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Improved functional recovery to I/R injury in hearts from lipocalin-2 deficiency mice: restoration of mitochondrial function and phospholipids remodeling

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Abstract: Aims: Recent clinical and experimental evidences demonstrate an association between augmented circulating lipocalin-2 [a pro-inflammatory adipokine] and cardiac dysfunction. However, little is known about the pathophysiological role of lipocalin-2 in heart. The present study was designed to compare the heart functions of mice with normal (WT) or deficient lipocalin-2 (Lcn2-KO) expression. Methods and results: Echocardiographic analysis revealed that the myocardial contractile function was significantly improved in hearts of Lcn2-KO mice, under both standard chow and high fat diet conditions. The heart function before and after I/R injury (20-min of global ischemia followed by 60-min of reperfusion) was assessed using the Langendorff perfusion system. Compared to WT littermates, hearts from Lcn2-KO mice showed improved functional recovery and reduced infarct size following I/R. Under baseline condition, the mitochondrial function of Lcn2-KO hearts was significantly enhanced, as demonstrated by biochemical analysis of respiratory chain activity and markers of biogenesis, as well as electron microscopic investigation of the mitochondrial ultrastructure. Acute or chronic administration of lipocalin-2 impaired cardiac functional recovery to I/R and dampened the mitochondrial function in hearts of Lcn2-KO mice. These effects were associated with an extensive modification of the fatty acyl chain compositions of intracellular phospholipids. For example, lipocalin-2 facilitated the redistribution of linoleic acid (C18:2) among different types of phospholipids, including cardiolipin, a structurally unique phospholipid located mainly on the inner membrane of mitochondria. Conclusions: Lack of lipocalin-2 improved the functional recovery of isolated mice hearts subjected to I/R, which is associated with restoration of mitochondrial function and phospholipids remodeling.

Keywords: Lipocalin-2, adipokine, heart, mitochondria, phospholipids

Introduction

Lipocalin-2 [also known as 24p3, neutrophil gelatinase-associated lipocalin and siderocalin] belongs to the diversified lipocalin family that generally binds small hydrophobic ligands [1-3]. It is a 25-kDa adipokine constitutively produced by adipocytes [4-7]. The expression of lipocalin-2 is upregulated in pathological conditions such as obesity, inflammation, hypertension and cancer [8-10]. Abnormal regulation and function of lipocalin-2 contribute to the development of obesity-related medical complications [11-16]. Recent evidences suggest that lipocalin-2 can be used as a diagnostic and prognostic marker for overt heart disease [17]. Circulating levels of lipocalin-2 are augmented and correlated closely with the severity of coronary heart disease [18, 19]. It is a biomarker for early detection of cardio-renal syndrome in patients with acute heart failure [20]. During systemic adaptation to chronic heart failure, patients with baseline lipocalin-2 > 783 ng/ml have a significantly higher mortality [21]. In both human and rodents, increased expression of lipocalin-2 is found in necrotic areas and the surrounding tissue of the infarcted heart [17, 22-24]. Results derived from animal studies suggest that lipocalin-2 may be actively involved in modulating the heart functions in response to acute injuries [23, 25, 26]. On the other hand, there is no information available...
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regarding the effects of this protein on cardiac contractile and hemodynamic functions.

The present study evaluated the heart functions of mice lacking lipocalin-2 at both baseline condition and in response to I/R injury. The results demonstrated that the presence or absence of lipocalin-2 could modulate cardiac performance and functional recovery to I/R. Mice without lipocalin-2 showed a better contractile activity and were partially protected from I/R-induced injury. The mitochondrial function was significantly improved in the heart tissues of lipocalin-2 knockout mice. Replacement with lipocalin-2 adversely modified the heart function and promoted remodeling of intracellular phospholipids. These findings collectively support a role of lipocalin-2 in the pathogenesis of heart diseases, especially under conditions such as obesity, metabolic syndrome and aging.

Materials and methods

Animals

All the animal experimental procedures were approved by the Committee on the Use of Live Animals for Teaching and Research, the University of Hong Kong (1852-09), and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Male wild type (WT) and Lcn2-KO mice of C57BL/6J background were randomized to receive a high-fat diet [D12451, 4.65 kcal/g, 23.6% fat from primarily lard composed of 45% of the caloric intake (Research Diet, New Brunswick, NJ, USA)] or a standard chow [LabDiet 5053, 3.41 kcal/g, 13.2% fats (Purina Mills Inc., Richmond, IN, USA)] from the age of four weeks onwards [12, 16]. Chronic treatment was performed by tail vein injection of the adenovirus encoding a FLAG-tagged murine lipocalin-2, while acute treatment was achieved by intraperitoneal injection of the murine lipocalin-2 recombinant protein [12, 16].

Evaluation of cardiac function

Echocardiographic studies were performed under light anesthesia with the use of intraperitoneal ketamine (100 mg/kg) and xylazine (1.25 mg/kg). A Vivid i cardiovascular ultrasound system with a 11.5 MHz probe (GE Healthcare, Milwaukee, WI) was used for echocardiographic examination. For Langendorff perfusion, mice were anaesthetized with an intraperitoneal (i.p.) injection of a mixture of fluanisonum (25 mg kg\(^{-1}\)) and fentanylum (0.78 mg kg\(^{-1}\)) (Hypnorm\(^{\circledR}\), Janssen Pharmaceutica, Beerse, Belgium) and midazolam (12.5 mg kg\(^{-1}\)) (Dormicum\(^{\circledR}\), Hoffman-La Roche, Basel, Switzerland). Loss of consciousness and suppression of reflexes (withdrawal reflex and tail pinch response absent; limb muscle tone absent; heart and respiratory rates regular) were checked to ensure the animal is anesthetized deeply enough. The hearts were excised, aorta cannulated and retrograde perfusion initiated immediately at a constant perfusion pressure of 80 mmHg. A polyethylene, fluid-filled balloon was introduced into the left ventricle (LV) cavity for assessing contractile functions. Global ischemia was achieved by clamping the inflow tubing. The isolated hearts were subjected to either continuous perfusion for 80 min or a period of 20 min global ischemia followed by 60 min or 120 min (for infarct size analysis) of reperfusion. The left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), heart rate (HR) and coronary flow (CF) were monitored continuously. Effluent from the perfused heart was collected for measuring lactate dehydrogenase (LDH) activity. After perfusion, the hearts slices stained with 2,3,5-triphenyl-tetrazolium chloride were analyzed for LV area and infarct area (IA) using Image J software (National Institutes of Health). The infarct size was expressed as a percentage of the total LV area [27].

Measurement of mitochondrial respiratory chain (MRC) activities

The mitochondria were isolated from freshly collected heart tissues by following the instruction of a commercial kit MITOISO1 (Sigma, St Louis, MO, USA). The MRC activity of individual complex was evaluated by biochemical assays [28]. The mitochondrial ultrastructure and DNA copy number was evaluated as previously described [28].

Extraction and fractionation of cardiac phospholipids

Total lipids were extracted from heart tissue by a modified Folch method [12, 28]. Solid phase extraction was performed for phospholipids fractionation using the aminopropyl bond col-
umn (Waters Milford, MA, USA) [29]. In brief, 1 ml chloroform/methanol (2:1, v/v) was added to 200 µl tissue lysates and the lower organic phase was collected. The organic solvents were evaporated by blowing nitrogen to reduce the volume to ~100 µl. The column was equilibrated with 1 ml hexane. Subsequently, the total lipid extract was applied to the column under moderate vacuum. Neutral lipids and free fatty acids were eluted with chloroform/isopropanol (2:1, v/v) and acetic acid/diethyl ether (2:98, v/v), respectively. In sequence, phosphatidylcholine (PC) was eluted with acetonitrile/n-propanol (2:1, v/v), followed by the elution of phosphatidylethanolamine (PE) and phosphatidylserine (PS), using methanol and isopropanol/3N HCl in methanol (4:1, v/v), respectively. Finally, phosphatidylinositol (PI) was eluted with methanol/3N HCl in methanol (9:1, v/v). Each fraction of the phospholipids was subjected to fatty acyl chain composition analysis by gas chromatography-mass spectrometry (GC-MS) as previously described [12]. For mitochondrial samples, phospholipids were extracted as above and all four fractions (PC, PE, PS and PI) were combined for GC-MS analysis. The results were normalized against the initial protein content in each sample.

Separation and comparison of cardiolipin contents using high-pressure liquid chromatography (HPLC)

Cardiolipin phospholipids fractions were prepared from isolated mitochondria (1.5 mg) as described [30]. After sequential extraction, phospholipids enriched with cardiolipin were eluted with methanol, evaporated under nitrogen flow and reconstituted in 150 µl methanol. Fifty microliters of the resulting sample were used for reversed-phase HPLC separation, which was performed on a Symmetry® C18 column (5 µm, 3.9 × 150 mm, Waters) with the mobile phase consisted of solvent A [methanol-acetonitril-0.01M KH₂PO₄/KOH (pH 7.5, 3:6:1, v/v/v)] and solvent B [methanol-acetonitrile (2/3)]. Chromatographic peaks detected by UV at 205 nm were collected for subsequent analysis of acyl-group composition. Cardiolipin standard (5.3 mg/mL in ethanol, ≥ 98%, Sigma) was used for comparison and quantification.

Data analysis

All results were derived from at least three sets of repeated experiments. The statistical calculations were performed by Student’s t-test and one-way analysis of variances followed by Tukey multiple comparisons using Prism version 5 (GraphPad Software; San Diego, CA, USA). All values are presented as means ± SEM. In all statistical comparisons, a P value less than 0.05 was considered to indicate significant differences.

Results

Lipocalin-2 deficiency improved the recovery of heart functions during ischemia/reperfusion (I/R) injury

Hearts collected from WT and Lcn2-KO mice displayed no overt abnormalities and the ratios of heart/body weight were similar (Supplementary Table 1). Echocardiography analysis revealed no significant differences of the systolic/diastolic LV posterior wall thickness (LVPW) and interventricular septum (IVS) thickness between the two groups of mice under both standard chow and high fat diet conditions. Compared to the values of WT mice, the percent LV ejection fraction (EF) and the percent LV fractional shortening (FS) of Lcn2-KO mice were elevated slightly but significantly by ~9% and ~15%, respectively (Supplementary Table 1). Next, the function of hearts isolated from high fat diet fed animals was evaluated by applying an acute global I/R injury with the use of Langendorff perfusion system. At the end of the equilibration period, LVDP was 99.4 ± 2.0 and 102.1 ± 4.5 mmHg, RPP was 361.9 ± 14.2 and 370.9 ± 13.5 mmHg/min/100 , CF was 4.5 ± 0.2 and 4.4 ± 0.5 ml/min and the average HR was 363.8 ± 10.5 and 363.3 ± 16.1 beats/min, for the hearts from WT and Lcn2-KO mice, respectively. Compared to those of the WT mice, post-ischemic recovery of LVDP and RPP was significantly improved in hearts isolated from Lcn2-KO mice (Figure 1A and 1B), whereas the recovery of CF and HR was not different between the two groups (data not shown). LVEDP increased during no flow global ischemia and decreased to 4-10 mmHg after reperfusion in both WT and Lcn2-KO hearts, suggesting that the systolic function might be different between the two groups of hearts. In addition to these observations, the release of LDH, an injury marker of myocardial infarction, and the ratio of infarct sizes (IA/LV) was significantly reduced in Lcn2-KO hearts (Figure 1C
Mitochondrial functions were significantly enhanced in hearts of Lcn2-KO mice

Electron microscopy was applied to evaluate the ultrastructures of hearts in WT and Lcn2-KO mice. As shown in Figure 2A, the alignments and morphology of interfibrillar mitochondria were markedly different between the two groups of hearts collected from mice fed with high fat diet for seven weeks. In WT hearts, the density of mitochondria significantly increased. There were small distinct crystalline or dense particles in the mitochondrial matrix. The aggregates of mitochondria disrupted myofibril arrays. In Lcn2-KO hearts, the interfibrillar mitochondria were arranged parallel to the myofibrils in a more stereotypic manner. The cristae area was much higher in Lcn2-KO hearts and no vacuoles could be observed in the matrix. To further evaluate the mitochondrial function, biochemical measurement of MRC activities was performed using the total mitochondria isolated from heart tissues of mice fed with a high fat diet for seven weeks (Figure 2B). The yield from Lcn2-KO hearts was slightly higher (46.37 ± 1.82 mg/g) than that from WT mice hearts (41.19 ± 3.04 mg/g). Compared to those of the WT hearts, the activities of complex I, II+III, IV and complex V of Lcn2-KO hearts were significantly increased by 26.94%, 162.39%, 43.50% and 60.02%, respectively.

The copy number of mitochondria mtDNA was not significantly different between the two groups of hearts, although the expression of genes involved in mitochondrial biogenesis were elevated in the heart tissues of Lcn2-KO mice (Supplementary Figure 1).

Treatment with lipocalin-2 attenuated heart performances during I/R injury

Lcn2-KO mice were treated with recombinant adenovirus encoding lipocalin-2 for cardiac function evaluation (Figure 3A). The augmented circulating lipocalin-2 levels (264.53 ± 18.69 ng/ml) were comparable to those in WT animals and lasted for over two weeks [12]. Overexpressing lipocalin-2 significantly suppressed the recovery of cardiac contractile function. In particular, the chronic treatment decreased the recoveries of LVDP by 33.98% and RPP by 47.28% at 5-min of reperfusion when compared to the luciferase control group. At 60-min of reperfusion, LVDP and RPP only recovered to ~70% of the control group. In addition to the dampened responses to I/R injury, recombinant adenovirus-mediated chronic overexpression of lipocalin-2 also led to a significant reduction of the activity of all MRC complexes in heart tissues of Lcn2-KO mice (Figure 3B).

Next, the acute effects of lipocalin-2 on heart functional recovery during I/R injury were investigated. 800 μg recombinant lipocalin-2 protein...
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Or PBS vehicle was injected intraperitoneally into Lcn2-KO mice that had been fed with a high fat diet for seven weeks [12, 16]. Compared to the circulating lipocalin-2 levels in WT mice (202.49 ± 21.07 ng/ml), those in Lcn2-KO mice were augmented by 10-15 folds at 1- and 2-h after injection and dropped to the control levels by 6-h [12]. In isolated Lcn2-KO heart tissues, the protein amount was transiently elevated to 122.79 ± 29.89 ng/mg (similar to that in WT hearts, 130.39 ± 40.26 ng/mg) for a short period and rapidly decreased to 15.20 ± 1.48 ng/mg at 2-h after injection. At 6-h of treatment, there were barely detectable levels of lipocalin-2 in Lcn2-KO hearts. Accordingly, the recovery of heart function to I/R was significantly inhibited by lipocalin-2 treatment in a time-dependent manner (Figure 4). At 1-h after protein injection, the values of LVDP and RPP at 5-min reperfusion were dropped by 59.61% and 72.24%, respectively, when compared to the vehicle group. At the end of reperfusion period, LVDP and RPP only recovered to ~60% of the control group. Time-dependent effects of lipocalin-2 on LDH release could also be observed. These effects were diminished after 6-h of treatment (data not shown). Echocardiography analysis revealed that acute treatment with lipocalin-2 decreased both EF (63.00 ± 2.35 vs 69.01 ± 3.58 of vehicle group, P < 0.05, n = 8) and FS (29.67 ± 2.89 vs 34.00 ± 3.68 of vehicle group, P < 0.05, n = 8) of the Lcn2-KO hearts by ~8% and ~12%, respectively.

Acute treatment with lipocalin-2 recombinant protein also inhibited the cardiac MRC activities of Lcn2-KO mice (Figure 5). As early as 1-h after intraperitoneal injection, a significant reduction of MRC activity was observed for complex II+III (by 35.65%), complex IV (by 23.66%) and complex V (by 32.61%). Four hours after injection, the activity of complex IV was raised to the control level, whereas those of the other three complexes remained significantly low.

Lipocalin-2 modulated the fatty acyl composition of cellular and mitochondrial phospholipids.
Lipocalin-2 belongs to a family of lipid carrier protein [2, 10]. Mice deficient with this protein show different free fatty acid (FFA) composition in adipose tissue [12]. GC-MS analysis, however, did not suggest a significant difference of FFA composition between the heart tissues of WT and Lcn2-KO mice (data not shown). On the other hand, the fatty acyl composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) was remarkably different between Lcn2-KO and WT hearts (Supplementary Figure 2). For example, the percentage composition of C16:0 was significantly higher in PI, but less in PE of WT hearts when compared to Lcn2-KO hearts. By contrast, the polyunsaturated fatty acyl compositions of C20:4 and C22:6 were increased in the PE fractions of WT hearts. The distribution of C18:2 acyl was shifted from PI (5.16%) in WT hearts to PC (5.48%) in Lcn2-KO hearts (Supplementary Figure 3). In addition, the fatty acyl composition of mitochondrial phospholipids was also significantly different between the two groups of hearts (Supplementary Figure 4). Further analysis of heart tissues from mice treated with vehicle or lipocalin-2 revealed that C18:2 is one of the major fatty acyl chains regulated by lipocalin-2 (Figure 6). Acute treatment with this protein down-regulated the percentage composition of C18:2 in PC by over 20-fold and elevated this fatty acyl species in PI by ~20-fold. In addition, the contents of C20:4 and C22:6 in PI and PC were altered in an opposite direction by the treatment (Figure 6A). The fatty acyl compo-
Position of mitochondrial phospholipids was also analysed using the isolated mitochondrial fractions. Lipocalin-2 significantly modified the percentage contents of several species by over 2-fold, including $C_{18:2}$, $C_{20:2}$, $C_{20:4}$ and $C_{24:0}$ (Figure 6A). Moreover, it facilitated the remodeling of fatty acyl composition in cardiolipin, a mitochondrial inner membrane phospholipid. Six cardiolipin species containing different combinations of fatty acyl chains could be reproducibly detected in mitochondria from both WT and Lcn2-KO hearts (Supplementary Figure 5). Integration analysis of the area under curve did not suggest a significant change of the total cardiolipin contents. However, compared to that in WT heart samples, the amount of $\text{(C18:2)}_{2}(\text{C22:6})_{2}$ and $\text{(C18:2)}_{3}(\text{C22:6})_{1}$ in Lcn2-KO mitochondria were decreased by ~20% and ~40%, respectively. In addition, the content of $\text{(C18:2)}_{2}(\text{C18:1})_{1}(\text{C22:6})_{1}$ species was ~1.7 fold higher than that of WT mitochondria. Administration of lipocalin-2 restored all three species to the level of WT samples (Figure 6B).

Discussion

Although a potential causal relationship between lipocalin-2 and cardiac dysfunction has been suggested [21, 22, 24, 25, 31], limited information is available regarding the role of this adipokine in regulating cardiac contractile function. Using both echocardiography and Langendorff perfusion system, the present study demonstrates that hearts derived from mice without lipocalin-2 show improved LV systolic performance (FS and EF) and a better functional recovery during I/R injury. On the contrary, administration of lipocalin-2 to these mice impaired cardiac contractile activity under both basal and I/R conditions.

Optimal cardiac contractile function requires mitochondria to maintain a sufficient energy supply [32]. Defects in mitochondrial function can cause energetic alterations and compromise the generation of sufficient amounts of ATP, in turn affecting the contractile apparatus and rendering the heart more susceptible to ischemic injury [33]. Furthermore, abnormal mitochondrial function increases the production of superoxide anions from MRC complex I and III, which contributes to the transition from reversible to irreversible injury during reperfusion [34]. The results of the present study demonstrate that due to the improved cardiac mitochondrial function, lipocalin-2 deficiency partially preserves myocardium from I/R injury in mice. Lipocalin-2 deficiency is associated with more organized mitochondrial ultra-structure, augmented cardiac MRC activity, as well as
enhanced mitochondrial biogenesis. Replacement with lipocalin-2 by both chronic overexpression and acute protein treatment attenuates MRC activity and the functional recovery to I/R.

Analysis of cellular phospholipids suggests that lipocalin-2 exerts its actions in heart at least in part by regulating specific phospholipids pools. In particular, those containing the linoleic acid (C18:2) are more sensitive to the presence or absence of lipocalin-2.
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absence of this protein. Lipocalin-2 modifies the composition of C18:2 acyl chains in the phospholipids sub-fractions PC and PI, as well as the mitochondrial inner membrane phospholipids, cardiolipin, a phospholipid critically involved in regulating mitochondrial structure, oxidative phosphorylation and biogenesis [35, 36]. Cardiolipin binding is essential for the stability of respiratory chain supercomplexes and oxidative generation of ATP [37]. Structurally, cardiolipin consists of two phosphatidyl residues linked by a glycerol moiety and attached with a total of four fatty acyl chains [38]. In mammalian hearts, cardiolipin enriched with symmetric linoleic acid (C18:2) provides optimal function [39]. The 18-carbon unsaturated acyl side-chain is essential for the high affinity binding of cardiolipin to membrane proteins, and an 18:2-rich configuration is particularly important for maintenance of mitochondrial respiration [36]. The loss of linoleic acid content due to cardiolipin remodeling occurs in various cardiac disorders, including I/R injury, heart failure, diabetic cardiomyopathy and aging-induced cardiac dysfunction [40-42]. Here, the results demonstrate that in Lcn2-KO mice hearts, the contents of linoleic acid (C18:2) in mitochondria are elevated when compared to WT hearts. Moreover, lipocalin-2 modulates the fatty acyl compositions of non-symmetric cardiolipin species, especially those containing both C18:2 and C22:6 fatty acyl chains. By promoting the fatty acyl remodeling of cardiolipin species, lipocalin-2 may adversely affect mitochondrial function and energy production, hence impairing cardiac performances during I/R injury.

Lipocalin-2 also alters the intracellular distribution of other fatty acyl, such as C20:4 and C22:6. The release of arachidonic acid (AA, C20:4) from phospholipid depot facilitates the subsequent production of eicosanoid metabolites, which play important roles in the regulation of myocardial physiology, bioenergetics, contractile function, and signaling pathways [43]. Our earlier studies suggest that in mice, lipocalin-2 deficiency attenuates the metabolism of arachidonic acid, which in turn alleviates aging- and obesity-associated metabolic dysfunctions [10, 12]. In fact, the effects of lipocalin-2 on phospholipid remodeling may also explain the observations that lack of this protein in mice is associated with enhanced insulin sensitivity in both metabolic organs [12] and endothelial cells [16]. Insulin signaling depends on inositol phospholipids, which recruit phosphoinositide 3-kinases (PI3K) to produce phosphatidylinositol(3,4,5)-triphosphate (PIP3) and activate the downstream signaling cascades [44, 45]. The affinity of PI3K increases when the membrane phospholipids contain an arachidonoyl fatty acyl chain in the sn-2 position [46]. In PI fraction of Lcn2-KO hearts, there is an elevated C20:4 content when compared to that in WT hearts. By alteration of the C20:4 acyl composition in this type of phospholipids, lipocalin-2 may attenuate signaling evoked by insulin or other factors.

The molecular events in lipocalin-2-induced phospholipids remodeling are not clear. It is not known whether this process is though a receptor-mediated signaling cascade or depends on the lipid carrier property of this protein. Nevertheless, the selective regulation of C18:2 acyl chain by lipocalin-2 suggests the involvement of a more specific pathway in heart tissue. Since the enzymes responsible for de novo phospholipid synthesis do not exhibit acyl-specificity, the 18:2-rich composition is mainly achieved via an acyl chain remodeling process [35]. Enzymes involved in the remodeling of acyl groups in phospholipids include phospholipase A1, phospholipase A2, acyltransferases and transacylases, as well as tafazzin [47-49]. Whether lipocalin-2 regulates the activity of these enzymes is currently under investigation in our laboratory. In addition, lipocalin-2 may act as a lipid chaperone to selectively facilitate linoleic acid transportation into and translocalization within cardiomyocyte. It may also pathologically remodel phospholipids and cardiolipin by oxidation through non-enzymatic (free-radical mediated) or enzymatic (lipoxygenases or peroxidases) mechanisms. Moreover, the detailed cellular mechanisms contributing to the regulatory effects of lipocalin-2 on cardiac function remain to be addressed, such that whether this adipokine primarily acts on cardiomyocytes or through vascular endothelium, or both at the same time. Conceptually, the effects of lipocalin-2 can be direct as well as indirect, like other circulating adipokines/hormones such as adiponectin. Phospholipid remodeling may represent a common mechanism underlying lipocalin-2’s actions in vasculature, heart, adipose tissues and others, and the involvement of cross-talks between these interrelated organs cannot be excluded. Despite
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these promises and gaps related to lipocalin-2’s action, it is intriguing to speculate that reverse remodeling to restore functional phospholipids pool of hearts by targeting lipocalin-2 may represent a potential strategy to improve the cardiac mitochondrial function and to prevent I/R injury.

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Supporting Information

Materials and Methods:

Echocardiography. Echocardiographic studies were performed under light anesthesia and spontaneous respiration. The chest was shaved and warmed acoustic coupling gel was applied. Subsequently, mice were fixed supine in a left lateral position on a heated pad to keep the body temperature at 37°C. A Vivid i cardiovascular ultrasound system with a 11.5 MHz probe (GE Healthcare, Milwaukee, WI) was used for the echocardiographic examination of cardiac structure and function. Echocardiographic parameters, including interventricular septum (IVS), left ventricular internal diameter (LVID), left ventricular posterior wall (LVPW), the long-axis percentage LV ejection fraction (EF) and percentage LV fractional shortening (FS) were recorded using M-mode measurement. The depth was set to 2 cm and zoomed to 1.2 cm. Wall thickness and LV dimensions were obtained from the parasternal long axis view at a frame rate of 260Hz. FS was calculated as $FS(\%) = \left(\frac{LVID_d - LVID_s}{LVID_d}\right) \times 100$, where LVIDd is LV internal dimension in diastole and LVIDs is LV internal dimension at end-systole. EF was calculated from the parasternal long-axis view using the equation $EF(\%) = \left(\frac{LV_A - LV_s}{LV_A}\right) \times 100$.

Heart preparation and perfusion. After injection of heparin (10,000 IU kg$^{-1}$, i.p.), the hearts were excised and placed in ice-cold Krebs-Henseleit (KH) buffer solution (118 mmol/L NaCl, 25 mmol/L NaHCO$_3$, 4.7 mmol/L KCl, 1.2 mmol/L MgSO$_4$, 1.2 mmol/L KH$_2$PO$_4$, 2.5 mmol/L CaCl$_2$, and 11 mmol/L D-glucose, pH 7.4), which immediately stopped the contractile activity of the heart. The aorta was rapidly slipped onto a grooved 20-gauge stainless steel cannula attached to the perfusion column, and retrograde perfusion initiated immediately with KH buffer oxygenated with 95% O$_2$ and 5% CO$_2$ at 37°C at a constant perfusion pressure of 80 mmHg. The total time from heart excision to
initiation of Langendorff perfusion was less than 2 min. A polyethylene, fluid-filled balloon was introduced into the left ventricle (LV) cavity through the left atrial incision. The balloon catheter was linked to a pressure transducer connected to a data acquisition system for assessing LV contractile functions. The volume of the balloon was tested before insertion into the heart, and it was large enough to be inflated with 30 µL saline without producing a pressure of more than 1 mmHg. After insertion, the balloon was adjusted to obtain a LV end diastolic pressure (LVEDP) of 4–10 mmHg during the equilibration period. Heart rate (HR) was obtained from the LV pressure signal. Coronary flow (CF) was monitored by collection of the coronary effluent into pre-cooled tubes prior to ischemia and over each 5 min during reperfusion.

**I/R injury and cardiac function evaluation.** Following a stabilization period of 30 min, the isolated hearts were subjected to either continuous perfusion for a further 80 min or a period of 20 min global ischemia followed by 60 min of reperfusion. Hearts were excluded from the protocol at the end of the stabilization period if they did not meet the inclusion criteria: HR > 300 beats min⁻¹, 1.5 ml < CF < 5 mL min⁻¹ and LV systolic pressure > 80 mmHg. Global ischemia was achieved by clamping the inflow tubing. During ischemia, the hearts were immersed in a 37 °C chamber to keep the temperature constant. Functional recoveries were recorded during reperfusion. The left ventricular systolic pressure (LVSP), LVEDP, HR and CF were monitored continuously. Rate-pressure product (RPP) was calculated as the product of LVDP (LVDP=LVSP-LVEDP) and HR using the formula (RPP=LVDP*HR/100). To assess the extent of myocardial tissue injury, 1 ml of effluent from the perfused heart was collected at 1, 5, 10, 20 and 30 min of reperfusion for measuring LDH activity by a biochemical analyzer (BS-120, Chemistry Analyzer, Midray, China). For infarct size analysis, after 120 min of reperfusion, the hearts were snap-frozen and cut into 0.7 mm slices for
staining with 1% (w/v) 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma, St Louis, MO, USA) to aid visualization of the infarcted area. Digital images of heart sections were analyzed for LV area and infarct area (IA) using Image J software (National Institutes of Health).

**Chronic treatment with recombinant adenoviruses or acute treatment with lipocalin-2 protein.** The adenovirus vector encoding FLAG-tagged murine lipocalin-2 was generated using the Adeno-X Expression System (Clontech, Mountain View, CA). The recombinant adenovirus was injected into the tail vein of mice two weeks prior to tissue collection. The amount of injected adenovirus (10⁸ plaque-forming units) caused no toxicity in the mice. The increased expression level of lipocalin-2 was confirmed by both Western blotting and enzyme linked immunosorbent assay as described. Recombinant murine lipocalin-2 was expressed, purified and endotoxin removed to less than 0.1 EU/ml. The purity of the protein was confirmed by SDS-PAGE and mass spectrometry analysis. No siderophore or iron was found to bind to the protein. Administration of lipocalin-2 was performed by intraperitoneal injection (800 µg protein per mouse). Mice were sacrificed under anesthesia at different time points (0, 1, 2, 4, 6 and 12 hours) after injection for experiment.

**Electron microscopy analysis of mitochondrial ultrastructures.** The electron micrographs and ultrathin sections were prepared from hearts freshly collected from WT and Lcn2-KO mice. Ultrastructural analysis was performed in the Electron Microscope Unit of our institution and examined by experienced electron microscopists blinded to the treatment groups. In brief, LV myocardium was cut to 1 mm³ and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for overnight at 4°C. After brief washing, the specimens were postfixed in 1% osmium tetroxide, dehydrated, embedded in epoxy resin, and polymerized overnight at 60°C. Ultra-thin sections (100 nm) were cut
and poststained with Reynold’s lead citrate and uranyl acetate to increase contrast. Sections were viewed and photographed using a Philips EM208S transmission electron microscopy (TEM) (Eindhoven, The Netherlands).

**Measurement of mitochondrial respiratory chain (MRC) activities.** Cardiomyocyte contains two distinct subpopulations of mitochondria, subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM). Short period incubation of heart tissue with trypsin could facilitate the separation of IFM from the fibrillar ². Here, the combined cardiac mitochondria fractions were isolated following the instruction of a commercial kit MITOISO1 (Sigma, St Louis, MO, USA) and the MRC activity of individual complex was evaluated as described ³. Briefly, freshly collected heart tissues were cut into small pieces and washed with extraction buffer (10 mM HEPES containing 200 mM manitol, 70 mM sucrose and 1 mM EGTA, pH 7.5). After incubating with 0.25 mg/ml trypsin in extraction buffer on ice for 20-min, 10 mg/ml BSA was added to quench the proteolytic activities. The heart tissue suspensions were subsequently homogenized using a Dounce glass homogenizer and briefly centrifuged at 600 × g for 5 min. The supernatant was collected and centrifuged at 11,000 × g for 10 min. The pellet was suspended in storage buffer (10 mM HEPES, containing 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K₂HPO₄ and 1 mM DTT). Preparations were used immediately for the assessment of MRC activity. The remainder of the samples was frozen at −80°C.

**Profiling and quantification of fatty acyl chains.** For fatty acid composition analysis, the total and fractionated lipids were evaporated to dryness by nitrogen flow, and reconstituted in a mixture of 0.5 ml 4% methanolic HCl, 0.5 ml methanol and 125 µl hexane. After the addition of internal standard I [5 µl, 20 mg/ml nonadecanoic acid C19:0 (Sigma)], the mixture was boiled at 100°C for 60 min and then mixed with 1ml hexane and 1 ml of deionized water. The upper layer was collected and the internal standard II [5
µl, 10 mg/ml tridecanoic acid methyl ester methyl C13:0 (Sigma)] added. Methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) using a 6890N Network GC system attached to a 5973 Network Mass Selective Detector (Agilent, Palo Alto, CA, USA). The concentration of each fatty acid species was quantified by relating the peak area to those of the fatty acid standards (Supelco® 37 Component FAME mix, 10 mg/ml). All results were normalized against the initial protein contents in each sample.

References


**Supplementary Table 1.** Evaluation of LV function by echocardiography\(^1\).

<table>
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<tr>
<th>Experimental groups</th>
<th>HW/ BW(^%)</th>
<th>LVIDd</th>
<th>LVIDs</th>
<th>FS</th>
<th>LVPWd</th>
<th>LVPWs</th>
<th>IVSd</th>
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<tr>
<td>7-wk HF(^2)</td>
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<tr>
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\(^1\)Data are presented as means ± SEM, \(n = 12\) animals/group.

\(^2\)Mice fed with high fat diet (HF) for seven weeks (starting from the age of 4-wk).

\(^3\)Mice (20-wk old) fed with standard chow (STC).

*, \(P < 0.05\) vs corresponding WT control group.
Supplementary Figure 1. Comparison of the mtDNA copy number and the mRNA expression of genes regulating mitochondrial biogenesis. DNA was extracted from heart tissues of mice under standard chow (NC) or high fat diet (HF). Gene copy number was measured as described in Method (A). The expression of genes involved in mitochondrial biogenesis was evaluated by quantitative PCR analysis (B). The results were expressed as fold change against the value of WT mice. *, \( P < 0.05 \); **, \( P < 0.01 \) vs WT, \( n = 5 \). mTFAM, mitochondrial transcription factor A; Pol\( \gamma \), mitochondrial DNA polymerase gamma; PGC-1\( \alpha \), peroxisome proliferators-activated receptor gamma co-activator-1\( \alpha \).
Supplementary Figure 2. GC-MS analysis of the fatty acyl composition in four major types of cardiac phospholipids. Heart tissues were collected from WT and Lcn2-KO mice after 7-wk of high fat diet. Solid-phase extraction was performed to obtain phospholipids fractions, including PC, PE PI and PS. Fatty acyl chain composition was analyzed using GC-MS. The compositions of fatty acyl chains in each of the four types of phospholipids are presented for both WT (inside circle) and Lcn2-KO (outside circle) samples, n = 4.
Supplementary Figure 3. GC-MS analysis of the fatty acyl composition in cardiac phospholipids. Heart tissues were collected from WT and Lcn2-KO mice after 7-wk of high fat diet. Solid-phase extraction was performed to obtain four major fractions of phospholipids, PC, PE, PS and PI. Fatty acyl chain composition was analyzed using GC-MS. The results were presented as the percent distribution of each fatty acyl chain among the four types of phospholipids for both WT (inside circle) and Lcn2-KO (outside circle) hearts, n = 4.
Supplementary Figure 4. GC-MS analysis of the fatty acyl composition in cardiac mitochondrial phospholipids. Heart mitochondria were prepared from WT and Lcn2-KO mice after 7-wk of high fat diet, and subjected to phospholipids fractionation using the solid-phase extraction protocol described in Methods. The four major fractions of phospholipids, PC, PE, PS and PI were subsequently combined for fatty acyl chain composition analysis using GC-MS. The results were presented as the percent distribution of each fatty acyl chain among the total phospholipids for both WT (inside circle) and Lcn2-KO (outside circle) hearts, n = 4.
Supplementary Figure 5. Reversed-phase HPLC analysis of different cardiolipin species in heart mitochondria isolated from WT mice, Lcn2-KO mice, or Lcn2-KO mice treated with lipocalin-2 as in Figure 6. The elution profile is presented and the six distinct species of cardiolipin indicated as numbers. Note that the elution profile of cardiolipin was not altered by vehicle treatment (data not shown). The fatty acyl compositions for the six species are: 1, (C18:2)2(C22:6)2; 2, (C18:2)3(C22:6)1; 3, (C18:2)4; 4, (C18:2)2(C18:1)1(C22:6)1; 5, (C18:2)3(C18:1)1 and 6, (C18:2)2(C18:1)2.