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SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans

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Non-technical summary Advancing age is a major risk factor for the development of cardiovascular disease. A key characteristic of older arteries that may lead to cardiovascular disease is reduced endothelial function, characterized by blunted endothelium-dependent dilatation. Sirtuins, specifically sirtuin-1, are proteins linked to increases in lifespan and lower incidence of age-related diseases. We hypothesized that diminished sirtuin-1 with advancing age may alter regulation of a key endothelium dilatory enzyme, nitric oxide synthase. Our findings provide novel translational evidence that sirtuin-1 expression and activity contribute to arterial endothelial dysfunction with ageing and that this may be due to altered nitric oxide synthase activation. Importantly, our results provide further compelling support for sirtuin-1 as a potential therapeutic target for lifestyle and pharmacological interventions aimed at the prevention and treatment of arterial ageing and age-associated cardiovascular diseases.

Abstract We tested the hypothesis that reductions in the cellular deacetylase, sirtuin-1 (SIRT-1), contribute to vascular endothelial dysfunction with ageing via modulation of endothelial nitric oxide synthase (eNOS) acetylation/activation-associated nitric oxide (NO) production. In older (30 months, $n = 14$) *vs.* young (5–7 months, $n = 16$) B6D2F1 mice, aortic protein expression of SIRT-1 and eNOS phosphorylated at serine 1177 were lower (both $P < 0.05$), and acetylated eNOS was 6-fold higher ($P < 0.05$), whereas total eNOS did not differ ($P = 0.65$). Acetylcholine (ACh)-induced peak endothelium-dependent dilatation (EDD) was lower in isolated femoral arteries with ageing ($P < 0.001$). Incubation with sirtinol, a SIRT-1 inhibitor, reduced EDD in both young and older mice, abolishing age-related differences, whereas co-administration with L-NAME, an eNOS inhibitor, further reduced EDD similarly in both groups. Endothelium-independent dilatation to sodium nitroprusside (EID), was not altered by age or sirtinol treatment. In older (64 ± 1 years, $n = 22$) *vs.* young (25 ± 1 years, $n = 16$) healthy humans, ACh-induced forearm EDD was impaired ($P = 0.01$) and SIRT-1 protein expression was 37% lower in endothelial cells obtained from the brachial artery ($P < 0.05$), whereas EID did not differ. In the overall group, EDD was positively related to endothelial cell SIRT-1 protein expression ($r = 0.44$, $P < 0.01$). Reductions in SIRT-1 may play an important role in vascular endothelial dysfunction with ageing. SIRT-1 may be a key therapeutic target to treat arterial ageing.

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Abbreviations ACh, acetylcholine; EDD, endothelium-dependent dilatation; EID, endothelium-independent dilatation; eNOS, endothelial nitric oxide synthase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; L-NAME, N^G-monomethyl-L-arginine; NO, nitric oxide; peNOS, phosphorylated endothelial nitric oxide synthase; SIRT, sirtuin; SNP, sodium nitroprusside; vWF, von Willebrand factor.

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Introduction

Arterial endothelial function declines with advancing age, as indicated by a reduction in endothelium-dependent dilatation (EDD) with ageing in humans and animals (Celermajer *et al.* 1994; Taddei *et al.* 1996; Spier *et al.* 2004; Lesniewski *et al.* 2009; Seals *et al.* 2011). The age-related decline in EDD is predictive of future cardiovascular events in older adults without cardiovascular disease (Yeboah *et al.* 2007; Lind *et al.* 2011). The decrease in EDD with ageing is mediated by reduced bioavailability of nitric oxide (NO) (van der Loo *et al.* 2000; Taddei *et al.* 2001), a dilating molecule produced by the enzyme endothelial NO synthase (eNOS) (Fleming & Busse, 1999). As such, understanding the mechanisms regulating eNOS and NO-mediated EDD with ageing has important implications for age-associated cardiovascular disease.

The sirtuin family of NAD^+-dependent protein deacetylases and ADP-ribosyltransferases are involved in the physiological responses to altered energy metabolism and stress (Cohen *et al.* 2004; Guarente, 2006; Dali-Youcef *et al.* 2007). In mammals, sirtuins (SIRT) 1–4 are observed in multiple tissues. SIRT-1 is expressed in the nucleus and cytoplasm, whereas SIRT-2, 3 and 4 are predominantly expressed in mitochondria (Guarente, 2006; Whittle *et al.* 2007; Rodgers *et al.* 2008). Arterial SIRT-1 expression is reduced in older rodents (Ungvari *et al.* 2008; Rippe *et al.* 2010), but no clear mechanistic link with endothelial dysfunction has been established. Recent evidence suggests that reductions in SIRT-1 deacetylation of eNOS can impair NO-dependent EDD (Mattagajasingh *et al.* 2007).

We tested the hypothesis that reductions in SIRT-1 contribute to impaired NO-mediated EDD with ageing. To do so, we used a translational approach to elucidate the role of SIRT-1 in age-related endothelial dysfunction. We first sought to establish that inhibiting SIRT-1 activity in excised murine arteries would eliminate age-related reductions in NO-dependent EDD. We then determined if impaired EDD with ageing is associated with reduced SIRT-1 expression in endothelial cells obtained from peripheral arteries of humans.

Methods

Experiments in young and older B6D2F1 mice

Animals. Sixteen young (5–7 months) and 14 older (30 months) male B6D2F1 mice were obtained from the National Institute on Aging rodent colony. All mice were housed in an animal care facility at the University of Colorado at Boulder on a 12:12 light–dark cycle and fed standard rodent chow *ad libitum*. All animal procedures conformed to the *Guide to the Care and Use of Laboratory Animals* (NIH publication no. 85–23, revised 1996), conform to the principles of UK regulations as described by Drummond (2009) and were approved by the University of Colorado at Boulder and University of Utah Animal Care and Use Committees.

EDD and endothelium-independent dilatation (EID). Measures of EDD and EID were made in isolated vessels studied *ex vivo* as described previously (Donato *et al.* 2005, 2007*b*, 2009; Lesniewski *et al.* 2009, 2011). Mice were killed by exsanguinations via cardiac puncture while under inhaled isoflurane anesthesia (1–5%). Femoral arteries were excised and placed in myograph chambers (DMT Inc., Aarhus, Denmark) containing EDTA-buffered physiological saline solution (PSS), cannulated onto glass micropipettes and secured with nylon (11–0) suture. Once cannulated, the femoral arteries were warmed to 37°C and pressurized to 50 mmHg intraluminal pressure and allowed to equilibrate for 1 h. All arteries then were submaximally preconstricted with phenylephrine (2 μM). Increases in luminal diameter in response to increasing concentrations of the endothelium-dependent dilator acetylcholine (Ach; 1×10^{-9} to 1×10^{-4} M) and endothelium-independent dilator sodium nitroprusside (SNP; 1×10^{-10} to 1×10^{-4} M) were determined. To assess the influence of SIRT-1, dose responses to ACh were repeated in the presence of the sirtinol, a SIRT-1 antagonist (10^{-4} M, 60 min incubation), (Ota *et al.* 2008, 2010; Zhang *et al.* 2008). These concentrations have been established to increase acetylated eNOS *in vitro* (Ota *et al.* 2010). To determine the contribution of NO to dilatation, responses to ACh and sirtinol were repeated in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M, 30 min incubation) (Durrant *et al.* 2009; Lesniewski *et al.* 2009).

Arterial protein expression. The thoracic aorta was excised, cleared of surrounding tissues while maintained in 4°C PSS, and frozen in liquid nitrogen. Whole artery lysates were prepared as previously described (Durrant *et al.* 2009; Lesniewski *et al.* 2009; Lesniewski *et al.* 2011). For measures of protein expression in aortic lysates, 15 μg of protein with 2 mol l^{-1} dithiothreitol were loaded into polyacrylamide gels, separated by electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween (TBS-T) overnight at 4°C. After blocking, the membrane was washed with TBS-T and incubated overnight at 4°C in primary antibody. eNOS (1:1000; 140 kDa; BD Biosciences, Franklin Lakes, NJ, USA), Ser1177-phosphorylated eNOS (peNOS, 1:1000; 140 kDa; Cell Signaling Technology, Inc., Danvers, MA, USA) and SIRT-1 (1:1000; 110 kDa; Abcam Inc., Cambridge, MA, USA) abundance were measured by standard Western blotting techniques

using an HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and Supersignal ECL (Pierce, Rockford, IL, USA). Bands were visualized using a digital acquisition system (ChemiDoc-It, UVP, Upland, CA, USA) and quantified using ImageJ software (NIH). Expression is presented normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1:1000; 37 kDa; Cell Signaling), to account for differences in protein loading. The quantifications of total protein were normalized to GAPDH of the same band after stripping and re-probing, and are expressed relative to the mean of the young control bands on a given blot. The ratio of phoshorylated to total protein was calculated from the bands for a given sample lysate run on the two gels/membranes, with each normalized to its own GAPDH. These data also are expressed as a ratio of the mean of the young control group within a given set of lysates.

eNOS acetylation expression. To determine acetylation state of eNOS for which there is no commercially available acetylated specific antibody, acetyl-lysine (1:1000 crosslinked to DYNAL (Invitrogen) magnetic beads for extraction) was immunoprecipitated from 20 μg of aortic protein lysate and the association of the acetyl-lysine with eNOS was determined by immunoblotting (as described above) with the primary antibody for eNOS (eNOS, 1:1000; 140 kDa; BD Biosciences).

Experiments in human subjects

Subjects. Data were obtained on 38 healthy men and women: 16 young (aged 18–30 years) and 22 older (59–76 years). All subjects had resting blood pressure less than 140/90 mmHg, body mass index less than 30 kg m^{-2}, and were free of cardiovascular diseases (CVD), diabetes, and other clinical disorders as assessed by medical history, physical examination, and blood chemistries. Subjects greater than 40 years of age were further screened for CVD using electrocardiogram and blood pressure responses to incremental treadmill exercise performed to volitional exhaustion. Subjects were non-smokers, not taking dietary supplements, and not regularly exercising. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers and their written informed consent was obtained prior to participation.

Study procedures. All measurements were performed at the University of Colorado at Boulder Clinical and Translational Research Center after an overnight fast and a 24 h abstention from alcohol and physical activity.

Subject characteristics. Body mass, body mass index, resting arterial blood pressure, fasting blood chemistries, habitual physical activity, oxidized low-density lipoprotein (LDL), total antioxidant status and C reactive protein were measured as previously described (Moreau *et al.* 2005; Donato *et al.* 2007a).

EDD and endothelium-independent dilatation. EDD and endothelium-independent dilatation were determined as the peak forearm blood flow (measured by venous occlusion plethysmography) responses to an incremental intra-brachial artery infusion of ACh (1.0, 2.0, 4.0 and 8.0 μg (dl forearm tissue)$^{-1}$ min^{-1}) and SNP (0.5, 1 and 2.0 μg (dl forearm tissue)$^{-1}$ min^{-1}), respectively, as described previously (Donato *et al.* 2008*b*, 2009; Pierce *et al.* 2008).

Endothelial cell protein expression. The procedures used for collection of endothelial cells and measurement of protein expression were originally described by Feng *et al.* (1999) and Colombo *et al.* (2002), and more recently by our laboratory (Eskurza *et al.* 2006; Gates *et al.* 2007; Silver *et al.* 2007; Donato *et al.* 2007*a*, 2008*a*, 2009). Briefly, J-wires were advanced into a brachial artery ~4 cm beyond the tip of the catheter and withdrawn, and cells were recovered by washing and centrifugation. Collected cells were fixed with 3.7% formaldehyde, plated on slides and stored at –80°C.

For immunofluorescence staining, two control cultured human umbilical vein endothelial cell (HUVEC: passage 6–9 processed identically to the human ECs) slides and eight subject slides were selected (balanced for age) for each staining batch. After blocking non-specific binding sites with 5% donkey serum (Jackson Immunoresearch, West Grove, PA, USA), cells were incubated with monoclonal antibody against SIRT-1 (1:150; Abcam, Cambridge, MA, USA) and a specific AlexaFlour555-conjugated secondary antibody (Research Diagnostics, Acton, MA, USA). Next, cells were incubated with vWF (von Willebrand factor; 1:1000; Dako, Carpinteria, CA, USA) and a specific AlexaFlour488-conjugated secondary antibody (Research Diagnostics). Slides were then cover slipped with a Vectashield DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) fluorescent mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and stored at 4°C overnight.

During analysis, slides were viewed using a fluorescence microscope (Eclipse 600, Nikon) and 30 individual endothelial cell images were digitally captured by a Photometrics CoolSNAPfx digital camera (Roper Scientific, Tuscon, AZ, USA). These endothelial cells were documented by cell staining of vWF and nuclear integrity was confirmed using DAPI staining. Once endothelial

cells with intact nuclei were identified, they were analyzed using Metamorph Software (Universal Imageing, Downingtown, PA, USA) to quantify the intensity of SIRT-1 dependent AlexaFlour555 staining (i.e., average pixel intensity). The software program allows for systematic quantification of staining intensity and eliminates the potential for subjective error during analysis.

Values are reported as ratios of subject's SIRT-1 EC protein expression to HUVEC control. Reporting ratios to a 'standard' (cultured HUVEC) control minimizes the possible confounding effects of differences in intensity of staining among different staining sessions. A single technician who was blinded to the subject's identity analysed each batch of slides.

Statistical analysis

Experiments in mice. For animal and vessel characteristics (maximum dilatation and EC_{50}), group differences were determined by one-way analysis of variance (ANOVA) or *t* test where appropriate. For all dose responses, group differences were determined by repeated measures ANOVA. Data are presented as means ± SEM. Significance was set at $P < 0.05$. Data analyses were performed with SPSS (version 16.0).

Experiments in human subjects. Group differences were determined by *t* tests for independent sample comparisons and forearm blood flow responses and *ex vivo* vascular responses to incremental doses of ACh and SNP were analyzed by repeated measures ANOVA. Pearson correlation analysis was used to determine relations of interest. Statistical significance for all analyses was set at $P < 0.05$. Data analyses were performed with SPSS (version 16.0).

Results

Studies in young and older B6D2F1 mice

Animal and artery characteristics. Ageing did not alter mouse body (older: 36 ± 1 *vs.* young: 34 ± 1 g, $P > 0.05$), soleus (older: 0.012 ± 0.003 *vs.* young: 0.014 ± 0.001 g, $P > 0.05$) or gastrocnemius (older: 0.188 ± 0.010 *vs.* young: 0.205 ± 0.009 g, $P > 0.05$) muscle masses. Femoral artery maximal diameter was similar in the two groups

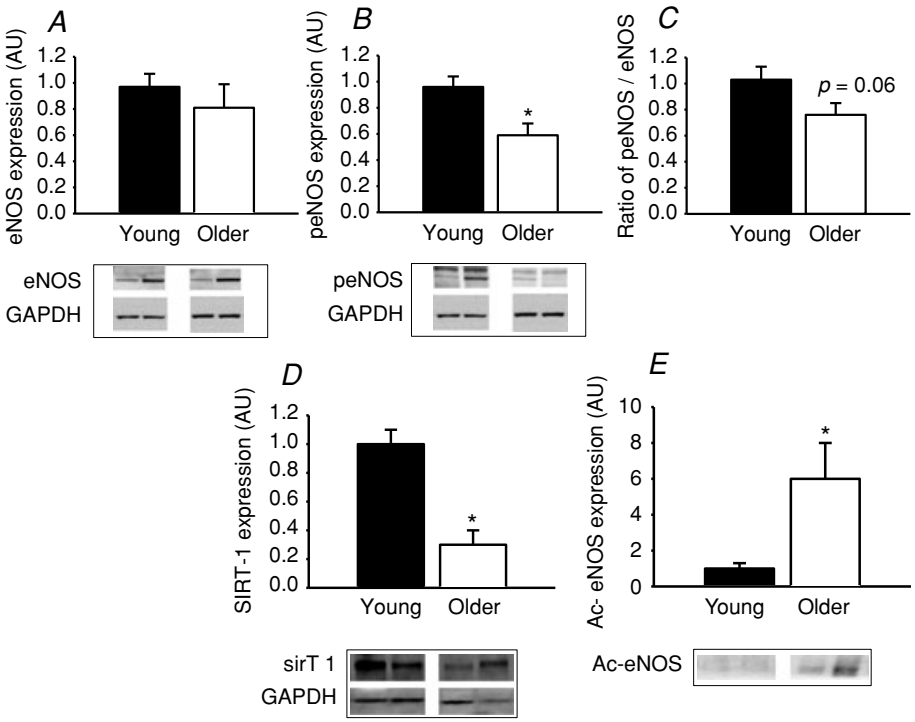

Figure 1. Protein expression in aortas from young and older mice for eNOS, eNOS phosphorylated at serine 1177, ratio of peNOS to eNOS, SIRT-1 and acetylated eNOS
Protein expression is shown in aortas from young and older mice ($n = 7$–9 per group) for eNOS (*A*), eNOS phosphorylated at serine 1177 (peNOS) (*B*), ratio of peNOS to eNOS (*C*), SIRT-1 (*D*) and acetylated (Ac)-eNOS (*E*). Protein expression of eNOS, peNOS and SIRT-1 is expressed relative to GAPDH and immunopreciptated (IP) Ac-eNOS is expressed relative to no antibody IP control eNOS expression. Representative images are shown below the summary data. GAPDH images are from the same membrane after stripping and re-probing. Data are shown normalized to the young control mean values. Values are means ± SEM. *$P < 0.05$ *vs.* young.

(older: 404 ± 10 *vs.* young: 409 ± 9 μm, $P > 0.05$), as was the vascular tone after preconstriction (older: 33 ± 3 *vs.* young: 33 ± 2% tone from maximum diameter, $P > 0.05$).

Arterial protein expression. Total eNOS did not differ with age (older: 0.81 ± 0.18 *vs.* young: 1.00 ± 0.09 eNOS arbitrary units (AU), $P > 0.05$; Fig. 1*A*), but phosphorylation of eNOS at serine 1177 (peNOS) was lower (older: 0.59 ± 0.09 *vs.* young: 1.00 ± 0.08 peNOS AU, $P < 0.05$; Fig. 1*B*) and the ratio of peNOS to eNOS tended to be lower (older: 0.81 ± 0.10 *vs.* young: 1.00 ± 0.10 peNOS/eNOS ratio, $P = 0.06$; Fig. 1*C*) in aorta from the older mice. SIRT-1 protein expression was markedly lower in aorta from the older mice (older: 0.31 ± 0.09 *vs.* young: 1.00 ± 0.13 SIRT-1 AU, $P < 0.05$; Fig. 1*D*) and this was associated with a 6-fold greater eNOS acetylation (older: 6.01 ± 1.95 *vs.* young: 1.00 ± 0.30 acetyl-eNOS AU, $P < 0.05$; Fig. 1*E*).

Femoral artery EDD and endothelium-independent dilatation. Peak ACh-mediated dilatation (EDD) was lower in femoral arteries of the older mice (maximum dilatation to ACh, older: 85 ± 1%, *vs.* young: 95 ± 1%, $P < 0.001$; Fig. 2*A*). Sensitivity to ACh (EC_{50}) was not different between young and older arteries (Table 1, $P = 0.12$). Inhibition of the SIRT-1 with sirtinol reduced EDD in both age groups ($P < 0.01$) and eliminated the age-related differences (maximum dilatation, older: 33 ± 7% *vs.* young: 33 ± 5%, $P > 0.05$; Fig. 2*A*). EC_{50} was significantly reduced with sirtinol treatment in young ($P < 0.05$), but not older arteries ($P > 0.05$) (Table 1). Addition of the eNOS inhibitor L-NAME combined with sirtinol pretreatment further reduced EDD ($P < 0.01$) similarly in the two age groups (maximum dilatation, older: 5 ± 6% *vs.* young: 10 ± 3%, $P > 0.05$; Fig. 2*A*), but did not alter EC_{50} ($P > 0.05$). There were no differences in femoral artery dilatation or EC_{50} to SNP in the young and older mice with or without sirtinol treatment (all $P > 0.05$; Fig. 2*B*).

Studies in young and older human subjects

Subject characteristics. Characteristics of the young and older subjects are shown in Table 2. Body mass index, resting blood pressure, fasting plasma glucose and total and LDL cholesterol concentrations were higher in the older adults (all $P < 0.05$), but all values were within clinically normal ranges. Body mass, plasma HDL cholesterol and habitual physical activity were not significantly different between the groups. Plasma oxidized LDL was greater ($P < 0.01$), total antioxidant status was lower ($P < 0.01$) and C-reactive protein was not different in the older *vs.* young subjects.

EDD and endothelium-independent dilatation. The forearm blood flow response to ACh was lower ($P < 0.05$) in the older subjects (area under the curve (AUC), older: 67 ± 9 *vs.* young: 99 ± 12 AU, $P < 0.01$; Fig. 3*A*), whereas

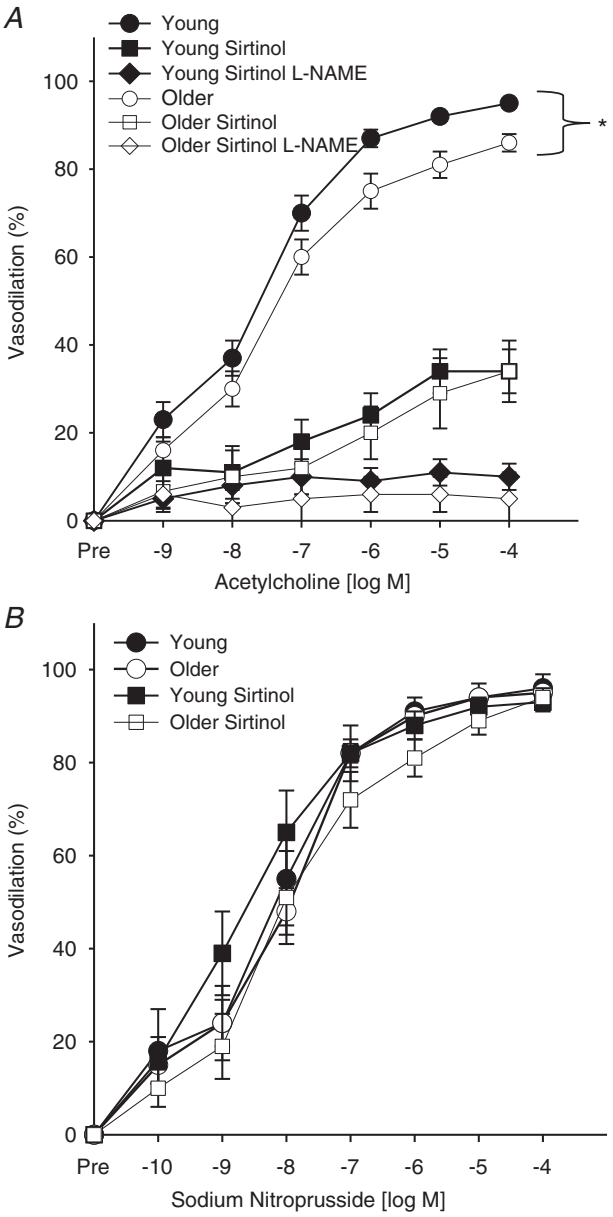

Figure 2. Endothelium-dependent and -independent dilatation shown in untreated, sirtinol treated and sirtinol and L-NAME treated femoral arteries from young and older mice
Endothelium-dependent dilatation (acetylcholine, 1×10^{-9} to 1×10^{-4} M; *A*) and endothelium-independent dilatation (sodium nitroprusside, 1×10^{-10} to 1×10^{-4} M; *B*) are shown in untreated ($n = 16$, young, filled circles; $n = 14$, older, open circles), sirtinol treated ($n = 14$, young, filled squares; $n = 13$, older, open squares) and sirtinol and L-NAME ($n = 15$, young, filled diamonds; $n = 13$, older, open diamonds) treated femoral arteries from young and older mice. Values are means ± SEM. *$P < 0.05$ *vs.* young.

Table 1. Sensitivity (EC_{50}) to ACh and SNP

EC_{50}	Young	Older
Acetylcholine	$(6.6 \pm 2.9) \times 10^{-8}$	$(41.3 \pm 34.9) \times 10^{-8}$
Acetylcholine + sirtinol	$(1.8 \pm 1.2) \times 10^{-6}$*	$(12.0 \pm 7.7) \times 10^{-6}$
Acetylcholine + sirtinol + L-NAME	$(1.2 \pm 0.5) \times 10^{-6}$	$(1.6 \pm 0.8) \times 10^{-6}$
Sodium nitroprusside	$(2.5 \pm 1.7) \times 10^{-8}$	$(1.2 \pm 0.2) \times 10^{-8}$
Sodium nitroprusside + sirtinol	$(8.7 \pm 5.1) \times 10^{-9}$	$(22.0 \pm 7.2) \times 10^{-9}$

Values are means ± SEM; EC_{50}, acetylcholine dose at which yields 50% vasodilation; L-NAME, N^G-monomethyl-L-arginine; *$P < 0.05$ *vs.* Young acetylcholine.

Table 2. Subject charateristics

	Young ($n = 16$)	Older ($n = 22$)	P
Male/female	12/4	15/7	
Age (years)	25 ± 1	64 ± 1	< 0.01
Body mass (kg)	75 ± 3	82 ± 3	0.06
BMI (kg m^{-2})	23.8 ± 0.7	27.6 ± 1.0	< 0.01
Blood Pressure (mmHg)			
Systolic	111 ± 3	126 ± 3	< 0.01
Diastolic	71 ± 2	78 ± 2	< 0.01
Cholesterol (mg dl^{-1})			
Total	162 ± 6	202 ± 5	< 0.01
LDL	92 ± 5	124 ± 4	< 0.01
HDL	51 ± 3	57 ± 3	0.08
Fasting glucose (mg dl^{-1})	87 ± 2	95 ± 2	< 0.01
Leisure physical activity (MET h wk^{-1})	24 ± 7	31 ± 6	0.23
Oxidized LDL (U l^{-1})	49.8 ± 4.3	64.7 ± 2.3	< 0.01
Total antioxidant status (mmol l^{-1})	1.46 ± 0.03	1.32 ± 0.04	< 0.01
C-reactive protein (mg l^{-1})	0.92 ± 0.30	1.19 ± 0.25	0.25

Values are means ± SEM; *n*, no. of subjects; BMI, body mass index; MET, metabolic equivalent; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

the response to SNP was similar in the groups (Fig. 3*B*) (AUC, older: 27 ± 2 *vs.* young: 31 ± 4 AU, $P > 0.05$).

SIRT-1 protein expression. Vascular endothelial cell expression of SIRT-1 was lower in the older subjects (older: 0.37 ± 0.03 *vs.* young: 0.51 ± 0.05, SIRT-1/HUVEC, $P < 0.05$; Fig. 4*A*). In the overall sample, forearm blood flow AUC to ACh was positively related to SIRT-1 expression in endothelial cells ($r = 0.44$, $P < 0.01$; Fig. 4*B*). There was no relation between forearm blood flow AUC to SNP and SIRT-1 ($r = 0.17$, $P > 0.05$). The age group differences in SIRT-1 expression were not related to age-associated differences in any other factor (all $P > 0.20$).

Discussion

The novel findings of the present studies are as follows. First, the reduced arterial expression of SIRT-1, a cellular deacetylase, with ageing is associated with increased acetylated eNOS. Second, a reduction in SIRT-1 activity in arteries is an important mechanism mediating the age-associated impairment in NO-dependent EDD, without affecting endothelium-independent dilatation. Third, expression of SIRT-1 is lower in endothelial cells from older compared with young healthy adults, extending previous observations in arteries of rodents to humans. Lastly, among healthy adults of increasing age, EDD is positively related to SIRT-1 protein expression in vascular endothelial cells. Collectively, these results provide experimental evidence that decreases in sirtuins, specifically SIRT-1 expression and activity, play a key role in vascular endothelial dysfunction with ageing and that this is associated with increases in eNOS acetylation.

The present results confirm previous observations of our laboratory (Rippe *et al.* 2010) and others (Ungvari *et al.* 2008) that arterial SIRT-1 expression is reduced with age in rodents, and that this occurs in the absence of changes in total eNOS (Woodman *et al.* 2002; Spier *et al.* 2004; Durrant *et al.* 2009; Lesniewski *et al.* 2009). The

present findings extend insight on this issue by showing that the reduction in arterial SIRT-1 with ageing is associated with an increase in acetylated eNOS. This is consistent with the fact that both pharmacological (sirtinol) and viral (siRNA) inhibition of SIRT-1 expression and activity directly acetylate eNOS, which inhibits eNOS activity and the production of NO in cultured endothelial cells (Mattagajasingh *et al.* 2007; Ota *et al.* 2010). Likewise, endothelial cells in culture that are exposed to shear stress, a potent physiological stimulus for NO production, exhibit enhanced colocalization of SIRT-1 and eNOS, resulting in deacetylation of eNOS and an associated increase in NO production (Chen *et al.* 2010). These results support the role of SIRT-1 expression and activity in regulation of eNOS (via eNOS acetylation) and NO bioavailability. In the present study, we found that reductions in SIRT-1 and increased acetylation of eNOS were associated with a reduction in peNOS and the peNOS:eNOS ratio, indicators of eNOS activation state, as we have reported previously in this model (Durrant *et al.* 2009; Rippe *et al.* 2010). Taken together, these results indicate that in mice, reductions in arterial SIRT-1 expression and activity are associated with acetylation and deactivation of eNOS, and impaired NO-mediated vascular endothelial dysfunction.

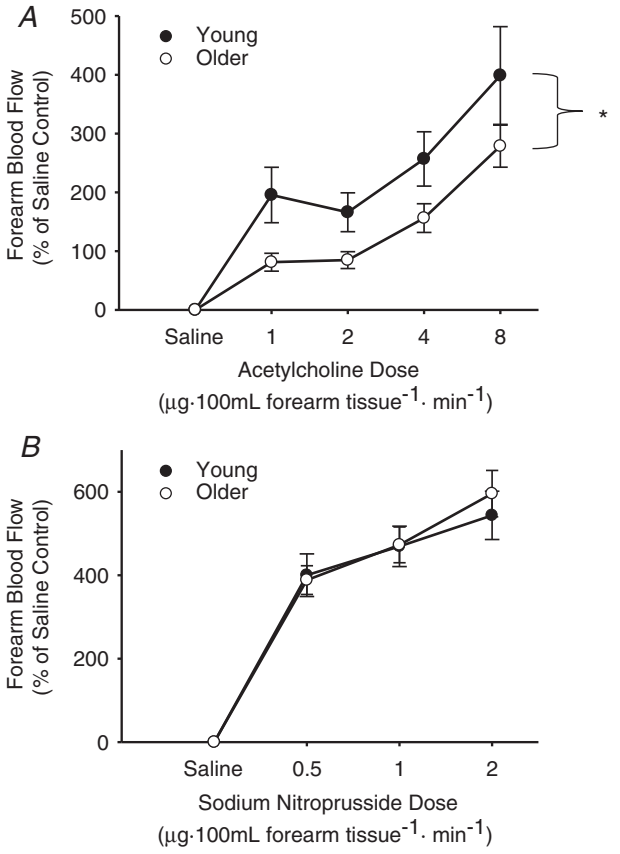

Figure 3. Endothelium-dependent and -independent dilatation shown in young and older healthy humans
Endothelium-dependent dilatation (forearm blood flow to intra-brachial artery infusion of acetylcholine; 1.0, 2.0, 4.0, and 8.0 μg 100^{-1} ml forearm tissue; *A*) and endothelium-independent dilatation (forearm blood flow to intra-brachial artery infusion of sodium nitroprusside; 0.5, 1.0, 2.0 μg 100 ml^{-1} forearm tissue; *B*) are shown in young ($n = 16$, filled circles) and older ($n = 22$, open circles) healthy humans. Values are means ± SEM. $^*P = 0.01$ *vs.* young.

To establish more direct evidence linking reductions in SIRT-1 to endothelial dysfunction with ageing, we utilized an *in vitro* artery model to inhibit SIRT-1 activity using sirtinol. Treatment with sirtinol eliminated the age-related difference in NO-dependent EDD, without affecting vascular smooth muscle sensitivity to NO (endothelium-independent dilatation). These results are the first to demonstrate that diminished SIRT-1 is involved in vascular endothelial dysfunction with ageing. Our data are consistent with other observations in models of SIRT-1 inhibition in which adenoviral transfection of the endothelium with a dominant negative SIRT-1 mutant in aortic rings was found to impair NO-mediated EDD (Mattagajasingh *et al.* 2007), and transgenic mice with SIRT-1 overexpression demonstrated preserved EDD compared with wild-type mice in response to a high fat diet (Zhang *et al.* 2008).

In the present study, the fact that SIRT-1 inhibition reduced EDD in both age groups of mice (with a greater reduction in young arteries) and that the addition of L-NAME further inhibited EDD, indicates that inhibition of SIRT-1 decreases eNOS activity, but does not completely eliminate it. This is in contrast to the results of a previous study that showed complete inhibition of eNOS-mediated EDD with the andenoviral knock down of SIRT-1 in rat aorta (Mattagajasingh *et al.* 2007). These differences could be due to the method of SIRT-1 inhibition used or differences in the species studied, but in both cases support a strong role of SIRT-1 in regulating NO-dependent endothelial function.

To provide translational insight into the potential role of SIRT-1 in age-associated vascular endothelial dysfunction, we determined the forearm blood flow responses to ACh, a measure of NO-mediated EDD with ageing (Taddei *et al.* 2001; Seals *et al.* 2011), and the expression of SIRT-1 in endothelial cells acquired from arteries of young and older healthy adults. Our results are the first to demonstrate in humans that SIRT-1 is lower in vascular endothelial cells of older adults and that this is positively related to *in vivo* differences in EDD. These results extend our *in vitro* observations in mice to suggest a possible role for reduced SIRT-1 in mediating vascular endothelial dysfunction with ageing in humans. These findings also provide evidence that SIRT-1 activation may have therapeutic potential for the treatment of age-associated vascular dysfunction in humans.

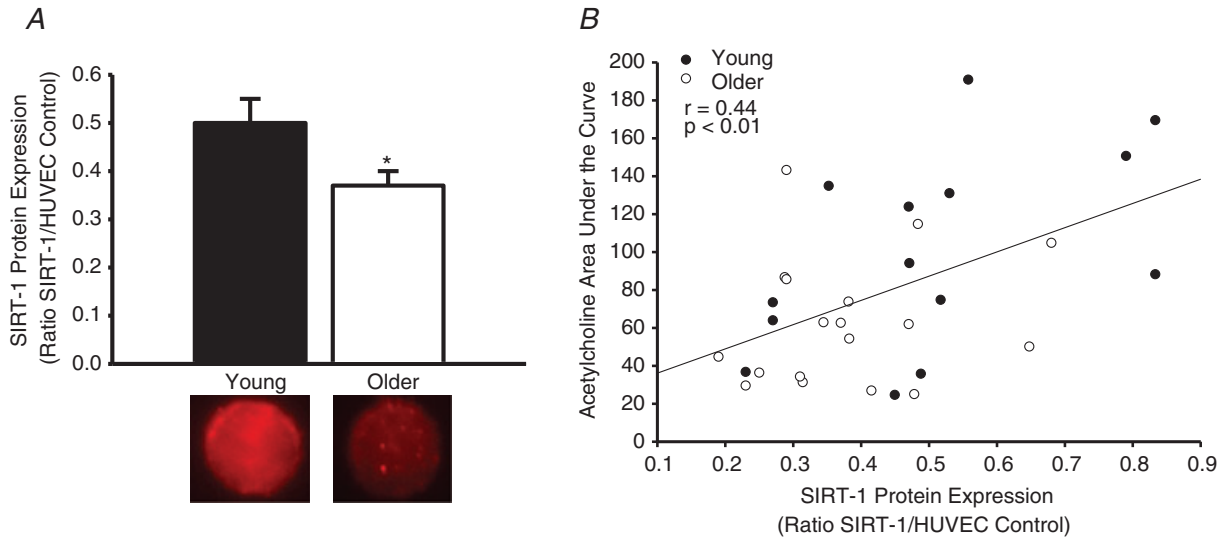

Figure 4. Arterial endothelial cell SIRT-1 protein expression and relation to endothelial dependent dilation
SIRT-1 protein expression is shown in endothelial cells obtained from brachial arteries of young ($n = 14$) and older ($n = 18$) healthy humans (*A*). Representative images of the immunofluorescence images of SIRT-1 from individual young and older subjects are shown below the group mean bars. The relation between acetylcholine-induced endothelium-dependent dilatation (area under the curve) and SIRT-1 endothelial cell protein expression is shown for all subjects (*B*: young, filled circles; older, open circles). Values are means ± SEM. $^*P < 0.05$ *vs.* young.

We recognize several limitations of our study. First, because of the limited number of cells available from our sampling procedure, we were not able to immuno-precipitate and measure acetylated eNOS in our human endothelial cell samples. Second, although we focused on the role of SIRT-1 in acutely modulating endothelial function and the putative role of acetylated eNOS, altering SIRT-1 function may have influenced the acetylation status of other proteins affecting NO bioavailability. Finally, we used different arteries in our mouse (femoral, thoracic aorta) and human (brachial) studies. It is possible that the modulatory influence of SIRT-1 differs in the vascular endothelium of these arteries, although we found reduced expression of SIRT-1 in arterial tissue from both mice and humans in the present study. Nevertheless, eNOS expression and activation with ageing can be specific to the model used and species studied (Muller-Delp, 2006; Donato *et al.* 2009), so we cannot rule out the possibility of such specificity for SIRT-1.

In conclusion, the results of the present study provide new insight into the molecular events that contribute to vascular endothelial dysfunction with ageing. Specifically, here we provide direct evidence for a role for reduced SIRT-1 expression and activity in age-associated endothelial dysfunction, and suggest that increased acetylation of eNOS may be among the key mechanisms involved. We also show for the first time that SIRT-1 expression is reduced in endothelial cells obtained from arteries of older compared with young adult humans and is related to differences in vascular endothelial function. Together, these findings provide novel translational evidence that SIRT-1 expression and activity contribute to vascular endothelial dysfunction with ageing and that this may be due to altered eNOS acetylation. Importantly, our results provide further compelling support for SIRT-1 as a potential therapeutic target for lifestyle, nutraceutical and pharmacological interventions aimed at the prevention and treatment of arterial ageing and age-associated cardiovascular diseases.

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Author contributions

A.J.D., K.A.M., B.R.L., J.R.D., L.A.L., and D.R.S. contributed to the conception and design, analysis and interpretation of data. A.J.D., L.A.L., and D.R.S. contributed to the drafting and revision of the article. All authors provided final approval of the version to be published. All experiments were carried out at the University of Colorado at Boulder and the University of Utah.

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