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The prevalence of cardio-metabolic risk factors is differentially elevated in obesity-prone Osborne-Mendel and obesity-resistant S5B/Pl rats

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ABSTRACT

Aims: Individual susceptibility to develop obesity may impact the development of cardio-metabolic risk factors that lead to obesity-related comorbid conditions. Obesity-prone Osborne-Mendel (OM) rats expressed higher levels of visceral adipose inflammation than obesity-resistant, S5B/Pl (S5B) rats. However, the consumption of a high fat diet (HFD) differentially affected OM and S5B rats and induced an increase in visceral adipose inflammation in S5B rats. The current study examined the effects of HFD consumption on cardio-metabolic risk factors in OM and S5B rats.

Materials & methods: Glucose regulation and circulating levels of lipids, adiponectin and C-reactive protein were assessed following 8 weeks of HFD or low fat diet (LFD) consumption. Left ventricle hypertrophy and mRNA expression of cardiovascular disease biomarkers were also quantified in OM and S5B rats.

Key findings: Circulating levels of triglycerides were higher, while HDL cholesterol, adiponectin and glycemic control were lower in OM rats, compared to S5B rats. In the left ventricle, BNP and CTGF mRNA expression were higher in OM rats and IL-6, IL-1β, VEGF, and iNOS mRNA expression were higher in S5B rats.

Significance: These findings support the hypothesis that cardio-metabolic risk factors are increased in obesity-prone individuals, which may increase the risk for the development of obesity-related comorbidities. In the current models, obesity-resistant S5B rats also exhibited cardiovascular risk factors supporting the importance of monitoring cardiovascular health in individuals characterized as obesity-resistant.

1. Introduction

Obesity is a chronic disease characterized by an excess of adiposity and is a known risk factor for the development of cardio-metabolic diseases [1–5]. Adipose inflammation is a crucial link between obesity, the development of type 2 diabetes and an increased risk for cardiovascular disease [6–13]. Recent studies from our laboratory reported that adipose tissue inflammation and adipose morphology were differentially affected by the susceptibility to develop obesity [14]. Higher levels of pro-inflammatory cytokines were found in visceral fat depots (i.e. epididymal fat) of obesity-prone Osborne-Mendel (OM) rats, however, the consumption of a high fat diet (HFD) exacerbated pro-inflammatory cytokine expression in obesity-resistant S5B/Pl (S5B) rats, but not OM rats [14].

The recruitment of inflammatory cells, such as macrophages and lymphocytes, in adipose tissue has been associated with systemic inflammation [14–17], which likely affects cardiac function via the development of glucose intolerance and insulin resistance [1,4,18,19]. Diet-induced obese rats have an increased myocardial protein expression of pro-inflammatory cytokines, such as TNFα and IL-6, and decreased circulating levels of adiponectin, an adipokine associated with insulin sensitivity [20]. Consumption of a high sucrose diet has been reported to increase left ventricle weight and expression of markers of cardiac function (VEGF, BNP and TGF-β1) in the left ventricle [21]. In a model of pre-diabetes, rats fed a high fat, high carbohydrate diet developed glucose intolerance, increased plasma lipids, hypertension, left ventricular hypertrophy and fibrosis [22].

The physiological, behavioral, and neurochemical mechanisms underlying individual susceptibility to developing obesity have been studied using obesity-prone OM and obesity-resistant S5B rats [14,23–30]. OM rats consume more calories from fat than carbohydrates and gain more weight than S5B rats. Adiposity levels are higher in OM rats and

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consumption of HFD further increases adiposity [14,23,28]. The visceral fat depots of OM rats contain a higher percentage of large adipocytes, more crown-like structures and higher expression levels of pro-inflammatory cytokines, compared to S5B rats. However, the consumption of a HFD in S5B rats significantly increased the expression of pro-inflammatory cytokines and increased the presence of crown-like structures in the epididymal fat depot [14]. Evidence on the effects of HFD consumption on cardio-metabolic dysfunction in OM and S5B rats is limited. Studies indicate that OM rats have higher basal levels of insulin than S5B rats [31] and decreased glucose clearance [32]. Consumption of HFD in OM rats has been shown to induce cardiac hypertrophy and a slight increase in mean arterial pressure [33]. Chronic consumption of a high cholesterol diet in OM rats induces hypertension, enhanced coagulation and platelet aggregation and reduces survival time [34].

The goal of the current study was to examine the effects of HFD intake on cardio-metabolic risk factors using two models that differ in their susceptibility to develop obesity. We hypothesized that obesity-prone OM rats would have an increased prevalence of risk factors associated with glucose intolerance and cardiovascular disease, irrespective of HFD consumption. However, based on our previous study [14], we further hypothesized that HFD intake would lead to a greater increase in cardio-metabolic risk factors in obesity-resistant S5B rats, than OM rats.

2. Material & methods

2.1. Animals

Male obesity-prone, Osborne-Mendel (OM) and obesity-resistant, S5B/PI (S5B) rats (10–11 weeks of age; $n = 6$/group) used in these studies were bred in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved LSU Health Sciences Center vivarium. Rats were individually housed on a 12/12 h light/dark cycle with food and water available ad libitum. Animals were given access to a pelleted high-fat diet (HFD, 60% kcal from fat, Research Diets, D12492, New Brunwick, NJ) or a pelleted low-fat diet (LFD, 10% from fat, D12450B, Research Diets) for 8 weeks. Body weight and estimated percent visceral adiposity ((epididymal weight (g) + retroperitoneal weight (g)/body weight (g)) × 100) was determined at sacrifice. At the time of sacrifice, blood was collected and the left ventricle of the heart (LV) was dissected, weighed, and processed. All procedures were approved by the LSU Health Sciences Center Institutional Animal Care and Use Committee and followed the Guide for the Care and Use of Laboratory Animals.

2.2. Glucose tolerance test

A glucose tolerance test (GTT) was performed to determine fasting glucose levels and glucose regulation, in a separate group of OM and S5B rats ($n = 4$–5/group), fed either HFD or LFD for 8 weeks. Prior to GTT, rats were subjected to an overnight fast (food removed at 1600 h). Following administration of glucose (2 g/kg, intraperitoneal), blood glucose levels were measured using the Contour Glucose Monitoring system (Bayer/Ascensia, Parsippany, NJ) at baseline and subsequently at 15-minute intervals for 90 min following the injection of glucose. Area under the curve was calculated.

2.3. Serum analyses

Serum triglyceride (TG) level was measured using the Triglyceride Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI), total cholesterol, low density lipoprotein cholesterol (LDL), and high-density lipoprotein cholesterol (HDL) levels were determined using Cholesterol Assay Kit - HDL and LDL/VLDL (Abcam, Cambridge, MA). Serum levels of adiponectin (Abcam) and C-reactive protein (CRP; Abcam) were measured using commercially available rat ELISAs. Assays were conducted as described in the protocol provided by the manufacturer.

2.4. Histochemical staining

Sections from the LV were placed in 4% paraformaldehyde, processed for paraffin imbedding and sectioned at a thickness of 5 μm. Hematoxylin and Eosin (H&E) staining and Picrosirius Red staining were performed by the Pennington Biomedical Research Center Cell Biology and Bioimaging Core based on standard protocols. Once stained, slides were scanned and images were processed using NanoZoomer Scanner and Viewer software (Hamamatsu, Bridgewater, NJ) for the assessment of LV thickness (H&E) and using Image J (https://imagej.nih.gov/ij/) for the assessment of relative fibrosis (Picrosirius Red). For LV thickness (mm), four evenly spaced measures of radial thickness of the LV were recorded for each LV section (2 sections/rat) and an average value per rat was determined. For the assessment of fibrosis, eight interstitial regions from two sections of each LV were analyzed. Perivascular collagen was excluded from the measurements. Images were captured (20×) and analyzed using ImageJ software. The mean interstitial collagen was expressed as a percentage of the total area for each LV section and then an average value per rat was determined.

2.5. Real-time polymerase chain reaction PCR

RNA was isolated from sections of the LV using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and RNeasy Mini Kit procedures (Qiagen, Valencia, CA, USA) and based on previous studies [25,26,35–37]. Reverse transcription (RT) was conducted using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). The following primers were used, IL-6: 5′-AAG CCAGAGTCATTCAGAGC-3′ and 5′-GTCCTTAGCCACTCCTTCTG-3′; TNFα: 5′-CCAACAAGGAGGAGAAGTTCCCAA and 5′GAGAAGATGATC TGAGTGTGAGGG-3′; IL-1β: 5′-AGCAGCTTTCGACAGTGAGGAGAA-3′ and 5′-TCTCCACAGCCACAATGAGTGACA-3′; BNP: 5′-GGGCTGTGACG GGCTGAGGTT-3′ and 5′-AGTTTGTGCTGGAAGTAAGA-3′; CTGF: 5′-CGTAGACGGTAA AGCAATGG-3′ and 5′-AGTCAAAGAAGCAGCAAA CAC-3′; ICAM-1: 5′-TTCAACCCGTGCCAGGC and 5′-GTTCGTCTTTCAT CCAGTTAGTCT-3′; VCAM-1: 5′-GAAGCCGGTCATGGTCAAGT-3′ and 5′-GACGGTCACCCTTGAACAGTT C-3′; iNOS: 5′-CAGAAGCAGAATGTG ACCATCAT-3′ and 5′-CGGAGGGACCAGCCAAATC-3′; VEGF: 5′-GAGA ATTCGGCCCCAACCATGAACTTTCTGCT-3′ and 5′-GAGCATGCCCTCCT GCCCGGCTCACCGC-3′ and Cyclophilin: 5′-CCCACCGTGTTCTTCGA CAT -3′ and 5′-CTGTCTTTGGAACTTTGTCCTGCAA-3′. For real-time PCR, SYBR Green Master Mix (Applied Biosystems), forward and reverse primers (10 M), and RT product (10 ng) were added to 384-well plates. The cycling parameters consisted of an initial 2 min incubation at 50 °C, followed by 10 min at 95 °C, then 15 s at 95 °C, and a 1 min annealing/extension step at 60 °C (40 cycles). The mRNA expression of the genes of interest was based on a standard curve and normalized to cyclophilin levels and are shown as fold change to S5B rats fed LFD (ABI Prism 7900 Sequence Detection System, Applied Biosystems).

2.6. Statistical analyses

The effects of individual susceptibility to obesity and consumption of a HFD on cardio-metabolic risk factors were assessed using by 2 × 2 ANOVA, with strain and diet as the experimental variables. For the GTT, a mixed ANOVA was used to assess changes in glucose values over the 90-min test period. A Bonferroni post-hoc test was used to determine differences between groups. A significance level of $p < .05$ was used for all tests.

Table 1
Effects of high fat diet intake on cardio-metabolic risk factors in obesity-resistant S5B/Pl (S5B) and obesity-prone Osborne-Mendel (OM) rats.

	Body weight (g)	Visceral fat (%)	Cholesterol/HDL (mg/dL)	Cholesterol/LDL (mg/dL)	Total cholesterol (mg/dL)	Triglycerides (mg/dL)
S5B/Pl (S5B)						
Low fat diet	345.0 ± 6.3	1.3 ± 0.1	19.6 ± 0.6	11.8 ± 1.1	61.3 ± 2.3	115.5 ± 8.4
High fat diet	356.8 ± 8.5	1.5 ± 0.1	20.4 ± 0.6	13.7 ± 0.9	63.3 ± 2.4	102.2 ± 16.1
Osborne-Mendel (OM)						
Low fat diet	442.6 ± 7.2*	2.8 ± 0.4*	14.3 ± 1.5*	17.3 ± 3.7	59.2 ± 1.8	133.5 ± 5.6*
High fat diet	528.4 ± 16.1*,+	5.8 ± 0.5*,+	15.6 ± 1.6*	18.6 ± 4.7	58.4 ± 1.4	149.2 ± 11.0*

Data are expressed as mean ± SEM.
* $p < .05$, strain effect.
+ $p < .05$, diet effect.

3. Results

Body weight and percent visceral adiposity were measured following 8 weeks of HFD or LFD consumption. An interaction between strain and diet was detected for final body weight ($F = 13.9, p < .001$) and percent visceral adiposity ($F = 27.8$, $p < .001$; Table 1). As previously reported, OM rats weighed more and had higher adiposity than S5B rats. Consumption of HFD increased weight and adiposity in OM rats. Circulating HDL levels were lower ($F = 18.1$, $p < .001$) and circulating triglyceride levels ($F = 8.81, p < .01$) were higher in OM rats, compared to S5B rats (Table 1).

A GTT was performed to assess glucose dysregulation and a significant time x strain interaction was detected ($F = 6.0$, $p < .01$; Fig. 1A). OM rats exhibited a higher peak circulating glucose level at 15 min. Calculated area under the curve in the GTT was higher in OM rats ($F = 7.6$, $p < .02$; Fig. 1B). Circulating adiponectin level was measured to assess insulin sensitivity and adiposity and an interaction between strain and diet was detected ($F = 11.7$, $p < .01$, Fig. 1C). Adiponectin levels were higher in S5B rats compared to OM rats. Consumption of HFD decreased circulating adiponectin levels in S5B rats, but not OM rats. Circulating levels of the inflammatory marker, CRP, was increased by HFD consumption in OM and S5B rats ($F = 4.9$, $p < .05$, Fig. 1D).

The LV was assessed for indicators of hypertrophy, fibrosis, and inflammation. Overall, OM rats had a lower heart to body weight ratio ($F = 76.7$, $p < .001$, Fig. 2A) and lower LV to body weight ratio ($F = 98.2$, $p < .001$, see Fig. 2B) than S5B rats. HFD consumption did not affect these parameters, suggesting that S5B rats had inherently larger hearts and LVs when normalized to their smaller body weight. An interaction between strain and diet was detected for the ratio of LV to heart weight ($F = 8.2$, $p < .01$, Fig. 2C). HFD consumption lowered the LV/heart weight ratio in S5B rats. LV thickness was assessed histologically and an interaction between strain and diet was observed ($F = 5.3$, $p < .05$, Fig. 2D). Post-hoc tests did not reveal any specific differences between groups, however there was a trend for S5B rats fed the HFD to have reduced LV thickness, which was similar to percentage of LV/heart weight. Relative collagen content in the LV, assessed following staining with picrosirius red, did not differ between strains or diets ($p > .05$, data not shown). Gene expression analysis of markers of cardiac collagen content and hypertrophy revealed that OM rats expressed higher levels of brain natriuretic peptide mRNA (BNP; $F = 7.0$, $p < .01$, Fig. 2E) and connective tissue growth factor (CTGF/CCN2; $F = 6.1, p < .05$, Fig. 2F) mRNA than S5B rats. The expression of these genes was not affected by HFD consumption.

Gene expression levels of markers of inflammation, angiogenesis, and oxidative stress were assessed in the LV. Consumption of HFD decreased TNF-α mRNA expression in both strains ($F = 4.7, p < .05$). OM rats had lower mRNA expression of IL-6 ($F = 4.3$, $p < .05$) and IL1-β ($F = 8.8$, $p < .01$) than S5B rats, regardless of diet (Fig. 3A). OM rats expressed lower mRNA levels of vascular endothelial growth factor (VEGF; $F = 17.95, p < .001$; Fig. 3B) than S5B rats. OM rats exhibited lower expression of inducible nitric oxide synthase (iNOS) mRNA, than S5B rats ($F = 22.4$, $p < .001$) and HFD consumption downregulated iNOS expression in both strains ($F = 5.3, p < .05$). Expression level of the adhesion molecule, intracellular adhesion molecule (iCAM), did not differ across strains and was not affected by HFD intake ($p > .05$), however, a significant strain x diet interaction was seen for vascular cell adhesion protein (vCAM) mRNA expression ($F = 4.3$, $p < .05$). Basal levels of vCAM mRNA were higher in OM rats, compared to S5B rats. Levels of vCAM mRNA were upregulated in S5B rats consuming HFD (See Fig. 3B).

4. Discussion

Obesity and excessive adiposity associated with obesity are risk factors for the development of cardio-metabolic dysfunction [1–5]. Obesity is chronic condition characterized by low levels of inflammation and numerous studies suggest that adipose tissue inflammation leads to the development of glucose intolerance and insulin resistance. Insulin resistance and inflammation are thought to be key players in the development of cardiovascular disease [6–13]. Our laboratory has recently investigated the effect of HFD consumption on adipose tissue inflammation in rat models that differ in their susceptibility to develop obesity [14]. As expected, these data indicated that obesity-prone rats expressed higher levels of pro-inflammatory cytokines in visceral fat depots (i.e. epididymal fat). Interestingly, HFD consumption did not further increase the expression of these cytokines. However, in the obesity-resistant S5B rats, HFD consumption significantly increased the expression of pro-inflammatory cytokines and the number of crown-like structures in epididymal fat depots. Therefore, even though the S5B rats did not become “obese” consuming the HFD, we hypothesized that S5B rats may be at risk for the development of obesity-related comorbidities, like CVD, due to increased visceral adipose inflammation [14].

Few studies have investigated cardio-metabolic risk factors in these obesity-prone and resistant rat models. Obesity-prone OM rats have higher basal levels of insulin than obesity-resistant S5B rats [31] and decreased glucose clearance using an insulin clamp [32]. Fitzgerald and colleagues [33] reported a trend toward a HFD-induced increase in mean arterial blood pressure in OM rats and HFD-induced cardiac hypertrophy and Asahina and colleagues [34] reported hypertension in OM rats. Neither of these studies investigated the effects of HFD consumption on cardiac biomarkers of inflammation, fibrosis, hypertrophy or oxidative stress in OM and S5B rats. Therefore, the current study examined the effects of HFD on the presence of cardio-metabolic risk factors in OM and S5B rats. We hypothesized that rats that are prone to developing obesity will have an increased prevalence of cardio-metabolic risk factors compared to obesity-resistant rats. However, based on our previous study, we further hypothesized that consumption of a HFD will exacerbate the development of these cardio-metabolic risk factors in S5B rats, compared to OM rats.

In the current study, elevated levels of several variables associated with an increased risk for the development of cardio-metabolic

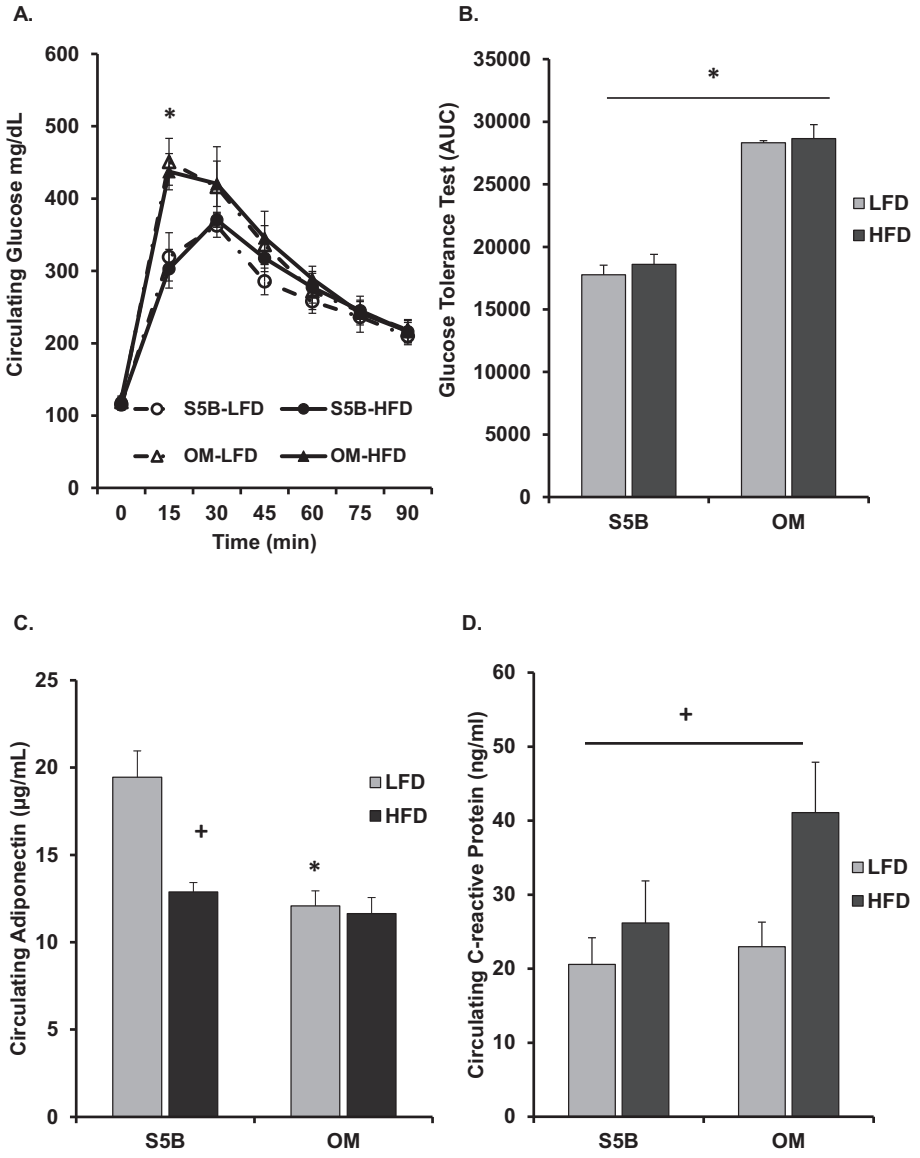

Fig. 1. OM and S5B rats were fed either a HFD or a LFD for 8 weeks. A. Circulating glucose levels were measured for 90 min following glucose administration. OM rats exhibited a higher peak in circulating glucose at 15 min post-glucose administration B. Area under the curve from the GTT was determined. OM rats had a higher AUC than S5B rats. C. Circulating adiponectin levels were lower in OM rats than S5B rats fed LFD. HFD intake decreased adiponectin in S5B rats. D. Circulating CRP levels were higher in rats consuming HFD. + $p < .05$, across diets; * $p < .05$, across strain. Data shown as mean ± SEM.

dysfunction were detected in the OM rats. These include an increase in body weight, body adiposity, decreased HDL cholesterol and increased triglycerides. Though the consumption of HFD increased body weight and adiposity in OM rats, cholesterol and triglycerides were not further exacerbated. The development of glucose tolerance and decreased insulin sensitivity were measured by GTT and circulating adiponectin levels. In the GTT, OM rats showed a significantly greater peak in circulating glucose levels early in the GTT, suggestive of glucose dysregulation. Circulating adiponectin levels, which have an inverse association with adiposity, were higher in S5B rats, compared to OM rats, suggestive of decreased insulin sensitivity and increased adiposity in OM rats. Though consumption of HFD decreased circulating adiponectin levels in S5B rats, HFD intake did not further reduce adiponectin levels in OM rats. Circulating CRP, which is used as a predictor of future coronary events [38], was elevated by HFD consumption in both strains and suggestive of increased systemic inflammation [39–41].

A relationship has been established between adipose tissue inflammation, insulin sensitivity and CVD. Our previous study indicated that adipose tissue inflammation is differentially affected by the susceptibility to develop obesity [14]. In the current study, indices of cardiovascular risk were measured by assessing biomarkers of cardiac hypertrophy, fibrosis and inflammation [42,43]. Interestingly, the S5B rats had larger left ventricles and hearts than the OM rats, when normalized to their smaller body weight. Additionally, LV weight and wall thickness were decreased by the consumption of HFD in S5B rats. Together these data suggest a HFD-induced thinning of the LV wall and reduction in mass in the S5B rats, which may affect cardiovascular health in this strain. To further assess markers of fibrosis and hypertrophy in OM and S5B rats, we measured the expression of CTGF and BNP mRNA. BNP is a peptide hormone released from the LV in response to volume overload and increased pressure. This peptide has been described as a marker of cardiac health and is one of the most relevant markers of cardiac hypertrophy. Overexpression of BNP mRNA in the LV has been detected in a rat model of diet-induced prediabetes [21,44]. In the current study, BNP mRNA expression was elevated in the OM rats, but not the S5B rats, suggesting that OM rats exhibit early signs of cardiac hypertrophy. In addition, CTGF mRNA, which is associated with the increased potential for fibrosis, was higher in the OM

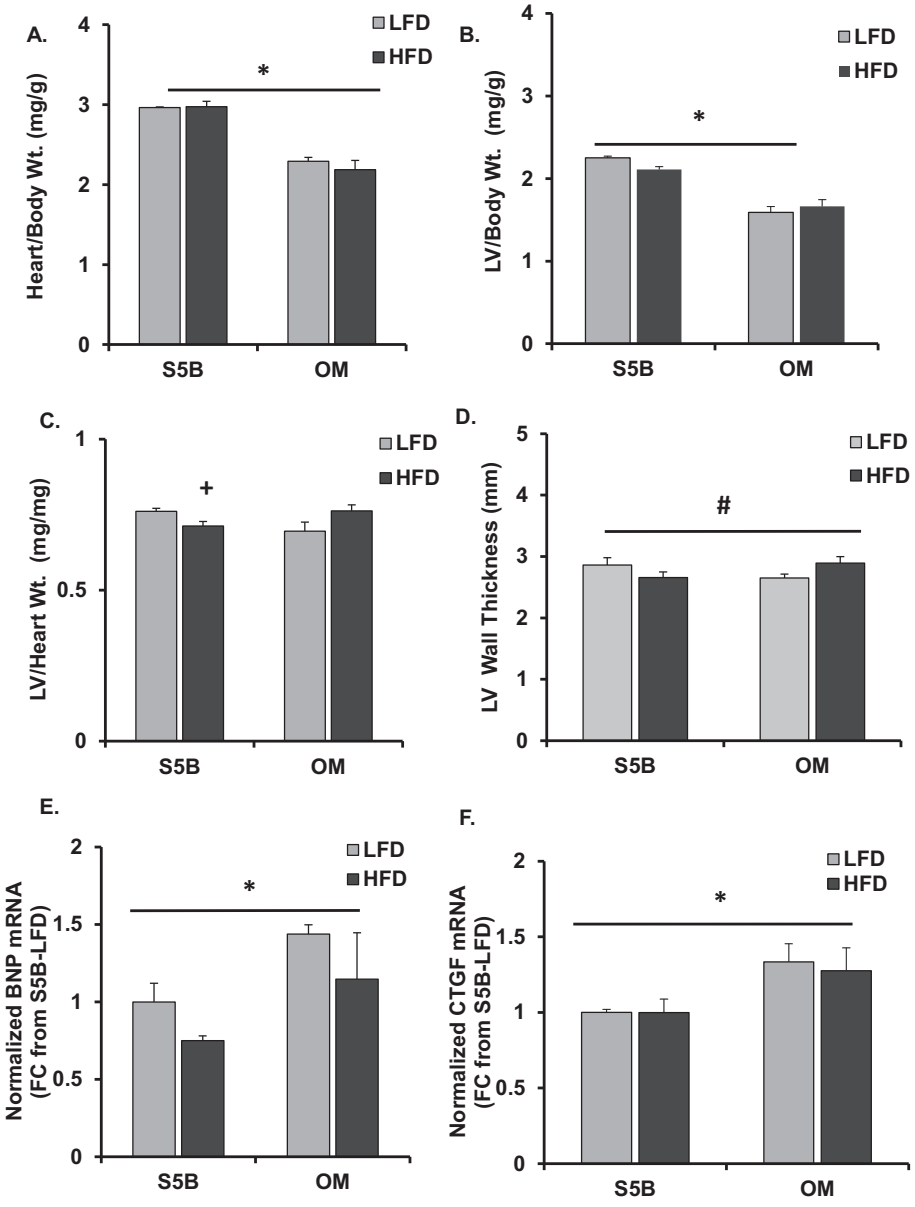

Fig. 2. HFD-induced increases in hypertrophy and fibrosis were assessed. A. The hearts of S5B rats were significantly larger than the hearts of OM rats, when normalized to body weight. B. The left ventricles of S5B rats were significantly larger than the left ventricles of OM rats, when normalized for body weight. C. Consumption of HFD decreased left ventricle weight, normalized to heart weight in S5B rats. D. Diet and strain differentially affected left ventricle wall thickness. E. BNP mRNA level in the left ventricle was higher in OM rats, compared to S5B rats. F. CTGF mRNA expression in the left ventricle was elevated in OM rats, compared to S5B rats. + $p < .05$, across diets; * $p < .05$, across strain. Data shown as mean ± SEM.

rats. Histological measures of collagen content were not indicative of fibrosis in OM rats, suggesting that an increase in gene expression of CTGF may preclude fibrosis.

Assessment of early markers of CVD in the LV of OM and S5B rats included measuring indicators of inflammation, angiogenesis, oxidative stress and endothelial dysfunction. Expression of pro-inflammatory cytokines IL-6 and IL-1β, angiogenic marker, VEGF, and oxidative stress marker, iNOS, were lower in OM rats, compared to the S5B rats. VEGF promotes angiogenesis and increased vascular permeability [45–47]. Previous studies indicate that VEGF mRNA expression in the myocardium is decreased in diabetic rats [46]. In the current study, lower levels of VEGF mRNA are suggestive of decreased angiogenesis and vascular permeability in the hearts of OM rats. Activation of iNOS contributes to the pathogenesis of CVD, most likely due to an increase in reactive oxygen species, but also may serve a protective role against enhanced inflammation and excessive contraction [18,19,48]. In the current study, iNOS mRNA levels were lower in OM rats. Endothelial dysfunction was assessed by measuring iCAM-1 and vCAM-1 mRNA levels in the LV. Both iCAM-1 and vCAM-1 have been implicated in the development of CVD [49,50] and are regulated by TNFα [51]. Basal levels of vCAM-1 mRNA were higher in OM rats and were upregulated by HFD intake in the S5B rats. Higher levels of pro-inflammatory cytokines, angiogenesis and oxidative stress in S5B rats maybe be reflective of their inherent ability to resist the development of obesity, which may, in conjunction with larger hearts and LV serve an adaptive function.

5. Conclusions

Based on our previous studies, OM rats have an increase in visceral adiposity and visceral adipose inflammation compared to S5B rats, however, when fed a HFD, adipose inflammation was increased only in the S5B rats [14]. Data from the current study indicates that cardiometabolic risk factors are differentially regulated by the susceptibility to develop obesity. Metabolic risk factors are more prevalent in OM rats, compared to S5B rats. Multiple measures of CVD were elevated in S5B rats (i.e. heart and LV weight, inflammation, angiogenesis, oxidative stress) and these may be related to their resistance to develop obesity. However, key biomarkers of CVD (i.e. BNP) were elevated in OM rats. Therefore, these data suggest that an increased susceptibility to develop obesity is linked to an increased risk for cardio-metabolic disease. However, the consumption of HFD did not significantly

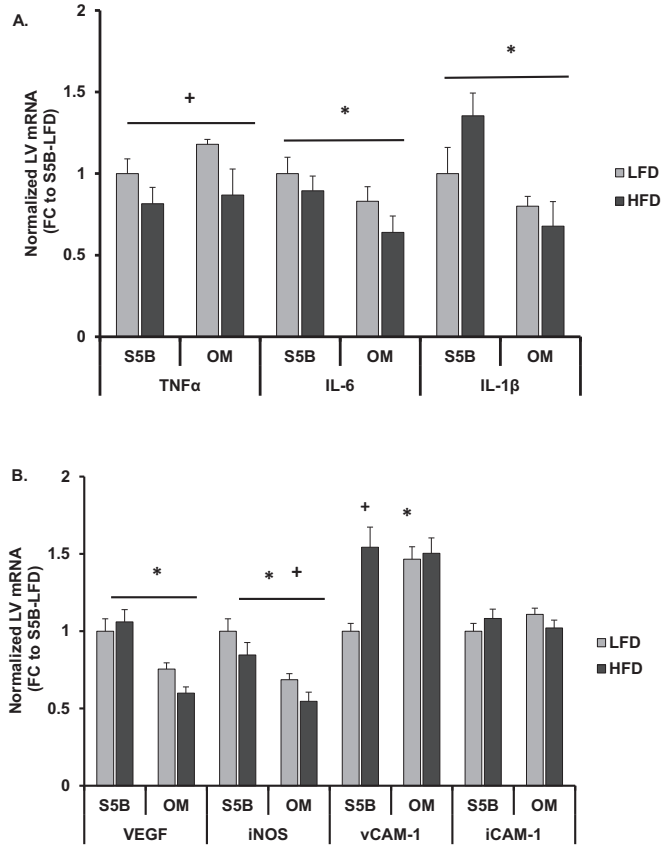

Fig. 3. mRNA levels of indicators of inflammation, angiogenesis, and oxidative stress were assessed in the left ventricle of OM and S5B rats. A. Consumption of HFD decreased expression of TNFα mRNA in the left ventricle of OM and S5B rats. IL-6 mRNA and IL-1β mRNA levels were lower in OM rats, compared to S5B rats. B. VEGF mRNA and iNOS mRNA levels were lower in OM rats compared to S5B rats. iNOS mRNA expression was decreased by HFD consumption. Basal levels of vCAM-1 mRNA were higher in OM rats, compared to S5B rats and were elevated by HFD intake in S5B rats. + $p < .05$, across diets; * $p < .05$, across strain. Data shown as mean ± SEM.

exacerbate these risk factors.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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