Female Rats Fed a High Fat Diet Were Associated with Vascular Dysfunction and Cardiac Fibrosis in the Absence of Overt Obesity and Hyperlipidemia: Therapeutic Potential of Resveratrol (trans-3,5,4′-Trihydroxystilbene)

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Running title: High fat diet and cardiac fibrosis

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Number of Text Pages: 35
Number of Tables: 6
Number of Figures: 4
Number of References: 33
Number of Words in Abstract: 248
Number of Words in Introduction: 385
Number of Words in Discussion: 1,541

Recommended Section: Cardiovascular

ABBREVIATIONS: ANP, atrial natriuretic peptide; BW, body weight; DBP, diastolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; LV, left ventricle; NO, nitric oxide; RT-PCR, reverse transcription – polymerase chain reaction; SBP, systolic blood pressure; SERCA2, sarcoplasmic reticulum Ca^{2+}-ATPase2; SNP, sodium nitroprusside
ABSTRACT

It remains presently unknown whether vascular reactivity is impaired and maladaptive cardiac remodeling occurs prior to the onset of overt obesity and in the absence of hyperlipidemia. Normal female rats were fed a high fat diet for 8 weeks and associated with a modest non-significant increase of body weight (standard diet, 300±10 versus high fat, 329±14 grams) and a normal plasma lipid profile. In rats fed a high fat diet, systolic (171±7 mmHg) and diastolic blood pressures (109±3) were increased compared to a standard diet (SBP, 134±8; DBP, 96±5 mmHg) and acetylcholine-dependent relaxation of isolated aortic rings (high fat, 22±5% versus standard diet, 53±8%) was significantly reduced. Furthermore, perivascular fibrosis was detected in the heart of rats fed a high fat diet. The exogenous addition of resveratrol (trans-3,5,4′-Trihydroxystilbene) (0.1 µM) to aortic rings isolated from rats fed a high fat diet restored acetylcholine-mediated relaxation (47±9%). The administration of resveratrol (20 mg kg⁻¹ d⁻¹ for 8 weeks) to rats fed a high fat diet prevented the increase in blood pressure and preserved acetylcholine-dependent relaxation of isolated aortic rings. However, resveratrol therapy failed to attenuate the perivascular fibrotic response. These data have demonstrated that a high fat diet fed to normal female rats can elicit a hypertensive response and induce perivascular fibrosis prior to the development of overt obesity and in the absence of hyperlipidemia. Resveratrol therapy can prevent the hypertensive response in female rats fed a high fat diet but is without effect on the progression of perivascular fibrosis.
Introduction

Obesity represents a growing health problem in industrialized countries contributing to the onset and/or development of various metabolic-associated disease states including dyslipidemia and type 2 diabetes. Furthermore, in patients diagnosed with metabolic disorders, hypertension and coronary artery disease are prevalent and the subsequent risk of myocardial infarction is increased (Grundy et al., 2006; Kenchaiah et al., 2002; Klein et al., 2004). In the experimental setting, animals provided with a high fat diet and associated with overt obesity were likewise documented with elevated blood pressure (Dobrian et al., 2000, 2001; Erdei et al., 2006). The study by Dobrian et al. (2000) demonstrated that the increase in systolic blood pressure in hypercholesterolemia obese rats that were fed a high fat diet may be related to the 2-fold increase of plasma renin. Furthermore, increased oxidative stress was also highlighted in obese patients and experimental animal models of obesity and delineated as a seminal event contributing to the development of hypertension (Dobrian et al., 2000; Erdei et al., 2006; Van Gaal et al., 2006; Roberts et al., 2001; Vachharajani and Vital, 2006; Yamato et al., 2007). The underlying mechanism attributed to the hypertensive action of oxidative stress is secondary to a reduction of biologically active nitric oxide (NO) via the interaction with superoxide anions (Dobrian et al., 2001; Erdei et al., 2006).

Despite the unequivocal relationship between obesity and cardiovascular disease, it remains presently unknown whether vascular reactivity is impaired and maladaptive remodeling of the heart occurs prior to an overt gain in body weight or a change in the plasma lipid profile. In this regard, the present study tested the hypothesis that an impairment of endothelial-mediated relaxation and a reactive fibrotic response in the heart are prevalent in normal adult female rats fed a high-fat diet prior to the manifestation of overt obesity and in the absence of
hyperlipidemia. Secondly, as previously discussed, elevated oxidative stress represents a putative feature of obesity and implicated in both vascular dysfunction and cardiac fibrosis (Dobrian et al., 2001; Erdei et al., 2006; Lu et al., 2008; Van Gaal et al., 2006). Thus, a second series of experiments tested the hypothesis that the co-administration of the natural antioxidant resveratrol (Baur et al., 2006; Das and Maulik, 2006) would counteract the deleterious effect of a high fat diet on endothelial-mediated relaxation and reactive fibrosis.
Methods

Animal Care. Female Sprague-Dawley strain rats (180-200 grams; Charles River, St-Constant, QC, Canada), were housed on arrival and had access to food and tap water *ad libitum*. The environment was controlled in terms of light (12:12-h light-dark cycle starting at 6:00 AM), humidity, and room temperature (20-23°C). All experiments were performed in compliance with recommendations of the guidelines on the care and use of laboratory animals issued by the Canadian Council on Animal Research and the guidelines of the Animal Care, and approved by the Animal Care Committee of the Montreal Heart Institute.

Diet and Treatment Protocol. One week after their arrival, rats were randomly assigned to either a standard or high fat diet for a period of 8-weeks. The high fat diet consisted of 42% lipids, 36% carbohydrates, and 22% proteins (kcal) and was provided in small pellets (ICN Pharmaceuticals, New York, NY). The standard rat diet consisted of 12.5% lipids, 63.2% carbohydrates, and 24.3% proteins (kcal) (Agribands Purina Canada, Woodstock, ON). To assess the therapeutic benefit of resveratrol, 20 mg kg\(^{-1}\) d\(^{-1}\) was added directly to rat chow and this dose was reported to achieve a peak serum concentration of 1.2 µM (Baur and Sinclair, 2006). Furthermore, an equivalent dose of resveratrol was recently shown to significantly prolong the health and survival of mice fed a high calorie diet (Baur et al., 2006). During the 8 week protocol, the quantity of resveratrol added to the rat chow was adjusted according to changes in body weight measured once a week.

Hemodynamic Measurements and Sirius Red Staining. At the end of the study, rats were anesthetized with a mixture of ketamine (50 mg kg\(^{-1}\); Rogarsetic, Toronto, ON, Canada) and
xylazine (10 mg kg\(^{-1}\); Rompun, Cambridge, ON, Canada). Systolic and diastolic blood pressures were measured with a Millar catheter (2F; model SPR-407, Millar Instrument, Houston, Texas) following insertion through the right carotid. The Millar catheter was then inserted into the left ventricle (LV) to measure left ventricular contractility. Following baseline measurements, a cannula was inserted into the right jugular vein and a submaximal dose of dobutamine (5 µg kg\(^{-1}\) min\(^{-1}\)) was injected at a rate of for a period of 3 minutes (Plante et al., 2005). Blood pressure and contractile indices were analyzed with the program IOX version 1.8.9 (Emka Technologies; Falls Church, VA). Plasma lipid and glucose concentration were determined by standard techniques in blood samples drawn from the carotid artery prior to sacrifice and subsequently placed in tubes containing sodium citrate. The heart was subsequently removed, separated into the right ventricle, LV and septum, weighed and stored at \(-80^0\)C.

In a separate series of experiments, collagen content was determined by Sirius red and staining detected with a Polarizer-Trans U-P110 filter (Olympus, Carson Group Inc., Markham ON, Canada), as previously described (Mercier et al., 2002). Ventricular function and cardiac morphology were not determined in these rats. Briefly, the heart was excised, immersed in 10% formalin and subsequently cut halfway between the base and apex. The apex and base sections of the heart were fixed, dehydrated and embedded in paraffin. Serial cryostat sections (6 µm) of ventricular tissue were prepared. At least 3 distinct regions from the LV were assessed for collagen \(\alpha_1\) type 1 content (normalized to the surface area; mm\(^2\)) and subsequently averaged. With regard to perivascular fibrosis, 4 vessels from each rat were assessed and the lumen area was subtracted from the surface area. In parallel, vascular wall thickness was determined by measuring four distinct regions of the vessel and subsequently averaged. Following the quantification of collagen \(\alpha_1\) type 1, Sirius red staining was visualized with either a 10X/0.3
PLAN-NEOFLUOR or 63X-oil/1.4 DIC PLAN-APOCHROMAT objective mounted on a Zeiss LSM 510 confocal microscope and the emitted signal detected between 560 and 615 nm. Figure 1 represents a maximum projection derived from a z-stack of 0.2 µm slices. The Z-stack was deconvolved with the Huygens Professional 3 software (SVI, Netherland) and the maximum projection rendered with LSM 510 software (Zeiss, Germany).

Vascular Reactivity. Following sacrifice, the aorta was harvested and placed in a modified Krebs bicarbonate solution (composition in mmol L⁻¹: NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, K₂HPO₄ 1.2, NaHCO₃ 25, EDTA 0.026, dextrose 11.1). The aorta was subsequently dissected free of adherent fat and connective tissue and divided into rings (∼ 4 mm in length). The aortic rings were suspended between 2 metal stirrups connected to an isometric force transducer in a chamber containing Krebs bicarbonate solution maintained at 37°C, and oxygenated with a mixture of 95% O₂/5% CO₂. Following 30 minutes of stabilization, tension was progressively increased to the optimal tension of its active length-tension curve (approximately 3.5 g), as determined by the contractile response to potassium chloride (KCl; 30 mmol L⁻¹) at increasing levels of stretch. Maximal contraction was determined with KCl (60 mmol L⁻¹) and aortic rings were excluded if the contractile response to potassium chloride was not observed (exclusion rate of less than 5%). Following extensive washing, aortic rings were incubated in the presence of indomethacin (10⁻⁵ mol L⁻¹) and propranolol (10⁻⁷ mol L⁻¹). Following a 45 minute period of stabilization, phenylephrine (2x10⁻⁹ to 10⁻⁸ mol L⁻¹) was added to achieve a contraction that was a 100% of the maximal contraction observed with KCl (60 mmol L⁻¹). Subsequent endothelial-dependent relaxation of isolated aortic rings was assessed with the addition of acetylcholine (10⁻¹⁰ to 10⁻⁵ mol L⁻¹) and the vascular response expressed as a
percentage of the maximal contraction to phenylephrine. In a separate series of experiments, resveratrol (0.1 µmol L\(^{-1}\)) was added to aortic rings isolated from both rats fed a high fat and standard diet 10 minutes prior to the addition of acetylcholine. Lastly, endothelial-independent relaxation was assessed via the addition of sodium nitroprusside and the vascular response expressed as a percentage of the maximal contraction to phenylephrine.

**Isolation of Total RNA, RT-PCR, and Real-Time PCR.** Total myocardial RNA was isolated by a modification of the guanidine thiocyanate-phenol-chloroform extraction method, as previously described (Calderone et al., 1995). The reverse transcriptase reaction contained 5 ng µL\(^{-1}\) total RNA (each sample), M-MLV reverse transcriptase (800 U), RNaseOUT (40 U), random-hexamer primers (0.04 U), dNTPs (0.5 mmol L\(^{-1}\)), and supplied optimal buffers. The reaction protocol consisted of 3 successive incubation steps at (1) 25°C for 10 minutes, (2) 37°C for 50 minutes, and (3) 70°C for 15 minutes. Real-time polymerase chain reaction (PCR) was performed on 2.5 ng of cDNA template containing the appropriate primers (300 nmol L\(^{-1}\)) and SYBR Green PCR master mix. Primers for each gene were obtained from distinct exons that spanned an intron by using the Ensembl Genome Browser program (http://www.ensembl.org). The sequence specificity of each primer was verified with the Blast program derived from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The primers used are as follow: rat atrial natriuretic peptide (ANP):

Forward 5’AGAGCGGACTAGGCTGCAACA-3’;

Reverse 5’ATTTGGCTGTATCTTCGGTA-3’; rat sarcoplasmic reticulum calcium ATPase (SERCA2):

Forward 5’-TGTATCGACAGGACAGAAAGAGT-3’,
Reverse 5'-TGATGAGCGAGACAGATTCACCTG-3'; rat transforming growth factor-β₁ (TGF β₁):
Forward 5'-CGTGCTAATGGTGACCAGCAACA-3',
Reverse 5'-AGCTCTGCACGGACAGCAAT-3'; rat transforming growth factor-β₃ (TGF-β₃):
Forward 5'-AGAGATCCATAATTCCGACAT-3',
Reverse 5'-ACACATTGAAACGGAAAAACCT; rat connective tissue growth factor (CTGF):
Forward 5'-AGGCCCTGTGAAGCTGACCTAGA-3',
Reverse 5'-TTTTAGGCGGTCCGGATGACT-3'; rat β-actin:
Forward 5'-CCCTAAGGCCAACCGTGGA-3',
Reverse 5'-GAGGCATACAGGGACAACACAG-3'.
Appropriate negative controls were used for each experiment.

**Drugs.** All reagents were prepared daily in ultrapure distilled water with the exception of indomethacin, and resveratrol prepared in ethanol and dimethyl sulfoxide, respectively. Acetylcholine, bradykinin, indomethacin and sodium nitroprusside were obtained from Sigma Chemical Co. (Mississauga, ON, Canada). Phenylephrine was obtained from Cayman Chemical Co. (Ann Arbor, MI), dobutamine from Sandoz Canada Inc. (Boucherville, QC, Canada), propranolol from Biomol Research Laboratories Inc. (Playmouth Meeting, PA). For real-time PCR, M-MLV reverse transcriptase and RNAse OUT were obtained from Invitrogen (Burlington, ON, Canada), random-hexamer primers from Amersham Biosciences (Baie-d’Urfe, QC, Canada), dNTPs from MBI Fermentas (Burlington, ON, Canada) and SYBR Green PCR master mix from Applied BioSystems (Foster, CA). Resveratrol was obtained from Royalmount Pharma (Montreal, QC, Canada).
**Statistical Analysis.** All values are expressed as the mean ± SEM. The half-maximum effective concentration (EC50) of acetylcholine-mediated relaxation of isolated aortic rings was measured from the individual dose-response curve using a logistic curve-fitting program (Allfit for Windows 2.12, Dr. DeLéan, Université de Montréal, Quebec, Canada). The $pD_2$ value, the negative log of the EC50, was likewise obtained with the program Allfit. A one-way analysis of variance was performed to assess differences in collagen content, vessel lumen area and wall thickness and a significant difference was determined by the Bonferroni post-hoc test and a $p$-value less than 0.05 was considered statistically significant (STATVIEW; SAS Institute Inc., Cary, North Carolina). The effects of a high-fat diet and resveratrol treatment on body weight, cardiac morphology, left ventricular contractility, TGF-β3 mRNA and acetylcholine-mediated relaxation (maximal response) of aortic rings were analyzed by a two-way ANOVA (STATISTICA; StatSoft, Tulsa, Oklahoma), applied to determine whether there was an interaction between the two main effects. In the case of a significant interaction, a Tukey (honest significant difference for equal or unequal n) post-hoc test was used to compare diet (high-fat or standard) and treatment (with or without resveratrol) and a $p$-value less than 0.05 was considered statistically significant.
Results

Morphometric Data and the Plasma Lipid Profile. A modest non-significant increase in body weight was observed in female rats fed a high fat diet for a period of 8 weeks as compared to female rats fed a standard diet (Table 1). Despite a modest change in body weight, absolute heart and left ventricular weight were similar in rats fed a high fat compared to rats fed a standard diet (Table 1). In rats fed a high fat diet, plasma cholesterol, HDL, and LDL concentrations were similar to rats fed a standard diet (Table 2). Likewise, plasma glucose levels were comparable between both groups (standard diet, 7.94 ± 1.07; high-fat diet, 7.31 ± 0.79 mmol L⁻¹). Lastly, in the high fat fed rats, plasma triglyceride levels were modestly elevated as compared to rats fed a standard diet, but did not reach statistical significance (Table 2).

Mean Arterial Pressure and Left Ventricular Function. In rats fed a high fat diet, systolic and diastolic blood pressure, mean arterial pressure and left ventricular systolic pressure were significantly (p<0.05) increased compared to rats fed a standard diet (Table 3). Furthermore, left ventricular rate of contraction (+dP/dt) and relaxation (-dP/dt) were likewise increased but did not reach statistical significance (Table 3). Lastly, to assess myocardial reserve, the contractile response to a dobutamine challenge was examined. In rats fed either a standard or high fat diet, the increase in contractile response to dobutamine was identical (Table 4).

Cardiac Remodelling. Despite an increase of mean arterial pressure, the steady-state mRNA levels of ANP and SERCA2 in the LV were comparable in rats fed a high fat and standard diet (Table 5). Employing the Sirius Red technique, collagen α₁ protein content in the LV of rats fed a high fat diet was not significantly increased compared to rats fed a standard diet (Table 6). By
contrast, extensive and significant perivascular fibrosis was detected in rats fed a high fat diet (Table 6 and Fig. 1). Vessel lumen area (standard diet, 0.0122 ± 0.0024 versus high fat diet, 0.0201 ± 0.003 mm²) and wall thickness (standard diet, 0.0128 ± 0.0014; high fat diet, 0.0156 ± 0.002 mm) were not significantly different between rats fed a standard or high fat diet. The reactive fibrotic response in rats fed a high fat diet was not associated with a change in the expression of putative pro-fibrotic peptides as the steady-state mRNA levels of connective tissue growth factor and transforming growth factor-β₁ in the LV were identical to that observed in rats fed a standard diet (Table 5). Moreover, transforming growth factor-β₃ mRNA levels were significantly decreased in the LV of rats fed a high fat diet compared to rats fed a standard diet (Table 5).

**Vascular Reactivity and the in vitro Effect of Resveratrol.** Endothelial-mediated relaxation may be compromised in hypertensive rats fed a high fat diet. Indeed, the maximal relaxation of isolated aortic rings to acetylcholine was significantly decreased (p<0.05) in rats fed a high fat diet (22 ± 5%) compared to rats fed a standard diet (53 ± 8%) (Fig. 2). By contrast, vascular sensitivity (pD₂) to acetylcholine was similar in rats fed a standard (7 ± 0.1) and high fat diet (6.9 ± 0.2). The impaired response to acetylcholine in rats fed a high fat diet was not related to an alteration in soluble guanylate cyclase responsiveness as the administration of the NO donor SNP promoted an identical dose-dependent relaxation of aortic rings from both groups (Fig. 3).

The impaired acetylcholine-mediated relaxation of aortic rings isolated from rats fed a high fat diet may be related to either a decrease of NO synthesis and/or a reduction of NO bioavailability secondary to an increase of vascular oxidative stress. In this regard, the antioxidant resveratrol (0.1 µmol L⁻¹) reported to also stimulate NO synthase activity was added
to aortic rings isolated from rats fed a high fat diet and acetylcholine-mediated relaxation was normalized (47 ± 9%) (Fig. 2). By contrast, the exogenous administration of resveratrol to aortic rings isolated from rats fed a standard diet had no effect on acetylcholine-mediated relaxation (53 ± 5%) (Fig. 2). Lastly, vascular sensitivity (pD2) to acetylcholine of resveratrol-treated aortic rings isolated from rats fed either a standard (7 ± 0.1) or high-fat diet (7 ± 0.1) was similar. Thus, the beneficial in vitro effect of resveratrol on acetylcholine-mediated relaxation of aortic rings isolated from high-fat fed rats provided the impetus to examine whether the in vivo administration of the antioxidant could exert a therapeutic effect.

Morphometric Data and Plasma Lipid Profile: the in vivo Effect of Resveratrol. The administration of resveratrol to rats fed either a standard or high fat diet was associated with a modest but non-significant decrease of overall body weight and body weight gain, as compared to the appropriate untreated group (Table 1). The modest decline of body weight was attributed in part to the reduction of food intake in rats receiving resveratrol (Table 1). Lastly, resveratrol administration did not alter either heart weight, LV weight or the lipid profile in rats receiving either a standard or high fat diet (Tables 1 and 2).

Blood Pressure and Left Ventricular Function: the in vivo Effect of Resveratrol. The administration of resveratrol to rats fed a standard diet caused a modest non-significant decrease in blood pressure, left ventricular systolic pressure and LV dP/dt indices, as compared to untreated rats fed a standard diet (Table 3). By contrast, in rats fed a high fat diet, the concomitant administration of resveratrol prevented the increase of systolic and diastolic blood pressure, mean arterial pressure and left ventricular systolic pressure (Table 3). Moreover, the
administration of the antioxidant to rats fed a high fat diet significantly decreased both LV dP/dt indices as compared to untreated rats. Lastly, resveratrol administration did not influence the dobutamine response in rats fed either a standard or high fat diet (Table 4).

**Cardiac Remodelling: the in vivo Effect of Resveratrol.** Resveratrol did not alter either vessel lumen area (high fat diet, 0.0201 ± 0.003 versus high fat diet + resveratrol, 0.0155 ± 0.003 mm²) or wall thickness (high fat diet, 0.0156 ± 0.002 versus high fat diet + resveratrol, 0.0164 ± 0.002 mm) in rats fed a high fat diet. Furthermore, resveratrol administration to rats fed a high fat diet failed to prevent the progression of perivascular fibrosis (Table 6). In the LV of rats fed a standard diet, resveratrol therapy did not influence TGF-β₃ mRNA expression (3.14 ± 0.29; n = 5). However, the downregulation of TGF-β₃ mRNA (1.49 ± 0.36, n = 8) in rats fed a high fat diet was prevented following the concomitant administration of resveratrol (3.49 ± 0.31; p<0.05 versus high fat; n = 5 rats).

**Vascular Reactivity: the in vivo Effect of Resveratrol.** The administration of resveratrol to rats receiving a standard diet enhanced acetylcholine-mediated relaxation of aortic rings, as compared to untreated rats (Fig. 4). Moreover, resveratrol treatment of rats fed a high fat diet significantly improved acetylcholine-mediated relaxation of isolated aortic rings and the magnitude of relaxation was comparable to that observed in rats fed a standard diet (Fig. 4). The vascular sensitivity (pD₂) to acetylcholine was unchanged in both resveratrol-treated rats groups (resveratrol+standard diet; 7 ± 0.02, resveratrol+high-fat diet; 7 ± 0.12), as compared to their respective untreated groups.
Discussion

The relationship between obesity and cardiovascular disease has been unequivocally established clinically and in experimental models. Despite these observations, it remains presently unknown whether vascular reactivity is impaired and maladaptive cardiac remodeling occurs prior to an overt gain in body weight and in the absence of hyperlipidemia. The present study has demonstrated that normal female rats provided with a high fat diet for a period of 8 weeks were associated with a significant elevation of blood pressure and extensive perivascular fibrosis in the myocardium despite a non-significant increase in body weight and a normal plasma lipid profile and glucose concentration. Furthermore, the blood pressure increase in rats fed a high fat diet was prevented by the co-administration of the antioxidant resveratrol. However, the therapeutic effect was limited to the vasculature as resveratrol treatment failed to attenuate the perivascular fibrotic response in the heart of rats fed a high fat diet.

An important paradigm established in the present study was that feeding normal female rats a high fat diet for a period of 8 weeks caused a modest non-significant increase in body weight and plasma triglyceride levels without a change in the plasma lipid profile or glucose concentration. The administration of a comparable high fat diet in a similar time frame to normal male rats led to a significant increase in body weight (Dobrian et al., 2000; Erdie et al., 2006; Roberts et al., 2001). Likewise, a similar sex-dependent relationship with regard to body weight gain was documented in various strains of mice fed a high fat diet (Nishikawa et al., 2007). Thus, these data further support the premise that the effect of a high fat diet on body weight gain is temporally delayed in female rats as compared to male rats.

Elevated blood pressure was reported in normal male rats fed a high fat diet characterized by a marked increase in body weight gain and hyperlipidemia (Dobrian et al., 2000; Erdie et al.,
In the present study, normal female rats fed a high fat diet were likewise associated with an increase in blood pressure and left ventricular systolic pressure. However, these changes occurred prior to a significant increase of body weight and in the absence of hyperlipidemia and hyperglycaemia. Previous studies have demonstrated that elevated mean arterial pressure in obese rats was related to an impairment of endothelium-mediated relaxation of vascular tissue (Dobrian et al., 2001; Erdie et al., 2006; Galili et al., 2006). In the present study, acetylcholine-mediated relaxation of aortic rings isolated from rats fed a high fat diet was significantly compromised, whereas sodium nitroprusside-mediated relaxation was intact. Collectively, these data suggest that either NO production was diminished and/or bioavailability was reduced secondary to the increased production of superoxide anions (Dobrian et al., 2001; Erdie et al., 2006; Roberts et al., 2006; Galili et al., 2006). In addition to its well established antioxidant property, resveratrol was also reported to increase NO synthesis (Wallerath et al., 2002; Wang et al. 2007). In the present study, the exogenous administration of resveratrol to aortic rings isolated from rats fed a high fat diet normalized acetylcholine-mediated relaxation. Thus, these data strongly support the premise that impaired vascular relaxation in female rats fed a high fat diet was related to either an increased oxidative stress and/or decreased synthesis of NO and occurred in the absence of overt obesity, hyperlipidemia or hyperglycaemia.

It has been well established that elevated systemic arterial hypertension promotes a concentric pattern of cardiac hypertrophy characterized by the increased expression of ANP mRNA and concomitant downregulation of SERCA2 mRNA (Grossman et al., 1975; Calderone et al., 1995). In the present study, despite an increase in blood pressure in rats fed a high fat diet, neither ANP nor SERCA2 mRNA levels were altered in the LV. These data were consistent with the lack of change of either absolute LV weight or LV/BW ratio in rats fed a high fat diet as
compared to rats fed a standard diet. Thus, at least in female rats fed a high-fat diet for a period of 8 weeks, elevated blood pressure was not associated with a concomitant hypertrophic response. It is possible that the increase in blood pressure was insufficient to promote hypertrophy or a more chronic exposure to a hypertensive state was required. By contrast, an extensive perivascular fibrotic response was observed in the heart of female rats fed a high-fat diet. Likewise, collagen α1 protein content was modestly increased in the myocardium of female rats fed a high-fat diet but did not reach statistical significance. Collectively, these data were consistent with previous studies demonstrating the presence of a reactive fibrotic response in the myocardium of various experimental animal models of obesity (Carrol and Tyagi, 2005; Tarikuz Zaman et al., 2004). However, the present study has revealed that the reactive fibrotic response in the heart of female rats fed a high-fat diet represents an early maladaptive event independent of overt obesity, hyperlipidemia and hyperglycaemia. Furthermore, the induction of putative pro-fibrotic peptides in the heart of obese animals were implicated in reactive fibrosis (Carrol and Tyagi, 2005; Tarikuz Zaman et al., 2004). Unexpectedly, a significant downregulation of transforming growth factor-β3 mRNA was observed in the LV of rats fed a high-fat diet. Presently, the underlying mechanism(s) attributed to the latter paradigm remains unknown. Moreover, neither connective tissue growth factor nor transforming growth factor-β1 mRNAs were upregulated in the myocardium of female rats fed a high-fat diet. Despite these findings, we cannot exclude the possibility that these peptide growth factors were selectively increased in the vasculature, thereby directly contributing to the perivascular fibrotic response (Tarikuz Zaman et al., 2004; Ruiz-Ortega et al., 2007). Lastly, the suppression or attenuation of anti-fibrotic events may likewise contribute to cardiac fibrosis. Indeed, a dysfunction of NO signaling is sufficient to promote both reactive fibrosis and the upregulation of putative pro-fibrotic peptides in the heart.
(Goto et al., 1999; Ruetten et al., 2005) and the present study has demonstrated that either a decrease in NO synthesis or bioavailability was reduced in the aorta of female rats fed a high fat diet.

The efficacy of exogenous resveratrol to normalize acetylcholine-mediated relaxation of aortic rings isolated from rats fed a high fat diet provided the impetus to assess whether the \textit{in vivo} administration would likewise attenuate the rise in blood pressure. Indeed, resveratrol administration to rats fed a high fat diet prevented the hypertensive response. Furthermore, the beneficial \textit{in vivo} hemodynamic effect of resveratrol was associated with the preservation of acetylcholine-mediated relaxation of aortic rings isolated from rats fed a high fat diet. Thus, these data support the premise that resveratrol represents an appropriate pharmacological approach to prevent vascular dysfunction in female rats fed a high fat diet. The scavenging of superoxide anions, increasing NO synthesis as well as the phytoestrogenic property of resveratrol represent underlying mechanisms that may have synergistically prevented the rise of mean arterial pressure in rats fed a high fat diet (Gehm et al., 1997; Wallerath et al, 2002; Baur and Sinclair, 2006; Wang et al, 2007).

The relationship between hypertension and cardiac fibrosis has been well established (Berk et al., 2007). Moreover, increased oxidative stress represents a putative feature of obesity and was further shown to participate in the progression of cardiac fibrosis either directly or indirectly via the suppression of NO synthesis (Dobrian et al., 2000; Erdei et al., 2006; Ruetten et al., 2005; Lu et al., 2008). In this regard, an attenuation of the perivascular fibrotic response would be expected in the heart of resveratrol-treated rats fed a high fat based on the beneficial effect on blood pressure and its established antioxidant property. Furthermore, a direct anti-fibrotic action was identified \textit{in vitro} as resveratrol suppressed cardiac fibroblast proliferation.
(Olson et al., 2005; Wang et al., 2007). Despite these observations, the \textit{in vivo} administration of resveratrol failed to prevent the progression of perivascular fibrosis in the heart of rats fed a high fat diet. Nonetheless, cardiac remodeling was sensitive to resveratrol therapy as the decreased expression of transforming growth factor-\(\beta_3\) mRNA in the heart of rats fed a high fat diet was prevented. The latter beneficial effect may be related in part to the phytoestrogenic property of resveratrol as 17\(\beta\)-estradiol treatment of either cardiac fibroblasts or osteoclasts increased transforming growth factor-\(\beta_3\) mRNA expression (Yang et al., 1996; Mercier et al., 2002a).

Thus, in female rats fed a high fat diet, the perivascular fibrotic response was at least independent of a rise in blood pressure. Furthermore, the normalization of acetylcholine-mediated relaxation of aortic rings isolated from resveratrol-treated female rats fed a high fat diet indirectly suggests that the increased perivascular fibrotic response in these hearts may not be secondary to a decreased bioavailability or synthesis of NO.

In conclusion, the present study has demonstrated that prior to the development of overt obesity and in the absence of hyperlipidemia and hyperglycaemia, female rats fed a high fat diet were associated with vascular dysfunction and perivascular fibrosis. Resveratrol administration preserved vascular function whereas the perivascular fibrotic response in the heart of female rats fed a high fat diet persisted. Thus, these data demonstrate that disparate events are linked to the development of hypertension and perivascular fibrosis in female rats fed a high fat diet.
Acknowledgements

The authors would like to thank Marie-Pierre Mathieu for her technical assistance and Antoinette Paolitto for excellent secretarial assistance.
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and Metabolism: endorsed by the American College of Cardiology Foundation. *Circulation* **110**:2952-2967.


Footnotes

Financial Support:
This work was supported by the Heart and Stroke Foundation of Canada and Quebec, Canadian Institutes of Health Research, and "Fonds de la Recherche de l’Institut de Cardiologie de Montréal" (FRICM). Marie-Claude Aubin is a PhD student funded by the Heart and Stroke Foundation of Canada. Louis P. Perrault is a Chercheur-Boursier Senior and Angelino Calderone is a Chercheur-Boursier National of the ‘Fonds de la Recherche en Santé du Québec (FRSQ).

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Legends for Figures

Figure 1. Sirius Red staining. Modest level of collagen $\alpha_1$ type 1 protein was detected surrounding blood vessels in rats fed a standard diet (Panels A1 & A2). By contrast, the perivascular deposition of collagen was markedly higher in rats fed a high fat diet (Panels B1 & B2). Furthermore, the administration to resveratrol to rats fed a high fat diet (Panels C1 & C2) did not prevent the perivascular fibrotic response. Panels A1, B1 and C1 were taken at 10X; panels A2, B2, and C2 taken at 63X. Vessel lumen area and wall thickness were not statistically different among the groups (please see Results section).

Figure 2. Acetylcholine-mediated relaxation of aortic rings and the in vitro effect of resveratrol administration. In rats fed a high fat diet (□), acetylcholine-mediated relaxation was significantly reduced compared to female rats fed a standard diet (◊). The exogenous administration of resveratrol (0.1 $\mu$mol L$^{-1}$) to aortic rings isolated from rats fed a standard diet (♦) did not influence acetylcholine-mediated relaxation. By contrast, acetylcholine-mediated relaxation was normalized following the exogenous administration of resveratrol to aortic rings isolated from rats fed a high fat diet (■). Acetylcholine-mediated relaxation is expressed as a percentage of the maximal contraction to phenylephrine. Results are presented as mean ± S.E.M. and (*) denotes p<0.05 versus all groups as determined by a two-way ANOVA.

Figure 3. Sodium nitroprusside mediated relaxation of aortic rings. Sodium nitroprusside mediated relaxation of aortic rings isolated from female rats fed either a standard (◊) or high fat diet (□) was equivalent. Sodium nitroprusside-mediated relaxation is expressed as a percentage of the maximal contraction to phenylephrine. Data are presented as mean ± S.E.M.
Figure 4. Acetylcholine-mediated relaxation of aortic rings and the in vivo effect of resveratrol administration. In rats fed a high fat diet (□), acetylcholine-mediated relaxation was significantly reduced compared to female rats fed a standard diet (◊). The in vivo administration of resveratrol for a period of 8 weeks significantly enhanced acetylcholine-mediated relaxation of aortic rings isolated from rats fed a standard diet (●). Moreover, acetylcholine-mediated relaxation remained intact in resveratrol-treated rats fed a high fat diet (■). Acetylcholine-mediated relaxation is expressed as a percentage of the maximal contraction to phenylephrine. Data are presented as mean ± SEM. (*) denotes p<0.05 versus standard diet; (**) denotes p<0.05 versus respective resveratrol-treated group as determined by a two-way ANOVA.
TABLE 1

Body and heart weight in female rats fed a standard or high fat diet and the effect of resveratrol (RES)

Data are presented as mean ± S.E.M. (n = number of rats per group)

<table>
<thead>
<tr>
<th></th>
<th>Standard (n = 9)</th>
<th>High Fat (n = 10)</th>
<th>Standard+RES (n = 10)</th>
<th>High Fat+RES (n =10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>222 ± 4</td>
<td>228 ± 3</td>
<td>227 ± 3</td>
<td>227 ± 2</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>300 ± 10</td>
<td>330 ± 14</td>
<td>289 ± 3</td>
<td>301 ± 7</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>78.0 ± 8</td>
<td>102 ± 17</td>
<td>62 ± 4</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>17.8 ± 0.2</td>
<td>17.7 ± 0.2</td>
<td>17.0 ± 0.3*</td>
<td>16.5 ± 0.2</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>0.41 ± 0.02</td>
<td>0.45 ± 0.02</td>
<td>0.41 ± 0.01</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.88 ± 0.02</td>
<td>0.93 ± 0.04</td>
<td>0.90 ± 0.02</td>
<td>0.91 ± 0.02</td>
</tr>
<tr>
<td>Ratio LV/body weight</td>
<td>1.36 ± 0.04</td>
<td>1.34 ± 0.05</td>
<td>1.42 ± 0.05</td>
<td>1.33 ± 0.04</td>
</tr>
<tr>
<td>Ratio heart/body weight</td>
<td>2.9 ± 0.1</td>
<td>2.81 ± 0.06</td>
<td>3.14 ± 0.04</td>
<td>3.04 ± 0.09</td>
</tr>
</tbody>
</table>

*p<0.05 versus standard diet as determined by a two-way ANOVA.
TABLE 2
The plasma lipid profile in female rats fed a standard or high fat diet and the effect of resveratrol (RES)

Data are presented as mean ± S.E.M.  \( (n) = \) number of rats per group

<table>
<thead>
<tr>
<th></th>
<th>Standard ((n = 9))</th>
<th>High Fat ((n = 10))</th>
<th>Standard+RES ((n = 6))</th>
<th>High Fat+RES ((n = 5))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol L(^{-1}))</td>
<td>2.15 ± 0.14</td>
<td>2.33 ± 0.19</td>
<td>1.84 ± 0.09</td>
<td>2.23 ± 0.17</td>
</tr>
<tr>
<td>HDL (mmol L(^{-1}))</td>
<td>0.75 ± 0.04</td>
<td>0.72 ± 0.05</td>
<td>0.66 ± 0.02</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>LDL (mmol L(^{-1}))</td>
<td>0.64 ± 0.16</td>
<td>0.54 ± 0.24</td>
<td>0.69 ± 0.13</td>
<td>0.61 ± 0.33</td>
</tr>
<tr>
<td>Triglycerides (mmol L(^{-1}))</td>
<td>1.69 ± 0.24</td>
<td>2.42 ± 0.44</td>
<td>1.11 ± 0.22</td>
<td>1.80 ± 0.46</td>
</tr>
</tbody>
</table>

HDL, high density lipoprotein; LDL, low density lipoprotein.
TABLE 3

MAP and left ventricular contractility in female rats fed a standard or high fat diet and the effect of resveratrol (RES)

Data are presented as mean ± S.E.M. (n = number of rats per group)

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>High Fat</th>
<th>Standard+RES</th>
<th>High Fat+RES</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 8)</td>
<td></td>
<td>(n = 10)</td>
<td>(n = 8)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>254 ± 5</td>
<td>242 ± 6</td>
<td>244 ± 12</td>
<td>244 ± 9</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>134 ± 8</td>
<td>171 ± 7*</td>
<td>115.0 ± 7</td>
<td>107 ± 6**</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>96 ± 5</td>
<td>109 ± 3*</td>
<td>89 ± 5</td>
<td>81 ± 5**</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>108 ± 6</td>
<td>130 ± 4*</td>
<td>97 ± 6</td>
<td>90 ± 5**</td>
</tr>
<tr>
<td>LV systolic pressure (mmHg)</td>
<td>122 ± 6</td>
<td>148 ± 9*</td>
<td>106 ± 5</td>
<td>106 ± 4**</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>9.1 ± 1.0</td>
<td>10.9 ± 2</td>
<td>6.3 ± 0.7</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>LV + dP/dt</td>
<td>6068 ± 289</td>
<td>6973 ± 282</td>
<td>5297 ± 520</td>
<td>5564 ± 249**</td>
</tr>
<tr>
<td>LV – dP/dt</td>
<td>-5189 ± 312</td>
<td>-6012 ± 333</td>
<td>-4257 ± 503</td>
<td>-4336 ± 263**</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure.

*p<0.05 versus standard diet.

**p<0.05 versus high fat diet as determined by a two-way ANOVA.
TABLE 4

Cardiac response to dobutamine infusion (5 µg kg⁻¹ min⁻¹) in female rats fed a standard or high fat diet and the effect of resveratrol (RES)

Data are presented as mean ± S.E.M.  (n = number of rats per group)

<table>
<thead>
<tr>
<th>Dobutamine Response (% Change versus Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
</tr>
<tr>
<td>(n = 7)</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
</tr>
<tr>
<td>+dP/dt (mmHg/sec)</td>
</tr>
<tr>
<td>-dP/dt (mmHg/sec)</td>
</tr>
</tbody>
</table>
TABLE 5
mRNA expression in the left ventricle of female rats fed a standard or high fat diet

Data are presented as mean ± S.E.M. (n = number of rats per group) and normalized to β-actin mRNA

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>High Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP (n = 8)</td>
<td>10.60 ± 1.97</td>
<td>11.65 ± 2.10</td>
</tr>
<tr>
<td>SERCA-2 (n = 8)</td>
<td>1.67 ± 0.21</td>
<td>1.43 ± 0.12</td>
</tr>
<tr>
<td>TGF β1 (n = 4)</td>
<td>0.36 ± 0.07</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>TGF β3 (n = 8)</td>
<td>3.38 ± 0.50</td>
<td>1.49 ± 0.36*</td>
</tr>
<tr>
<td>CTGF (n = 4)</td>
<td>7.57 ± 0.98</td>
<td>6.32 ± 1.05</td>
</tr>
</tbody>
</table>

*p<0.05 versus standard diet.
TABLE 6

Left ventricular and perivascular collagen α₁ type 1 content in rats fed a standard or high fat diet and the effect of resveratrol (RES)

Data are presented as mean ± S.E.M. (n = number of animals per group) and normalized to surface area (mm²).

<table>
<thead>
<tr>
<th></th>
<th>Standard (n = 3)</th>
<th>High Fat (n = 3)</th>
<th>High Fat+RES (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left ventricle</td>
<td>0.0057 ± 0.002</td>
<td>0.0092 ± 0.002</td>
<td>0.0129 ± 0.001</td>
</tr>
<tr>
<td>Perivascular</td>
<td>0.1141 ± 0.03</td>
<td>0.267 ± 0.02*</td>
<td>0.324 ± 0.02*</td>
</tr>
</tbody>
</table>

*p<0.05 versus standard diet as determined by a one-way ANOVA.
Figure 3

Relaxation

Log SNP (M)