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CARD9 Knockout Ameliorates Myocardial Dysfunction Associated with High Fat Diet-Induced Obesity

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Abstract

Obesity is associated with chronic inflammation which plays a critical role in the development of cardiovascular dysfunction. Because the adaptor protein caspase recruitment domain-containing protein 9 (CARD9) in macrophages regulates innate immune responses via activation of pro-inflammatory cytokines, we hypothesize that CARD9 mediates the pro-inflammatory signaling associated with obesity en route to myocardial dysfunction.

C57BL/6 wild-type (WT) and CARD9^{-/-} mice were fed normal diet (ND, 12% fat) or a high fat diet (HFD, 45% fat) for 5 months. At the end of 5-month HFD feeding, cardiac function was evaluated using echocardiography. Cardiomyocytes were isolated and contractile properties were measured. Immunofluorescence was performed to detect macrophage infiltration in the heart. Heart tissue homogenates, plasma, and supernatants from isolated macrophages were collected to measure the concentrations of pro-inflammatory cytokines using ELISA kits. Western immunoblotting analyses were performed on heart tissue homogenates and isolated macrophages to explore the underlying signaling mechanism(s). CARD9 knockout alleviated HFD-induced insulin resistance and glucose intolerance, prevented myocardial dysfunction with preserved cardiac fractional shortening and cardiomyocyte contractile properties. CARD9 knockout also significantly decreased the number of infiltrated macrophages in the heart with reduced myocardium-, plasma-, and macrophage-derived cytokines including IL-6, IL-1 β and TNF α . Finally, CARD9 knockout abrogated the increase of p38 MAPK phosphorylation, the decrease of

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LC3BII/LC3BI ratio and the up-regulation of p62 expression in the heart induced by HFD feeding and restored cardiac autophagy signaling.

In conclusion, CARD9 knockout ameliorates myocardial dysfunction associated with HFD-induced obesity, potentially through reduction of macrophage infiltration, suppression of p38 MAPK phosphorylation, and preservation of autophagy in the heart.

Keywords

Autophagy; Myocardial Dysfunction; Heart Disease; Inflammation; Obesity; Diabetes

Introduction

As obesity has emerged as an epidemic, strategies to curb this disease are limited. Obesity is often associated with a cluster of metabolic diseases including insulin resistance, glucose intolerance, dyslipidemia, type II diabetes, hypertension, as well as cardiac abnormalities. As one of the hall markers of high fat diet (HFD)-induced obesity, low grade chronic inflammation has detrimental consequences on metabolism and cardiovascular function [1, 2]. This so-called “metabolic inflammation” is due to heightened infiltration of pro-inflammatory cells such as macrophages and has been linked to obesity-associated metabolic syndromes and cardiovascular dysfunction [3–6]. The infiltrated pro-inflammatory cells secrete a number of cytokines as potential risk factors for the development of insulin resistance and glucose impairment as well as subsequent tissue damage [3, 7, 8]. Therefore, dissecting the specific signaling pathways that lead to production of pro-inflammatory cytokines would be critical for the understanding of HFD-induced metabolic diseases.

With the prevalence of obesity and associated chronic inflammatory responses, myocardial dysfunction is still the major cause of mortality. However, the specific signaling pathways responsible for HFD-induced and obesity-associated inflammation and cardiovascular dysfunction are still not completely understood. The adaptor protein caspase recruitment domain-containing protein 9 (CARD9), which is highly expressed in immune cells, activates pro-inflammatory cytokines [9–11]. As a central regulatory protein, CARD9 plays a critical role in the innate and adaptive immune responses against pathogen invasion [9, 12, 13]. CARD9 is specifically expressed in immune cells and is activated in the presence of infections induced by a number of pathogens [3, 14, 15]. CARD9 has been attributed to the activation of transcriptional factors including p38 MAPK and NF κ B and the transcription of a number of pro-inflammatory cytokines including IL6, IL-1 β , and TNF α from immune cells [9]. However, whether or not CARD9 participates in HFD-induced and obesity-associated chronic inflammation and myocardial dysfunction remains unknown. We hypothesize that CARD9 signaling is involved in the development of myocardial dysfunction associated with HFD-induced obesity.

Over-nutrition as in HFD feeding has been linked to a pro-inflammatory state and the development of metabolic syndrome associated with myocardial abnormalities [8]. Sustained systemic inflammation poses a potent risk to the development of heart failure [14]. These pro-inflammatory cues often lead to dysregulation of intrinsic cellular homeostasis.

As one of the critical homeostatic regulators, autophagy plays a pivotal role in the maintenance of subcellular organelle biogenesis and degradation [15, 16]. Autophagy also regulates cellular bio-component and quality in immune responses [17–19]. However, whether or not there is a link between CARD9 signaling and obesity-associated dysregulation of myocardial autophagy is unknown.

Using a HFD-induced obese mouse model and a homozygous CARD9^{-/-} mouse strain, the current study was designed to dissect the specific mechanisms underlying HFD-induced and obesity-associated myocardial dysfunction and CARD9-regulated pro-inflammatory signaling pathways. As CARD9 serves as an upstream regulator to a number of pro-inflammatory cytokines, the outcome of the current study should be important for potential pharmacological interventions that aim at reducing obesity-associated chronic inflammation and improving heart function and health.

Materials and Methods

Animals and HFD Feeding Regimen

Mice were bred and housed in the animal care facility of University of Wyoming College of Health Sciences. All animal studies were approved by Institutional Animal Care and Use Committee (IACUC) at the University of Wyoming and the investigation conforms to the federal guidelines for the humane and appropriate care of laboratory animals, Federal Law (89–544, 91–579) and all NIH regulations.

C57BL/6 wild-type (WT) and CARD9^{-/-} mice were fed normal diet (ND, 12% fat) and/or a HFD (D12451, 45% fat, Research Diets, NJ) for 5 months starting at 4–6 weeks of age. At the end of 5-month HFD feeding, body weight, epididymal adipose tissue weight, heart weight, and tibia length were measured before tissue was harvested. In a sub-group of HFD-fed mice, peritoneal macrophages were isolated.

Glucose Tolerance and Insulin Resistance Tests

Mice were fasted for 16 hours and the fasting blood glucose levels were measured using an automated blood analyzer (Bayer Contour Next EZ, NJ). Then mice were given an intraperitoneal injection of glucose (2 g/kg body weight), and tail vein blood was drawn at 30, 60, 120 min to determine blood glucose levels. Before sacrificing the mice, blood samples were collected into 1.5 ml plastic tubes containing ethylenediaminetetraacetic acid (EDTA), and plasma was obtained. Plasma samples were analyzed for cytokines and insulin by routine ELISA assay with commercial kits (CRYSTAL CHEM, IL). Insulin resistance was evaluated by homeostasis model assessment (HOMA). HOMA insulin resistance index (HOMA-IR) was calculated as [plasma glucose (GLU; mmol/l) × insulin (mIU/l)]/22.5 [20].

Echocardiographic Assessment of Cardiac Function

Cardiac geometry and function were evaluated in isoflurane-anesthetized mice using the 2-D guided M-mode echocardiography (Philips HP Sonos 5500) equipped with a 15–6L linear transducer. Left ventricular (LV) anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M-mode using methods adopted by

the American Society of Echocardiography. Fractional shortening (FS) was calculated from LV end-diastolic diameter (EDD) and end-systolic diameter (ESD) as previously reported [21].

Isolation of Cardiomyocytes and Measurement of Contractile Properties

Cardiomyocytes were isolated from WT and CARD9^{-/-} mice fed on ND or HFD for 5 months as described previously [22]. Briefly, after anesthesia with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (15 mg/kg), hearts were removed, perfused with Ca²⁺-free Tyrode's solution containing (in mM): 135.0 NaCl, 4.0 KCl, 1.0 MgCl₂, 10.0 HEPES, 0.3 NaH₂PO₄, 10.0 glucose, 10.0 butanedione monoxime purged with 95% O₂ plus 5% CO₂, and digested with Liberase Blendzyme 4 (Hoffmann-La Roche, Indianapolis, IN).

Cell contractile properties were measured with SoftEdge MyoCam system (IonOptix, Milton, MA) using contraction buffer containing (in mM): 131.0 NaCl, 4.0 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 10.0 glucose, and 10.0 HEPES at pH 7.4. The following lengthening and relengthening indices were assessed: resting cell length, peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR₉₀), and maximal velocity of shortening/relengthening ($\pm$ dL/dt).

Masson's Trichrome Staining

Following 5-month ND or HFD feeding, hearts were excised from anesthetized WT and CARD9^{-/-} mice and fixed in 10% neutral buffered formalin (NBF) for 48 hours as described previously [23]. The specimens were then embedded in paraffin and sliced in 6- μ m sections. The slides were deparaffinized and stained using a Masson's trichrome staining kit (HT15-1KT, Sigma-Aldrich) to detect fibrosis in the heart according to manufacture's specification (Procedure No. HT15).

Isolation of Macrophages

Mice were injected intraperitoneally with 2 ml of 4% thioglycollate broth (Sigma Aldrich, St Louis, MO). Four days later, peritoneal lavage was performed with 5 ml ice-cold phosphate buffered saline (PBS) as described [9, 24]. Any residual red blood cells were separated out by centrifugation.

Immunofluorescence Analyses of Macrophage Infiltration into the Heart

Frozen heart tissue was cryo-sectioned and permeabilized with 0.1% Triton X-100/PBS and incubated in blocking buffer. The sections were incubated in 3% bovine serum albumin (BSA)-PBS solution containing primary antibody against CD68 (ABCAM, ab955) overnight at 4 °C. Then the sections were incubated with the secondary antibodies for 1 hour and DAPI for 5 min. Fluorescence staining was visualized using a microscope (OLYMPUS, BX51TRF). The CD68 positive cells were counted from at least 10 random fields of a minimum of 5 sections using the ImageJ software (NIH).

Western Immunoblotting Analyses

Heart tissue or macrophages were lysed in ice cold RIPA buffer. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gels and were transferred to nitrocellulose

membranes. Membranes were incubated with specific primary antibodies overnight at 4°C against CARD9 (abcam, ab124922), p38 MAPK (Cell Signaling, 9212), phospho-p38 MAPK (Cell Signaling, 4631), LC3BI/II (Cell Signaling, 2775), p62 (PROGEN, GP62-C), α -tubulin (Cell Signaling, 2148), and β -actin (Cell Signaling, 8475). Membranes were incubated with respective secondary antibodies prior to the detection of immunoreactive bands using enhanced chemiluminescence detection solution. Densities of protein bands were analyzed using ImageJ software (NIH).

Cytokine Detection in Heart Tissue, Plasma, and Macrophage Culture Supernatant

Heart tissue was homogenized in ice cold PBS with a homogenizer to yield a 10% (wt/vol) heart homogenate. The homogenate was then centrifuged (1200 rpm) at 4°C for 10 min and the supernatant was collected. Blood samples were centrifuged and plasma was collected. The isolated macrophages were cultured in RPMI-1640 media (R8758, Sigma Aldrich) under 5% CO₂ at 37°C for 24 hours and the supernatant was collected. Both plasma and macrophage supernatant samples were prepared without further dilution. The samples were then used for measuring IL-1 β , TNF α and IL-6 by ELISA assay following specifications by the manufacture (IL-1 β , MLB00C; TNF α , MTA00B; IL-6, M6000B, R&D SYSTEMS).

Statistical Analyses

Data are shown as Mean $\pm$ SEM. At least 3 mice were used for experiments in each group if not mentioned otherwise. Significance is set at $p < 0.05$. All statistical analyses were performed with GraphPad Prism Software (Inc. San Diego, CA) by One-Way ANOVA followed by a Tukey test for post hoc analyses where appropriate. The dependability of HOMA-IR was performed by straight-line correlation analyses. HOMA-IR was normally distributed after logarithmic transformation and the logarithms of these parameters were used.

Results

CARD9 Knockout Had No Effect on HFD-Induced Adiposity

As shown in Fig. 1A–C, at the end of 5-month HFD feeding, body weight and epididymal adipose tissue weight of WT and CARD9^{-/-} mice were significantly elevated compared to the respective ND-fed groups with little difference between the two HFD-fed groups. The ratio of heart weight over tibia length showed no significant difference among all four groups indicating that these HFD-induced obese mice had no sign of cardiac hypertrophy (Fig. 1D). To correlate adiposity with metabolic activity, metabolic rates were measured in the open-circuit indirect calorimetry cages (Supplemental Data). As shown in Supplemental Figure 1, HFD feeding significantly decreased oxygen consumption (V_{O2}), respiratory ratio (RER), and energy expenditure compared to the ND-fed mice. Consistently, there is no significant difference between WT and CARD9^{-/-} mice whether the mice were on ND or HFD feeding regimen. These results indicated that CARD9 knockout had no effect on HFD-induced adiposity.

CARD9 Knockout Attenuated HFD-Induced Insulin Resistance and Glucose Intolerance

At the end of 5-month HFD feeding, WT obese mice developed characteristic features of type II diabetes with hyperglycemia, hyperinsulinemia, and glucose intolerance as shown in Fig. 2. Specifically, fasting glucose and insulin levels in the plasma were significantly increased in HFD-fed WT mice and the increase was significantly attenuated in CARD9^{-/-} mice (Fig. 2A–B). Furthermore, HFD feeding impaired glucose disposal and CARD9 knockout improved glucose tolerance (Fig. 2C). In addition, insulin resistance was evaluated by HOMA index. As shown in Fig. 2D, HFD feeding induced insulin resistance in HFD-fed WT mice while CARD9 knockout significantly improved insulin sensitivity following HFD-feeding.

CARD9 Knockout Protected against Myocardial Dysfunction

Sustained obesity is potentially associated with cardiovascular dysfunction [25, 26]. To determine if CARD9 knockout protects against obesity-associated myocardial anomalies, cardiac geometry, function and cardiomyocyte contractile properties were evaluated. As shown in Fig. 3, while there were no significant differences among other parameters including: heart rate, LVEDD, LVESD, interventricular septal thickness at diastole and systole (IVSD and IVSS), LV posterior wall thickness at diastole and systole (LVPWD and LVPWS), cardiac FS was significantly compromised in HFD-fed WT mice, and the effect of which was ameliorated by CARD9 knockout. Representative 2D guided M-mode echocardiograph images were provided in Supplemental Figure 2. Furthermore, as demonstrated in Fig. 4, cardiomyocytes from HFD-fed WT mice showed significant reduction in peak shortening and $\pm$ dL/dt, and significant increase in TR₉₀ with little change in resting cell length and TPS, indicating myocardial diastolic and systolic dysfunction. CARD9 knockout totally ameliorated myocardial anomalies associated with HFD-induced obesity.

CARD9 Knockout Blunted Obesity-Associated Interstitial Fibrosis

To determine potential intrinsic defects in cardiomyocyte shortening/relaxation associated with obesity, collagen formation in myocardial tissues was detected using Masson's trichrome staining. As shown in Fig. 5, very weak staining was present in the hearts of WT-ND and CARD9^{-/-}-ND mice. Strong and diffusive staining was clearly present in the hearts of WT-HFD, but not CARD9^{-/-}-HFD mice. The strong collagen staining of fibrosis formation may represent a potential link between HFD-induced obesity and cardiomyocyte contractile dysfunction.

CARD9 Knockout Attenuated HFD-Induced Macrophage Infiltration into the Heart

Uncorrected obesity is associated with chronic inflammation with a hallmark of macrophage infiltration in adipose and other tissues [4, 5, 8]. To determine the protective mechanisms of CARD9 knockout on myocardial function, heart sections were stained with immunofluorescence antibody against CD68, a specific marker of macrophages. As shown in Fig. 6, 5-month HFD feeding dramatically increased macrophage infiltration in the heart of HFD-fed WT mice compared to that of the ND-fed WT mice. Interestingly, CARD9

knockout significantly decreased the number of infiltrated macrophages in the heart of HFD-fed CARD9^{-/-} mice compared to that of HFD-fed WT mice.

HFD-Induced Obesity Is Associated with Up-regulated CARD9 Protein Expression in Macrophages and Heart Tissue

Given that CARD9 knockout offers protective effects on glucose tolerance, insulin sensitivity, and myocardial function, we next examined how CARD9 signaling is regulated following HFD feeding and the onset of obesity. To determine whether or not CARD9 protein expression is altered in obese mice, Western immunoblotting analyses were performed on heart tissue and isolated macrophages. As shown in Fig. 7, CARD9 protein was significantly up-regulated in the heart and isolated macrophages of HFD-fed WT mice compared to that of the ND-fed WT mice. As expected, there was little expression in the heart tissue and isolated macrophages from CARD9^{-/-} mice.

CARD9 Knockout Abrogated HFD-induced Up-Regulation of p38 MAPK Phosphorylation

It has been demonstrated that p38 MAPK is involved in a number of pathological conditions [27–29]. Furthermore, p38 MAPK is a transcriptional factor regulated by CARD9 signaling complex during innate and adaptive immune responses [9, 12, 13]. As demonstrated in Fig. 7, CARD9 protein was significantly up-regulated in HFD-fed WT mice, therefore we hypothesize that CARD9 and its activated p38 MAPK pathway is involved in the development of HFD-induced myocardial dysfunction. As shown in Fig. 8, the ratio of p-p38 MAPK/p38 MAPK was significantly increased in the heart and isolated macrophages of HFD-fed WT mice. CARD9 knockout completely ameliorated the increase of the phosphorylated p38 MAPK in both heart and macrophages following HFD feeding. In addition, phosphorylation of NFκB, another potential transcriptional factor downstream of CARD9, was also evaluated and no difference was observed between the WT and CARD9^{-/-} groups (data not shown).

CARD9 Knockout Ameliorated HFD-induced Cytokine Production

Obesity-associated pro-inflammatory cytokines are key risk factors to the development of metabolic dysfunction [7, 30, 31]. To determine how CARD9 regulates obesity-associated pro-inflammatory cytokine production, cytokines IL-6, TNFα, and IL-1β were measured in heart tissue homogenate, plasma, and isolated macrophage culture supernatant using ELISA Kits as described in the **Method** section. As shown in Fig. 9, in heart tissue homogenate, HFD feeding significantly increased IL-6 and TNFα concentrations in WT mice compared to those fed with ND. CARD9 knockout completely ameliorated these increases. Though HFD feeding did not affect IL-1β concentrations, CARD9 knockout showed significantly lower cytokine levels compared to WT mice. In plasma, HFD feeding significantly increased concentrations of IL-6 and IL-1β compared to those of ND-fed WT mice, and CARD9 knockout significantly attenuated such rises. Plasma TNFα also showed a trend towards increasing following HFD feeding. Interestingly, the cytokine levels were several-fold lower in plasma than that in the heart tissue which was already diluted (10% wt/vol). In the supernatant of cultured macrophages, all three cytokine concentrations were significantly increased in HFD-fed WT mice compared to ND-fed WT mice, and CARD9 knockout

completely ameliorated the increases of cytokine production to the ND-fed control levels or with a pronounced reduction below baseline. These results indicated that HFD-induced obesity is accompanied with increased pro-inflammatory cytokine production in WT mice and these cytokines were significantly reduced in CARD9^{-/-} mice.

CARD9 Knockout Restored Dysfunctional Myocardial Autophagy Associated with HFD-Induced Obesity

Myocardial autophagy is reported to be altered in experimental HFD-induced obesity [21]. To determine the potential mechanisms of CARD9-afforded protection against obesity-associated myocardial dysfunction, we examined the effect of CARD9 knockout on myocardial autophagy signaling. As markers of autophagy initiation and maturation, LC3BII/I and p62 protein expressions were determined with Western immunoblotting analyses. As shown in Fig. 10, HFD-induced obesity is associated with decreased ratio of LC3BII/LC3BI and increased p62 expression in the heart of HFD-fed WT mice, indicating dysfunctional maturation of myocardial autophagy. CARD9 knockout completely restored the decreased ratio of LC3BII/I and the increased expression level of p62 in HFD-fed CARD9^{-/-} mice, indicating a potential tie between CARD9 signaling and HFD-induced dysfunctional myocardial autophagy.

Discussion

Obesity has become an epidemic and is often associated with a constellation of metabolic abnormalities including impairments of insulin and glucose homeostasis [8, 32]. Moreover, as a modifiable risk factor, obesity is associated with increased cardiovascular anomalies [33–36]. On the other hand, “obesity paradox” has also been noticed from several clinical studies with a cohort of obese patients displaying better outcomes of heart failure prognosis [37, 38]. Therefore, understanding the mechanisms underlying obesity-associated pathophysiology is pertinent for development of therapeutic interventions to curb this epidemic. Results from the current study demonstrated that deficiency of CARD9, a critical cytosolic regulatory protein of innate and adaptive immune responses, protected the heart from myocardial dysfunction associated with HFD-induced obesity.

There are several salient findings in the current study. First, 5-month 45% HFD feeding resulted in significant elevation of body weight, insulin resistance and glucose intolerance characteristic of type II diabetes, as well as myocardial dysfunction in WT mice, which is consistent with a number of recent publications [21, 39, 40]. Interestingly, CARD9 knockout significantly attenuated HFD-induced metabolic abnormalities including insulin resistance and glucose intolerance, without affecting HFD-induced adiposity as shown in Fig. 1. In addition, CARD9 knockout did not affect metabolic activities following HFD feeding as shown in Supplemental Figure 1. These results suggest that CARD9 knockout-afforded protection is independent of HFD-induced adiposity. Interestingly, such adiposity-independent protective effects were also reported on cJun NH₂-terminal kinase (JNK) and macrophage migration inhibitory factor (MIF) [21, 41]. The adiposity-independent protection of CARD9 knockout not only provided an advantageous model for the study of obesity-associated underlying mechanisms such as chronic inflammation without the

confounding effect of adiposity, but also suggested that adiposity alone may not be enough to be responsible for obesity-associated metabolic abnormalities. In addition, the WT obese mice did not show signs of cardiac hypertrophy which presented another advantage for studying the role of obesity-associated underlying inflammation on myocardial dysfunction.

Secondly, CARD9 knockout protected against HFD-induced metabolic syndrome, cardiac fibrosis and myocardial dysfunction, possibly through reduction of pro-inflammatory cytokines including IL-6, TNF α , and IL-1 β (Fig. 9). It is noteworthy that levels of HFD-induced cytokines are several-fold higher in the heart tissue than that in circulation. Even though circulatory cytokines are considered risk factors for the development of metabolic syndrome [7, 31], our results suggest that cytokines in the myocardium may play a major role in the development of myocardial dysfunction as levels of these cytokines are several-fold higher than that in the plasma. Furthermore, CARD9 knockout significantly attenuated macrophage infiltration in the heart. These data strongly suggest that HFD-induced and obesity-associated myocardial dysfunction is potentially through macrophage infiltration and heightened secretion of pro-inflammatory cytokines in a paracrine manner. In addition, 5-month HFD feeding significantly up-regulated CARD9 protein expression in the isolated macrophages as well as heart tissue (Fig. 7) which is also indicative of increased macrophage infiltration into myocardium, as CARD9 was demonstrated to be only expressed in immune cells [9, 12, 13].

Thirdly, as one of the downstream transcriptional factors of CARD9 signaling [9, 12, 13], phosphorylation levels of p38 MAPK were significantly increased in the isolated macrophages as well as heart tissue which may be responsible for the up-regulation of a number of pro-inflammatory cytokines in heart tissue such as IL-6, TNF α , and IL-1 β as shown in Fig. 8 and 9.

Finally, HFD-induced obesity is associated with systolic and diastolic dysfunction as illustrated by echocardiography (decreased FS) and cardiomyocyte contractile properties (depressed PS, $\pm$ dL/dt, and prolonged TR₉₀). Interestingly, CARD9 knockout totally ameliorated myocardial dysfunction. A potential underlying mechanism responsible for CARD9 knockout-afforded protection against myocardial anomalies may be mediated through restoration of cardiac autophagy signaling, an essential conservative organelle degradation machinery to maintain cellular homeostasis [17, 18, 21]. As recently reported [18], p38 MAPK interacting protein (p38IP) plays a critical role in the initiation and maturation of autophagy through association with autophagy protein 9 (Atg9). Phosphorylated p38 MAPK interacts with p38IP, therefore, interferes with the p38IP-Atg9 autophagy signaling. Our data demonstrated that CARD9 knockout suppressed HFD-induced p38 MAPK phosphorylation. Therefore, CARD9 knockout could potentially protect and promote p38IP-dependent autophagy signaling in the heart. This may represent a new signaling control niche in obesity-associated dysregulation of autophagy and cardiac dysfunction.

In summary, the current study demonstrated that CARD9 knockout protected against HFD-induced and obesity-associated myocardial dysfunction through attenuation of macrophage infiltration, reduction of pro-inflammatory cytokines, and restoration of cardiac autophagy

signaling. As CARD9 situates at a higher signaling control niche with respect to the downstream cytokine production, these results may provide a potential new therapeutic target for the management of HFD-induced metabolic syndrome, and in particular obesity-associated myocardial dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights of the Core Findings

- CARD9 knockout significantly attenuated HFD-induced metabolic abnormalities
- CARD9 knockout had no effect on HFD-induced adiposity
- CARD9 knockout significantly reduced HFD-induced pro-inflammatory cytokines
- CARD9 knockout ameliorated HFD-induced myocardial dysfunction
- CARD9 knockout restored HFD-induced down-regulation of cardiac autophagy signaling

Figure 1. The effect of CARD9 knockout on HFD-induced adiposity

WT and CARD9^{-/-} mice were fed ND or HFD for 5 months starting at 4–6 weeks of age. At the end of 5-month feeding, body weight, epididymal adipose tissue weight, heart weight and tibia length were measured. The ratio of heart weight over tibia length was used as an assessment of potential cardiac hypertrophy. (A) Body weight increase over 5-month feeding; (B) body weight at the end of 5-month feeding; (C) epididymal adipose tissue weight at the end of 5-month feeding; (D) heart weight over tibia length at the end of 5-month feeding. Five-month HFD feeding induced obesity and CARD9 knockout showed no effect on HFD-induced adiposity. Mean $\pm$ SEM, $n = 12/\text{group}$, * $p < 0.05$, *** $p < 0.001$, HFD vs. ND in WT and CARD9^{-/-} groups.

Figure 2. The effect of CARD9 knockout on glucose tolerance and insulin sensitivity

At the end of 5-month ND or HFD feeding, WT and CARD9^{-/-} mice were fasted overnight and blood glucose and insulin concentrations were measured. Intraperitoneal glucose tolerance test (IPGTT) was followed with i.p. injection of glucose (2 g/kg body weight). (A) Fasting blood glucose concentrations; (B) fasting blood insulin concentrations; (C) blood glucose levels within 120 min following acute glucose challenge; (D) HOMA-IR index. HFD feeding increased fasting blood glucose and insulin concentrations and impaired glucose disposal and insulin sensitivity. CARD9 knockout improved both glucose tolerance and insulin sensitivity following HFD feeding. Mean $\pm$ SEM, $n = 12$ /group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, HFD vs. ND; # $p < 0.05$, ### $p < 0.001$, CARD9^{-/-} vs. WT.

Figure 3. The effect of CARD9 knockout on HFD-induced cardiac dysfunction

At the end of 5-month ND or HFD feeding, mice were anesthetized with isoflurane and cardiac geometry and function were evaluated using echocardiography. (A) Heart rate; (B) fractional shortening (FS); (C) LV end-diastolic diameter (LVEDD); (D) LV end-systolic diameter (LVESD); (E) interventricular septum thickness at diastole (IVSD); (F) interventricular septum thickness at systole (IVSS); (G) LV posterior wall thickness at diastole (LVPWD); (H) LV posterior wall thickness at systole (LVPWS). Five-month HFD feeding induced systolic dysfunction with significant decrease of FS compared to ND-fed mice. CARD9 knockout protected the heart from HFD-induced cardiac dysfunction. Mean $\pm$ SEM, n = 6/group, *** p < 0.001, HFD vs. ND; ### p < 0.001, CARD9^{-/-} vs. WT.

Figure 4. CARD9 knockout preserved cardiomyocyte contractility following HFD feeding

At the end of 5-month ND or HFD feeding, myocytes were isolated from WT and CARD9^{-/-} mice and cell shortening and relengthening were measured. (A) Resting cell length; (B) peak shortening (PS % of resting cell length); (C) maximal velocity of shortening (+ dL/dt); (D) maximal velocity of relengthening (− dL/dt); (E) time-to-peak shortening (TPS); and (F) time-to-90% relengthening (TR₉₀). CARD9 knockout protected myocytes from HFD-induced contractile dysfunction in both diastolic and systolic phases. Mean ± SEM, n = 3 mice per group from a total of 100 – 120 cardiomyocytes, *p < 0.05, WT-HFD vs. WT-ND.

Figure 5. Representative Masson's trichrome staining for detecting cardiac fibrosis

At the end of 5-month ND or HFD feeding, hearts from WT and CARD9^{-/-} mice were excised and fixed in 10% NBF, paraffinized and sectioned (~6 μm), and then deparaffinized and stained with Masson's trichrome. Collagen is stained blue (pointed at by black arrows) and the scale bar is 40 μm. (A) Weak staining in the WT-ND mouse heart; (B) strong and diffusive interstitial fibrosis staining is present in the WT-HFD mouse heart; (C) weak staining in the CARD9^{-/-}-ND mouse heart; and (D) weak staining in the CARD9^{-/-}-HFD mouse heart.

Figure 6. CARD9 knockout reduced HFD-induced macrophage infiltration in heart tissue

At the end of 5-month ND or HFD feeding, heart tissues of WT and CARD9^{-/-} mice were collected. (A) Frozen sections (~7 μ m) of heart tissue were stained with antibodies against CD68 (red) and DAPI (blue) and fluorescence microscopy was performed (scale bar is 100 μ m); (B) CD68 positive cell numbers. HFD feeding induced significant increase of macrophage infiltration into heart tissue and CARD9 knockout dramatically attenuated the number of infiltrated macrophages. Mean $\pm$ SEM, n = 6/group; ** p < 0.01, *** p < 0.001, HFD vs. ND; ### p < 0.001, CARD9^{-/-} vs. WT.

Figure 7. The effect of HFD feeding on CARD9 protein expression

At the end of 5-month ND or HFD feeding, peritoneal macrophages were isolated and heart tissues were collected. Western immunoblotting analyses were performed with β-actin as the loading control. CARD9 protein expression in the heart (A) and macrophages (B). HFD feeding significantly up-regulated CARD9 protein expression in the heart and macrophages from WT mice. Mean ± SEM, n = 6/group; * $p < 0.05$, HFD vs. ND.

Figure 8. The effects of HFD feeding and CARD9 knockout on p38 MAPK phosphorylation

At the end of 5-month HFD feeding, peritoneal macrophages were isolated and heart tissue was collected. Representative Western blots and densitometric values of the ratio of p-p38 MAPK over p38 MAPK were analyzed using α-tubulin as the loading control.

Representative Western bands and density analyses from heart tissue homogenate (A) and isolated macrophages (B). HFD feeding significantly increased phosphorylation levels of p38 MAPK in the heart and macrophages. CARD9 knockout prevented the increase of the phosphorylated p38 MAPK. Mean ± SEM, n = 6/group; **p < 0.01, ***p < 0.001, HFD vs. ND; ##*p* < 0.01, ###*p* < 0.001, CARD9^{-/-} vs. WT.

Figure 9. The effect of CARD9 knockout on HFD-induced cytokine production

At the end of 5-month ND or HFD feeding, heart tissue and blood samples were collected. Macrophages were isolated and cultured for 24 hours. Commercial ELISA kits were used to measure the production of IL-6, TNFα, and IL-1β from the homogenized heart tissue, plasma, and macrophage supernatant. IL-6, TNFα, and IL-1β concentrations from heart tissue (A–C), plasma (D–F), and macrophage supernatant (G–I). HFD feeding significantly increased cytokine production in the heart, blood, and macrophages. CARD9 knockout ameliorated HFD-induced increase of cytokine production. Mean ± SEM, n = 6/group; * $p < 0.05$, HFD vs. ND; # $p < 0.05$, CARD9^{-/-} vs. WT.

Figure 10. The effects of HFD feeding and CARD9 knockout on cardiac autophagy signaling (A) Representative Western blots of LC3BI/II and p62 using β -actin as the loading control; Densitometric values of LC3BI/ β -actin (B), LC3BII/ β -actin (C), LC3BII/LC3BI (D), and p62/ β -actin (E). HFD feeding impaired the maturation of autophagy signaling as indicated by reduced ratio of LC3BII/LC3BI and increased p62 expression. CARD9 knockout ameliorated HFD-induced dysfunctional autophagy signaling. Mean $\pm$ SEM, $n = 6$ /group; ** $p < 0.01$, *** $p < 0.001$, HFD vs. ND; ### $p < 0.001$, CARD9^{-/-} vs. WT.