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

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Abstract

Arterial stiffness, a major cardiovascular risk factor, develops within two months in mice fed a high fat, high sucrose diet (HFHS), serving as a model of human metabolic syndrome, and is associated with activation of pro-inflammatory 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 (PWV), in mice fed HFHS for 2 or 8 months, but not in mice lacking SirT1 in VSM (SMKO). Similarly, VSM specific (SMTG) genetic SirT1 over-expression prevented PWV increases induced by HFHS feeding, over 8 months. Administration of resveratrol or S17834, two polyphenolic compounds known to activate SirT1, prevented HFHS-induced arterial stiffness and were mimicked by global SirT1 over-expression (SirBACO), without evident metabolic improvements. Additionally, HFHS-induced PWV increases were reversed by one-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 NFκB activation and 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 anti-oxidant 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.

Keywords

arterial stiffness; metabolic syndrome; animal model cardiovascular disease; obesity; sirtuin-1

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Disclosures. None.

Introduction

Among non-communicable diseases, cardiovascular diseases (CVD) remain the leading cause of mortality, in the US and worldwide [1]. Arterial stiffness is characterized by the progressive loss of compliance of large conduit arteries, and is a major risk factor for CVD. 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, as 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 cardio-metabolic risk factors associated with overweight/obesity and insulin resistance, significantly increases the risk of developing CVD [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 two months [16]. Aortic wall stiffening was associated with increased inflammation, as measured by cytokines TNFα, MCP-1, MIP1α, 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, TNFα-induced activation of the oxidant-generating enzyme NADPH oxidase, Nox2, is a major determinant of inflammation associated with functional impairment of aortic smooth muscle cells [17]. 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 [16], suggesting that aortic wall stiffening is, at least in part, a dynamic process, amenable to therapy and/or life-style 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 [21]. Mice over-expressing SirT1, when fed a high caloric diet or inbred into an obesity-prone *db/db* genetic background, are protected from diabetes [22]. However, the vascular-specific effects of SirT1 on the cardiovascular complications of diabetes 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 [23], 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 the present 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-NFκB activation and VCAM-1 upregulation and by decreasing p47phox, a major functional subunit of Nox2, in the aortic wall and VSM cells.

Notably, anti-inflammatory and anti-oxidant effects of SirT1 in the aortic wall occurred even after a short treatment of obese mice with a specific SirT1 activator, SRT1720, 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 US, where 68% of Americans, including children, are overweight or obese [1], 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 online-only data supplements at http://hyper.ahajournals.org.

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, as described in details in the online supplemental material. Mice over-expressing 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 normal diet (ND) or high fat, high sucrose diet (HFHS), 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, two polyphenolic compounds known to activate SirT1 [24, 25], 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 [24], at a daily dose of 100 mg/kg.

Glucose tolerance test (GTT)

Glucose tolerance was assessed in HFHS-fed WT (n=5-9), SMTG (n=6), SirBACO (n=5) and S17834-treated (n=9) mice, as we previously described [16].

Pulse wave velocity (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 [16, 23].

Aortic tissue isolation and *ex vivo* treatments

Whole aortas from WT and SMTG mice were cultured at 37°C overnight in 1 ml 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 snap-frozen in liquid nitrogen and kept at -80°C until further processing.

Isolation of vascular smooth muscle cells

Aortic smooth muscle cells were isolated by enzymatic dissociation, as we previously described [17, 23].

Western blot and qRT-PCR on aortas and VSM cells were performed by standard methods, described in details in the online supplemental material.

NFκB activity assay

WT and SMTG VSM cells were co-infected with adenoviral constructs containing a firefly luciferase reporter gene under the control of specific NFκB DNA responsive elements (1×10^9 PFU/ml) and a renilla luciferase (1×10^9 PFU/ml). Two days post-infection, cells were treated with TNFα (10 ng/ml) or vehicle control (PBS). Firefly and renilla luciferase activities were assayed with a standard luciferase assay, as per manufacturer's instructions (Promega).

Oxidant measurement

mainly superoxide anion, was performed in freshly frozen aortic sections with dihydroethidine (DHE), as we previously described [23].

Statistical analysis

All data are expressed as mean ± SEM. Repeated measures two-way ANOVA with Bonferroni's 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 GTT 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 one-way ANOVA with Bonferroni's multiple comparisons. PWV in SirBACO mice and quantification of DHE staining were analyzed by nonparametric one-way ANOVA Kruskal-Wallis test with Dunn's multiple comparisons. PWV and body weights in mice treated with or without SRT1720 were analyzed by unpaired t-test. For Western blots and qRT-PCR, non parametric Whitney-Mann t test was used. P values < 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 a normal diet, returns PWV, the *in vivo* index of arterial stiffness, to normal, over a 4 month period [16], indicating that arterial stiffness in settings of obesity is reversible. Caloric restriction is known to increase SirT1 activity through increased NAD^+ production [26] and SirT1 expression [27]. Here, we used overnight fasting to mimic acute, but severe, caloric restriction in mice fed a HFHS diet for 2 or 8 months as a means of increasing SirT1 activity in the aorta, measured by acetylated histone H3 [28] (Figure 1A), in accordance to what has been reported for other tissues [29, 30]. 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 (pVASP), an eNOS-NO-cGMP downstream effector in VSM cells [31], involved in cytoskelatal protein dynamics [32]. Fasting-induced SirT1 and pVASP were abrogated in aortas of SMKO mice (Figure 1D). 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 over-expression prevents HFHS-induced arterial stiffness

To directly study the effects of SirT1 on arterial stiffness in settings of obesity, we used mice over-expressing SirT1 in VSM (SMTG), fed HFHS for up to 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 qRT-PCR in aortic homogenates (Supplemental Figure S1). Specific VSM SirT1 over-expression in SMTG mice prevented HFHS-induced arterial stiffness (Figure 2A), without notable differences in weight gain (Supplemental Figure S2A) or glucose tolerance (Supplemental Figure S2B), compared with HFHS-fed WT littermate controls.

Polyphenols and global SirT1 over-expression prevent HFHS-induced arterial stiffness

Similar to HFHS-fed SMTG mice, treatment with the polyphenols resveratrol (Figure 2B) or S17834 (Supplemental Figure S3), each at a dose of 130 mg/kg/day, completely prevented the development of HFHS-induced arterial stiffness over 8 months. These results were mimicked in mice globally over-expressing 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 experimental groups (Supplemental Figures 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 (Supplemental Figures 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/day) to obese mice, when obesity and arterial stiffness were well established. After 8 months of HFHS diet, one 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 vs 57 ± 2 g in HFHS-fed mice treated with SRT1720, n=5; NS).

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 TNFα [16, 17]. Therefore, we assessed whether the amelioration of arterial stiffness by SirT1 activators and genetic overexpression of SirT1 were mediated by anti-inflammatory and anti-oxidant effects.

As shown in Figure 3, VSM SirT1 over-expression prevented TNFα-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 TNFα 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, TNFα-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).

Similarly, incubation of aortas *ex vivo* with SRT1720 (50 μmol/L) prevented TNFα-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 (Figures 1A and 1D).

Oxidant production, mainly superoxide anion, assessed by DHE 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 [17], we first assessed whether the anti-oxidant effects of SirT1 on the aortic wall were mediated by downregulation of Nox2. We found no significant changes in Nox2 expression in VSM of aortic sections of HFHS-fed SMTG (n=6), compared with HFHS-fed controls (Supplemental Figure S4). Nox2 remained abundantly expressed in the endothelium and adventitia of both WT and SMTG mice fed HFHS (Supplemental Figure S4), with limited expression in the media. However, protein expression of p47phox, a major functional subunit of Nox2 [34], was significantly decreased in SMTG aortas (Figure 4B).

Discussion

High fat, high sucrose (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 two months in mice fed HFHS [16] and that, at the molecular level, obesity-induced vascular remodeling and stiffening are associated with impaired NO bioavailability and TNFα-induced inflammatory responses in VSM, leading to functional impairment of VSM cells [17] and increased extracellular matrix remodeling [16]. VSM cell functional alterations, such as increases in VSM cell contractility and stiffness [18-20], and extracellular matrix remodeling [35] are major contributors to aortic wall stiffening. Building upon our previous studies, here we demonstrate, that sirtuin-1 (SirT1), 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 non-polyphenolic 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 over-expressing 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, over-expressing 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 [24], improves survival and insulin-resistance in mice fed high caloric diets [36]. However, the therapeutic potential of resveratrol and other polyphenolic compounds as anti-diabetic drugs has been controversial because of non-SirT1-mediated actions [37, 38]. Several small molecule activators of SirT1 have been developed, which exert more specific and/or potent effects on SirT1 than resveratrol [24], and are currently in clinical trials for type 2 diabetes and metabolic disorders (ClinicalTrials.gov identifier NCT01018628). However, very limited studies have elucidated the effects of SirT1 activators in the cardiovascular complications of the metabolic syndrome. Specifically, only one recent study used resveratrol in high fat, high sucrose-fed nonhuman primates to study arterial stiffness as primary endpoint [39].

In our study, the beneficial effects of resveratrol against HFHS-induced aortic stiffness were mimicked in mice over-expressing SirT1 globally (SirBACO), or specifically in VSM (SMTG), as well as in mice treated one week with SRT1720. These effects were locally mediated in the aorta and not a consequence of a metabolic amelioration, as weight gain and glucose intolerance were similar in obese mice whether or not pharmacological or genetic intervention was used to increase SirT1. This is in apparent disagreement with previous studies, in which global SirT1 over-expression [22, 40] or treatment with resveratol [36] were associated with improvement of glucose intolerance and prevention of diabetes. These

discrepancies are likely explained by different experimental approaches, mainly the composition of the diets [22, 40], as pointed out by others [41].

We previously showed that HFHS-induced arterial stiffness associated with aortic oxidants and inflammation can be normalized by returning obese mice to a normal diet [16]. 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 VASP, a NO downstream effector in VSM cells [31], involved in cytoskeletal protein dynamics [32], as the PWV lowering effect of fasting and VASP 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 [27], normalizes age-related endothelial dysfunction and arterial stiffness by reducing oxidative stress and preserving NO function in aging mice [42]. 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 anti-oxidants effects in obese mice by inhibiting VCAM1 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 [43] and activates Nrf2 [44], a transcription factor which upregulates anti-oxidant enzymes, including Cu-Zn superoxide dismutase, suggesting that similar pathways may be at play in VSM.

The expression of NADPH oxidase Nox2 in the aortic media, which we previously showed as a major source of oxidants in aortic smooth muscle cells in HFHS-fed mice [17], did not seem to change significantly in aortas of mice over-expressing 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/or other oxidant-generating enzymes, such as Nox1, Nox4 [34] and xanthine oxidases [45], known to be abundantly expressed in the aortic wall in the setting of diabetes 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 co-dependently ameliorate the complex vascular phenotype of arterial stiffness associated with obesity. SirT1 directly deacetylates a number of transcription factors involved in cellular processes linked to metabolic perturbations, including Nrf2, FOXO1 and FOXO3, p53, PGC-1α, STAT3, SREBP-1 and PPAR-γ, to name a few (reviewed in [46]). Additionally, SirT1, a class III HDAC, can epigenetically modulate heterochromatin remodeling via histone H3 deacetylation, as shown in the aorta of SMTG mice, thereby affecting multiple genomic programs [28]. Nonetheless, we used multiple and complementary approaches to demonstrate the role of SirT1 in vascular homeostasis *in vivo* and on isolated aortas. Treatment with three different pharmacological SirT1 activators, including two polyphenols (resveratrol, S17834) and one non-polyphenolic small molecule (SRT1720), global or VSM-specific SirT1 genetic over-expression, and *in vivo* and *ex vivo* approaches, all indicate that SirT1 protects against HFHS-induced arterial stiffness by

stimulating anti-inflammatory and anti-oxidant molecular pathways in the aorta. These findings of a protective role of SirT1 in the aortic wall are corroborated by our previous work in which lack of SirT1 in VSM caused aortic dissection in response in angiotensin II [23]. Angiotensin II has been causally linked to arterial stiffness [47], suggesting that the anti-inflammatory and anti-oxidants effects of SirT1 may antagonize angiotensin II-mediated deleterious effects on aortas of obese mice.

In conclusion, considering the epidemic proportions of overweight and obesity in US, it is crucial to develop novel therapies to prevent cardiovascular events in individuals affected by metabolic syndrome. Our studies indicate that SirT1 activators may represent a potential therapeutic means of preventing or ameliorating arterial stiffness and associated cardiovascular complications in this vast patient population.

Perspectives

Arterial stiffening, a vascular condition characterized by remodeling of large conduit arteries, increases the risk of cardiovascular events. Arterial stiffness is increased in obese and overweight individuals with metabolic syndrome, further increasing their cardiovascular risk. Mice, fed a high fat, high sucrose (HFHS) diet closely mimic the human metabolic syndrome and develop arterial stiffness within two months of HFHS, compared to normal diet (ND)-fed mice. Sirtuin-1 (SirT1) is an evolutionarily conserved NAD^+-dependent deacetylase, considered an important metabolic regulator and the putative molecular target of caloric 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 over-expressing SirT1 globally or in vascular smooth muscle (VSM) cells in mice, prevented or reversed HFHS-induced arterial stiffness over a course of 8 months, without evident metabolic improvements. At the molecular level, SirT1 over-expression in VSM prevented cytokine-induced NFκB activation, VCAM-1 upregulation and decreased p47phox protein 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 anti-oxidant pathways in the aortic wall. Considering that 68% Americans are overweight or obese, even at a 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

1) 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 anti-oxidant pathways in the aortic wall.

2) 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.

3) SUMMARY

Sirtuin-1 (SirT1), an NAD^+-dependent deacetylase regulated by the cellular metabolic status, may protect against the development of diabetes 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 anti-oxidants 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.

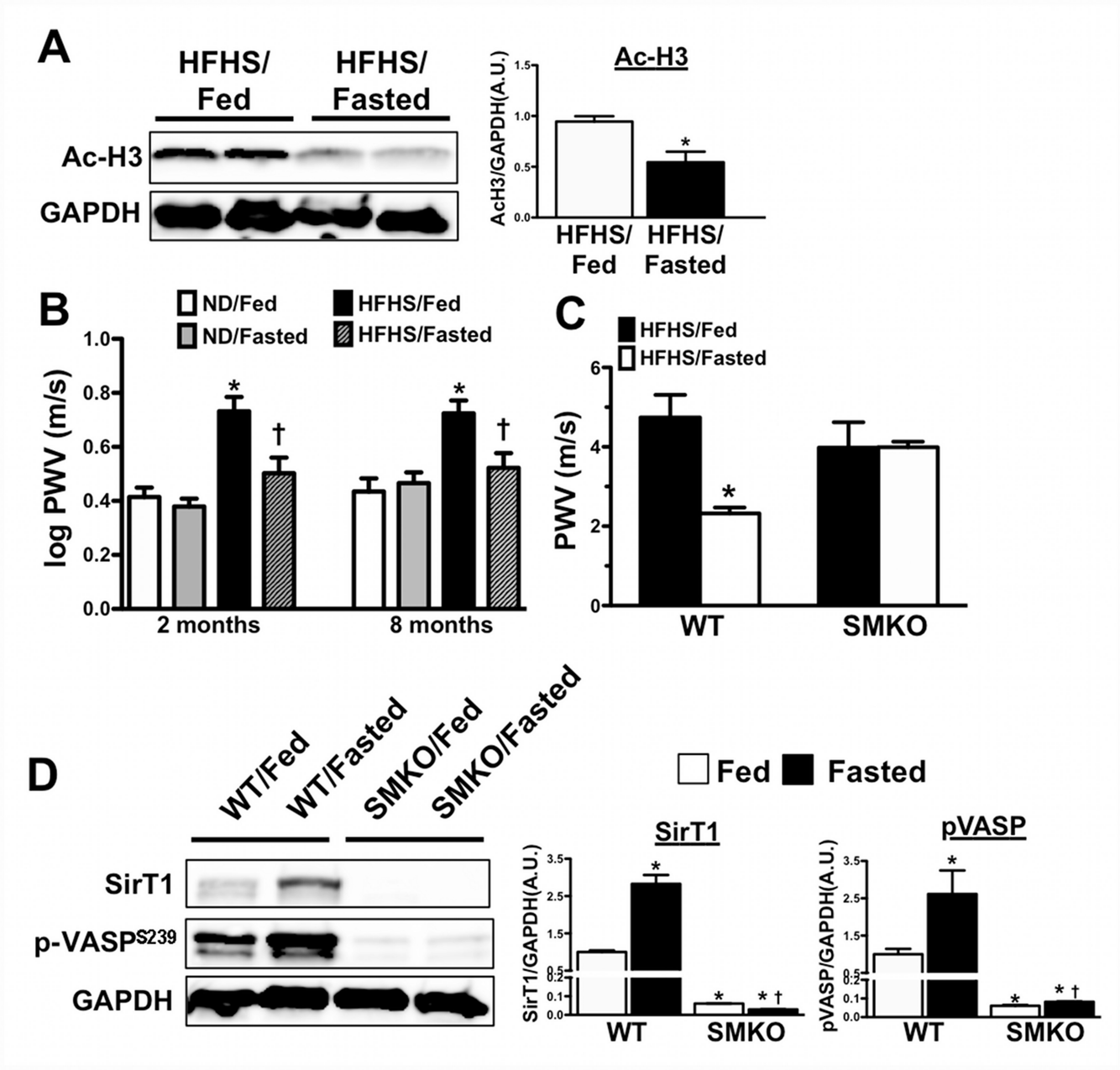

Figure 1. Fasting acutely normalizes HFHS-induced arterial stiffness via VSM SirT1

(**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 (HFHS/fasted, n=4). Each lane represents one aorta from one 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 months of HFHS feeding, but it did not have an effect on PWV in mice fed ND. PWV values were logarithmically transformed to fit a normal distribution. 2 months: *, $p<0.05$ HFHS/fed (n=11) vs ND/fed (n=11); † , $p<0.05$ HFHS/fasted vs HFHS/fed (n=11); 8 months: *, $p<0.05$ 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 WT/HFHS/fasted (n=4) vs WT/HFHS/fed (n=4); SMKO/HFHS/fasted (n=4) vs SMKO/HFHS/fed (n=4), NS. (**D**) Representative Western blots of aortic homogenates indicate that overnight fasting increases SirT1 expression and VASP phosphorylation, but not in mice lacking SirT1 in VSM (SMKO). GAPDH serves as loading control. Western blots were repeated 3 times. Each lane represents one aorta from one mouse. Quantitation of band intensities in graph. *, p<0.05 vs WT/fed; † , p<0.05 vs HFHS/fed.

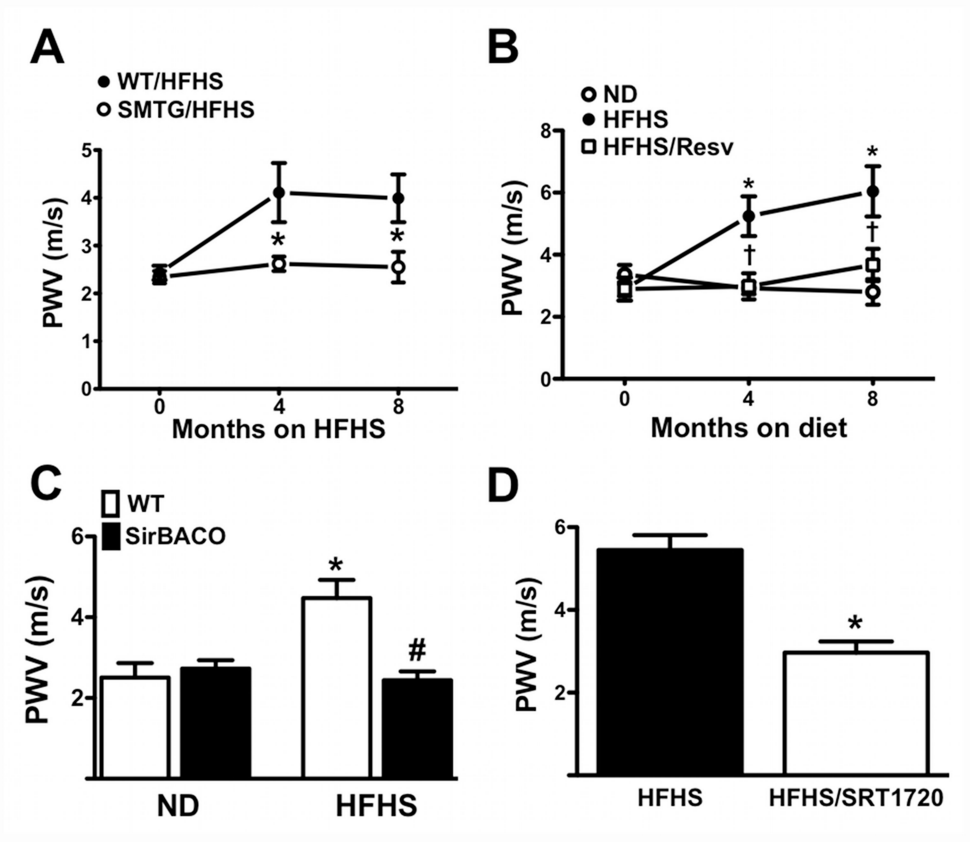

Figure 2. VSM SirT1 protects against high fat, high sucrose (HFHS)-induced arterial stiffness

(**A**) Mice over-expressing SirT1 in VSM (SMTG; n=6) were protected from HFHS-induced PWV increases compared with HFHS-fed littermate controls (n=4), over a 8 month period. *, p<0.05 WT/HFHS vs SMTG/HFHS. (**B**) The polyphenolic compound resveratrol, administered in food for 8 months at a dose of 130 mg/kg/day, prevented HFHS-induced arterial stiffness. ND, normal diet. *, p<0.05 ND (n=6) vs HFHS (n=6); † , p<0.05 HFHS/Resv (n=6) vs HFHS (n=6). (**C**) Whole body SirT1 over-expression in SirBACO mice prevented HFHS-induced PWV increases, compared with WT littermate controls, after 8 months 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/day) decreased HFHS-induced arterial stiffness, measured by PWV (m/s). *, p<0.05 HFHS/SRT1720 (n=5) vs HFHS (n=4).

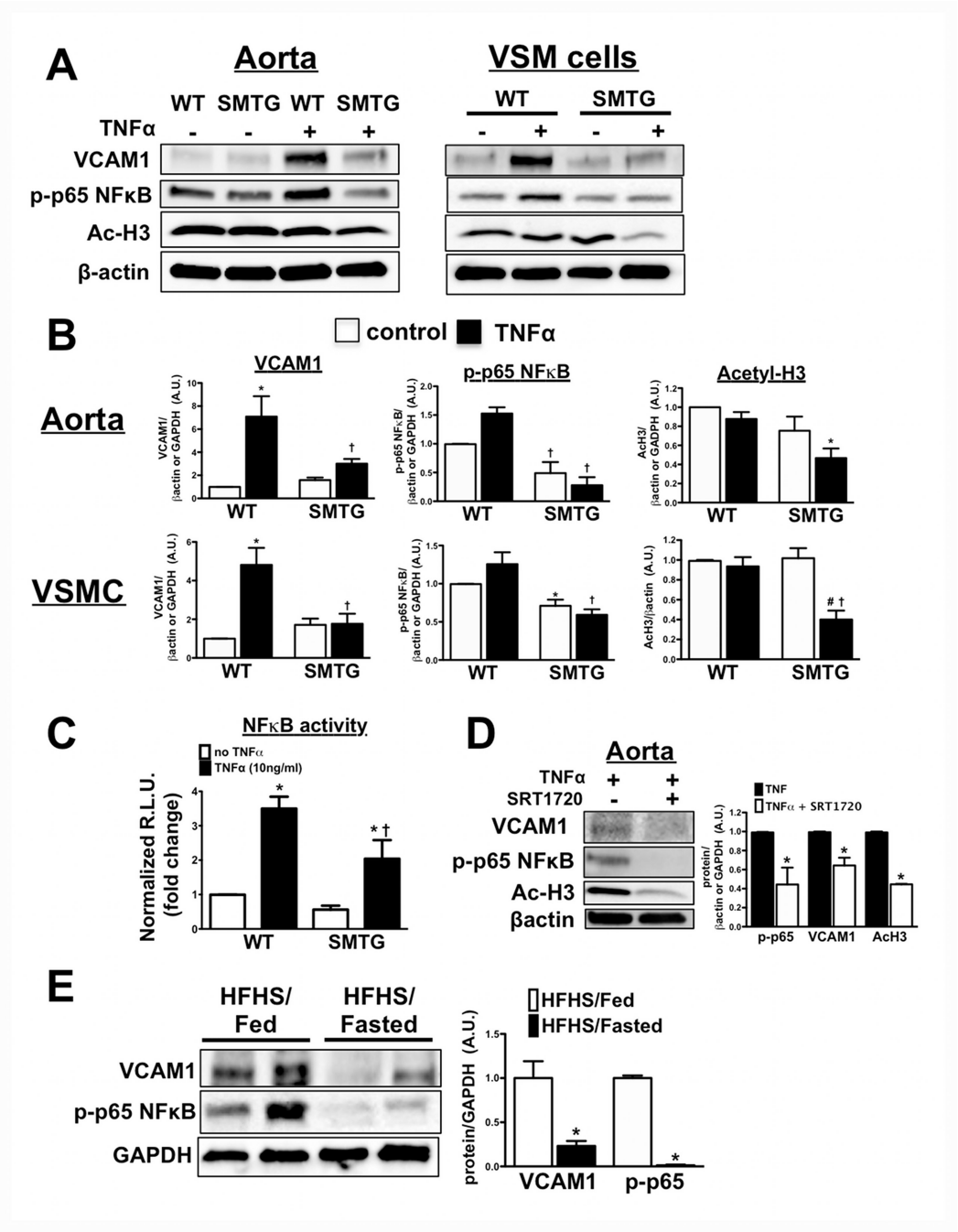

Figure 3. SirT1 prevents VCAM1 upregulation and NFκB activation in the aorta and VSM cells

(**A**) Representative Western blots for VCAM1, phosphorylated p65 (active NFκB subunit), acetylated histone H3 and β-actin in aortas (left panels) and aortic smooth muscle cells (right panels) from WT and SMTG mice and treated overnight with or without TNFα (10 ng/ml). Each lane on the left panels represents one aorta from one mouse. Western blots were repeated 4-6 times. (**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 non-

treated WT controls. *, p<0.05 vs WT/control; †, p<0.05 vs WT/TNFα; #, 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 TNFα (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 VCAM1, phosphorylated p65 (active NFκB subunit), acetylated histone H3 and β-actin in aortas treated overnight with TNFα (10 ng/ml), with or without SRT1720 (50 μmol/L). Each lane represents one aorta from one mouse. Western blots were repeated 3 times. *, p<0.05. (**E**) Western blots for VCAM1, phosphorylated p65 NFκB and β-actin in aortas of mice fed HFHS for 8 months (HFHS/fed, n=4) or fasted overnight after 8 months of HFHS (HFHS/fasted, n=4). Each lane represents one aorta from one mouse. Quantitation of band intensities in graph. *, p<0.05 vs HFHS/fed.

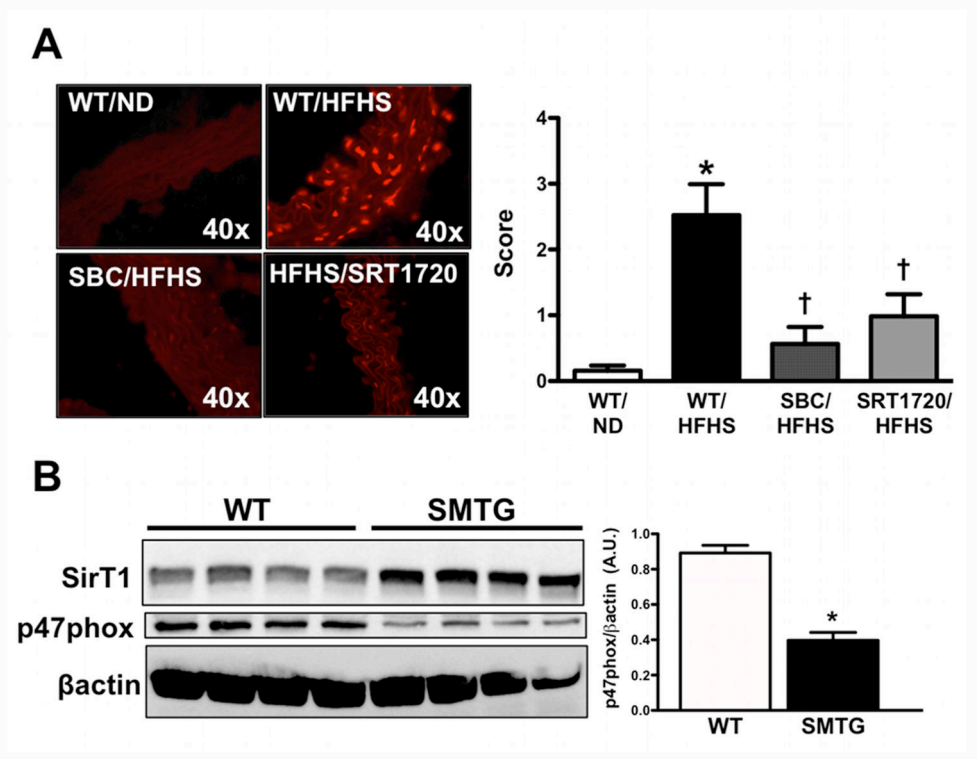

Figure 4. SirT1 decreases HFHS-induced oxidants in the aorta

(**A**) Representative images of DHE-stained aortic sections from WT/ND (n=5), WT/HFHS (n=10), SirBACO/HFHS (n=5) and WT/HFHS/SRT1720 (n=4) mice. Quantification of red fluorescence intensities in graph. A scoring system, with a scale of 0-4, with 0 corresponding to the lowest red fluorescence intensity and 4 to the highest, was used by 6 investigators, blinded to the treatment groups, to quantify the red fluorescence. *, $p<0.05$ WT/HFHS vs WT/ND; †, $p<0.05$ SirBACO/HFHS or WT/HFHS/SRT1720 vs WT/HFHS. SBC, SirBACO. (**B**) Representative Western blots for SirT1 and p47phox, the functional subunit of Nox2, in aortas of WT and SMTG mice. Each lane represents one mouse (WT, n=4; SMTG, n=4). Graph indicates p47phox band intensity quantitation. *, $p < 0.05$.

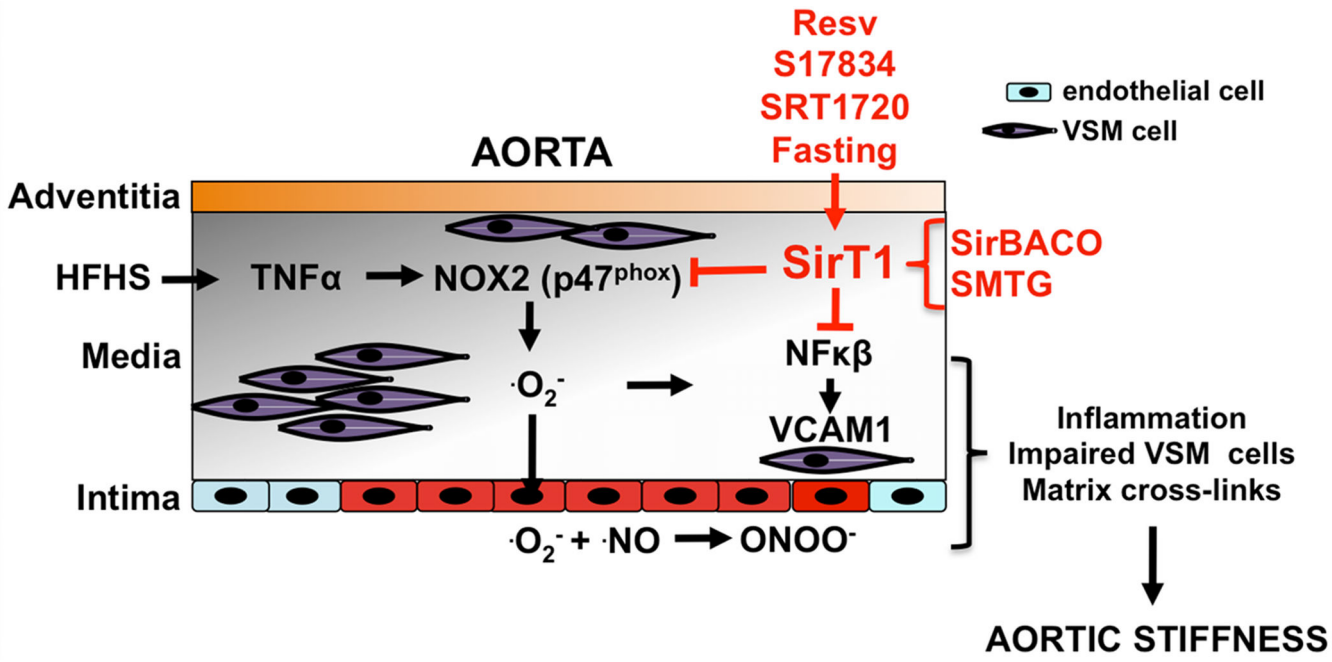

Figure 5. Summary of the postulated mechanisms by which SirT1 protects the aorta from HFHS-induced aortic stiffness

HFHS increases TNFα in the aorta [16, 17], which activates, among others, the superoxide-generating enzyme NADPH oxidase Nox2 in VSM cells [17]. Superoxide anions can impair VSM cells by inactivating endothelial-derived NO, forming peroxynitrite ($ONOO^-$) and stimulating NFκB to induce VCAM1 in VSM cells [17]. Decreased NO also stimulates the formation of enzymatic extracellular matrix cross-links in the aortic wall [16]. These pathways co-dependently increase aortic stiffness. VSM SirT1, which is activated by resveratrol (resv), S17834, SRT1720 and overnight fasting, or in mice genetically engineered to over-express SirT1 (SirBACO, SMTG), prevents HFHS-induced aortic stiffness by inhibiting NFκB, VCAM1 and p47phox, thus opposing oxidants.