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Gender Differences in Adiponectin Modulation of Cardiac Remodeling in Mice Deficient in Endothelial Nitric Oxide Synthase

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ABSTRACT

Left ventricular hypertrophy (LVH) is a risk factor for cardiovascular disease, a leading cause of death. Alterations in endothelial nitric oxide synthase (eNOS), an enzyme involved in regulating vascular tone, and in adiponectin, an adipocyte-derived secretory factor, are associated with cardiac remodeling. Deficiency of eNOS is associated with hypertension and LVH. Adiponectin exhibits vaso-protective, anti-inflammatory, and anti-atherogenic properties. We hypothesized that increased levels of adiponectin would alleviate cardiac pathology resulting from eNOS deficiency, while decreased levels of adiponectin would exacerbate the pathology. Male and female mice, deficient in eNOS, and either lacking or over-expressing adiponectin, were fed high fat diet (HFD) or normal chow. Cardiac magnetic resonance imaging was performed to serially assess heart morphology and function up to 40 weeks of age. Thirty-two weeks of HFD feeding led to significantly greater LV mass in male mice deficient in eNOS and either lacking or over-expressing adiponectin. Heart function was significantly reduced when the mice were deficient in either eNOS, adiponectin or both eNOS and adiponectin; for female mice, heart function was only reduced when both eNOS and adiponectin were lacking. Thus, while over-expression of adiponectin in the eNOS deficient HFD fed male mice preserved function at the expense of significantly increased LV mass, female mice were protected from decreased function and increased LVH by over-expression of adiponectin. Our results demonstrate a sexual dimorphism in response of the heart to alterations in eNOS and adiponectin during high fat feeding and suggest that adiponectin might require eNOS for some of its metabolic effects. J. Cell. Biochem. 113: 3276–3287, 2012.

KEY WORDS: ADIPONECTIN; ENOS; LEFT VENTRICULAR HYPERTROPHY; MRI; CARDIAC REMODELING; HYPERTENSION

Left ventricular hypertrophy (LVH) is a powerful independent risk factor for cardiovascular morbidity and mortality. However, cardiac mass is variable in humans, influenced by lifestyle (diet, exercise, and smoking), chronic disease (hypertension, diabetes, and obesity), and genetic factors. Hypertensive patients show significant prognostic changes in left ventricular (LV) mass, size and geometry; all of which define LV remodeling [Koren et al., 2002; Verdecchia et al., 2006]. Inhibition, deficiency or mutation of endothelial nitric oxide synthase (eNOS) has been shown to be associated with LVH in mice [Ichinose et al., 2004] while restoration of eNOS has been shown to inhibit hypertrophy and preserve function [Janssens et al., 2004; Buys et al., 2007]. Mice lacking the coding gene for eNOS develop hypertension, lose weight, and exhibit heart rate variability, decreased plasma renin, impaired

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angiogenesis, insulin resistance, and age-dependent LV hypertrophy [Mungrue et al., 2002]. These mice develop a progressive and prolonged compensatory LV hypertrophy that eventually shows signs of decompensation at 1 year of age [Flaherty et al., 2007]. In humans, eNOS gene mutations are associated with increased risk of LVH [Xin et al., 2009]. Thus, eNOS plays a critical role in cardiac remodeling and development of cardiac hypertrophy.

Previously, we observed cardiac remodeling in mice in response to adiponectin over-expression or upon upregulation of adiponectin induced by treatment with PPARγ agonist [Durand, 2008]. Adiponectin is an adipocyte-secreted hormone involved in energy metabolism and endothelial activation [Wang and Scherer, 2008], and it has been associated with vaso-protective, anti-inflammatory, and anti-atherogenic properties [Berg and Scherer, 2005; Nawrocki et al., 2006; Vecoli et al., 2011; Wong et al., 2011]. Adiponectin deficiency in mice leads to the development of a mild phenotype with insulin resistance and loss of cardiovascular protection, while overexpression of adiponectin improves insulin sensitivity [Combs et al., 2004]. Over-expression of adiponectin in the ob/ob mouse resulted in significantly larger (>30%) hearts when compared to ob/ob mice with normal levels of adiponectin expression [Kim et al., 2007], suggesting a link between excess adiponectin and cardiac remodeling. Results of several studies of mice deficient in adiponectin suggest that adiponectin, like eNOS, exerts cardioprotective actions against pressure overload, hypertrophy and ischemia-reperfusion injury [Shibata et al., 2005, 2007]. A more detailed analysis recently revealed that at least some of the adiponectin actions can be explained on the basis of an adiponectin receptor-associated ceramidase activity that leads to changes in sphingolipid levels that are generally associated with anti-inflammatory, anti-apoptotic, and insulin-sensitizing properties [Holland et al., 2011]. In other studies, APPL1 has been implicated as an effector of adiponectin-induced NO production. Adiponectin stimulated production of eNOS in human umbilical vein endothelial cells has been shown to be reduced when APPL1 (an adapter protein containing a pleckstrin homology domain) expression is suppressed and in diabetic db/db mice the expression of APPL1 and adiponectin-induced vasodilation are reduced compared with lean littermates [Cheng et al., 2007]. Altered APPL1 expression might also be involved in the adiponectin-insensitivity observed in Zucker diabetic rats compared to Zucker lean rats even though eNOS expression is not significantly different in these rats [Schmid et al., 2011].

In humans, serum adiponectin levels correlate inversely with the incidence of obesity, type-2 diabetes, coronary artery disease and hypertension, LVH, and risk of coronary heart disease [Scherer et al., 1995; Iwashima et al., 2004; Hopkins et al., 2007; Ai et al., 2011; Pischon et al., 2011]. Nevertheless, in advanced heart failure cases adiponectin levels are increased and positively correlate with the severity of disease [George et al., 2006; Kizer et al., 2008]. This suggests that adiponectin may protect against CVD under healthy conditions, but in older adults already afflicted with CVD or in patients with underlying metabolic risk factors, such as hypertension and diabetes, there may be compensatory up-regulation of adiponectin and, therefore, levels of adiponectin may correlate with mortality risk.

We undertook an in vivo approach to elucidate the effects of adiponectin expression on hypertension and cardiac remodeling in eNOS deficient mice. Adiponectin levels reveal a sexual dimorphism, with female humans and mice having higher serum levels than males; therefore, we studied both male and female mice. A previous study of naïve male eNOS deficient mice revealed that LVH was compensatory in these mice until approximately 1 year of age [Flaherty et al., 2007]. Thus, in order to accelerate development of symptoms, we challenged the mice in our study by feeding with a high fat diet (HFD). Consumption of a HFD contributes to heart disease, obesity, and the development of the metabolic syndrome. Non-invasive cardiac magnetic resonance imaging (MRI) permitted longitudinal evaluation of cardiac function and the progression of cardiomyopathy in the same animal. BP measurement, serum metabolite analyses and histopathology were also performed. This study constitutes the first in vivo assessment of the effects of variations in plasma levels of adiponectin in the setting of eNOS deficiency (as a model for hypertension and endothelial dysfunction) and HFD challenge.

MATERIALS AND METHODS

All experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the Institute for Animal Studies of the Albert Einstein College of Medicine and were conducted under the guidelines of the Care and Use of Laboratory Animals published by the Office of Science and Health Reports, National Institutes of Health.

ANIMAL TREATMENT

Male and female mice were housed in groups of 2–5 in filter top cages, and maintained in a pathogen-free AALAC-accredited facility under controlled environment settings (22–25°C, 40–50% humidity). Mice were kept on a 12-hour light and dark cycle with ad libitum access to water and food. C57BL/6J (control wild-type) and eNOS-KO mice ($Nos3^{tm1Unc}$) were bred in-house from a parent line purchased from Jackson Lab (Bar Harbor, ME) and used thereafter as matching control mice. We crossed eNOS-KO mice with adiponectin-KO mice (Scherer lab) [Nawrocki et al., 2006] to generate the double knockout model adiponectin-KO/eNOS-KO. In a similar way we generated the adiponectin-TG/eNOS-KO mouse by crossing male adiponectin-TG [Combs et al., 2004] mice (fertility is challenged in females) with female eNOS-KO mice. Thus, all groups (Control, eNOS-KO, adiponectin-KO, adiponectin-TG, adiponectin-KO/eNOS-KO, and adiponectin-TG/eNOS-KO) were bred in house. Each group was then randomly separated (at 8 weeks of age) approximately in halves and fed either normal chow (ND) or a high fat/high cholesterol diet (HFD). The ND (PicoLab Rodent Diet 20, #5053) contained 23.6 kcal% from protein, 64.5 kcal% from carbohydrate, and 11.9 kcal% from total fat. The HFD (Research Diets Inc., D12089) contained 1% added cholesterol and 17.0 kcal% from protein, 42.7 kcal% from carbohydrate and 40.3 kcal% from total fat. Although this extensive study comprised data acquisition at 8, 12, 16, 24, 32, and 40 weeks of age for each group, here we focus on data at 8 and 40 weeks of age, except when otherwise stated.

CARDIAC MAGNETIC RESONANCE IMAGING

MRI measurements were performed using a GE Omega 9.4T vertical bore nuclear magnetic resonance spectrometer (Fremont, CA) operating at a proton (^{1}H) frequency of 400 MHz and equipped with 50 mm shielded gradients with strength of 75 G/cm, and a 40 mm inner diameter-60 mm long ^{1}H quadrature birdcage imaging coil that was custom designed for our laboratory (RF Sensors, LLC; NYC, NY). Mice were anesthetized with isoflurane inhalation anesthesia (2% in medical air) administered via a nose cone. A set of Gould ECG leads with thin silver wire contacts were attached under the skin to the four limbs and the ECG signal was fed to a Gould ECG amplifier linked to the Omega system and a PC. Mice were positioned head-up in the imaging coil and temperature within the gradient coils was maintained at ~30°C with a NESLAB gradient water cooling system, to prevent hypothermia. The ECG was monitored continuously and used as the gating signal triggering the MR spectrometer acquisition, as previously described [Durand et al., 2006]. Isoflurane anesthesia results in a very stable heart rate and cardiac gating using the R wave as a trigger minimizes any motion due to the high heart rate of the mice. We have found that there is minimal respiratory motion and no need for respiratory gating in this system as the mice fit snugly into the MRI coil. Body composition was measured by proton spectroscopy using the well-resolved water and fat proton resonances used to determine fat mass as described by Stein et al. [1998].

Diastolic and systolic cardiac gated MRI images were acquired with four scans in sets of eight contiguous slices of 1 mm thickness with a field of view (FOV) of 51.2 mm and a 128 × 256 matrix size (interpolated to 256 × 256). These parameters result in an in-plane resolution of approximately 100 μm. We used a spin echo sequence, which gives very good contrast between blood and heart wall muscle [Siri et al., 1997]. All images were acquired with a repetition time (TR) of approximately 140 ms (depending on the cardiac R–R interval and whether we were acquiring images in diastole or systole) and an echo time (TE) of 14 ms. Images were analyzed with MATLAB-based software and diastolic and systolic dimensions of the hearts were measured. Routine heart measurements obtained by centerline analysis [Durand et al., 2006] of heart short axis images include the left ventricle (LV) and right ventricle (RV) inner and outer diameters and wall thicknesses in diastole and systole. MR images were converted to JPEG format and sequentially loaded into Amira software and were merged for 3D reconstructions.

Fractional shortening (FS) was used to evaluate cardiac function in the mice and was calculated using the diastolic and systolic LV inner diameters ($LVID_{diastole}$ and $LVID_{systole}$) in the following formula:

$$FS = \frac{(LVID_{diastole} - LVID_{systole})}{LVID_{diastole}}$$

BLOOD PRESSURE AND HEMODYNAMICS

Following MRI sessions, systolic, mean and diastolic BPs (SBP, MBP and DBP) were measured with an indirect tail-cuff volume pressure recording sensor. This method incorporates a specially designed differential pressure transducer that measures the systolic and diastolic BP by determining the blood volume (BV) in the tail. Up to six mice were placed in individual tube restrainers on a temperature-controlled platform. Tails remained exposed for positioning of volume–pressure sensor and tail-cuff inflation/deflation. BP (in mmHg) was recorded every 20 s using a PC-integrated CODA6 system (Kent Scientific Corp.). Mice were trained at least twice before measurements. Each measurement session consisted of 15–17 cycles (one cycle = one BP reading) and the 5–8 acclimation cycles, which assist in animal adaptation and allow the system to automatically obtain the correct deflation time. There are typically 10 s between cycles, an interval that includes the calculation time (3–5 s). With the same integrated system, we recorded mouse heart rate (BPM), BV (ml), and peripheral blood flow (BF; ml/cycle).

SERUM METABOLITE MEASUREMENT

Plasma glucose was determined using a OneTouch II Blood glucose monitoring system (Lifescan, Johnson & Johnson). A small section of the tail was nicked with a sterilized razor blade and a drop of blood was placed on the glucose test strip.

Blood was collected from the mouse tail and serum was separated by centrifugation. Cholesterol and triglycerides were measured by an enzymatic endpoint method, using an Infinity Kit (Thermo-Fisher Corp.). Reagents and cholesterol/triglycerides standards were prepared following kit instructions. The assay was performed in a 96-well plate adding 2 μl of samples (standards, water, and serum) and 200 μl of cholesterol or triglyceride Infinity reagent. After incubation at 37°C, absorbance was measured at 550 and 620 nm in a Multiskan Plus spectrophotometer (Thermo Electron Corp.) using the Ascent Software for Multiskan (v2.6). Absorbance difference was calculated with EXCEL (MS-OFFICE 2002).

Serum insulin and adiponectin concentrations were measured with mouse respective RIA kits (LINCO Research, St. Charles, MO).

HISTOLOGY

Upon completion of the 40-week imaging sessions mice were deeply anesthetized with isoflurane. After cervical dislocation, heart and aorta were either snap frozen or perfused with saline solution (PBS) and dissected. Tissue was stained with hematoxylin and eosin (H&E), Oil red-O, Trichrome, or CD34. The tissue used for non-Oil-red O analysis was fixed in 10% neutral buffered formalin for 24 h and then rinsed and stored in 70% ethanol at room temperature. Tissue was embedded in paraffin and sections of 6–10 μm thickness were cut in the Histopathology Core Facility, where all of the histological staining was performed. The tissue designated for Oil-red O staining (lipids content) was fixed in OCT instead of ethanol.

STATISTICAL ANALYSIS

Data were analyzed with Minitab 14 (Minitab Inc., State College, PA) and GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA). Data and results are expressed as mean ± SEM. The statistical comparisons of different mouse groups and the effects of diet were performed using one-way and two-way ANOVA analysis. In addition, for pairwise multiple comparisons (more than two groups), Tukey's post hoc test was performed. Differences were considered significant when P-values < 0.05.

RESULTS

METABOLIC PROFILE

As expected, adiponectin-TG (TG–WT) mice of either sex had significantly elevated levels of circulating adiponectin at 40 weeks regardless of diet (Table I). Male adiponectin-TG/eNOS-KO (TG–KO) mice showed a statistically significant decrease in adiponectin when fed HFD, compared to the same genotype on ND (Table I). Female TG–KO mice did not exhibit this effect of HFD and maintained high levels of circulating adiponectin on either diet. Plasma glucose levels were significantly decreased in male HFD fed TG–KO (132.5 ± 15.0 mg/dl) mice compared to eNOS-KO (164.2 ± 8.2 mg/dl) mice at 40 weeks of age. Male adiponectin-KO (KO–WT) mice fed HFD became hyperglycemic at 40 weeks (262.5 ± 28.6 mg/dl compared to 147.0 ± 6.5 for the same genotype fed ND) and a similar trend was observed in female adiponectin-KO mice, where glucose was 180 ± 11.4 mg/dl with HFD and 140.5 ± 13.6 mg/dl with ND.

At 40 weeks of age cholesterol was significantly increased in all HFD fed male mice lacking eNOS (289 ± 30 mg/dl in eNOS-KO (WT–KO), 314 ± 55 mg/dl in adiponectin-KO/eNOS-KO (KO–KO), and 321 ± 52 mg/dl in TG–KO compared with 168 ± 26 mg/dl in wild type (WT–WT) male mice). Cholesterol was lower in HFD fed female mice (118 ± 15 mg/dl in WT–WT, 147 ± 11 mg/dl in WT–KO, and 207 ± 39 mg/dl in KO–KO). Overexpression of adiponectin in the HFD fed female TG–KO maintained cholesterol at the levels of the WT–WT female mice fed the same diet (118 ± 12 mg/dl). Triglyceride levels at 40 weeks of age were variable, with the highest values for male TG–KO (142 ± 20 mg/dl), WT (137 ± 15), and adiponectin-TG (108 ± 15) mice fed HFD. Triglyceride levels remained low and were unaffected by diet in male KO–KO mice while WT–KO and KO–WT mice exhibited decreased triglyceride levels with HFD (HFD vs. ND: 78 ± 11 vs. 112 ± 9 mg/dl and 46 ± 5 vs. 100 ± 16 mg/dl, respectively). In female mice triglycerides were typically <100 mg/dl regardless of diet, except for HFD fed TG–KO mice (119 ± 12 mg/dl). Insulin was higher in WT–KO mice fed ND (2.7 ± 0.5 ng/ml in males and 2.4 ± 0.2 ng/ml in females) than in other groups fed ND (range: 0.3–1.6 ng/ml). At 40 weeks of age HFD feeding of the male WT–KO mice resulted in an increase of 67% in the insulin level (to 4.5 ng/ml) while there was no difference among female WT–KO mice associated with the diet. Both male and female KO–WT mice exhibited a 1.5-fold increase in insulin with HFD (1.1 and 1.0 to 2.7 and 2.5 ng/ml, respectively). While insulin levels of female KO–KO mice were unaffected by diet (1.3–1.4 ng/ml) the combined effect of the deficiency of both adiponectin and eNOS and the challenge of HFD led to the largest increase in insulin in male mice (1.6–5.5 ng/ml). The female and male TG–KO mice exhibited the lowest insulin levels with ND (0.3–0.5 ng/ml). In those mice, HFD resulted in greater effect on male than on female mice (increase to 2.5 and 0.9 ng/ml, respectively).

TABLE I. Levels of Circulating Adiponectin in Female and Male Mice With Different Genotype and Fed Two Different Diets at 40 Weeks of Age (After 32 Weeks of HDF or ND Feeding)

APN-eNOS	Male adiponectin (μg/ml)	Female adiponectin (μg/ml)
WT–WT ND	8.5 ± 1.0	13.1 ± 1.0
HFD	7.5 ± 1.0	12.7 ± 2.1
WT–KO ND	$14.0 \pm 2.1^*$	$31.6 \pm 4.3^*$
HFD	$10.3 \pm 1.1^*$	$18.5 \pm 0.8^*$
KO–WT ND	BQL	BQL
HFD	BQL	BQL
KO–KO ND	BQL	BQL
HFD	BQL	BQL
TG–KO ND	$27.3 \pm 5.0^*$	$37.0 \pm 0.2^*$
HFD	$3.5 \pm 0.4^*$	$26.4 \pm 4.2^*$
TG–WT ND	$22.2 \pm 3.4^*$	$33.3 \pm 16.0^*$
HFD	$18.5 \pm 1.8^*$	$30.3 \pm 4.6^*$

Control wildtype (WT–WT), APN-KO/eNOS-KO (KO–KO), eNOS-KO (WT–KO) and APN-KO (KO–WT), APN-TG/eNOS-KO (TG–KO), and APN-TG (TG–WT) mice fed either normal diet (ND) or high fat diet (HFD). APN, adiponectin. BQL, below quantifiable level.
$^*P < 0.05$ compared to WT–WT on respective diet.

BODY MASS PROFILE

We measured bodyweight (BW) along the timeline of the MRI experiments to evaluate the effect of HFD feeding on the different genotypes. Before random separation into the two diet groups, 8-week-old mice lacking eNOS but not adiponectin (the WT–KO and TG–KO groups) had decreased BW compared to control wildtype (WT–WT) mice, while mice lacking adiponectin had BW values similar to WT–WT regardless of eNOS level (data not shown). At 40 weeks, diet more strongly influenced BW in male KO–KO mice compared to either of the parent lines, WT–KO or KO–WT (Table II), suggesting that the lack of adiponectin in the eNOS null mice is associated with BW gain under the challenge with HFD. While TG–KO male mice also gained significantly more weight on HFD only WT–WT female mice gained a significant amount of weight when challenged with HFD feeding (Table III). Lean body mass (total BW − fat mass) was calculated as described by Stein et al. [1998] and no difference was observed among genotypes fed the same diet (data not shown). The percent increase in body mass and fat mass from 8 to 40 weeks of age for the different groups is shown in Figure 1.

CARDIAC MORPHOLOGY AND FUNCTION

The absence of eNOS was associated with a significant increase in LV mass during HFD feeding only in male mice (compare Tables II and III). At 40 weeks of age, LV mass was increased in the hearts of WT–KO, KO–KO, and TG–KO male mice when fed HFD (Table II). LV mass was not significantly influenced by diet or genotype in female mice (Table III and Fig. 2), except in the case of female KO–KO.

At 40 weeks of age the LVs of male TG–WT and TG–KO mice were more dilated (with LVID values similar to WT mice) than the LVs of WT–KO fed either diet or KO–KO fed HFD (Table II). While the LVID of male KO–WT mice was unaffected by diet, higher levels of adiponectin were associated with increased LVID (exacerbated by HFD) regardless of eNOS levels in the male mice. On the contrary, none of the female mice exhibited significant LV dilation. LV wall thickness was increased in male mice lacking eNOS, particularly those over-expressing adiponectin and fed HFD compared to WT–WT mice (1.37 ± 0.02 mm vs. 1.02 ± 0.03 mm, respectively).

Percent fractional shortening (%FS), a measure of cardiac function, was robustly diminished by prolonged feeding of HFD in WT–KO and KO–KO male mice compared with the same genotypes fed ND or with WT mice (Table II, compare %FS at 8 and 40 weeks of

TABLE II. Bodyweight (BW), Diastolic Left Ventricle Inner Diameter (LVID), LV Wall Thickness (LV Wall), and LV Mass at 40 weeks of Age

Male Adiponectin-eNOS-Diet	n	BW (g)	LVID (mm)	LV mass (mg)	FS−8 w.o. (%)	FS−40 w.o. (%)
WT–WT ND	10	32.5 ± 0.8	4.3 ± 0.2	193.3 ± 19.6	55.5 ± 2.0	55.7 ± 3.0
WT–WT HFD	12	$46.1 \pm 1.0^{\#}$	4.5 ± 0.3	189.4 ± 6.0	57.9 ± 3.0	58.1 ± 2.0
KO–KO ND	9	31.5 ± 1.9	$3.9 \pm 0.1^{*,\dagger}$	179.0 ± 8.4	53.3 ± 1.0	$49.1 \pm 3.0^{*}$
KO–KO HFD	7	$44.4 \pm 2.2^{\#}$	4.0 ± 0.4	$235.9 \pm 16.3^{*,\dagger\#}$	54.3 ± 3.0	$38.5 \pm 4.0^{*,\dagger,\#,\diamond}$
TG–KO ND	4	34.5 ± 4.1	$4.3 \pm 0.2^{\ddagger}$	212.3 ± 20.6	56.2 ± 1.0	50.5 ± 3.0
TG–KO HFD	6	$46.4 \pm 1.7^{\#}$	$4.70 \pm 0.2^{\ddagger}$	$231.8 \pm 8.4^{*,\ddagger,\S}$	57.1 ± 1.0	$48.6 \pm 3.0^{\ddagger,\diamond}$
WT–KO ND	9	31.2 ± 1.6	3.7 ± 0.1	191.5 ± 9.1	52.3 ± 3.0	$48.8 \pm 3.0^{*,\dagger}$
WT–KO HFD	7	$38.1 \pm 2.5^{*,\#}$	$3.8 \pm 0.2^{*}$	$207.5 \pm 13.5^{*}$	54.1 ± 2.0	$37.6 \pm 4.0^{*,\#,\diamond}$
KO–WT ND	8	$28.7 \pm 0.8^{*}$	4.1 ± 0.2	$160.9 \pm 9.6^{*}$	50.9 ± 3.0	55.6 ± 2.0
KO–WT HFD	6	$37.6 \pm 1.3^{*,\#}$	4.1 ± 0.1	$163.1 \pm 9.8^{*}$	50.0 ± 3.0	$39.5 \pm 6.0^{\#}$
TG–WT ND	3	31.7 ± 1.1	4.3 ± 0.2	211.2 ± 13.9	56.3 ± 4.0	59.0 ± 5.0
TG–WT HFD	6	$44.3 \pm 1.5^{\#}$	$4.8 \pm 0.1^{\#}$	$204.9 \pm 7.5^{*}$	57.2 ± 2.0	56.4 ± 4.0

Fractional shortening (FS) at 8 and 40 weeks of age for male control wildtype (WT–WT), APN-KO/eNOS-KO (KO–KO), eNOS-KO (WT–KO) and APN-KO (KO–WT), APN-TG/eNOS-KO (TG–KO), and APN-TG (TG–WT) mice fed either normal diet (ND) or high fat diet (HFD). *, †, ‡, and § refer to $P < 0.05$ in comparison with same diet WT–WT, APN-KO, eNOS-KO, and APN-TG, respectively. $^{\#}P < 0.05$ compared with same genotype on normal diet ND diet. $^{\diamond}P < 0.05$ compared with same genotype at 8 weeks of age on same diet.

age). Challenge with HFD led to a significant decrease in %FS in both male and female KO–WT mice (Tables II and III). Although %FS tended to be reduced in female KO–KO mice, statistical significance was not achieved. While challenge with HFD compromises cardiac function in the absence of either eNOS or adiponectin, the effects are not cumulative; loss of function in the double knockout mice is no worse than that in either of the single knockouts. Over-expression of adiponectin in the female eNOS deficient mice fully preserved %FS but only partially rescued the male eNOS deficient mice from effects of prolonged HFD on %FS and did little to influence the effects of aging on %FS in the male mice (Fig. 3).

Hypertension was observed in eNOS deficient mice as expected, and was further exacerbated by challenge with HFD and aging with the combined effect of diet and aging being worse for the male mice (Table IV). Male and female mice lacking both adiponectin and eNOS fed ND were hypertensive at 8 weeks of age; BP decreased as male mice aged but was unaffected by age in female mice. When fed HFD, male KO–KO mice exhibited slightly higher BP. The female KO–KO mice did not show an age related increase in BP when fed ND but the combined influence of HFD and aging resulted in the highest systolic BP for this group compared with the other female mice fed HFD. TG–KO mice exhibited an increase in BP with aging and by 40 weeks, the difference became statistically significant for male mice, regardless of the diet (Table IV). The level of hypertension in TG–KO mice was not as severe as that in eNOS-KO or KO–KO mice until 32 weeks of age (data not shown), when their BP approached the levels of those groups. The increase in BP with aging was attenuated by over-expression of adiponectin in the female TG–KO mice. These results together with the fact that KO–WT mice fed HFD had elevated BP, suggest that adiponectin plays a protective role against hypertension. This protection seems stronger when eNOS is normally expressed, as no sign of hypertension was seen in the TG–WT mice. In fact, those mice tended to have lower BP than WT mice. This suggests that some cardioprotective actions of adiponectin are eNOS dependent. Although KO–WT mice fed HFD became hypertensive by 32 weeks of age they did not develop LV hypertrophy (Fig. 2), suggesting that lack of eNOS plays a greater role in cardiac remodeling.

At the end of in vivo studies, histological samples were prepared. Samples from male mice were extensively studied since they exhibited greater hypertrophy and loss of function than female mice. Because LV hypertrophy can depend on angiogenesis we

TABLE III. Bodyweight (BW), Diastolic Left Ventricle Inner Diameter (LVID), LV Wall Thickness (LV Wall), and LV Mass at 40 weeks of Age

Female adiponectin-eNOS-Diet	n	BW (g)	LVID (mm)	LV mass (mg)	FS−8 w.o. (%)	FS−40 w.o. (%)
WT–WT ND	16	25.3 ± 0.6	3.8 ± 0.1	157.2 ± 7.1	56.7 ± 3.0	54.3 ± 3.0
WT–WT HFD	8	$31.2 \pm 1.7^{\#}$	3.7 ± 0.3	155.7 ± 9.6	56.4 ± 3.0	55.2 ± 4.0
KO–KO ND	8	26.4 ± 1.5	3.9 ± 0.1	156.1 ± 13.7	53.1 ± 6.0	46.1 ± 6.0
KO–KO HFD	4	30.1 ± 4.5	$3.3 \pm 0.1^{\#}$	167 ± 18.8	55.3 ± 4.0	43.1 ± 5.0
TG–KO ND	4	24.9 ± 0.9	3.5 ± 0.2	145.8 ± 17.8	57.2 ± 3.0	55.1 ± 4.0
TG–KO HFD	7	28.6 ± 1.4	3.9 ± 0.2	146.5 ± 12.3	56.6 ± 3.0	59.9 ± 3.0
WT–KO ND	5	26.2 ± 1.3	3.2 ± 0.2	162.6 ± 10.7	53.3 ± 4.0	51.9 ± 4.0
WT–KO HFD	10	24.4 ± 2.0	3.5 ± 0.2	146.3 ± 12	54.6 ± 5.0	47.6 ± 8.0
KO–WT ND	6	23.3 ± 0.7	3.7 ± 0.2	124.5 ± 7.5	54.6 ± 2.0	56.5 ± 2.0
KO–WT HFD	9	25.7 ± 2.0	3.5 ± 0.2	134.8 ± 10	57.0 ± 4.0	$42.8 \pm 4.0^{\#,\diamond}$
TG–WT ND	7	25.1 ± 0.2	3.6 ± 0.1	142.5 ± 6.4	57.9 ± 2.0	54.3 ± 3.0
TG–WT HFD	5	25.7 ± 0.2	3.7 ± 0.1	137.5 ± 1.0	57.4 ± 2.0	53.5 ± 8.0

Fractional shortening (FS) at 8 and 40 weeks of age for female control wildtype (WT–WT), APN-KO/eNOS-KO (KO–KO), eNOS-KO (WT–KO) and APN-KO (KO–WT), APN-TG/eNOS-KO (TG–KO), and APN-TG (TG–WT) mice fed either normal diet (ND) or high fat diet (HFD). $^{\#}P < 0.05$ compared with same genotype on ND diet. $^{\diamond}P < 0.05$ compared with same genotype at 8 weeks of age on same diet.

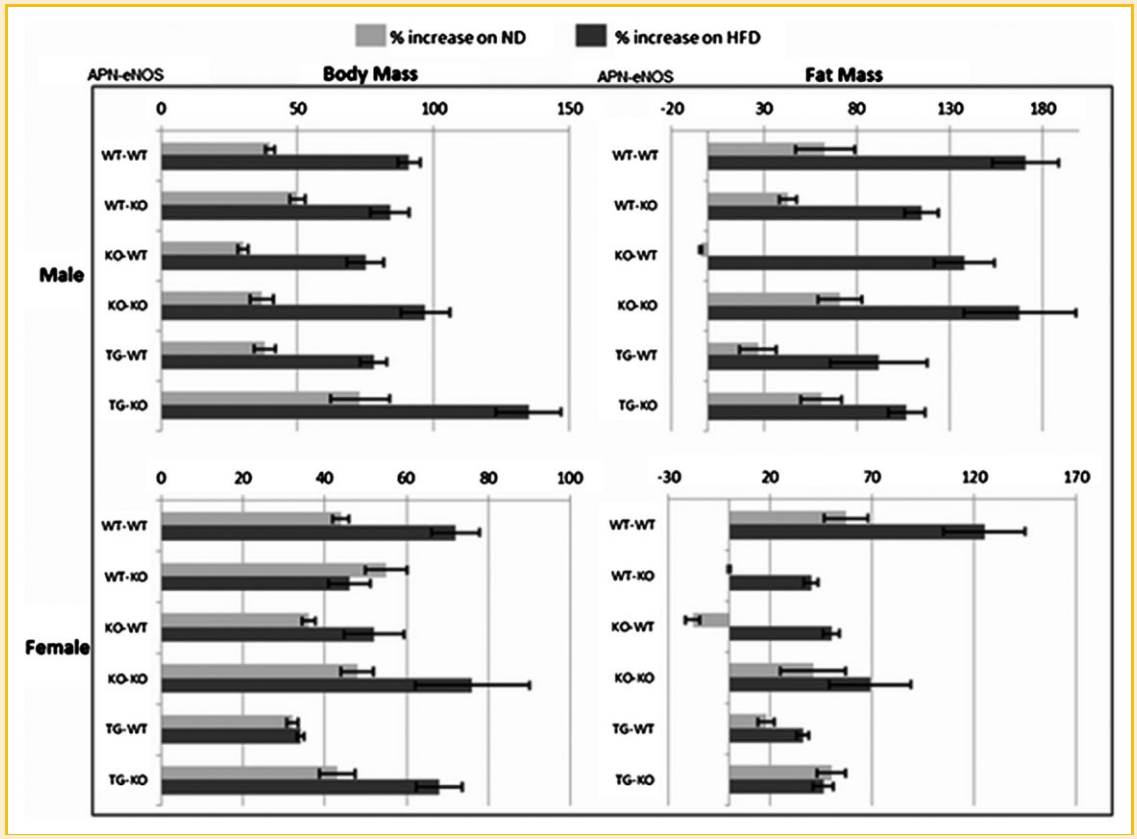

Fig. 1. Percent increase in body mass (BM) and fat mass (FM) from 8 to 40 weeks of age for all genotypes, male (m) and female (f) on normal diet (ND) or high fat diet (HFD).

stained samples with CD34, a marker used to evaluate capillary density and angiogenesis (see Fig. 4A). The absence of adiponectin was associated with decreased CD34 staining regardless of the diet (data not shown), and it was similar to that shown for control WT mice fed ND. In hearts of TG–KO mice fed HFD, CD34 staining was significantly increased compared to that in WT–KO, KO–WT (data not shown), and control WT mouse hearts, as shown quantitatively (see Fig. 4B).

Excessive deposition and accumulation of extracellular matrix proteins, such as collagen, is associated with disruption in the normal structure of the cardiac myocytes and with tissue hardening that affects contractility and/or relaxation [Burlew and Weber, 2002]. Trichrome staining was used to assess collagen content and relate the loss of contractility or relaxation with increased fibrosis in the LV tissue of mice lacking eNOS. On ND, KO–KO mice showed little collagen accumulation but, when fed HFD, KO–KO mice showed greater collagen accumulation (see Fig. 5). Loss of myocardial contractility may be related to the increased fibrosis observed in the LV of KO–KO and WT–KO mice, compared with control WT mice. TG–KO mice also exhibited an increase in Trichrome staining as a consequence of HFD, which may be associated with the LV wall thickening and hypertension observed in these mice.

Oil red-O stained sections (see Supplementary Fig. 1) show that lipid accumulation in cardiac tissue was increased in KO–KO mice fed HFD compared with same genotype and WT mice fed ND. The H&E stained sections of LV tissue from TG–KO mice fed HFD were more similar to those of WT–KO mice than to the LV of WT mice fed HFD, and there was an increase in the number of nuclei per field of view, a marker for LV hypertrophy (data not shown).

Tail BV and BF, indicators of peripheral endothelial function, were decreased in all mice lacking eNOS except for HFD-fed female TG–KO and HFD-fed female eNOS-KO mice (see Supplementary Fig. 2 for data for 40-week-old mice).

DISCUSSION

The effect of diet on BW was significant in all male groups, with those genotypes fed HFD showing increased BW and fat mass compared to respective groups fed ND. Among female mice, WT–WT, KO–KO, and KO–WT mice fed HFD showed a significant increase in BW and fat mass within their groups. However, female WT–KO and TG–WT mice showed an attenuated BW gain as a consequence of HFD. It is interesting to note that over-expression of adiponectin prevented the gain in fat mass in female mice throughout the HFD feeding period, and that the concomitant absence of both adiponectin and eNOS resulted in the largest gain in fat mass after control female WT–WT mice.

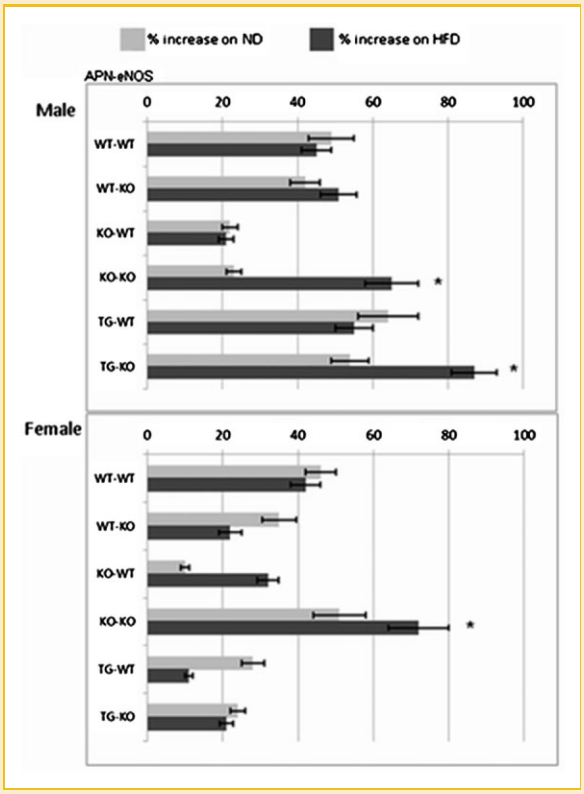

Fig. 2. Percent increase in heart mass (HM) from 8 to 40 weeks of age for all genotypes, male (m) and female (f) on normal diet (ND) or high fat diet (HFD).

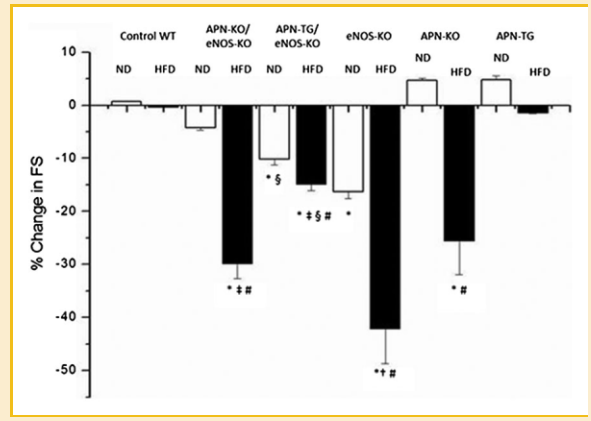

Fig. 3. Percent change of fractional shortening from 8 to 40 weeks of age in male control wildtype (WT–WT), KO–KO, TG–KO, WT–KO, KO–WT, and TG–WT mice, fed either ND or HFD.

The HFD-induced morphological changes (LV mass, LVID, LV wall thickness) observed in the hearts of mice lacking eNOS (WT–KO, KO–KO, and TG–KO) provide evidence that eNOS is a protective factor against cardiac hypertrophy, particularly in male mice. For male mice, the size of the LV chamber also correlates with the levels of adiponectin when mice are fed HFD, since male adiponectin-TG mice had larger chambers than adiponectin-KO and control WT male mice. Female mice deficient in eNOS but expressing normal or increased levels of adiponectin are protected from HFD-induced increase in LV mass, as shown in Figure 2 where only female mice deficient in both adiponectin and eNOS exhibit significantly increased heart mass when fed HFD. El Akoum et al. recently reported a study of effects of HFD composition on male and female wild type (C57B6/J) mice that included echocardiographic evaluation of cardiac parameters [El Akoum et al., 2011]. They observed a sexual dimorphism in response to HFDs containing animal or vegetable fat with male mice exhibiting greater diastolic dysfunction and hypertrophy and suggest that susceptibility to develop HFD-linked type 2 diabetes may be related to sex hormone modulation of adiponectin production. We suspect that sex hormone modulation of adiponectin provides cardioprotection in the female mice in our study.

In eNOS deficient male mice, cardiac remodeling (LVID) is amplified when adiponectin levels are elevated regardless of the diet. This suggests that other mechanisms besides hypertension are

TABLE IV. Baseline and End-Of-Study Blood Pressure Values for Male and Female Mice Fed Normal and High-Fat Diets

	Male	Male	Female	Female
APN-eNOS	Systolic BP 8–40 w.o.	Diastolic BP 8–40 w.o.	Systolic BP 8–40 w.o.	Diastolic BP 8–40 w.o.
WT–WT ND	111.1 (4.2)–101.6 (2)	90.7 (5.7)–77.0 (2.3)	116.9 (3.5)–102.4 (3)	85.0 (3.0)–81.3 (2.6)
HFD	105.3 (3.2)–105.2 (1.9)	82.5 (2.9)–79.0 (1.6)	104.9 (1.3)–99.4 (2.2)	80.0 (3.4)–75.9 (2)
WT–KO ND	136.2 (5)–136.4 (5.2)	102.0 (6)–105.2 (6.1)	121.7 (12.1)–143.6 (4.3)	108.5 (7.1)–131.1 (9.3)
HFD	137.8 (8.9) –152.1 (13.4)	92.3 (0.3)–109.9 (3.6)	128.8 (12.5) –136.6 (6.3)	102.2 (11.4)–104.6 (4.5)
KO–WT ND	115.5 (8.7)–97.6 (4.5)	104.7 (5.7)–71.0 (3.6)	117.8 (6.3)–100.0 (1.4)	88.9 (2.5)–71.6 (2.2)
HFD	108.5 (3.8)–157.3 (6.8)	100.0 (5)–121.6 (7.8)	102.0 (3.2)–136.5 (4.2)	89.0 (2.5)–102.7 (4.7)
KO–KO ND	129.1 (5.6)–120.3 (2.6)	92.6 (7.8)–87.8 (2.8)	127.6 (5.4)–129.7 (6.2)	105.9 (2.5)–97.9 (8.2)
HFD	138.0 (6.7)–139.9 (8.6)	110.2 (9.8)–111.8 (3.6)	126.1 (2.4) –145.1(14.4)	96.5 (6.1)–113.8 (13.5)
TG–KO ND	122.2 (15) –145.1(17.7)	99.0 (5)–117.9 (18)	120.0 (4.0) –132.8(12.9)	96.0 (5)–103.2 (16.5)
HFD	118.6 (2.2)–141.7 (6)	90.6 (3.5)–113.2 (4.7)	124.0 (6.6)–129.1 (3)	94.4 (7.2)–98.8 (2.4)
TG–WT ND	89.0 (15)–102.0 (4.3)	63.0 (8)–76.7 (1)	102.0 (2.7)–98.4 (3.7)	83.0 (3.9)–75.3 (4)
HFD	87.0 (1.7)–102.1 (0.7)	61.1 (1.7)–74.9 (3.4)	100.9 (3)–98.7 (2.5)	71.7 (4.1)–73.5 (2.1)

Control wildtype (WT–WT), APN-KO/eNOS-KO (KO–KO), eNOS-KO (WT–KO) and APN-KO (KO–WT), APN-TG/eNOS-KO (TG–KO), and APN-TG (TG–WT) mice fed either normal diet (ND) or high fat diet (HFD).

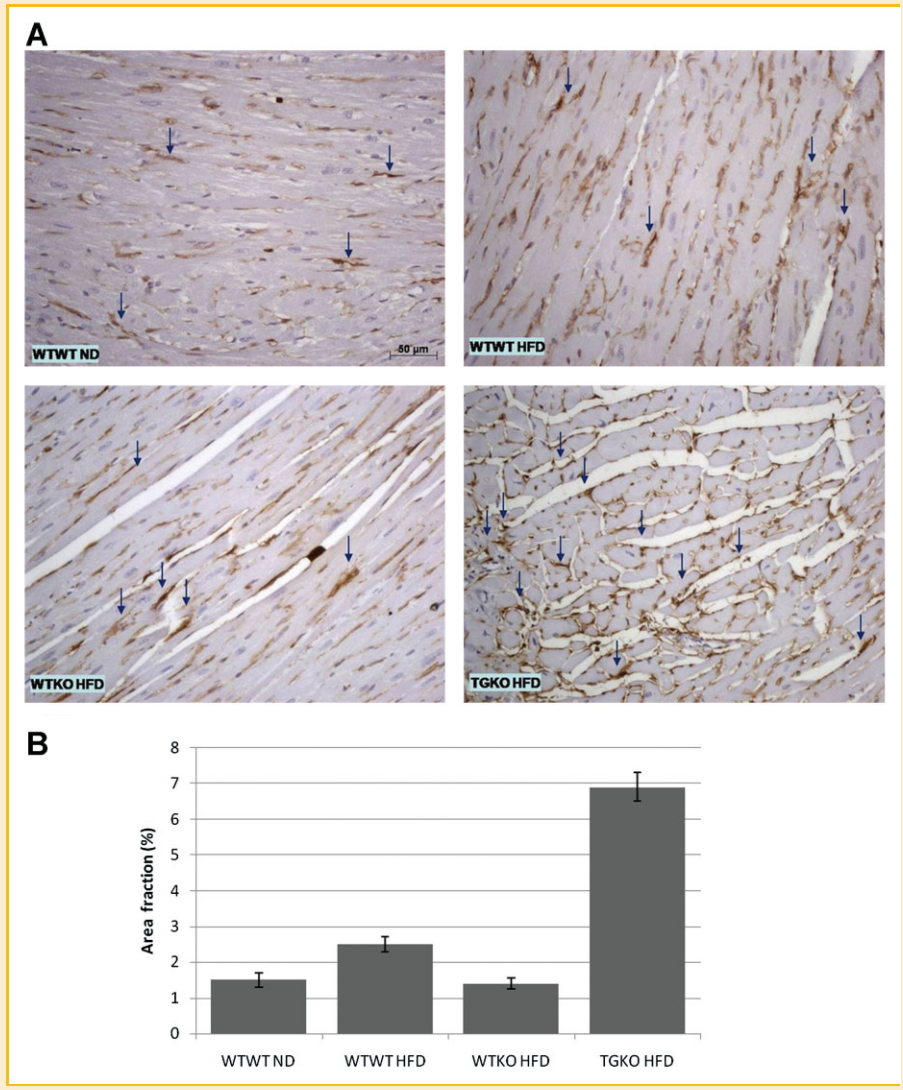

Fig. 4. (A) Left ventricle cross sections stained with CD34 staining (20×) in LV tissue of 40-week-old wild type mice (fed normal (ND) and high fat/high cholesterol diet (HFD)) and WT–KO and TG–KO mice fed HFD. B: Densitometry of CD34 staining (ImageJ). Increase in vessel density (brown) is dramatic in TG–KO mice ($n > 3$ in all groups). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

mediating the development of LV hypertrophy. It has been reported that myocardial hypertrophy in the absence of hypertension is induced by angiogenesis in mice [Tirziu et al., 2007] which could explain the increase in LV mass when adiponectin is over-expressed in mice. Hearts of male mice over-expressing adiponectin and stained for CD34 had significantly higher vessel density than adiponectin-KO, eNOS-KO, KO-KO, or WT mice (Fig. 4A). This supports a role for adiponectin as a promoter of angiogenesis as previously suggested [Brakenhielm et al., 2004; Ouchi et al., 2004; Shibata et al., 2004; Silha et al., 2005]. Vascular endothelial growth factor (VEGF) is the main inducer of angiogenesis, via cellular proliferation and activation of key signaling enzymes, including mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), Akt, and RhoA [van Nieuw Amerongen et al., 2003; Matsumoto and Mugishima, 2006]. While in vitro studies [Mahadev et al., 2008] show that adiponectin inhibits VEGF-stimulated human coronary artery endothelial cell migration via cAMP/PKA-dependent signaling, and therefore does not induce angiogenesis, in vivo cancer studies suggest that adiponectin has a direct role in the angiogenic response. Landskroner-Eiger et al. [2009] have shown that adiponectin supports the release of angiogenic factors such as VEGF-A and VEGF-B and can promote blood vessel formation in the PyMT mammary tumors relative to PyMT/adiponectin KO-derived tumors.

In male mice loss of cardiac function (%FS) was exacerbated by feeding with HFD in the absence of either eNOS or adiponectin (Fig. 3). Excessive deposition and accumulation of extracellular matrix proteins such as collagen are associated with disruption in the normal structure of the cardiac myocytes and with tissue hardening, thereby affecting contractility and/or relaxation [Burlew and Weber, 2002]. Thus, we investigated whether the loss of myocardial contractility or relaxation was related to an increase in

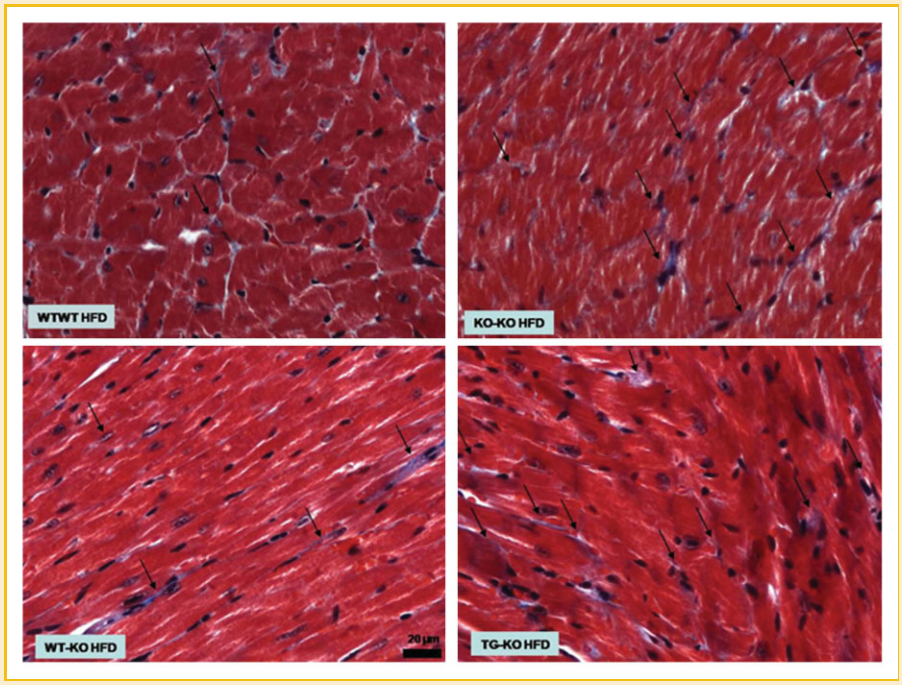

Fig. 5. Trichrome stained left ventricle cross sections (40×). The accumulation of collagen (blue, indicated by arrows) is increased in KO–KO and TG–KO mice fed high fat/high cholesterol diet (HFD) compared to control mice fed high fat diet (HFD). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

LV fibrosis. Our histology results show a clear correlation between accumulation of collagen and loss of cardiac function (Fig. 5). In addition, our histology data suggest that lipid accumulation in cardiac tissue of male KO-KO mice was exacerbated with HFD, and that lipid accumulation in male TG-KO and male eNOS-KO mice fed HFD was similar (data not shown).

Over-expression of adiponectin in the female TG-KO mice challenged with HFD resulted in complete preservation of cardiac function (%FS) while a reduced loss of cardiac function was observed in male TG-KO mice challenged with HFD compared to male KO-KO or eNOS-KO mice suggesting a compensatory adaptation in response to the absence of eNOS that may be provided by angiogenesis. We also explored the possible compensation derived from neuronal NOS (nNOS) activity in the heart, since NO is normally synthesized by both eNOS and nNOS in the heart [Sumeray et al., 2000]. It has been reported that nNOS compensates flow- and shear stress-induced responses by balancing NO production in the endothelium of coronary arteries of eNOS-KO mice [Huang et al., 2002]; however, we did not see a change in nNOS expression levels in TG-KO mice on either diet (data not shown). Thus, over-expression of adiponectin does not induce the upregulation of nNOS in our mouse model. Therefore, the proangiogenic properties of adiponectin in challenged hearts might be partially independent of NO, at least NO derived from eNOS or nNOS activation. The possibility that adiponectin over-expression indirectly increases iNOS activity in the heart, and thereby partially rescues the hypertension, and cardiac dysfunction observed in the eNOS-KO phenotype, warrants further investigation to measure the levels of expression and/or activity of iNOS in the cardiac tissue. In addition, adiponectin may activate other angiogenic pathways of the VEGF signaling cascade, such as the p38 MAPK mediated cell migration and Src mediated permeability responses. Furthermore, during exercise, it has been shown that NO production is not the sole factor involved in the VEGF-mediated angiogenic response. Elevated shear stress can activate the VEGFR2 pathway, independent of VEGF, and therefore is likely an important mechanical signal prompting angiogenesis within muscle [Prior et al., 2004].

It is interesting to note that both male and female adiponectin-deficient mice with normal eNOS levels exhibit a significant loss of cardiac function and increased BP when challenged with HFD, although these mice exhibit no significant changes in LVID or LV mass. This suggests that a protective role of adiponectin against hypertension may be modulated by dietary fat and likely involves eNOS since increased adiponectin cannot prevent hypertension in mice that lack eNOS (TG–KO). Male mice lacking both adiponectin and eNOS exposed to HFD, exhibit an age-related increase in LV mass that is accompanied by increased BP and considerably reduced FS; whereas male mice lacking eNOS and over-expressing adiponectin exhibit age-related increases in LV mass and BP accompanied by reduced FS and LV chamber dilation. Female mice lacking both adiponectin and eNOS also exhibit increased BP, without a significant increase in LV mass or loss of function, but with a smaller LVID, suggesting that mild hypertrophy observed at 40 weeks of age is still compensatory in the female mice. Female mice lacking eNOS and over-expressing adiponectin exhibit no loss of cardiac function, a smaller increase in BP, and no significant change in LV mass or LVID suggesting that adiponectin over-expression in the female eNOS deficient mice is sufficient to preserve

cardiac function and prevent detrimental cardiac remodeling. This may be due to sex hormone modulation of adiponectin that is independent of eNOS. It is possible that decompensation is delayed in the female mice. The development of peripheral endothelial dysfunction is intensified by HFD in mice lacking eNOS. This fact is coincident with the observed hypertension in these mice, and implies an association between high fat feeding and an increase in the production of reactive oxygen species, which has been indicated as a contributor to endothelial dysfunction [Munzel et al., 2008]. Interestingly, adiponectin-KO mice fed either diet did not show a decrease in BV or flow compared to WT mice, in contrast to the hypothesis that adiponectin deficiency causes severe endothelial dysfunction as previously reported [Cao et al., 2009]. Nonetheless, these discrepancies might be explained by the differences in ages of the mice studied and methodology; the previously reported studies were done ex vivo on descending aortic vascular segments while ours were performed in vivo using tail blood measurements.

With high fat feeding the absence of eNOS alone is not associated with an increase in triglyceride levels compared to those of WT mice. However, over-expression of adiponectin in HFD fed eNOS KO and WT mice resulted in increased triglyceride levels, suggesting that adiponectin overexpression is associated with the elevation of circulating triglycerides. Female mice in our study exhibited lower triglyceride levels than males consistent with results observed in rats [Amengual-Cladera et al., 2012]. In that study 26 weeks of HFD had little effect on total adiponectin levels in males and resulted in a small elevation of adiponectin levels in females. El Akoum et al. [2011] observed a decrease in circulating adiponectin levels in WT male mice fed HFD with little effect on levels in females, however, in their study adiponectin level in males was much lower than in our study (1.2–1.4 μg/ml vs. 7.5 μg/ml). This difference may be the result of the different ages of the mice: the mice in their study were 25-week-old and had been on HFD for 20 weeks whereas our mice were studied at 40 weeks of age after 32 weeks of HFD. Age-related changes in adiponectin levels remain controversial. In humans adiponectin levels appear to increase with age in both sexes [Schautz et al., 2012].

In our study, serum levels of adiponectin in eNOS-KO mice fed ND were lower than those of mice over-expressing adiponectin, but were higher than those of WT mice fed ND, suggesting that absence of eNOS induces the upregulation of adiponectin, likely as a compensatory response. The effects are even larger in females suggesting modulation by sex hormones as well. Higher levels of adiponectin correlated with lower insulin levels as expected. Similar to what we observed with triglyceride levels, our results of serum adiponectin levels differ from those of a previous study [Nakata et al., 2008], possibly due to age, diet and strain differences.

In summary, our results show that absence of both eNOS and adiponectin is associated with cardiac dysfunction. While over-expression of adiponectin fully rescues %FS in female mice lacking eNOS, possibly through an eNOS-independent pro-angiogenesis pathway, this rescue effect is blunted in male mice. Our study also suggests that the ability of adiponectin to protect the vasculature through the induction of NO production might be exclusively associated with the activation of eNOS (or iNOS) not nNOS. The cardioprotective function of adiponectin seems to be limited to the reduction of BP and the improvement of cardiac contraction and endothelial function, despite the dilation of the LV in eNOS deficient male mice. Thus, adiponectin might require active eNOS to prevent cardiomyopathies. Further work at the molecular level will offer new insights into the mechanisms, featuring adiponectin and eNOS, through which adiposity affects vascular health and will help to identify new therapeutic targets for prevention of the morbidity and mortality associated with obesity, type-2 diabetes, and hypertension.

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