Vascular Smooth Muscle Sirtuin-1 Protects Against Diet-Induced Aortic Stiffness

Jessica L. Fry, Leona Al Sayah, Robert M. Weisbrod, Isabelle Van Roy, Xiang Weng, Richard A. Cohen, Markus M. Bachschmid, Francesca Seta

Abstract—Arterial stiffness, a major cardiovascular risk factor, develops within 2 months in mice fed a high-fat, high-sucrose (HFHS) diet, serving as a model of human metabolic syndrome, and it is associated with activation of proinflammatory and oxidant pathways in vascular smooth muscle (VSM) cells. Sirtuin-1 (SirT1) is an NAD+-dependent deacetylase regulated by the cellular metabolic status. Our goal was to study the effects of VSM SirT1 on arterial stiffness in the context of diet-induced metabolic syndrome. Overnight fasting acutely decreased arterial stiffness, measured in vivo by pulse wave velocity, in mice fed HFHS for 2 or 8 months, but not in mice lacking SirT1 in VSM (SMKO). Similarly, VSM-specific genetic SirT1 overexpression (SMTG) prevented pulse wave velocity increases induced by HFHS feeding, during 8 months. Administration of resveratrol or S17834, 2 polyphenolic compounds known to activate SirT1, prevented HFHS-induced arterial stiffness and were mimicked by global SirT1 overexpression (SirT1 bacterial artificial chromosome overexpressor), without evident metabolic improvements. In addition, HFHS-induced pulse wave velocity increases were reversed by 1-week treatment with a specific, small molecule SirT1 activator (SRT1720). These beneficial effects of pharmacological or genetic SirT1 activation, against HFHS-induced arterial stiffness, were associated with a decrease in nuclear factor kappa light chain enhancer of activated B cells (NFκB) activation and vascular cell adhesion molecule (VCAM-1) and p47phox protein expressions, in aorta and VSM cells. In conclusion, VSM SirT1 activation decreases arterial stiffness in the setting of obesity by stimulating anti-inflammatory and antioxidant pathways in the aorta. SirT1 activators may represent a novel therapeutic approach to prevent arterial stiffness and associated cardiovascular complications in overweight/obese individuals with metabolic syndrome. (*Hypertension*, 2016;68:775-784. DOI: 10.1161/HYPER.116.07622.) • Online Data Supplement

Key Words: aorta ■ arterial stiffness ■ cardiovascular disease ■ metabolic syndrome ■ obesity ■ sirtuin-1 ■ vascular smooth muscle

Among noncommunicable diseases, cardiovascular disease remains the leading cause of mortality in the United States and worldwide.1 Arterial stiffness is characterized by the progressive loss of compliance of large conduit arteries, and it is a major risk factor for cardiovascular disease. Pulse wave velocity (PWV), the gold standard in vivo index of aortic wall stiffness, is predictive of incident hypertension,2–4 heart attack,5–8 cardiac failure,9 stroke,10 cognitive impairment,11–13 and kidney failure.14 Large artery compliance is necessary to dampen the pulsatility of cardiac contraction, and to ensure a steady blood supply to peripheral organs, and for optimal cardiac perfusion. However, when the aortic wall stiffens, this Windkessel effect is lost, causing a widening of pulse pressure and increased cardiac afterload, with deleterious functional consequences to the heart and downstream organs.

Metabolic syndrome, a cluster of cardiometabolic risk factors associated with overweight/obesity and insulin resistance, significantly increases the risk of developing cardiovascular disease.1 Arterial stiffness is increased in obese and diabetic individuals, even at a young age (10–24 years).15

We previously demonstrated that mice fed a diet rich in fat and sucrose closely resemble the human metabolic syndrome and develop arterial stiffness within 2 months.16 Aortic wall stiffening was associated with increased inflammation, as measured by cytokines tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and p47phox protein expressions, in aorta and VSM cells. In conclusion, HFHS SirT1 activation decreases arterial stiffness in the setting of obesity by stimulating anti-inflammatory and antioxidant pathways in the aorta. SirT1 activators may represent a novel therapeutic approach to prevent arterial stiffness and associated cardiovascular complications in overweight/obese individuals with metabolic syndrome. We previously demonstrated that mice fed a diet rich in fat and sucrose closely resemble the human metabolic syndrome and develop arterial stiffness within 2 months.16 Aortic wall stiffening was associated with increased inflammation, as measured by cytokines tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein isoform 1α (MIP-1α), decreased nitric oxide (NO) bioavailability, and increased extracellular matrix cross-linking in the aortic wall.16 We further demonstrated that, in the setting of dietary obesity, TNF-α-induced activation of the oxidant-generating enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, Nicotinamide adenine dinucleotide phosphate oxidase isoform 2 (Nox2), is a major determinant of inflammation associated with functional impairment of aortic smooth muscle cells.17 Vascular smooth muscle (VSM) cell functional alterations have recently emerged has important determinants of vascular stiffening, contributing ≈50% to aortic stiffness.18–20 Notably, arterial stiffness and associated aortic oxidants and inflammation were normalized by returning obese mice to a normal...
diet (ND), suggesting that aortic wall stiffening is, at least in part, a dynamic process, amenable to therapy, and lifestyle interventions, such as caloric restriction.

Sirtuin-1 (SirT1, mammalian homolog of silent information regulator [Sir2] in yeast), also known as the longevity gene, is an evolutionarily conserved NAD+-dependent deacetylase, considered an important metabolic regulator and the putative molecular target of caloric restriction. Mice overexpressing SirT1, when fed a high-caloric diet or inbred into an obesity-prone db/db genetic background, are protected from diabetes mellitus. However, the vascular-specific effects of SirT1 on the cardiovascular complications of diabetes mellitus and insulin resistance, and specifically on arterial stiffness, are not fully understood.

We previously demonstrated that lack of SirT1 in VSM leads to aortic dissection in response to angiotensin II, indicating a pivotal role of VSM SirT1 in maintaining the structural and functional integrity of the aortic wall in response to inflammatory and oxidant stimuli. In this study, we sought to explore the role of SirT1 in diet-induced arterial stiffness, where inflammation and excess oxidants are the culprit, and to evaluate the therapeutic potential of SirT1 activators against arterial stiffness. Using genetic, pharmacological, and lifestyle interventions (overnight fasting) along with our established model of diet-induced obesity, here we demonstrate that SirT1 ameliorates diet-induced arterial stiffness by preventing p65-nuclear factor kappa light chain enhancer of activated B cells (NFκB) activation and vascular cell adhesion molecule (VCAM)-1 upregulation and by decreasing p47phox, a major functional subunit of Nox2, in the aortic wall and VSM cells.

Notably, anti-inflammatory and antioxidant effects of SirT1 in the aortic wall occurred even after a short treatment of obese mice with a specific SirT1 activator, SIRT1720, yet without a concomitant metabolic improvement, and in ex vivo experiments on isolated aortas, indicating that the vasculature is a direct target for the biological functions of SirT1.

Considering the epidemic proportions of metabolic syndrome in the United States, where 68% of Americans, including children, are overweight or obese, it is imperative to find novel therapies to prevent arterial stiffness and associated adverse cardiovascular outcomes in this vast patient population. To this end, SirT1 activators may represent promising therapeutic means of ameliorating arterial stiffness and preventing cardiovascular complications in the setting of metabolic syndrome.

Materials and Methods
A detailed description of materials and methods can be found in the online-only Data Supplement.

Experimental Animal Models
All animal experimental procedures were approved by the institutional animal care and use committee (IACUC) at Boston University Medical Campus. We generated SirT1 VSM knockout (SMKO) and transgenic (SMTG) mice on C57Bl/6J genetic background, as described in detail in Data Supplement. Mice overexpressing SirT1 globally (SirT1 bacterial artificial chromosome overexpressor [SirBACO]) were obtained from Dr. Wei Gu, Columbia University, New York, NY.

SMTG, SMKO, SirBACO mice, and littermate controls were fed ND or high-fat, high-sucrose (HFHS) diet for 8 months. Fasting was performed by removing food overnight.

Subsets of male C57Bl/6J mice were fed HFHS diet supplemented with resveratrol or S17834, 2 polyphenolic compounds known to activate SirT1, at a daily dose of 130 mg/kg for 8 months. In reversal studies, subsets of mice were fed HFHS for 8 months before receiving HFHS supplemented with SRT1720, a small molecule SirT1 activator, at a daily dose of 100 mg/kg.

Glucose Tolerance Test
Glucose tolerance was assessed in HFHS-fed wild-type (WT, n=5–9), SMTG (n=6), SirBACO (n=5), and S17834-treated (n=9) mice, as we previously described.

PWV Measurements
PWV, the gold standard in vivo measurement of arterial stiffness, was assessed in the different experimental groups, using methods that we previously described.

Aortic Tissue Isolation and Ex Vivo Treatments
Whole aortas from WT and SMTG mice were cultured at 37°C overnight in 1 mL of DMEM containing 1 g/mL of glucose, with or without TNF-α (10 ng/mL, Peprotech) or with or without SRT1720 (50 μmol/L, Sellekchem). At the end of the overnight incubation, aortas were snap frozen in liquid nitrogen and kept at −80°C until further processing.

Isolation of VSM Cells
Aortic smooth muscle cells were isolated by enzymatic dissociation, as we previously described.

Western Blot and Quantitative Real Time Polymerase Chain Reaction on Aortas and VSM Cells
Western blot and quantitative real time polymerase chain reaction (qRT-PCR) on aortas and VSM cells were performed by standard methods, as described in detail in Data Supplement.

NFκB Activity Assay
WT and SMTG VSM cells were coinfected with adenoviral constructs containing a firefly luciferase reporter gene under the control of specific NFκB DNA-responsive elements (1×10⁷ PFU/mL) and a renilla luciferase (1×10⁷ PFU/mL). Two days post infection, cells were treated with TNF-α (10 ng/mL) or vehicle control (phosphate buffered saline). Firefly and renilla luciferase activities were assayed with a standard luciferase assay, as per manufacturer’s instructions (Promega).

Oxidant Measurement
Oxidants, mainly superoxide anion, were measured in freshly frozen aortic sections with dihydroethidine, as we previously described.

Statistical Analysis
All data are expressed as means±SEM. Repeated measures 2-way ANOVA with Bonferroni multiple comparison test was used to analyze PWV and body weights in SMTG and in mice treated or untreated with resveratrol or S17834, during the time course of ND or HFHS diets, and to analyze glucose tolerance test in HFHS-fed WT, SMTG, SirBACO, and S17834-treated mice. Body weights in SirBACO mice, PWV in fasting experiments, and NFκB activity assay were analyzed by 1-way ANOVA with Bonferroni multiple comparisons. PWV in SirBACO mice and quantification of dihydroethidine staining were analyzed by nonparametric 1-way ANOVA Kruskal–Wallis test with Dunn multiple comparisons. PWV and body weights in mice treated with or without SRT1720 were analyzed by unpaired t test. For Western blots and quantitative real time polymerase chain
reaction (qRT-PCR) nonparametric Whitney–Mann t test was used. P values of <0.05 were considered significant.

Results

Fasting Decreases HFHS-Induced Arterial Stiffness via VSM SirT1

We previously showed that returning HFHS-fed obese mice to an ND, returns PWV, the in vivo index of arterial stiffness, to normal, over a 4-month period, indicating that arterial stiffness in settings of obesity is reversible. Caloric restriction is known to increase SirT1 activity through increased NAD+ production and SirT1 expression. Here, we used overnight fasting to mimic acute, but severe, caloric restriction in mice fed an HFHS diet for 2 or 8 months as a means of increasing SirT1 activity in the aorta, measured by acetylated histone H3 (Figure 1A), in accordance to what has been reported for other tissues. In mice fed HFHS diet, which have elevated PWV, fasting acutely decreased PWV without having an effect in ND-fed controls (Figure 1B). Deletion of SirT1 activity in the VSM of SMKO mice completely abrogated the PWV-lowering effect of fasting (Figure 1C). In addition of increasing SirT1 in the aorta, overnight fasting increased phosphorylated vasodilator-stimulated phosphoprotein, an endothelial nitric oxide synthase–NO–cGMP downstream effector in VSM cells, involved in cytoskeletal protein dynamics. Fasting-induced SirT1 and phosphorylated vasodilator-stimulated phosphoprotein were abrogated in aortas of SMKO mice.

Figure 1. Fasting acutely normalizes high-fat, high-sucrose (HFHS) diet–induced arterial stiffness via vascular smooth muscle (VSM) SirT1-1 (SirT1). A, Representative Western blots for acetylated histone H3, indicative of SirT1 deacetylase activity, and GAPDH loading control, in aortas of mice fed HFHS for 8 months (HFHS/fed, n=4) or fasted overnight after 8 months of HFHS feeding (HFHS/fasted, n=4). Each lane represents 1 aorta from 1 mouse. Quantitation of band intensities in graph. *P<0.05 HFHS/fed. B, Overnight fasting decreased arterial stiffness, measured in vivo by pulse wave velocity (PWV, m/s) and induced by 2 or 8 mo of HFHS feeding, but it did not have an effect on PWV in mice fed normal diet (ND). PWV values were logarithmically transformed to fit a normal distribution. Two months: *P<0.05 HFHS/fed (n=11) vs ND/fed (n=11); †P<0.05 HFHS/fasted vs HFHS/fed (n=11); 8 mo: *P<0.05 HFHS/fed (n=9) vs ND/fed (n=9); †P<0.05 HFHS/fasted vs HFHS/fed (n=9). C, The PWV-lowering effect of fasting was lost in mice lacking SirT1 in VSM (SMKO). *P<0.05 wild-type (WT)/HFHS/fasted (n=4) vs WT/HFHS/fed (n=4); SMKO/HFHS/fasted (n=4) vs SMKO/HFHS/fed (n=4), not significant. D, Representative Western blots of aortic homogenates indicate that overnight fasting increases SirT1 expression and vasodilator-stimulated phosphoprotein (VASP) phosphorylation but not in mice lacking SirT1 in VSM (SMKO). GAPDH serves as loading control. Western blots were repeated 3×. Each lane represents 1 aorta from 1 mouse. Quantitation of band intensities in graph. *P<0.05 vs WT/ fed; †P<0.05 vs HFHS/fed; pVASP indicates phosphorylated VASP.
Our data suggest that fasting-induced acute activation of VSM SirT1 can decrease arterial stiffness in mice fed HFHS diet, at least in part, by acutely increasing NO bioactivity in VSM cells.

**VSM-Specific SirT1 Overexpression Prevents HFHS-Induced Arterial Stiffness**

To directly study the effects of SirT1 on arterial stiffness in settings of obesity, we used mice overexpressing SirT1 in VSM (SMTG), fed HFHS for ≤8 months. SMTG mice are characterized by ≈4-fold increase in SirT1 expression, as demonstrated by Western blotting in aortic media and isolated VSM cells, and quantified by quantitative real time polymerase chain reaction (qRT-PCR) in aortic homogenates (Figure S1). Specific VSM SirT1 overexpression in SMTG mice prevented HFHS-induced arterial stiffness (Figure 2A), without notable differences in weight gain (Figure S2A) or glucose tolerance (Figure S2B), compared with HFHS-fed WT littermate controls.

**Polyphenols and Global SirT1 Overexpression Prevent HFHS-Induced Arterial Stiffness**

Similar to HFHS-fed SMTG mice, treatment with the polyphenols resveratrol (Figure 2B) or S17834 (Figure S3), each at a dose of 130 mg/kg per day, completely prevented the development of HFHS-induced arterial stiffness >8 months. These results were mimicked in mice globally overexpressing SirT1 (SirBACO) and fed HFHS for 8 months (Figure 2C), suggesting that polyphenols may prevent HFHS-induced arterial stiffness by targeting SirT1.

Weight gains induced by the high-caloric diet were similar in all the experimental groups (Figure S2C, S2D, and S2E). Glucose intolerance, indicative of insulin resistance, was similarly impaired in HFHS-fed SirBACO mice or in mice treated with S17834, as HFHS-fed littermate controls (Figure S2F and S2G). Taken together, these findings further suggest that vascular-specific effects, and not a global improvement in metabolism, are responsible for the prevention of arterial stiffness by SirT1.

**The SirT1-Specific Activator, SRT1720, Ameliorates HFHS-Induced Arterial Stiffness**

To further explore the therapeutic potential of global activation of SirT1 in a clinically relevant scenario, we administered a small molecule SirT1 activator (SRT1720, 100 mg/kg per day) to obese mice, when obesity and arterial stiffness were well established. After 8 months of HFHS diet, 1 week of SRT1720 treatment was sufficient to significantly decrease PWV in obese mice to a normal range (Figure 2D), without altering body weight (55±1 g in HFHS-fed mice, n=4 versus 57±2 g in HFHS-fed mice treated with SRT1720, n=5; not significant).

**SirT1 Decreases Inflammation and Oxidants in the Aorta**

Our previous work showed a link between HFHS feeding, arterial stiffness, and the production of oxidants in the aortas of obese mice, leading to inflammation and functional impairment in VSM cells, associated with increases in TNF-α. Therefore, we assessed whether the amelioration of arterial stiffness by SirT1 activators and genetic overexpression of SirT1 was mediated by anti-inflammatory and antioxidant effects.

As shown in Figure 3, VSM SirT1 overexpression prevented TNF-α–induced VCAM-1 upregulation and p65-NFκB phosphorylation in whole aortas and cultured VSM cells (Figure 3A and quantitations in Figure 3B). SirT1 activity in SMTG was significantly increased after TNF-α

**Figure 2.** Vascular smooth muscle (VSM) sirtuin-1 (SirT1) protects against high fat, high sucrose (HFHS)–induced arterial stiffness. **A**, Mice-overexpressing SirT1 in VSM (SMTG; n=6) were protected from HFHS-induced pulse wave velocity (PWV) increases compared with HFHS-fed littermate controls (n=4) during an 8-mo period. *P<0.05 wild-type (WT)/HFHS vs SMTG/HFHS. **B**, The polyphenolic compound resveratrol (Resv), administered in food for 8 mo at a dose of 130 mg/kg per day, prevented HFHS-induced arterial stiffness. *P<0.05 normal diet (ND, n=6) vs HFHS (n=6); †P<0.05 HFHS/Resv (n=6) vs HFHS (n=6). **C**, Whole-body SirT1 overexpression in SirT1 bacterial artificial chromosome overexpressor (SirBACO) mice prevented HFHS-induced PWV increases, compared with WT littermate controls, after 8 mo of diet. *P<0.05 WT/HFHS (n=5) vs WT/ND (n=5); †P<0.05 SirBACO/HFHS (n=7) vs WT/HFHS (n=5). **D**, One-week treatment with the SirT1 activator SRT1720 (100 mg/kg per day) decreased HFHS-induced arterial stiffness, measured by PWV (m/s). *P<0.05 HFHS/SRT1720 (n=5) vs HFHS (n=4).
stimulation, as indicated by decreased acetylated histone H3, a SirT1 deacetylation target,28 (Figure 3A and quantitation in Figure 3B), consistent with the fact that SirT1 is activated in response to stress stimuli.33 In addition, TNF-α-stimulated NFκB activity, assessed with an adenoviral luciferase construct containing specific NFκB DNA-responsive elements, was significantly decreased in SMTG VSM cells compared with WT (Figure 3C).

Figure 3. Sirtuin-1 prevents VCAM-1 upregulation and nuclear factor kappa light chain enhancer of activated B cells (NFκB) activation in the aorta and vascular smooth muscle (VSM) cells. A, Representative Western blots for vascular cell adhesion molecule (VCAM)-1, phosphorylated p65 (active NFκB subunit), acetylated histone H3, and β-actin in aortas (left), and aortic smooth muscle cells (right) from wild-type (WT) and smooth muscle transgenic (SMTG) mice and treated overnight with or without tumor necrosis factor-α (TNF-α, 10 ng/mL). Each lane on the left panels represents 1 aorta from 1 mouse. Western blots were repeated 4× to 6×. B, Quantitation of band intensities of Western blots depicted in A. For each protein, the ratio with β-actin or GAPDH, used as loading controls, was calculated, averaged for each treatment group, and expressed as fold change relative to nontreated WT controls. *P<0.05 vs WT/control; †P<0.05 vs WT/TNF-α; #P<0.05 vs SMTG/control. C, NFκB activity, measured with an adenoviral construct containing NFκB-responsive elements and a firefly luciferase reporter, in WT and SMTG aortic smooth muscle cells, with or without treatment with TNF-α (10 ng/mL). Firefly luciferase luminescence intensities, normalized to renilla luciferase luminescence, are expressed in relative light units (R.L.U.) and as fold change of WT controls. n=4; *P<0.05. D, Western blots for VCAM-1, phosphorylated p65 NFκB, acetylated histone H3, and β-actin in aortas treated overnight with TNF-α (10 ng/mL), with or without SRT1720 (50 μmol/L). Western blots were repeated 3×. *P<0.05. E, Western blots for VCAM-1, phosphorylated p65 NFκB, and β-actin in aortas of mice fed high-fat, high-sucrose (HFHS) diet for 8 mo (HFHS/fed, n=4) or fasted overnight after 8 mo of HFHS (HFHS/fasted, n=4). Each lane represents 1 aorta from 1 mouse. Quantitation of band intensities in graph. *P<0.05 vs HFHS/fed. VSMC indicates VSM cell.
Similarly, incubation of aortas ex vivo with SRT1720 (50 μmol/L) prevented TNF-α–induced VCAM-1 upregulation and p65-NFκB phosphorylation, and decreased histone H3 acetylation (Figure 3D), indicating that SRT1720 efficiently activates SirT1 in the aorta. Likewise, overnight fasting, after 8 months of HFHS, decreased VCAM-1 expression and p65-NFκB phosphorylation (Figure 3E), consistent with the fact that SirT1 expression and activity are increased in the aorta after an overnight fast (Figure 1A and 1D).

Oxidant production, mainly superoxide anion, assessed by dihydroethidine staining in freshly frozen aortic sections, was significantly decreased in HFHS-fed SirBACO (n=5) and SRT1720-treated (n=4) mice compared with HFHS-fed mice (n=10; Figure 4 and quantitation in graph). As we previously reported that, in our model of obesity, VSM Nox2 is a major source of oxidants in the aorta, we first assessed whether the antioxidant effects of SirT1 on the aortic wall were mediated by downregulation of Nox2. We found no significant changes in Nox2 expression in VSM sections of HFHS-fed SMTG (n=6), compared with HFHS-fed controls (Figure S4). Nox2 remained abundantly expressed in the endothelium and adventitia of both WT and SMTG mice fed HFHS (Figure S4), with limited expression in the media. However, protein expression of p47phox, a major functional subunit of Nox2, was significantly decreased in SMTG aortas (Figure 4B).

**Discussion**

HFHS-fed obese mice closely mimick the human metabolic syndrome and represent an excellent model in which to study the cardiovascular complications of the metabolic syndrome, including arterial stiffness, which has been previously studied mainly in the context of aging. We previously demonstrated that arterial stiffness develops within 2 months in mice fed HFHS and that, at the molecular level, obesity-induced vascular remodeling and stiffening are associated with impaired NO bioavailability and TNF-α–induced inflammatory responses in VSM, leading to functional impairment of VSM cells and increased extracellular matrix remodeling. VSM cell functional alterations, such as increases in VSM cell contractility and stiffness, and extracellular matrix remodeling are major contributors to aortic wall stiffening. Building upon our previous studies, here we demonstrate, that sirtuin-1, an NAD+-deacetylase important for the cellular response to metabolic stresses, is effective in decreasing diet-induced arterial stiffness by inhibiting inflammatory and oxidant pathways.

We used multiple approaches to study the effects of SirT1 on aortic stiffness in HFHS diet-induced obesity. Two polyphenolic compounds (resveratrol and S17834) and a nonpolyphenolic small molecule (SRT1720), known to activate SirT1 in a specific manner, respectively, prevented and normalized HFHS-induced aortic stiffness after
8 months of HFHS feeding. These effects were mimicked in mice-overexpressing SirT1 globally (SirBACO), indicating that the beneficial effects of polyphenols, against obesity-induced aortic stiffness, may be, at least in part, mediated by SirT1. Importantly, overexpressing SirT1 specifically in VSM cells (SMTG) completely prevented HFHS-induced PWV increases, indicating a pivotal role of VSM SirT1 in preserving vascular function in settings of metabolic syndrome.

Resveratrol, a polyphenol found in red grapes, improves survival and insulin resistance in mice fed high-caloric diets. However, the therapeutic potential of resveratrol and other polyphenolic compounds as antidiabetic drugs has been controversial because of non–SirT1-mediated actions. Several small molecule activators of SirT1 have been developed, which exert more specific and potent effects on SirT1 than resveratrol, and are currently in clinical trials for type 2 diabetes mellitus and metabolic disorders. Limited studies have elucidated the effects of SirT1 activators in the cardiovascular complications of the metabolic syndrome. Specifically, only 1 recent study used resveratrol in HFHS-fed nonhuman primates to study arterial stiffness as primary end point.

In our study, the beneficial effects of resveratrol against HFHS-induced aortic stiffness were mimicked in mice overexpressing SirT1 globally (SirBACO), or specifically in VSM (SMTG), and in mice treated for 1 week with SRT1720. These effects were locally mediated in the aorta and not a consequence of a metabolic amelioration because weight gain and glucose intolerance were similar in obese mice whether pharmacological or genetic intervention was used to increase SirT1. This is an apparent disagreement with previous studies, in which global SirT1 overexpression or treatment with resveratrol were associated with improvement of glucose intolerance and prevention of diabetes mellitus. These discrepancies are likely explained by different experimental approaches, mainly the composition of the diets, as pointed out by others.

We previously showed that HFHS-induced arterial stiffness associated with aortic oxidants and inflammation can be normalized by returning obese mice to a ND. In this study, we found that overnight fasting acutely decreased arterial stiffness in obese mice. This beneficial effect of fasting against obesity-induced arterial stiffness were mediated by increased SirT1 activity and phosphorylated vasodilator-stimulated phosphoprotein, an NO downstream effector in VSM cells, involved in cytoskeletal protein dynamics, as the PWV-lowering effect of fasting and vasodilator-stimulated phosphoprotein phosphorylation were completely abrogated in mice lacking SirT1 in VSM (SMKO).

Consistent with our previous and current findings, life-long calorie restriction, whose beneficial effects are attributed to SirT1, normalizes age-related endothelial dysfunction and arterial stiffness by reducing oxidative stress and preserving NO function in aging mice. However, to the best of our knowledge, this is the first report showing that fasting acutely decreases arterial stiffness in obese mice, and that links SirT1 activity to the acute effect of fasting on the vasculature.

We found that SirT1 exerts anti-inflammatory and antioxidative effects in obese mice by inhibiting VCAM-1 and NfκB activation in the aorta and VSM cells (illustrated in the scheme in Figure 5). Our data are consistent with the fact that SirT1-mediated deacetylation inhibits NfκB activation and activates Nrf2, a transcription factor that upregulates antioxidant enzymes, including Cu–Zn superoxide dismutase, suggesting that similar pathways may be at play in VSM.

The expression of nicotinamide adenine dinucleotide phosphate oxidase Nox2 in the aortic media, which we previously showed as a major source of oxidants in aortic

Figure 5. Summary of the postulated mechanisms by which sirtuin-1 (SirT1) protects the aorta from high-fat, high-sucrose (HFHS) diet–induced aortic stiffness. HFHS increases tumor necrosis factor-α (TNF-α) in the aorta, which activates, among others, the superoxide-generating enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Nicotinamide adenine dinucleotide phosphate oxidase isofrom 2 (Nox2) in vascular smooth muscle (VSM) cells. Superoxide anions can impair VSM cells by inactivating endothelial-derived nitric oxide (NO), forming peroxynitrite (ONOO−) and stimulating nuclear factor kappa light chain enhancer of activated B cells (NfκB) to induce vascular cell adhesion molecule (VCAM)-1 in VSM cells. Decreased NO also stimulates the formation of enzymatic extracellular matrix cross-links in the aortic wall. These pathways coevitably increase aortic stiffness. VSM SirT1, which is activated by resveratrol (Resv), S17834, SRT1720 and overnight fasting, or in mice genetically engineered to overexpress SirT1 (SirT1 bacterial artificial chromosome overexpressor [SirBACO] and SMTG), prevents HFHS-induced aortic stiffness by inhibiting NfκB, VCAM-1, and p47phox, thus opposing oxidants.
smooth muscle cells in HFHS-fed mice, did not seem to change significantly in aortas of mice overexpressing SirT1 (SMTG). However, the Nox2 major functional subunit, p47phox, was decreased in SMTG aortas compared with WT controls. Therefore, we cannot exclude that Nox2-specific activity and other oxidant-generating enzymes, such as Nox1, Nox4, and xanthine oxidases, known to be abundantly expressed in the aortic wall in the setting of diabetes mellitus and insulin resistance, may have been decreased by VSM SirT1.

In addition to NFκB, multiple and synergistic SirT1 downstream molecular targets are likely involved and may independently ameliorate the complex vascular phenotype of arterial stiffness associated with obesity. SirT1 directly deacetylates several transcription factors involved in cellular processes linked to metabolic perturbations, including Nrf2, forkhead box protein O1 and forkhead box protein O3, p53, peroxisome proliferator-activated receptor gamma coactivator 1-alpha, signal transducer and activator of transcription 3, sterol regulatory element-binding protein 1, p47phox, was decreased in SMTG aortas compared with ND-fed mice. Sirtuin-1 (SirT1) is an evolutionarily conserved NAD+-dependent deacetylase, considered an important metabolic regulator and the putative molecular target of calorific restriction. Using multiple approaches, here we demonstrate that overnight fasting, which induced SirT1 in the aorta, pharmacological treatment with SirT1 activators (resveratrol, S17834, and SRT1720) or genetically overexpressing SirT1 globally or in VSM cells in mice, prevented or reversed HFHS-induced arterial stiffness during a course of 8 months, without evident metabolic improvements. At the molecular level, SirT1 overexpression in VSM prevented cytokine-induced NFκB activation, VCAM-1 upregulation, and decreased p47phox expression and oxidants in aorta and VSM cells. In summary, VSM SirT1 activation decreases arterial stiffness in the setting of obesity by stimulating anti-inflammatory and antioxidant pathways in the aortic wall. Considering that 68% of Americans are overweight or obese, even at their young age, SirT1 activators may represent a viable therapeutic approach to ameliorate arterial compliance in obese and overweight individuals with metabolic syndrome, as means of preventing cardiovascular events later in life.

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**Disclosures**

None.

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**Novelty and Significance**

**What Is New?**

- Pharmacological, genetic and lifestyle (fasting) interventions aimed at increasing sirtuin-1 (SirT1) in the aorta prevent or reverse arterial stiffness, in the setting of obesity, independently of metabolic effects.
- Increasing vascular smooth muscle SirT1 prevents aortic stiffness induced by high-fat, high-sucrose diet, by stimulating anti-inflammatory and antioxidant pathways in the aortic wall.

**What Is Relevant?**

- SirT1 activators may represent a potential therapeutic avenue to prevent arterial stiffness and associated cardiovascular complications in overweight/obese individuals with metabolic syndrome.

**Summary**

SirT1, an NAD+-dependent deacetylase regulated by the cellular metabolic status, may protect against the development of diabetes mellitus and insulin resistance. However, the effects of SirT1 on the vascular complications of the metabolic syndrome have not been fully elucidated. Using complementary in vivo and ex vivo approaches, here we demonstrate that vascular smooth muscle SirT1 exerts anti-inflammatory and antioxidants effects on the aortic wall, thereby decreasing arterial stiffness in obese mice. Pharmacological, genetic and lifestyle (fasting) approaches aimed at boosting SirT1 in the aortic wall prevent obesity-induced arterial stiffness, in the setting of metabolic syndrome.
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Vascular Smooth Muscle Sirtuin-1 Protects Against Diet-Induced Aortic Stiffness

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Short Title: Smooth muscle sirtuin-1 prevents aortic stiffness

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MATERIALS AND METHODS

Experimental animal models. All animal experimental procedures were approved by the institutional animal care and use committee (IACUC) at Boston University Medical Campus. We generated SirT1 vascular smooth muscle knockout (SMKO) and transgenic (SMTG) mice on C57Bl/6J genetic background, in house, as described and characterized in 1. Specifically, mice with two loxP sites flanking SirT1 exon 4, required for the enzyme catalytic activity, were obtained from Dr. David Sinclair at Harvard University, Cambridge, MA, USA. Floxed SirT1<sup>loxP</sup> mice were bred with mice in which Cre-recombinase expression is driven by the endogenous smooth muscle 22 α protein (SM22α, transgelin) promoter (Jackson Laboratories; endogenous locus SM22α Cre Knock-In; B6.129S6 Tagln<sup>tm2(cre)Yec</sup>/J) to generate a mouse with SirT1 exon 4 deletion in smooth muscle (SMKO).

Similarly, mice with SirT1 gene inserted downstream of the collagen type I locus and preceded by loxP sites flanking a Stop codon (obtained from Dr. David Sinclair), were intercrossed with the SM22α-Cre males, described above, to generate mice over-expressing SirT1 in VSM (SMTG).

Mice over-expressing SirT1 globally (SirT1 Bacterial Artificial Chromosome Overexpressor, SirBACO) 2 were obtained from Dr. Wei Gu, Columbia University, New York, NY, and maintained on a C57Bl/6J genetic background. Mice were housed in rooms with 12-hour light/dark cycle and in groups of 3-4, with food and water ad libitum. In each experiment the appropriate littermate controls (floxed SirT1<sup>loxP</sup>/Cre- or floxed Stop-SirT1/Cre-) were used and indicated as WT.

Diet and drug treatments. At 8 weeks of age, SMTG, SMKO, SirBACO mice and littermate controls were fed normal or high fat, high sucrose diets for up to 8 months. Diets were as follows: normal diet, ND: 4.5 % fat, 0% sucrose; high fat, high sucrose diet, HFHS: 35.5 % fat from lard, representing 60 % calories, 16.4 % sucrose (catalog numbers D09071702 and D09071703, Research Diets, New Brunswick, NJ, USA). To note, the control diet (ND) was custom-formulated to match the micronutrients contained in HFHS, except for fat and sucrose.

Fasting was performed in subsets of WT or SMKO mice, fed ND or HFHS, for 2 or 8 months, by removing food overnight and measuring arterial stiffness, in the same mice, before (fed) and after food removal (fasted).

Subsets of male C57Bl/6J mice (stock number 00664, The Jackson Laboratory, Bar Harbor, ME, USA) were fed HFHS diet supplemented with resveratrol or S17834, two polyphenolic compounds known to activate SirT1 3, 4. Resveratrol and S17834 were dissolved in ethanol and admixed with food to obtain a daily dose of 130 mg/kg, allowing the ethanol to evaporate for 48 hrs before feeding the diet to mice.

In reversal studies, subsets of mice were fed HFHS for 8 months before receiving HFHS supplemented with SRT1720, a small molecule SirT1 activator, more specific than resveratrol 3, 5, for one week. SRT1720 was suspended in ethanol, sonicated until complete dissolution and mixed into HFHS diet, followed by ethanol evaporation, to achieve a dose of 100 mg/kg/day. Body weights were recorded at baseline and every month after diet initiation.

Glucose tolerance test (GTT). Glucose tolerance was assessed in HFHS-fed WT (n=5-9), SMTG (n=6), SirBACO (n=5) mice and mice treated with S17834 (n=9) after an overnight fast, as we previously described 6. Briefly, plasma glucose levels were measured with a strip meter (Accu-Check, Roche Diagnostics, Indianapolis, IN, USA) before and every 30 min after intraperitoneal injection of a
freshly prepared glucose solution (1.5 mg/kg of body weight), over a 120-minute period. Data are expressed as curves of glucose plasma concentration (mg/dl) plotted against time (min) for each experimental group.

**Pulse wave velocity (PWV) measurements.** PWV is the gold standard *in vivo* measurement of arterial stiffness. PWV was assessed in the following experimental groups, where WT represents appropriate littermate controls, using methods that we previously described 1, 6, 7:

- experiment 1) WT/ND before and after fasting (n=11), WT/HFHS before and after fasting (n=11) at 2 months of diet;
- experiment 2) WT/ND before and after fasting (n=9), WT/HFHS before and after fasting (n=9), at 8 months of diet;
- experiment 3) WT/HFHS before and after fasting (n=4), SMKO/HFHS before and after fasting (n=4) at 8 months of diet;
- experiment 4) WT/HFHS (n=4), SMTG/HFHS (n=6) at baseline, 4 and 8 months of diet;
- experiment 5) WT/ND (n=6), WT/HFHS (n=6), WT/HFHS/resveratrol (n=6) at baseline, 4 and 8 months of diet;
- experiment 6) WT/ND (n=6), WT/HFHS (n=6), WT/HFHS/S17834 (n=6) at baseline, 4 and 8 months of diet;
- experiment 7) WT/ND (n=5), WT/HFHS (n=5), SirBACO/ND (n=6), SirBACO/HFHS (n=7) at 8 months of diet;
- experiment 8) WT/HFHS (n=4), WT/HFHS/SRT1720 (n=5) at 8 months of diet and after one week of drug treatment.

Briefly, mice were kept recumbent on a heated pad and attached to electrocardiogram (ECG) pads. A high-resolution Doppler echocardiography transducer (VEVO770, FujiFilm) was used to acquire 20 seconds of continuous recording of blood flow waveforms from 2 locations along the aorta, one proximal and one distal to the heart. The time between the R peak of ECG and the foot of each flow waveform were manually measured post-acquisition on at least 5 cardiac cycles, for the proximal and distal locations, for each mouse to compute the foot-to-foot arrival times. PWV (m/s) was calculated by dividing the distance between the proximal and distal locations by the difference in the proximal and distal arrival times. To note, PWV measurements were previously optimized by us 1, 6, 7 to maintain heart rates and mean arterial pressures at comparable values (heart rate 450 ± 30 bpm and mean arterial pressure 95 ± 5 mmHg) in all animals during Doppler waveform acquisitions.

**Aortic tissue isolation and *ex vivo* treatments.** Whole aortas were cleaned of periaortic fat and removed from PBS-perfused WT and SMTG mice. Aortas were cultured at 37°C overnight in 1 ml culture medium (DMEM containing 1g/mL glucose), with or without TNFα (10 ng/ml, Peprotech) or with or without SRT1720 (50 μmol/L, Sellekchem). At the end of the overnight incubation, aortas were briefly washed in cold PBS, snap-frozen in liquid nitrogen and kept at -80°C until further processing.

**Isolation of vascular smooth muscle cells.** Vascular smooth muscle (VSM) cells were isolated by enzymatic dissociation, as we previously described 1, 8. Aortas from WT and SMTG mice were cleaned of blood and periaortic fat and incubated at 37°C for 12 min in 3 mg/mL collagenase II (Worthington, Lakewood, NJ, USA), in serum-free DMEM. Adventitia was removed manually and the
medial layer was further dissociated in 3 mg/mL collagenase and 1 mg/mL elastase at 37°C for 30 min, with gentle agitation every 10 min. Cells were centrifuged and resuspended in complete growth medium (DMEM, 1 g/L glucose supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic) and used until passage 5.

**Western blot on aortas and VSM cells.** Aortas and VSM cells were manually homogenized in a glass-glass grinder (Kontes, number 20) in RIPA buffer (200 μL/1 mg tissue, Cell Signaling Technology, Danvers, MA, USA), freshly supplemented with protease inhibitor cocktail and trichostatin A (5 μM). Homogenates were sonicated (10 seconds, x3, on ice) then cleared of non-soluble material by centrifugation at 14,000 rpm for 10 min, 4°C. Protein concentration was assessed using Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA), as per manufacturer’s instructions.

VSM cell or aortic lysates were resolved by SDS-PAGE in 4-20% or 7.5% polyacrylamide gels and transferred to PVDF membranes (100V, 120 min). Membranes were blocked in 5% non-fat milk in 1% Tween-20 in TBS (TBS-T) for 1 hour and incubated with one of following antibodies, overnight at 1:1000 dilution: SirT1 (catalog # ab110304, Abcam, Cambridge, MA, USA), VCAM-1 (catalog # 14694, Cell Signaling Technologies), phosphorylated p-65 NFκB (catalog# 3033S, Cell Signaling Technologies), acetylated histone H3 (catalog# 9649S, Cell Signaling Technologies), p47phox (catalog# BS4600, Bioworld Technologies), phosphorylated VASP (catalog# 3114, Cell Signaling Technologies), β-actin (catalog# A5441, Sigma-Aldrich) and GAPDH (catalog # 2118S, Cell Signaling Technologies). Membranes were then washed in TBS-T and incubated with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technologies) for 1 hour then washed in TBS-T and exposed to the chemiluminescent substrate ECL (GE Healthcare, Boston, MA, USA) to visualize protein bands. Band images were documented with Imager model #: LAS4000 (General Electric). Protein band intensities were quantified with Image J (www.nih.gov), with band intensities pertaining to the same experimental group averaged and normalized to β-actin or GAPDH, used as loading controls, and expressed as fold change relative to WT controls.

**SirT1 mRNA quantitation by qRT-PCR.** Aorta, isolated from WT (n=4) and SMTG (n=4) mice, were homogenized in Trizol and kept at -80°C until RNA extraction by chloroform separation/isopropanol precipitation. Messenger RNA was retrotranscribed into cDNA with High capacity RNA-to-DNA kit (Life Technologies), as per manufacturer’s instructions. SirT1 gene expression was analyzed by quantitative RT-PCR with validated Taqman assays multiplexed for β-actin, in AB750 instrument (Applied Biosystems). SirT1 mRNA expression was calculated by the ΔΔCt method, using β-actin as endogenous control and expressed as fold ratio versus WT controls.

**NFκB activity assay.** WT and SMTG VSM cells, isolated as described above, were plated at a density of 5,000 cells/well in 96-well plates, in triplicate for each treatment. Cells were infected with adenoviral construct containing a firefly luciferase reporter gene under the control of specific NFκB DNA responsive elements (1 x 10⁹ PFU/ml) and a renilla luciferase (1 x 10⁹ PFU/ml), as internal control, in DMEM medium without serum or antibiotics. Four hours post-infection, complete growing medium (DMEM with 10%FBS and 1% antibiotics) was added. Two days later, cells were starved overnight in minimal medium (DMEM with 0.5% FBS) then treated overnight with TNFα (10 ng/ml)
or vehicle control (PBS). Cells were then lysed and the firefly and renilla luciferase enzymatic activities were assayed with a standard luciferase assay, as per manufacturer’s instructions (Promega). The experiment was repeated four times.

**Oxidant measurement in the aorta.** Oxidants were assessed in fresh frozen aortic sections with dihydroethidine (DHE), as we previously described \(^1\). Briefly, ~ 1 cm of thoracic aorta from ND- and HFHS-fed WT, SirBACO and SRT1720-treated mice (n=5, WT/ND; n=10, WT/HFHS; n=5, SirBACO/HFHS and n=4, WT/HFHS+SRT1720) were frozen in OCT and kept at -80°C until sectioning with a cryotome at a thickness of 10 μm. Aortic sections were washed on ice with cold PBS and incubated with DHE solution, freshly prepared at 10 μM working solution, at 37°C for 45 min. Sections were then washed in PBS and cover-slipped with Prolong Gold Antifade mounting medium (Life Technologies, Grand Island, NY). Digital images were captured with an epifluorescent microscope (Nikon Eclipse 80i), a CDD camera (DQ Qi Mc), and NIS-Elements 3.22 software (Nikon, Melville, NY, USA). DHE red fluorescence intensity was quantified by 6 independent observers, blinded to the experimental groups with a scoring system on a scale 0-4, with 0 corresponding to the lowest intensity and 4 to the highest. Scores pertaining to the same experimental group were averaged before statistical analysis.

**Immunohistochemistry on aortic sections.** We evaluated the expression of NAPDH oxidase Nox2 in freshly frozen aortic sections prepared from HFHS-fed WT (n=5) and HFHS-fed SMTG (n=6), by standard immunohistochemical methods using Vactastain ABC kit (Vector), as we previously described \(^8\).

**Statistical analysis.** All data are expressed as mean ± SEM and analyzed with GraphPad Prism v.5.0c. Repeated measures two-way ANOVA with Bonferroni’s multiple comparison post-hoc test was used to analyze PWV and body weights in SMTG (Figure 2A and S2A) and in mice treated or untreated with resveratrol or S17834 (Figures 2B, S2C and S2D), during the time course of ND or HFHS diets, and to analyze GTT in HFHS-fed WT, SMTG, SirBACO and S17834-treated mice (Figures S2B, S2F and S2G). Body weights in SirBACO mice (Figure 2E), PWV values in fasting experiments (Figure 1A), logarithmically transformed to fit a normal distribution, PWV values in fasted SMKO mice (Figure 1B) and NFκB activity luciferase assay (Figure 3C) were analyzed by one-way ANOVA with Bonferroni’s multiple comparison post test. PWV in SirBACO mice (Figure 2C) and quantification of DHE staining (Figure 4A) were analyzed by nonparametric one-way ANOVA Kruskal-Wallis test with Dunn’s multiple comparisons post test. PWV and body weights in mice treated with or without SRT1720 (Figure 2D) were analyzed by unpaired t-test. When experiments had n < 7, such as Western blots and qRT-PCR, non parametric Whitney-Mann t test was used to compare the means of two groups. P values < 0.05 were considered significant.
AUTHORS CONTRIBUTIONS.

JLF contributed to design the study and conducted experiments; LAS genotyped mice and performed Western blots and Western blots quantitations; RW contributed to design the study and performed PWV measurements; IVR performed Western blot, Western blot quantitations, and DHE immunostainings; XW supervised mouse feeding and recorded body weights over time; RAC and MB provided critical comments to the study and the manuscript; FS contributed to the study design, coordinated the study, designed and performed experiments, analyzed the data and wrote the manuscript.
REFERENCES FOR ONLINE SUPPLEMENTS.


Figure S1. Characterization of SMTG mice. (A) Representative Western blots of aortas (with adventitia removed; right panel) and VSMC isolated from aortas (left panel) of WT and SMTG mice, confirming SirT1 over-expression in VSM. Each lane represents one mouse. (B) Quantitative RT-PCR for SirT1 indicates ~4-fold increase in SMTG aortas (n=4) compared with WT (n=4). *, p<0.05.
Figure S2

Figure S2. SirT1 activation does not have metabolic effects in HFHS-fed mice. (A) Body weights in WT (n=4) and SMTG (n=6) fed HFHS up to 8 months. (B) Glucose tolerance test in HFHS-fed WT (n=4) and SMTG (n=6) mice, performed as described in methods. Curves plot glucose plasma concentration over time, for the two groups. Body weight gains over 8 months in mice fed (C) ND (n=6), HFHS (n=6) or HFHS supplemented with resveratrol (130 mg/kg/day; n=6) or (D) ND (n=6), HFHS (n=6) or HFHS supplemented with S17834 (130 mg/kg/day; n=6). *, p<0.05 WT/HFHS vs WT/ND. †, p<0.05 WT/HFHS/resv or WT/HFHS/S17834 vs WT/ND. (E) Body weights were similarly increased in HFHS-fed SirBACO mice as in HFHS-fed WT littermate controls, after 8 months of HFHS. *, p<0.05 WT/HFHS (n=5) or SirBACO/HFHS (n=6) vs WT/ND (n=5). (F-G) Glucose tolerance test in HFHS-fed WT (n=5-9), SirBACO (n=5) and S17384-treated (n=9) mice, performed as described in methods.
Figure S3. The polyphenol S17834 protects against HFHS-induced arterial stiffness. The polyphenolic compound S17834, administered in food for 8 months at a dose of 130 mg/kg/day, prevented HFHS-induced arterial stiffness, measured by PWV (m/s). ND, normal diet. *, p<0.05 ND (n=6) vs HFHS (n=6); †, p<0.05 HFHS/S17834 (n=6) vs HFHS (n=6).
Figure S4

Figure S4. Nox2 expression in the aorta of HFHS-fed mice. Representative images, at 40x magnification, of aortic sections from HFHS-fed WT (n=5) and SMTG (n=6) mice immuno-stained with an antibody against Nox2. An aortic section processed in absence of the primary antibody served as a negative control, to prove the specificity of the signal. E, endothelium; M, media; A, adventitia.