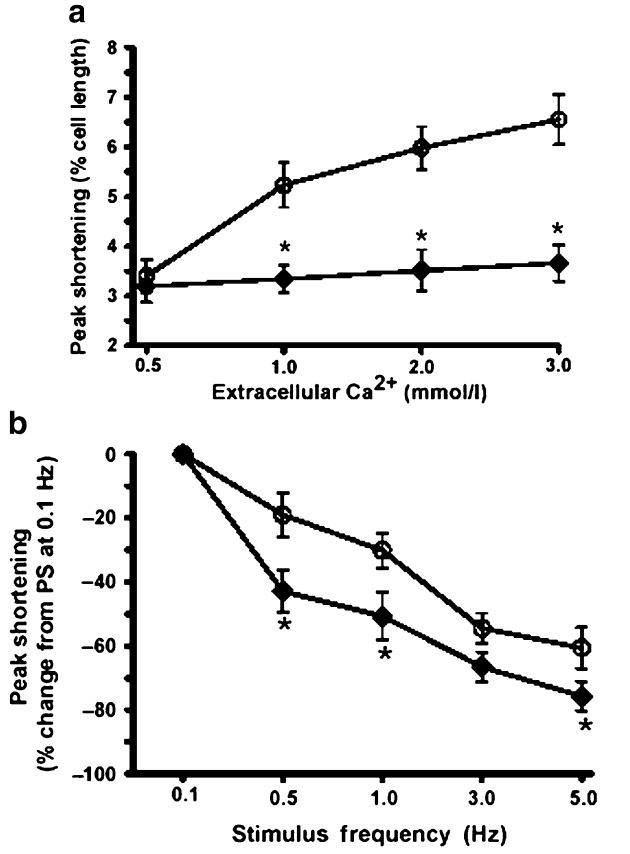

Fig. 4 Effect of increased extracellular Ca^{2+} concentration (**a**) and field electrical stimulus frequency (**b**) on peak shortening (PS) amplitude in myocytes from lean (*open symbols*) and *Lep*/*Lep* obese (*filled symbols*) mouse hearts. For frequency response, PS is shown as percentage change from respective PS value obtained at 0.1 Hz. Mean±SEM, n=25–29 cells from four mice per group, *p<0.05 vs lean control

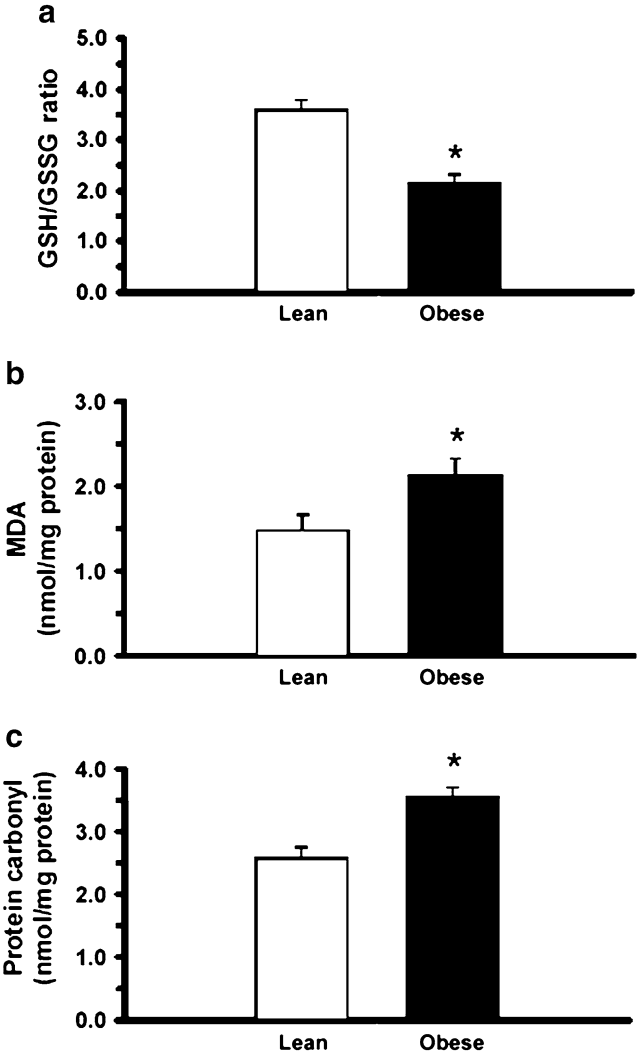

Fig. 5 Oxidative stress, lipid peroxidation level and protein carbonyl content in cardiac tissue. **a** Ratio of GSH to GSSG. **b** Level of the lipid peroxidation end-product malondialdehyde. **c** Protein carbonyl content. Mean±SEM, n=6, *p<0.05 vs lean control

and maximal velocity of contraction/relaxation, prolonged relaxation, elevated resting intracellular Ca^{2+} levels, slowed intracellular Ca^{2+} extrusion, impaired responsiveness to extracellular Ca^{2+} and diminished contractile capacity at higher stimulus frequencies. In addition, elevated oxidative stress, lipid peroxidation and protein carbonyl formation were observed in *Lep*/*Lep* hearts in conjunction with activation of NADPH oxidase. Our further evidence indicated overt oxidative modification to SERCA2a, diminished SERCA activity and MHC isozyme switch in *Lep*/*Lep* obesity. The fact that apocynin abolished the cardiac contractile defect and $pg91^{phox}$ subunit membrane distribution in *Lep*/*Lep* myocytes supports a role of NAPDH oxidase in obesity-associated cardiac dysfunction. However, the observation that apocynin fails to reverse obesity-induced oxidative modification of SERCA2a and reduced $^{45}Ca^{2+}$ uptake indicates that SERCA modification may not be reversed with short-term apocynin treatment. It is also possible that SERCA may not be a permissive player downstream of NADPH oxidase activation en route to cardiac mechanical defects in *Lep*/*Lep* obesity.

Leptin deficiency in *Lep*/*Lep* mice is associated with obesity, insulin resistance and mild to moderate diabetes

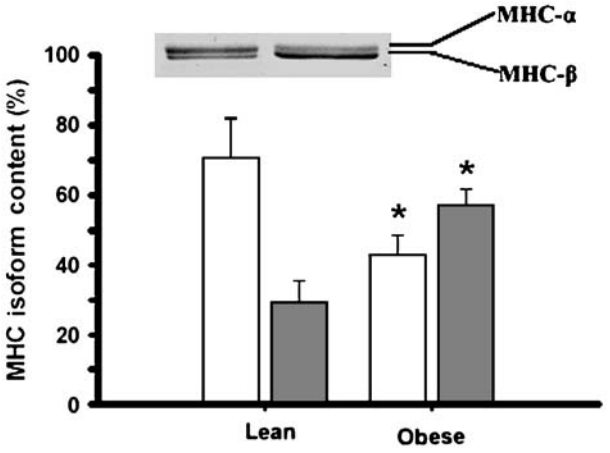

Fig. 6 Levels of myosin heavy chain α and β isoforms (MHC-α , *open bars*; MHC-β, *shaded bars*) in myocardium from lean control and *Lep*/*Lep* obese mice. Mean±SEM, n=6, *p<0.05 vs lean control

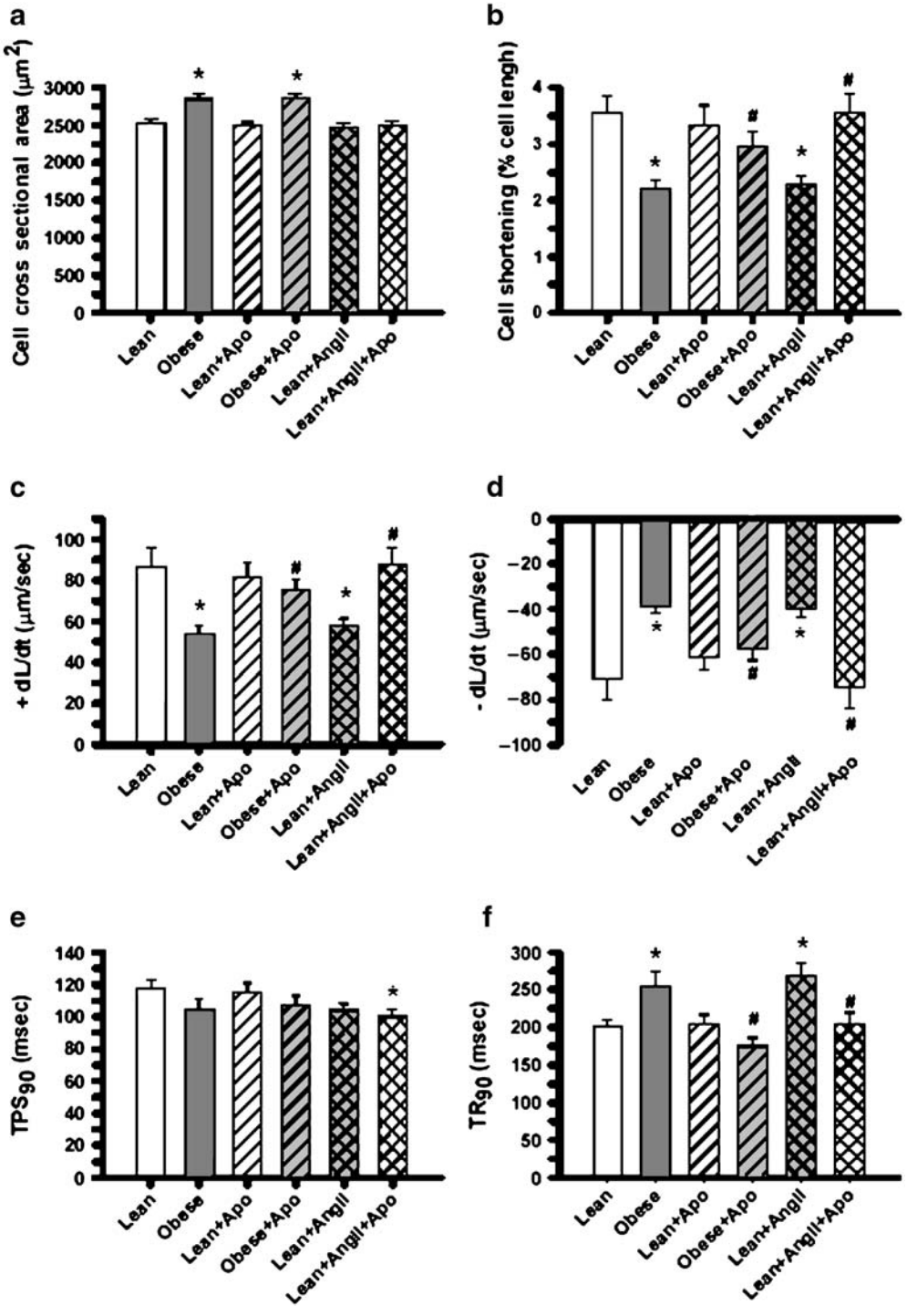

Fig. 7 Contractile properties of lean and *Lep*/*Lep* obese cardiomyocytes incubated for 4 h with angiotensin II (AngII, 100 nmol/l), the NADPH oxidase inhibitor apocynin (Apo, 100 μmol/l) or both. **a** Cross-sectional area. **b** Peak shortening (PS) normalised to resting cell length. **c** Maximal velocity of shortening (+dL/dt). **d** Maximal velocity of relengthening (−dL/dt). **e** Time to 90% peak shortening (TPS_{90}). **f** Time to 90% relengthening (TR_{90}). Mean± SEM, n=84–88 cells from three mice per group, *p<0.05 vs lean control, #p<0.05 vs respective apocynin-free group

[38, 39]. The 9-week-old *Lep*/*Lep* mice used in our study exhibited impaired glucose tolerance, indicative of prediabetic insulin resistance. Tibia length was similar between the lean and *Lep*/*Lep* mice, excluding potential contribution from developmental factor. Cardiac hypertrophy has been shown in *Lep*/*Lep* hearts from 8- and 12-week-old mice [40, 41], in agreement with our observation of increased absolute heart weight, ratio of heart weight to tibia length and enlarged cross-sectional area of myocytes. Myocardial remodelling is a critical factor in the transition of the heart from a compensated to a decompensated state and may contribute to compromised cardiac function and the MHC isozyme switch [42]. Reduced contractile capacity and prolonged duration of relaxation have been indicated in obesity and type 2 diabetes [2, 16]. Our present study demonstrated a larger cross-sectional area of the cells, reduced PS, lessened ±dL/dt and prolonged TR_{50}/TR_{90} in association with normal TPS_{50}/TPS_{90}, compared with lean control myocytes. Consistently, myocytes from *Lep*/*Lep* mice showed elevated resting and peak $[Ca^{2+}]_i$,

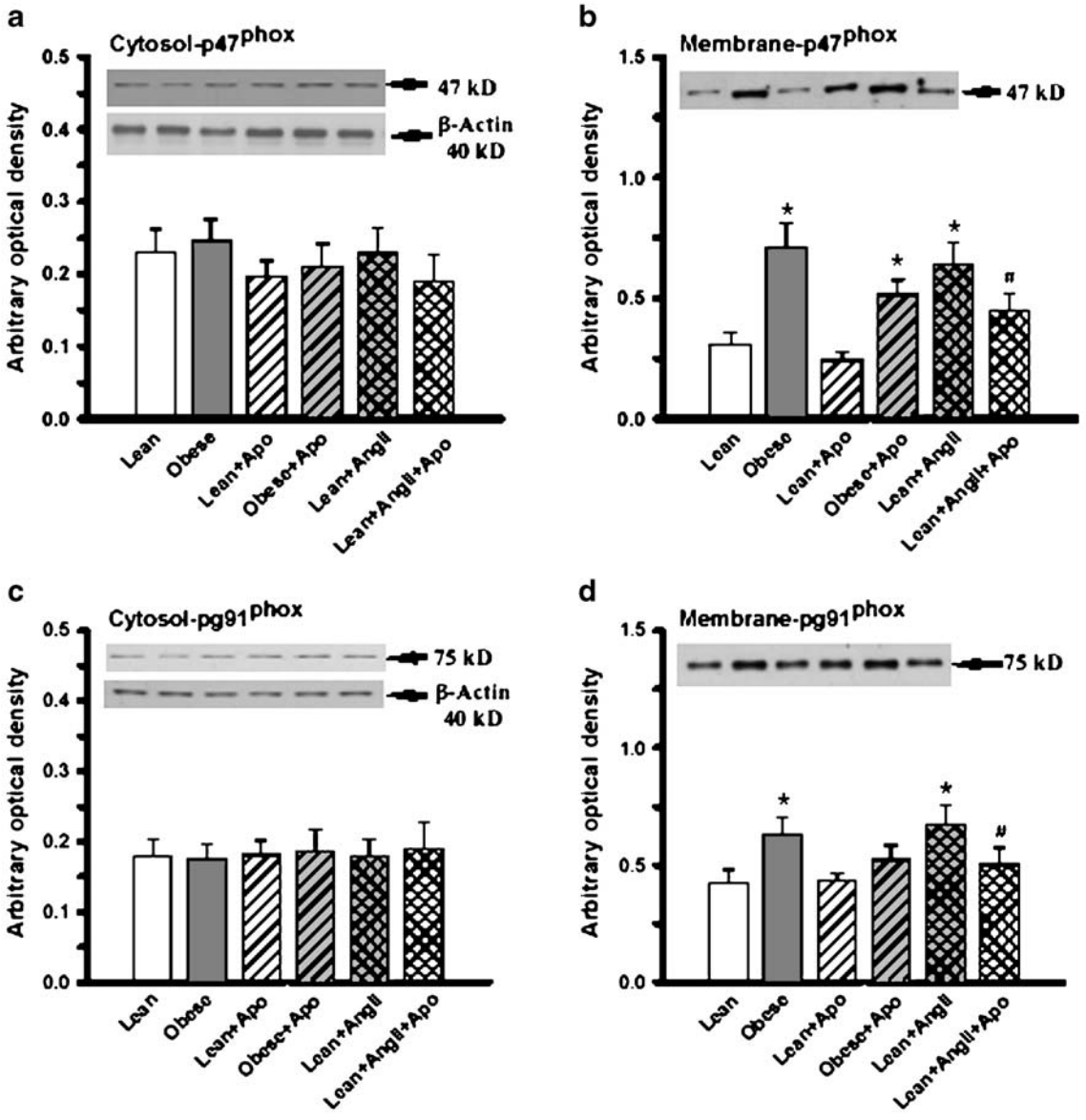

Fig. 8 NADPH oxidase expression and distribution in cytosolic and membrane fractions of lean and *Lep*/*Lep* obese mouse myocytes with or without treatment with angiotensin II (AngII, 100 nmol/l) or apocynin (Apo, 100 μmol/l). **a**, **b** The $p47^{phox}$ subunit of NADPH oxidase in cytosolic and membrane fractions. **c**, **d** The $gp91^{phox}$ subunit of NADPH oxidase in cytosolic and membrane fractions. *Insets*: representative immunoblots using specific antibodies against $p47^{phox}$ and $gp91^{phox}$ subunits. Mean±SEM, n=6, *p<0.05 vs lean control, #p<0.05 vs angiotensin II treatment group

prolonged τ, increased area integration underneath the intracellular Ca^{2+} fluorescence curve, reduced contractile capacity at higher field stimulus frequency, and reduced responsiveness to extracellular [Ca^{2+}]. Several mechanisms may be postulated for obesity-associated defects in *Lep*/*Lep* cardiac contractility and intracellular Ca^{2+} handling. It has been reported that prolonged duration of relaxation may simply be a consequence of impaired SERCA and Na^{+}/Ca^{2+} exchanger function [43]. This is supported by enhanced SERCA oxidative modification, reduced $^{45}Ca^{2+}$ uptake and slowed intracellular Ca^{2+} clearing rate (τ) in *Lep*/*Lep* mouse cardiomyocytes. The prolongation of both TR_{50} and TR_{90} seen in *Lep*/*Lep* myocytes indicates defects in both the rapid phase (usually controlled by SERCA) and the slow phase (controlled by Na^{+}/Ca^{2+} exchanger, sarcolemmal and mitochondrial Ca^{2+} pumps) of diastole. Our study revealed depressed myocyte shortening ability associated with a normal increase in intracellular Ca^{2+} in response to electrical stimuli in *Lep*/*Lep* myocytes. This discrepancy may be attributed to reduced myofilament Ca^{2+} sensitivity and MHC isozyme switch in the *Lep*/*Lep* group. Our data indicate that the contractile response of myocytes to increased extracellular Ca^{2+} was reduced in *Lep*/*Lep* mouse myocytes, indicative of poor myofilament Ca^{2+} responsiveness, and that the low sarcoplasmic reticulum Ca^{2+} load resulted from defective SERCA function. In addition, the *Lep*/*Lep* mice displayed interrupted leptin signalling and reduced leptin receptor LEPR

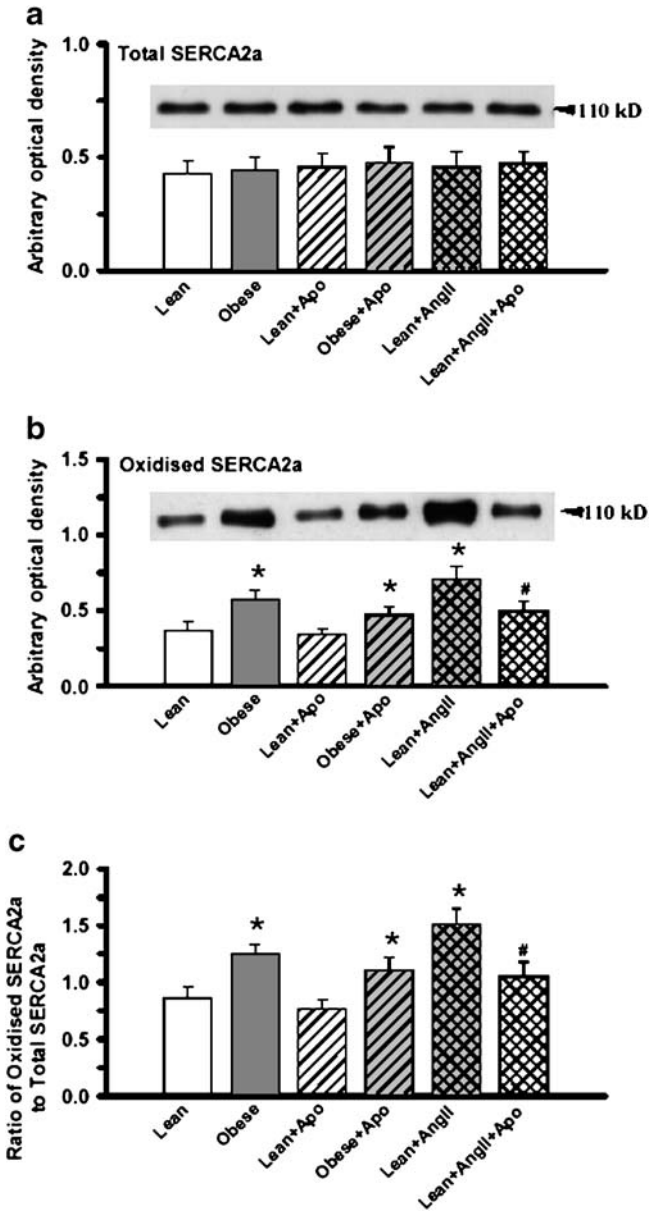

Fig. 9 Oxidative modification of SERCA2a protein in lean and *Lep*/*Lep* obese mouse myocytes with or without treatment with angiotensin II (AngII, 100 nmol/l) or apocynin (Apo, 100 μmol/l). **a** Total SERCA2a expression. **b** Oxidative modification of SERCA2a detected by immunoprecipitation followed by protein carbonyl immunoprobing. **c** Oxidised SERCA2a normalised to total SERCA2a abundance. Mean±SEM, n=6, *p<0.05 vs lean control, #p<0.05 vs angiotensin II treatment

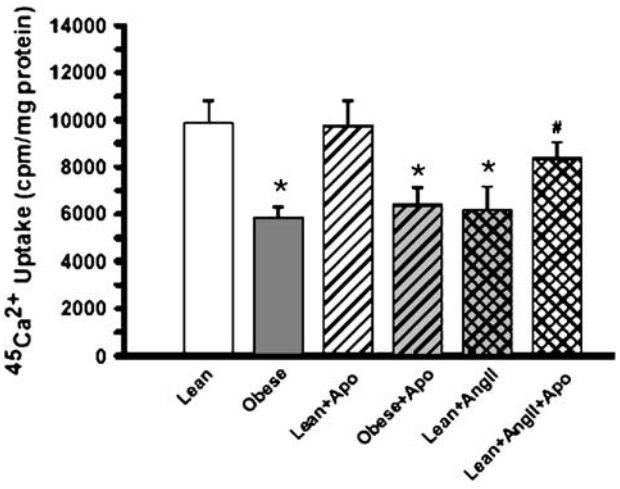

Fig. 10 SERCA2 activity measured by SERCA-dependent $^{45}Ca^{2+}$ uptake in lean control and *Lep*/*Lep* obese mouse myocytes with or without treatment with angiotensin II (AngII, 100 nmol/l) or apocynin (Apo, 100 μmol/l). Mean±SEM, n=6, *p<0.05 vs lean control, #p<0.05 vs angiotensin II treatment

[41], representing a unique model of obesity. Most obese or overweight individuals display enhanced rather than reduced plasma levels of leptin, contrary to the leptin-deficient status in *Lep*/*Lep* obesity [4]. In a recent study, Belke and colleagues found decreased rates of contraction, relaxation and pressure development in hyperleptinaemic *Lepr*/*Lepr* diabetic mouse hearts [44]. While cardiac mechanical findings and the slowed intracellular Ca^{2+} decay in *Lepr*/*Lepr* mice are consistent with our present observation in *Lep*/*Lep* mice, *Lepr*/*Lepr* diabetic myocytes displayed significantly reduced resting and peak Ca^{2+} levels [44], contrary to our finding in *Lep*/*Lep* myocytes. Decreased, increased and unchanged resting intracellular Ca^{2+} levels have been seen in diabetes, insulin resistance and obesity [2, 45]. The resting intracellular Ca^{2+} levels appear to be model- and age-dependent.

Increased oxidative stress plays an important role in obesity-related cardiac complications [4, 14], in which NADPH oxidase may serve as the predominant source of ROS [14]. Our results showed that the cardiac GSH/GSSG ratio was decreased in the *Lep*/*Lep* group, associated with elevated lipid peroxidation and protein carbonyl content. More importantly, $p47^{phox}$ and $gp91^{phox}$ were significantly elevated in membrane fractions of *Lep*/*Lep* mouse myocytes. Activation of NADPH oxidase and subsequent accumulation of ROS are key to oxidative injury in cardiovascular diseases [32, 33, 46]. Oxidative stress has been shown to result in oxidative damage to functional proteins [35]. SERCA2a has been shown to be a vulnerable target for oxidative insult [27, 36]. In this study we demonstrated a marked oxidative modification of SERCA2a in *Lep*/*Lep* mouse myocytes, which may contribute to reduced SERCA activity and subsequent cardiac contractile dysfunction in *Lep*/*Lep* mouse myocytes. Excessive and irreversible SERCA oxidation may account for heart dysfunction in heart failure, atherosclerosis, ageing and other cardiovascular diseases [27]. Our observation that apocynin abolished angiotensin II and *Lep*/*Lep* obesity-induced cardiac mechanical defects and NADPH oxidase subunit membrane distribution (with the exception of $p47^{phox}$ in *Lep*/*Lep* myocytes) further consolidated the important role of NAPDH oxidase in cardiac contractile dysfunction in *Lep*/*Lep* obesity. Although the discrepancy between apocynin-elicited effects on $p47^{phox}$ and $pg91^{phox}$ is not fully clear at this point, routes of NADPH oxidase activation and the inhibition of this by apocynin may contribute to the disparity in NADPH oxidase subunit response in *Lep*/*Lep* myocytes. First, apocynin interrupts

the activation of NADPH oxidase through inhibition of the NADPH subunit assembly, its post-translational modification and translocation to the membrane (especially the cytosolic subunit $p47^{phox}$) [32, 47]. It is possible that apocynin exerts a predominant inhibitory effect on protein assembly of the cytosolic subunit $p47^{phox}$ and the membrane-bound $gp91^{phox}$, whereas it has little effect on $p47^{phox}$ translocation to the membrane in *Lep*/*Lep* myocytes. As a result, apocynin failed to significantly suppress the increase in the membrane distribution of $p47^{phox}$ in *Lep*/*Lep* myocytes seen in our present study. Secondly, an association between $gp91^{phox}$ and $p47^{phox}$ was deemed an essential step for NADPH oxidase activation [33]. Therefore, inhibition of apocynin on membrane-bound $gp91^{phox}$ (but not $p47^{phox}$) in *Lep*/*Lep* myocytes may be sufficient to disrupt the association between these two subunits and the subsequent activation of NADPH oxidase in *Lep*/*Lep* myocytes. It is also possible that the enhanced membrane distribution of $p47^{phox}$ due to prolonged obesity may not be readily reversed by short-term apocynin treatment. In addition, activation of NADPH oxidase is known to stimulate protein synthesis and cardiac hypertrophy [32], although our 4 h incubation of angiotensin II was too short for the enlargement of cardiomyocytes.

Perhaps the most puzzling result of our study is that apocynin fails to restore the *Lep*/*Lep* obesity-induced oxidative modification of SERCA2a and reduced SERCA activity. This observation tends to indicate that the SERCA pump may not be a permissive player downstream of NADPH oxidase activation en route to cardiac mechanical defects in *Lep*/*Lep* obesity. However, it may be argued that enhanced oxidative modification of SERCA and impaired SERCA function as a result of the 9-week insulin resistance and mild hyperglycaemia in *Lep*/*Lep* obesity cannot be readily reversed by our short-term in vitro apocynin treatment. Nonetheless, a contribution from other SERCA-independent machineries cannot be ruled out at this time. For example, apocynin may counteract certain signalling pathways downstream of angiotensin II. This is supported by our finding that apocynin abolished angiotensin II-elicited mechanical defects and SERCA oxidative modification and reduced $^{45}Ca^{2+}$ uptake in lean control myocytes, all of which are reminiscent of the effects seen in *Lep*/*Lep* myocytes. Last but not least, it is plausible to speculate that insulin resistance and oxidative stress in *Lep*/*Lep* mice may both contribute to cardiac dysfunction. It is likely that ROS precedes Ca^{2+} overload since the former is known to induce Ca^{2+} overload in cardiomyocytes [48].

An altered distribution of MHC isoforms has been shown in myocardial remodelling, cardiac hypertrophy and cardiomyopathy [17, 28–31], indicating that the regulation of cardiac contractile function is directly related to the relative amounts of MHC-α and MHC-β isozymes [25]. Even a small shift in the relative expression of these isoforms may significantly alter cardiomyocyte power output [30, 31]. Although the predominant MHC isoform in healthy human hearts is β rather than α, overexpression of MHC-β in transgenic mice suggests that such an MHC-α to MHC-β switch is a maladaptive response to preserve energy [30]. The MHC isozyme switch from the fast type α isoform to the slow type β isoform (lower ratio of MHC-α to MHC-β) is believed to play a significant role in cardiac dysfunction in diabetes, cardiac hypertrophy and senescence [28, 29, 49, 50]. This is supported by the fact that an increase in MHC-α and a decrease in MHC-β mRNA expression (increased ratio of MHC-α to MHC-β) is deemed beneficial in the improvement of left ventricular function in patients with idiopathic dilated cardiomyopathy [31]. Similarly, our results reveal a significant isozyme switch from MHC-α to MHC-β in *Lep*/*Lep* mice, which may contribute to contractile dysfunction. This is supported by our observation of impaired SERCA function in *Lep*/*Lep* cardiomyocytes. So far, little information is available regarding the ratio between MHC-α and MHC-β in human obesity. To the best of our knowledge, this is the first report to correlate cardiac contractile dysfunction with cardiac MHC isozyme switch in a *Lep*/*Lep* model of obesity. However, it should be mentioned that factors such as cardiac hypertrophy, which exists in the *Lep*/*Lep* mouse model of obesity, may independently trigger the MHC isozyme switch.

In summary, our findings revealed reduced cardiac contractile capacity, a prolonged duration of relaxation, an elevated resting intracellular Ca^{2+} level, reduced intracellular Ca^{2+} extrusion, diminished stress tolerance and depressed Ca^{2+} responsiveness in *Lep*/*Lep* obese mouse myocytes. Our data showing that the NADPH oxidase inhibitor apocynin rescues cardiac contractile dysfunction in *Lep*/*Lep* myocytes suggest a causal relationship between oxidative stress and cardiac mechanical dysfunction. Moreover, our data demonstrated increased oxidative modification of SERCA2a, reduced SERCA activity and overt MHC isozyme switch in *Lep*/*Lep* mouse myocytes, indicating that both decreased SERCA function and the MHC isozyme switch may participate in cardiac mechanical dysfunction in the *Lep*/*Lep* model of obesity. Our short-term in vitro treatment of apocynin failed to reverse SERCA2a oxidative modification and the SERCA defect in *Lep*/*Lep* myocytes, making long-term apocynin treatment necessary if we are to understand the roles of NADPH oxidase and SERCA in cardiac contractile dysfunction in *Lep*/*Lep* obesity. To validate the role of NADPH oxidase activation in the increased oxidative modification of SERCA2a and reduced SERCA activity in obesity, in vivo treatment with an NADPH oxidase inhibitor using *Lep*/*Lep* mice or another genetic or diet-induced model of obesity is needed for future studies. Given what we know about obesity-associated ventricular dysfunction obesity and the high prevalence of juvenile obesity, the use of antioxidants to antagonise obesity-associated cardiac dysfunction should have a promising future.

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