Arterial Stiffening Precedes Systolic Hypertension in Diet-Induced Obesity


See Editorial Commentary, pp 1003–1004

Abstract—Stiffening of conduit arteries is a risk factor for cardiovascular morbidity. Aortic wall stiffening increases pulsatile hemodynamic forces that are detrimental to the microcirculation in highly perfused organs, such as the heart, brain, and kidney. Arterial stiffness is associated with hypertension but presumed to be due to an adaptive response to increased hemodynamic load. In contrast, a recent clinical study found that stiffness precedes and may contribute to the development of hypertension although the mechanisms underlying hypertension are unknown. Here, we report that in a diet-induced model of obesity, arterial stiffness, measured in vivo, develops within 1 month of the initiation of the diet and precedes the development of hypertension by 5 months. Diet-induced obese mice recapitulate the metabolic syndrome and are characterized by inflammation in visceral fat and aorta. Normalization of the metabolic state by weight loss resulted in return of arterial stiffness and blood pressure to normal. Our findings support the hypothesis that arterial stiffness is a cause rather than a consequence of hypertension. (Hypertension. 2013;62:1105-1110.) • Online Data Supplement

Key Words: hypertension □ inflammation □ obesity □ pulse wave velocity □ vascular stiffness

The aorta and its major branches stiffen with age and obesity, independently of atherosclerosis. In recent epidemiological and clinical studies, arterial stiffness emerged as an independent predictor of cardiovascular events, even after adjusting for risk factors such as age, sex, body mass index, and blood pressure.

Hypertension is associated with arterial stiffness, and conventional thinking suggests that hypertension stimulates aortic remodeling and stiffening, in addition to vascular smooth muscle cell hypertrophy, as an adaptive process to increase wall-to-lumen width ratio in response to long-term changes in hemodynamic forces. However, a large longitudinal study of the general population recently established that arterial stiffness precedes an increase in systolic blood pressure and newly diagnosed hypertension. Initial blood pressure was not independently predictive of subsequent aortic stiffening measured in the same individuals 4 to 10 years later. Although stiffness-induced hemodynamic changes have been implicated in the development of hypertension, lack of animal models for studying the relationship between arterial stiffness and hypertension has hampered the discovery of mechanisms and has led to a recent effort by the National Heart, Lung, and Blood Institute of the National Institutes of Health to establish such models.

Because of the rising prevalence of obesity-associated type 2 diabetes mellitus and the cardiovascular complications thereof, we sought to determine whether arterial stiffness occurs in a mouse model of diet-induced obesity. Arterial stiffness is increased in obese and diabetic individuals, even at a young age (10–24 years), and in a genetic model of obesity (ob/ob mice). Conversely, weight loss in overweight and obese individuals is associated with a reduction in arterial stiffness. Understanding the mechanisms underlying the development and regression of arterial stiffness might lead to additional options for prevention and treatment of hypertension and associated complications.

The goals of this study were to determine the temporal relationship between the development of arterial stiffness and hypertension and the potential reversal of stiffness and hypertension by return to normal diet (ND), as well as the mechanisms thereof.

Received May 20, 2013; first decision June 6, 2013; revision accepted August 22, 2013.
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The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.113.01744/-/DC1.
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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.113.01744

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Methods
A detailed description of Methods can be found in online-only Data Supplements.

Results
High-Fat/High-Sucrose Diet Recapitulates Human Metabolic Syndrome in Mice
High-fat/high-sucrose (HFHS) feeding significantly increased body weight (Figure S1A in the online-only Data Supplement) and fat mass compared with ND by 2 months, whereas lean mass remained unchanged over time (Figure S1B). Also within 2 months, mice became glucose intolerant (Figure S1C) and insulin resistant (Figure S1D). In addition to obesity and glucose intolerance, HFHS-fed mice developed chronic inflammation, as evidenced by substantial infiltration of activated macrophages in the visceral fat surrounding the kidneys (Figure S2A). Microalbuminuria, an indicator of kidney damage, was significantly increased after 4 months of HFHS diet (Figure S2B).

Arterial Stiffness Is Increased by HFHS Diet and Precedes Systolic Hypertension
Pulse wave velocity (PWV), an index of arterial stiffness measured by ultrasonography, was significantly increased by 2.4-fold after 1 month of HFHS compared with ND, and it remained elevated ≤8 months (Figure 1A). PWV was similarly elevated when assessed from the aortic arch to abdominal aorta as it was in the abdominal aortic segment (6.3±1.3 versus 3.4±0.6 mm/ms in ND and 5.7±0.9 versus 2.8±1.0 mm/ms in ND, respectively; P<0.05). The increase in PWV was confirmed by invasive hemodynamic measurements both at baseline and after phenylephrine (Figure 1B and Table S1).

To address the temporal relationship between arterial stiffness and hypertension, young mice (8 weeks old) were implanted with radiotelemetry pressure transducers and followed after starting HFHS for ≤1 year. The first statistically significant increase in systolic blood pressure (Figure 1C) and mean arterial pressure (Figure S3A) was observed after 6 months, whereas diastolic blood pressure was not significantly different (Figure S3B). Pulse pressure, an indirect index of arterial stiffness, gradually increased and reached statistical significance at 6 months (from baseline 26.4±2.0 to 37.8±2.2 mm Hg, n=4–6; Figure S3C). Systolic blood pressure (Figure 1D), mean arterial pressure (Figure S3D), and pulse pressure (Figure S3F) were also significantly and consistently elevated in HFHS-fed mice (n=11) compared with ND-fed mice (n=9) when blood pressure transducers were implanted between 5 and 6 months of diet and values were recorded for 1 month thereafter.

Spectral analysis of high-frequency telemetry recordings, performed to assess whether obesity-associated activation of the sympathetic nervous system21 could contribute to the hypertension observed after 6 months of HFHS diet, showed increased sympathetic modulation of blood pressure and heart rate in mice fed HFHS diet for 8 months compared with ND (Table S2). This was accompanied by significantly increased plasma norepinephrine in mice fed HFHS for 8 months compared with ND but not for 2 months (Table S3). Consistent with HFHS-associated sympathetic activation, heart rate was slightly but significantly elevated in HFHS-fed mice (Figure S3G) compared with ND-fed mice (Figure S3H), and this was reduced to normal values when obese mice were reversed to ND (Figure S3I).

Early Vascular Changes in HFHS-Fed Mice
Within 2 months of diet when the increase in PWV because of HFHS diet was fully developed, the aortas from HFHS mice had impaired relaxation to acetylcholine (Figure 2A), indicating reduced endothelial nitric oxide (NO) function. In addition, the activity of tissue transglutaminase-2 (TG-2), an NO-sensitive enzyme that contributes to arterial stiffening in aged mice and rats by increasing extracellular matrix cross-linking,22 was significantly increased in aortic lysates from HFHS-fed mice, consistent with reduced NO bioavailability and mean arterial pressure (Figure S3A) was observed after 6 months, whereas diastolic blood pressure was not significantly different (Figure S3B). Pulse pressure, an indirect index of arterial stiffness, gradually increased and reached statistical significance at 6 months (from baseline 26.4±2.0 to 37.8±2.2 mm Hg, n=4–6; Figure S3C). Systolic blood pressure (Figure 1D), mean arterial pressure (Figure S3D), and pulse pressure (Figure S3F) were also significantly and consistently elevated in HFHS-fed mice (n=11) compared with ND-fed mice (n=9) when blood pressure transducers were implanted between 5 and 6 months of diet and values were recorded for 1 month thereafter.

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Figure 1. Arterial stiffness precedes hypertension in diet-induced obese mice. Pulse wave velocity (PWV, mm/ms), an index of arterial stiffness, measured by Doppler echocardiography, mean±SD, n=4 to 10 each group (A) and invasively with high-fidelity pressure catheters, mean±SEM, n=8 each group (B) is increased in high-fat/high-sucrose (HFHS)–fed mice within 2 months. Mean arterial pressure was modulated by intravenous infusion of phenylephrine (0.1 μg/g body weight). *P<0.05 vs normal diet (ND) or ND baseline; #P<0.05 vs HFHS baseline; $P<0.05 vs ND-phenylephrine. Mice develop systolic hypertension after 6 months of HFHS (C) compared with ND (D). *P<0.05 vs baseline (time 0) or ND. SBP indicates systolic blood pressure.
weeks, obese mice lost 12.5% of body weight and returned to the body weight of ND-fed mice within 2 months of diet reversal (Figure 3A, left). A 50% loss in fat mass occurred with no significant change in lean mass and was accompanied by normalization of hyperinsulinemia (Figure 3B), indicating amelioration of metabolic state.

Similarly, PWV was reduced to control values within 2 months of diet reversal (Figure 3A, right). At this time point, systolic blood pressure and mean arterial pressure were significantly reduced to values not significantly different from those in control mice (Figure 3C).

In Vitro Aortic Stiffness, Aortic Inflammation, and Oxidant Stress Are Reduced After Return to ND

The force required for a fixed indentation of the basement membrane of endothelium-denuded aorta showed that HFHS significantly increased stiffness by 2-fold (52±4.8 kPa, n=7 versus 24±2.8 kPa in ND, n=10). This returned to normal levels after diet reversal in HFHS/ND (30.5±3.7 kPa, n=8; Figure 4A).

HFHS diet stimulated aortic hypertrophy compared with ND (medial area: 0.135±0.014 in HFHS versus 0.100±0.005 mm² in ND; P<0.05). Although this might have contributed to aortic stiffness, medial area remained unchanged after reversal to ND (0.14±0.02 mm², n=8; Figure 4B).

HFHS-induced upregulation of inflammatory genes in aortas was normalized after reversal to ND (Figure 4C). In addition, immunostaining of aortic sections with a specific anti-N-ε-(γ-glutamyl)-lysine antibody, indicative of TG-2 activity, revealed reduced extracellular matrix cross-links after diet reversal (Figure 5A) despite no change in TG-2 expression between different diet groups (Figure S5). Aortic immunostaining with a specific antisulfonylated sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) showed that HFHS increased SERCA oxidation, used as an index of oxidant stress,23–25 compared with ND, and this was decreased by reversal to ND (Figure 5B).

Discussion

Here, we report for the first time that in an animal model of diet-induced obesity arterial stiffness develops rapidly and precedes the onset of hypertension. In mice fed a diet rich in fat and sucrose, PWV, measured both by Doppler ultrasonography and invasively, was elevated coincident with increased fat mass, glucose intolerance, and hyperinsulinemia but preceded the gradual onset of kidney damage, manifested by albuminuria, cardiac diastolic dysfunction,26 and hypertension. In accordance with increased PWV, we found evidence of increased material stiffness of the aortic intimal extracellular matrix, measured in vitro by atomic force microscopy, in response to HFHS feeding that was normalized by reversal to ND. Extracellular membrane stiffening of the intima can increase endothelial permeability, potentially contributing to the development of atherosclerosis.27 Material stiffness of the aortic extracellular matrix28 or vascular cells29 have recently emerged as novel biomechanical paradigms of aging-associated arterial stiffness. Tachycardia-associated sympathetic hyperactivity is found in obese hypertensive individuals and is explained, in part,
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by impaired baroreflex.21 Similar to obese individuals, we found sympathetic activation and tachycardia in HFHS-fed mice that could, in part, contribute to hypertension in these mice. Notably, aortic stiffening could impair the activation of carotid and aortic baroreceptors because artery distensibility is the main stimulus for a baroreflex. Although the causal link between arterial stiffness and the development of hypertension is lacking partially because of the inherent functional relationship between arterial wall stiffness and distending pressure,30 our findings support the hypothesis that arterial stiffness contributes to later hypertension in our model of diet-induced obesity. Interestingly, normalization of metabolic state achieved by weight loss after reversing obese mice to ND was associated with a rapid reduction in HFHS-induced arterial stiffness and systolic hypertension, suggesting that normalization of metabolic state may contribute to amelioration of abnormal vascular function.

HFHS-fed mice recapitulate many aspects of the metabolic syndrome, including insulin resistance, increased plasma triglycerides, and hypertension,31,32 making them an excellent model to study obesity-linked cardiovascular complications. Proinflammatory cytokines TNF-α, MCP-1, and MIP1α were upregulated by ≈3-fold in aortas from HFHS-fed mice and returned to control levels after reversing obese mice to ND. Inflammation and reduced vascular NO bioavailability are known important determinants of cardiovascular diseases, including arterial stiffness, particularly in settings of aging and diabetes mellitus.33,34 In patients with rheumatoid arthritis, TNF-α is associated with an increase in carotid–femoral PWV and l-arginine/asymmetric dimethylarginine ratio, indicative of reduced cellular NO-producing capacity. Thus, TNF-α may contribute to aortic stiffness, at least in part, by regulating NO bioavailability in settings of chronic inflammation.35 Aortas of HFHS-fed mice had both increased expression of TNF-α and impaired aortic relaxation to acetylcholine, indicative of reduced endothelial NO production, as early as 2 months on HFHS diet. The reduced NO bioavailability was also evident by an increase in tissue TG-2 activity in aortic lysates from HFHS-fed mice (Figure 2B).

Figure 3. Reversal to normal diet (ND) reduces arterial stiffness and hypertension in high-fat/high-sucrose (HFHS)–fed mice. A, Body weights (BW, g) and pulse wave velocity (PWV, mm/ms) in HFHS-fed mice decreased to control values after reversal to ND, n=8 each group; *P<0.05 vs ND baseline; #P<0.05 versus HFHS baseline. B, Reversal to ND rapidly reduced fat mass (g, left y axis) and plasma insulin levels (μg/L, right y axis) in obese mice (n=4–14 in each group). C, Systolic blood pressure (SBP) and mean arterial pressure (MAP) of obese mice were significantly decreased after reversal to ND, whereas diastolic blood pressure (DBP) did not change (n=6). *P<0.05 vs baseline. Baseline indicates 5 months on diet (ND or HFHS), and reversal indicates 4 months of ND after 5 months of HFHS or ND.

Figure 4. In vitro aortic stiffness and medial area are increased in diet-induced obesity. A, Stiffness modulus (kPa) on aortic rings subjected to 0.1 μm indentation was increased in high-fat/high-sucrose (HFHS)–fed mice and reduced to normal values after reversal to normal diet (ND; HFHS/ND; n=7–10 each group; ***P<0.0005 vs ND). B, Medial area of thoracic aortas from HFHS-fed mice was significantly increased compared with ND-fed mice and was not affected by reversal to ND (n=4–8; *P<0.05 vs ND). C, HFHS-induced inflammatory cytokines tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1α mRNA in aortic extracts were reduced to normal levels after reversal to ND (n=8 each group). Data are expressed as fold change over ND. *P<0.05 vs ND; #P<0.05 versus HFHS.
which was decreased after reversal to ND (Figure 5A). TG-2 is ubiquitously expressed in vascular cells and catalyzes a transamidation reaction to form strong N-ε-(γ-glutamyl)-lysine bonds between extracellular matrix proteins. TG-2 activity is inhibited by S-nitrosylation such that in settings of low NO bioavailability, it translocates to the extracellular space and becomes more active in forming matrix cross-links associated with arterial stiffness.\textsuperscript{22} Furthermore, vascular extracellular matrix cross-links known as advanced glycosylation end products could form nenzymatically in settings of chronic hyperglycemia,\textsuperscript{36} possibly contributing to arterial stiffness in HFHS-fed mice.

In addition to decreased NO bioavailability, we found increased sulfonylated SERCA, a marker of oxidant stress,\textsuperscript{24,25,37} in aortas from obese mice compared with ND-fed mice or mice reversed to ND. Oxidative post-translational modifications, such as sulfonylation of SERCA at cysteine 674, prevent NO-mediated SERCA-dependent Ca\textsuperscript{2+} uptake into the sarco/endoplasmic reticulum and, therefore, impair vascular smooth muscle cell relaxation.\textsuperscript{38} Decreased SERCA activity and endothelial NO in settings of HFHS-induced oxidant stress could therefore contribute to HFHS-induced arterial stiffness, at least in part, by increasing vascular smooth muscle tone. Taken together, our findings indicate that inflammation and decreased NO function could play a causative role in aortic and extracellular matrix remodeling and tone in obesity-induced arterial stiffness.

In conclusion, we showed for the first time in an animal model of diet-induced obesity that arterial stiffness precedes hypertension. Multiple mechanisms involving inflammatory mediators, matrix cross-linking, and decreased NO bioactivity may contribute to vascular remodeling and stiffness in advance of hypertension that may be, at least in part, a result of arterial stiffness causing undue stress on resistance vessels. The finding that reversal to ND improves vascular function in addition to the metabolic state indicates that arterial stiffness can be a novel target for early pharmacological or lifestyle interventions to prevent hypertension and associated complications in settings of obesity.

Perspectives
Arterial stiffness is an independent risk factor for cardiovascular events. Hypertension is associated with arterial stiffness although their temporal relations remain controversial. Here, we report that in diet-induced obesity, arterial stiffness precedes the onset of hypertension. Weight loss improves not only the diet-induced metabolic impairment in obese mice but also arterial stiffness and hypertension. When considering the epidemic incidence of obesity in the United States, mainly because of excessive consumption of fat- and sucrose-rich diets, arterial stiffness could represent a novel therapeutic target to prevent obesity-associated cardiovascular complications including hypertension.

Acknowledgments
We thank Pratibha Chauhan and Xiuyun Hou for their technical assistance.

Sources of Funding
This work was supported by National Heart, Lung, and Blood Institute (NHBLI) R01 grants HL105287 to R.A. Cohen and HL07296 to C.A. Reinhart-King and was partially supported by the Evans Center for Interdisciplinary Biomedical Research ARC (Affinity Research Collaborative) on Molecular, Biomechanical and Genetic mechanisms of Arterial Stiffness at Boston University (http://www.bumc.bu.edu/evanscenteribr/), as well as NHBLI Contract Number HHSN26820100031C to R.A. Cohen.

Disclosures
G. Mitchell is the owner of Cardiovascular Engineering, Inc, a biomedical device manufacturer from which some instrumentation, used to conduct this study, was purchased. The other authors report no conflicts.

References

Figure 5. Aortic extracellular matrix cross-links and oxidant stress are induced by high-fat/high-sucrose (HFHS) diet and reduced after diet reversal. Representative pictures of (A) extracellular matrix N-ε-(γ-glutamyl)-lysine cross-links (×10 magnification), indicative of transglutaminase-2 activity, and (B) sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) sulfonylated at cysteine 674 (OxSERCA; ×40 magnification), used as index of oxidants, in aortas from normal diet (ND)-fed mice and HFHS-fed mice and after reversal to ND (HFHS/ND); n=8–16; *P<0.05 vs ND; #P<0.05 vs HFHS. Graphs indicate immunostaining intensities (expressed in millions of pixels). For a color version, see Figure S6.


**Novelty and Significance**

**What Is New?**

- Arterial stiffness is increased in a diet-induced model of obesity and precedes hypertension.

- Arterial stiffness and high blood pressure are reversed to normal when the metabolic state of obese mice is normalized by weight loss.

**What Is Relevant?**

- Arterial stiffness could represent a novel therapeutic target to prevent obesity-associated cardiovascular complications including hypertension.

**Summary**

Whether arterial stiffness could develop in advance of hypertension is unknown. Here, we report that in a model of diet-induced obesity, arterial stiffness precedes the onset of hypertension. Normalization of metabolic state by weight loss in obese mice returned arterial stiffness and high blood pressure to normal. Arterial stiffness could be a novel therapeutic target to prevent cardiovascular complications, including hypertension, in settings of obesity.
Arterial Stiffening Precedes Systolic Hypertension in Diet-Induced Obesity

Hypertension. 2013;62:1105-1110; originally published online September 23, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.01744

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/62/6/1105

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Arterial Stiffening Precedes Systolic Hypertension in Diet-induced Obesity.


Short title: Arterial stiffness in obesity
5 figures and online supplements
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METHODS.

**Diet-induced obese mice.** All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University Medical Campus. Male C57Bl/6J mice (stock number 00664, The Jackson Laboratory, Bar Harbor, ME, USA) were obtained at 8 weeks of age. After one week of acclimation, mice were fed control (normal diet, ND: 4.5 % fat, 0% sucrose) or high fat, high sucrose diet (HFHS: 35.5 % fat (lard) representing 60 % calories, 16.4 % sucrose) $^1$ ad libitum (catalog numbers D09071702 and D09071703, Research Diets, New Brunswick, NJ, USA). The control diet was custom-formulated to match the micronutrients contained in HFHS except for fat and sucrose. Mice were housed in rooms with 12-hour light/dark cycle and in groups of 3-4 whenever possible.

A subset of mice were fed HFHS for 5 months and then reverted to ND for the following 4 months (referred to as HFHS/ND) with bi-weekly assessment of body weights, arterial stiffness and blood pressure (“diet reversal” study).

Urine samples were collected for 24 h in 96-well plates placed on the bottom of the cage, centrifuged to remove any particulate matter and stored at -80°C until further analysis. Blood samples were collected by tail bleed into EDTA containing vessels (BD Microtainer, BD, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation at 14,000 rpm for 20 minutes and stored at -80°C until measurement of insulin or norepinephrine.

**Glucose tolerance test (GTT), Insulin and HOMA-IR.** GTT was measured in ND- and HFHS-fed mice following standard methods. Briefly, after overnight fasting, baseline blood glucose levels were measured with a strip meter (Accu-Check, Roche Diagnostics, Indianapolis, IN, USA). Freshly prepared glucose solution was injected intraperitoneally (1.5 mg/kg of body weight) into ND- and HFHS-fed mice and blood was drawn by tail bleed at 30 minutes intervals to measure glucose concentrations over 120 minutes. Data are expressed as area under the curve of the glucose concentration plotted against time for each experimental group.

Insulin was assessed with Ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden) on plasma collected from fasted animals, as per manufacturer’s instructions.

Homeostasis model assessment (HOMA-IR) was estimated from plasma glucose and insulin levels in fasted ND- and HFHS-fed mice as an index of insulin resistance$^2$.

**Non-invasive body composition analysis (NMR).** Fat and lean body mass content was assessed on anesthetized ND-, HFHS- and HFHS/ND-fed mice with EchoMRI 900 instrument in the Metabolic Phenotyping Core at Boston University Medical Center.

**Albumin, creatinine and norepinephrine measurements.** Microalbuminuria (Albuwell, Exocell Inc., Philadelphia, PA, USA) and urine creatinine (The creatinine
companion, Exocell Inc., Philadelphia, PA, USA) were assessed by colorimetric ELISA, as per manufacturer’s instructions. Albumin concentrations were normalized by urine creatinine (mg/L).

Norepinephrine was assessed after boronate affinity gel-based extraction and acylation of 100 µL plasma, followed by ELISA as per manufacturer’s recommendations (Abnova, Taipei, Taiwan).

**Radiotelemetry blood pressure measurements.** Heart rate (HR), systolic (SBP), mean (MAP), diastolic (DBP) and pulse (PP) pressures were measured in conscious, freely moving mice using radiotelemetry (Data Sciences International, DSI, St Paul, MI, USA). Radiotelemetry hardware includes: the Data Exchange Matrix, PhysioTel receivers (RCP-1) and a calibrated ambient pressure reference monitor (APR-1).

Implantation of the wireless transmitters was performed in sterile conditions following manufacturer’s recommendations. Briefly, the pressure-sensing region (4 mm) of the catheter (model TA11PA-C10) was situated in the aortic arch via catheterization of the left carotid artery in isofluorane-anesthetized mice. The transmitter body was placed in a subcutaneous pocket obtained with blunt scissors in the right flank. Weekly recordings (1 every 4 min, 360 recordings/mouse per day) started after complete recovery from surgery (1-2 weeks). Data are expressed as mean ± SEM for ND and HFHS after averaging 360 recordings/day for each mouse. The weight of the transmitter (1.4g) was subtracted for body weight calculations.

In the first study, male C57Bl/6J mice underwent surgery for radiotelemetry implantation at 8 weeks of age (n=6) and were followed longitudinally for up to 1 year while on HFHS. For the second study, we compared mice fed ND (n=9) or HFHS (n=11) already for 5-6 months at the time of surgery that were followed for 1 month thereafter. Mice with erratic pressure waveforms, due to clotting or transmitter failure, were excluded from the study (n=6).

In a subset of telemeter-implanted mice, 2 hours of continuous recordings with a sampling rate of 2000 Hz were collected at 8 months of ND or HFHS to perform a power spectral analysis, as an indication of sympathetic modulation of blood pressure and heart rate variations, as described below.

**Power spectral analysis.** Data collected at 2000 Hz from radiotelemetry-implanted mice were extracted using the Hemolab Software Suite Version 14.8 (http://www.haraldstauss.com/HemoLab). Artifact free heart rate and mean arterial pressure beat-to-beat data were re-sampled at a frequency of 25 Hz and converted from non-equidistant to equidistant time series. From this equidistant data, a power spectral analysis was performed using a fast Fourier transformation as previously described.

**Pulse wave velocity measurements.** Pulse wave velocity (PWV), the standard *in vivo* measure for arterial stiffness, was assessed by Doppler ultrasound (VEVO770,
Visualsonics, Toronto, ON, Canada) adapted from previous methods. PWV calculation is based on the difference in arrival times of a pressure or flow wave at two locations along the aorta a known distance apart (transit time or TT method). The R-wave of the ECG is used as a fiducial point for signal averaging and resynchronization of sequential recordings. The TT method by Doppler ultrasound has been previously described in rats and mice.

Briefly, the mouse was anesthetized by 2% isofluorane/air inhalation and maintained under light anesthesia (0.5-1% isofluorane/air; heart rate: 450-500 bpm; respiration rate ~130 bpm) during the procedure. Mice were in the recumbent position on a heated platform (38°C) and with paws in contact with pad electrodes for continuous ECG recording. After gentle hair removal, the high-resolution RMV 704 scan-head (30MHz) was placed over the mid-abdominal area. A cross-section of abdominal aorta was imaged at the level of renal vein, used as anatomical landmark, and then the scan-head was rotated of 90°C to visualize the aorta longitudinally. In some mice, in order to compare thoracic-abdominal vs abdominal aorta PWV, the proximal Doppler flow was sampled from the right parasternal view of the aortic arch using the subclavian artery as landmark while the distal site was in the abdominal aorta at the level of the renal vein. Flow waveforms were consecutively recorded at a proximal and a distal location along the aorta in Pulse Wave Doppler mode. The distances between the foot of the flow waveform and the R-wave of the ECG (arrival times) for the proximal and distal locations and the distance (d) between the two points were estimated post-acquisition with the Vevo770 software and averaged over 5-10 cardiac cycles. For the thoracic-abdominal PWV, the distance between the two sites was measured on the surface after marking the exact points of probe location. PWV (mm/ms) was calculated by dividing the distance (d) by the difference among the two arrival times (transit time).

In order to validate PWV measurements obtained with Doppler echocardiography, blood pressure waveforms were recorded from two locations along the aorta simultaneously, using solid-state high-fidelity pressure catheters (Mikro-tip catheter transducers, SPR-1000, Millar Instruments, Houston, TX, USA) interfaced with an acquisition and analysis workstation (NIHem, Cardiovascular Engineering, Norwood, MA, USA) with 1 kHz bandwidth and 5 kHz sampling rate. Briefly, the mouse was prepared as described above. A pressure catheter was inserted in the aortic arch (proximal site) via the left carotid artery and a second pressure transducer was advanced retrogradely into the aorta (distal site) from the right femoral artery. Recordings were obtained at baseline and after phenylephrine infusion (0.1 µg/g BW at a rate of 50 µl/min) through a polyurethane tapered catheter (FunnelCath, Harvard Apparatus, Boston, MA, USA) inserted in the right jugular vein. The distance between the proximal and distal site, was measured postmortem by inserting a piece of P10 tubing though the left ventricle and advanced along the aorta until it touched the distal catheter. The tubing was then cut at the level of the proximal catheter and its length measured with mm scale under microscope magnification. The foot-to-foot transit time was assessed automatically from signal-averaged proximal and distal pressure waveforms over 20 seconds continuous recording using the R-wave of ECG as a fiducial point (NIHem software v. 4.99, Cardiovascular Engineering, Norwood, MA, USA).
Quantitative PCR on aortic extracts. mRNA expression of inflammatory genes TNFα (tumor necrosis factor α), MCP-1 (macrophage chemoattractant protein 1) and MIP1α (macrophage infiltrating protein 1α) in aortic extracts was assessed using standard procedures. Briefly, total RNA from aortas of mice fed ND, HFHS or HFHS/ND (n=8 each group) was extracted with Trizol-chloroform, precipitated with isopropanol (Sigma-Aldrich, St Louis, MO, USA) and purified with RNA specific clean-up spin columns (RNeasy MinElute Cleanup kit, Qiagen, Valencia, CA, USA). After DNase treatment, to ensure removal of genomic DNA, and total RNA retro-transcription (High capacity RNA-to-cDNA, Applied Biosystems, Grand Island, NY, USA), RNA expression of genes of interest was analyzed by quantitative PCR with verified Taqman assays and AB750 instrument (Applied Biosystems). Relative mRNA quantitation was calculated by the $\Delta\Delta$Ct method, expressed as relative to ND and normalized by GADPH.

Aortic wall thickness measurement and immuno-staining. Aortic samples were fixed in formalin overnight and passaged in 70% ethanol before embedding in paraffin. To assess aortic medial area, sections (5 µm) were stained with hematoxylin and eosin, digitally photographed and analyzed using ImageJ (available at www.nih.gov).

Visceral fat sections (5 µm) were processed for detection of activated macrophages with anti-galectin-3 primary antibody (Abcam, Cambridge, MA, USA) in an automated immunostainer as per manufacturer’s recommendations (IntelliPath FLX Autostainer, Biocare, Concord, CA, USA).

Aortic collagen content from ND- and HFHS-fed mice was detected by staining sections with Picrosirius red (PolyScientific, Bay Shore, NY, USA) and visualized under a polarized light microscope (Olympus, Center Valley, PA, USA). Aortic oxidant stress in ND-, HFHS- and HFHS/ND-fed mice was assessed by immuno-staining aortic sections with a custom antibody that specifically detects sulphonylated cysteine 674 on sarco/endoplasmic reticulum Ca++-ATPase (SERCA), previously validated and routinely used in our laboratory as index of oxidant stress7-9. Quantitation of collagen or oxidized SERCA immuno-staining intensity (pixels above a fixed threshold in RGB images) was carried out with Image J.

In vitro arterial stiffness. Novascan© atomic force microscope (AFM) cantilevers (with a 5 µm spherical tip) were used to measure stiffness of endothelium-denuded mouse aorta, as previously described10. Aortas harvested from ND- (n=10), HFHS- (n=7) and HFHS/ND-fed (n=8) mice, were carefully cleaned of fat and connective tissue. Endothelial cells were removed by wiping the vessel lumen with a cotton applicator. A three mm-long aortic segment was positioned on a plastic cell culture dish modified for the atomic force microscope carrier and sufficiently covered with PBS to avoid artifacts due to hydrodynamic effects. Multiple indentations (up to 30 for each aorta) were carried out with PicoPlus© AFM (Veeco, Plainview, NY, USA) using a large scanner operating at 1 µm/sec indentation speed. To avoid that exact same locations were probed multiple times, the stage was shifted by ~ 100 µm after each indentation. Each aortic indentation was followed by re-calibration of the cantilever so that the stiffness constant of the tips did not vary after repeated indentations. The
force-displacement curve of each indentation was analyzed using the Hertz model for stiffness of biological samples, taking 0.5 as the Poisson’s ratio of the material and using the radius of cantilever tip as indentation sphere radius. Indentation points exhibiting stiffness modulus higher than 200 kPa, indicative of regions of punctured intima, were excluded from the analysis. Data are expressed as mean ± SEM of the different treatment groups, after averaging the stiffness modulus (kPa) of all probed locations for each aorta.

**Isometric tension measurements.** Detailed methods were previously described\textsuperscript{11}. Briefly, four mm-long aortic rings from ND- and HFHS-fed mice (n=5 each group) with intact endothelium, were mounted on 0.005 inch diameter metal stirrups in organ chambers for force measurement. Rings were maintained at 37°C and bubbled continuously with 95% oxygen/5% carbon dioxide in a physiological salt solution (PSS) of the following composition (mmol/L): KCl 4.7, CaCl\textsubscript{2} 2.5, NaCl 118.3, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 0.6, NaHCO\textsubscript{3} 25, and dextrose 5.5. Rings were stretched incrementally to an optimal tension of 2 g during one hour with repeated washing then, after a 30-min equilibration, the rings were contracted to PSS containing 50 mmol/L KCl and rinsed. After further 30-min equilibration, rings were contracted with phenylephrine to approximately 1 g. Relaxations to cumulative increasing concentrations of acetylcholine (10\textsuperscript{-9} – 10\textsuperscript{-5} mol/L) or NO donor sodium nitroprusside (SNP, 10\textsuperscript{-10} – 10\textsuperscript{-6} mol/L) were determined and expressed as percent decrease in the phenylephrine-induced force.

**Transglutaminase activity and expression.** A nonradioactive dot blot assay based on the incorporation of 5-(biotinpentyl)-amine (BPA), a transglutaminase substrate, into proteins was used on aortic extracts from ND- and HFHS-fed mice (n=4 each group). Details of the assay have been previously described\textsuperscript{12, 13}. Aortic sections from ND-, HFHS- and HFHS/ND-fed mice were prepared as described above and stained with a specific anti-N-\varepsilon-(\gamma-glutamyl)-lysine antibody (ab424, Abcam, Cambridge, MA, USA), indicative of TG-2 activity (9). Aortic TG-2 expression was assessed by immuno-staining sections with a specific anti-TG-2 antibody (cat. #RB-060, Thermo Scientific, Fremont, CA, USA). Quantitation of immuno-staining intensities was carried out with the same methodology as the one described above for collagen and oxidized SERCA quantitation.

**Statistical analysis.** Unless otherwise specified, all data are expressed as mean ± SEM and analyzed by Student’s t-test for comparisons between groups (ND, HFHS and HFHS/ND). In longitudinal studies, repeated measures of blood pressure and PWV over time were analyzed by one-way ANOVA with Dunnett’s multiple comparison post hoc test. P values <0.05 were considered significant.
AUTHORS CONTRIBUTIONS.
RMW designed the study, conducted experiments and wrote the manuscript; TS prepared aortic and fat sections and measured aortic medial area; LAS performed collagen analysis; LS performed the transglutaminase assay; HEL performed the spectral analysis; SB performed the material stiffness measurements; JLF, CRK and GM provided critical comments to the study and manuscript; RAC designed the study and wrote the manuscript; FS designed and coordinated the study, performed experiments, analyzed the data and wrote the manuscript.

REFERENCES FOR ONLINE SUPPLEMENTS.


<table>
<thead>
<tr>
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<th>ND (n=8)</th>
<th>HFHS (n=8)</th>
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<tbody>
<tr>
<td>PWV baseline (mm/ms)</td>
<td>2.89 ± 0.07</td>
<td>3.41 ± 0.10*</td>
</tr>
<tr>
<td>MAP baseline (mmHg)</td>
<td>80.7 ± 3.7</td>
<td>83.1 ± 2.6</td>
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<tr>
<td>PWV phenylephrine (mm/ms)</td>
<td>3.82 ± 0.15*</td>
<td>4.36 ± 0.16††</td>
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<tr>
<td>MAP phenylephrine (mmHg)</td>
<td>114.1 ± 1.1*</td>
<td>116.0 ± 0.5†</td>
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**Table S1. Arterial stiffness is increased in obese mice.** PWV measured invasively with high-fidelity pressure catheters, shown graphically in Fig. 1B, was significantly elevated in HFHS-fed mice compared to ND, at baseline and after phenylephrine infusion (0.1 μg/g BW). MAP, mean arterial pressure. Number of mice indicated in parenthesis. *, p<0.05 vs ND-baseline; †, p<0.05 vs HFHS-baseline; ††, p<0.05 vs ND-phenylephrine.
<table>
<thead>
<tr>
<th></th>
<th>ND (n=7)</th>
<th>HFHS (n=9)</th>
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<tbody>
<tr>
<td>SBP (mmHg²)</td>
<td>1.9 ± 0.4</td>
<td>4.4 ± 1.1*</td>
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<tr>
<td>MAP (mmHg²)</td>
<td>1.4 ± 0.3</td>
<td>3.2 ± 0.7*</td>
</tr>
<tr>
<td>Heart rate (mmHg²)</td>
<td>1.6 ± 0.3</td>
<td>3.5 ± 1.4*</td>
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Table S2. Sympathetic modulation of blood pressure and heart rate in obese mice. Results of spectral analysis on high frequency (2000 Hz) radiotelemetry recordings from mice fed ND or HFHS for 8 months. SBP, systolic blood pressure; MAP, mean arterial pressure; HR, heart rate. Number of mice indicated in parenthesis. *, p<0.05.
<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>HFHS</th>
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<tbody>
<tr>
<td>NE after 2 months diet (ng/ml)</td>
<td>3.9 ± 0.8 (n=12)</td>
<td>4.9 ± 0.9 (n=12)</td>
</tr>
<tr>
<td>NE after 8 months diet (ng/ml)</td>
<td>5.9 ± 1.3 (n=6)</td>
<td>11 ± 2.9*† (n=7)</td>
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</table>

**Table S3. Norepinephrine in HFHS-fed mice.** Plasma norepinephrine (NE) measured after 2 and 8 months of ND or HFHS diet. Number of mice in each group indicated in parenthesis. *, p<0.05 vs ND; †, p<0.05 vs HFHS-2months.
Figure S1. Model of diet-induced obesity. HFHS-fed mice had increased body weights (A), fat mass (B, left; lean mass shown on right; both expressed as percentage of BW) and glucose intolerance measured by glucose tolerance test (C) and HOMA-IR (D) compared to ND. n=4-6 each group; *, p < 0.05; **, p<0.005 vs ND.
Figure S2. HFHS diet stimulates fat inflammation and albuminuria. (A) Activated macrophages (brown staining, galectin-3+ cells) are more abundant in visceral fat surrounding the kidneys of HFHS-fed mice compared to ND. Representative pictures with indicated magnification (10X or 20X). (B) Albumin urinary excretion, expressed as μg per mg creatinine, increased after 4 months of HFHS and thereafter. n=4-8 each group; *, p< 0.05 vs ND.
Figure S3. Mean, diastolic and pulse pressures and heart rate in dietary obesity. Mean (MAP) (A) and pulse pressures (PP) (C) were significantly increased after 6 months of HFHS while diastolic blood pressure (DBP) (B) did not change significantly over time. n=6; *, p< 0.05 vs baseline (time 0). MAP (D) and PP (F) were significantly higher in obese (n=11) than lean mice (n=9) while DBP were similar (E). *, p< 0.05 vs ND. Heart rate (HR) was significantly increased by HFHS (G) compared to ND (H) and reduced to control values after diet reversal (I). *, p< 0.05 vs baseline (5 months HFHS).
Figure S4. Aortic collagen did not change after HFHS. Representative pictures of Picosirius red-stained aortic sections, visualized under polarized light illumination at 10X magnification, from ND- and HFHS-fed mice (n=8 each group). Old indicates 29 month old mice, shown for comparison (n=3). Quantitation of collagen intensities shown in graph.
Figure S5. Transglutaminase-2 expression. Representative pictures (10X magnification) and scatterplot of intensity quantitation (brown staining, expressed in millions of pixels) for TG-2 immunostained aortic sections from ND-, HFHS- and HFHS/ND-fed mice. TG-2 expression remained unchanged among diet groups (n=8-16).
Figure S6. Color version of Figure 5. Representative pictures of (A) extracellular matrix N-ε-(γ-glutamyl)-lysine crosslinks (10X) and (B) SERCA sulphonylated at cysteine 674 (OxSERCA) (40X) in aortas from ND-, HFHS-fed mice and after reversal to ND (HFHS/ND). Same as Figure 5 but shown in color.
Figure S7. Aortic relaxation to sodium nitroprusside. Relaxation of aortic rings to NO donor sodium nitroprusside (SNP, 1x10^{-10}-1x10^{-6} mol/L) was similar between ND- and HFHS-fed mice. n=4 each group, mean ± SEM.