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(-)-Epicatechin induced reversal of endothelial cell aging and improved vascular function: Underlying mechanisms

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(-)-Epicatechin induced reversal of endothelial cell aging and improved vascular function: Underlying mechanisms

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Abstract

The consumption of cacao products rich in (-)-epicatechin associates with reduced cardiovascular risk and improved vascular function. However, little is known about (-)-epicatechin effects on aged endothelium. In order to characterize the health restoring effects of (-)-epicatechin on aged endothelium and identify underlying mechanisms, we utilized high passage number (i.e. aged) bovine coronary artery endothelial cells and aortas of 3 and 18 month old rats. We evaluated cell senescence (β-galactosidase), nitric oxide (NO) production through the endothelial nitric oxide synthase pathway, mitochondria related endpoints, citrate synthase activity and vascular relaxation. Cells were treated with water or (-)-epicatechin (1 μM) for 48 h and rats orally with either water or (-)-epicatechin (1 mg/kg/day) for 15 days. Senescence associated β-galactosidase levels doubled in aged cells while those treated with (-)-epicatechin only evidenced ~40% increase. NO levels in cells decreased by ~33% with aging and (-)-epicatechin normalized them. Endothelial nitric oxide synthase phosphorylation levels paralleled these results. Aging increased total protein and synthase acetylation levels and (-)-epicatechin partially restored them to those of young by stimulating sirtuin-1 binding to the synthase. Phosphorylated sirtuin-1, mitofilin, oxidative phosphorylation complexes and transcriptional factor for mitochondria were reduced by ~40% with aging and were restored by (-)-epicatechin. (-)-Epicatechin enhanced acetylcholine induced aged aorta vasodilation and stimulated NO levels while reducing blood pressure. In conclusion, (-)-epicatechin reverses endothelial cell aging and restores key control elements of vascular function. These actions may partly explain the epidemiological evidence for the beneficial effects of cocoa consumption on the incidence of cardiac and vascular diseases.

Introduction

Cardiovascular diseases (CVD) are a prominent, worldwide cause of morbidity and mortality. It is well recognized that the health of the endothelium is central to the underlying pathophysiology of many CVD and that aging is an important contributor to the development of endothelial dysfunction (1,2). The identification of safe and effective agents that can promote the preservation and/or restoration of endothelial health is thus, of high priority as their use may reduce CVD incidence.

Central to the preservation of vascular health, is the endothelial nitric oxide synthase (eNOS), which under physiological conditions is responsible for the production of nitric oxide (NO) (3). It has been demonstrated that vascular and endothelial cell function deteriorates with aging and it appears to be mainly due to a decrease in NO bioavailability (4). The causes for reduced bioavailability of NO as a function of aging are not completely understood. However, the acetylation of specific amino acid residues present in eNOS can trigger the loss of function by interfering with their phosphorylation (3). Aging is known to correlate with higher protein acetylation levels and loss of sirtuin-1 deacetylation activity, which may partly underlie decreased eNOS activity (5,6).

The stimulation of NO synthesis by flavonoid-containing compounds has received widespread attention, as their effects appear to reduce CVD. (7,8). Epidemiological studies indicate that the regular intake of cacao derived products such as dark chocolate, is inversely associated with the incidence of CVD including myocardial infarction, heart failure and stroke (9-11). We have reported on the capacity of the most abundant cacao flavanol, (-)-epicatechin (Epi) to stimulate the production of NO and ascribed this effect to the ability of the flavanol to increase eNOS protein expression and activity levels (12,13). We have also reported on the capacity of Epi to

notably mitigate markers of tissue aging and mitochondrial deterioration in senile mice (14). So

far, no studies have examined the effects of Epi on aged endothelium NOS/NO levels and

determine its ability to restore mitochondrial number and function.

With these goals in mind, we evaluated the effects of Epi on markers of cell senescence, NO

production (via eNOS), acetylation levels, mitochondrial biogenesis and function as well as

vasodilatory responses using bovine coronary artery endothelial cells and aortas isolated from

young and aged animals. Results indicate that Epi is capable of reversing aging associated

endothelial dysfunction and loss of mitochondrial density through effects on NO levels, which

appear dependent on the acetylation/phosphorylation status of eNOS.

Materials and Methods

Reagents

Primary bovine coronary artery endothelial cells were obtained from Cell Applications, Inc. Dulbeco's modified Eagle medium low glucose cell culture media, trypsin, antibiotic/antimycotic solution, fetal bovine serum, and Hank's balanced salt solution (phenol red free) were from HyClone. Nonessential amino acids solution was from Mediatech Cellgro Inc. Phenol red free media, low glucose cell culture media was from Life Technologies, Inc. Bovine serum albumin, tween-20, protease and phosphatase inhibitor cocktail, NG-nitro-L-arginine methyl ester hydrochloride (L-NAME), and Epi were from Sigma Aldrich, Inc. Polyvinylidene difluoride transfer membranes were from Millipore Inc. and Bradford assay reagent and acrylamide gels were from Bio-Rad, Inc. Enhanced chemiluminescence Plus Western blot detection kit and MitoTracker Green FM were from Thermo Fisher Scientific Inc. Citrate synthase activity assay kit and MitoBiogenesis In-Cell ELISA kit (Colorimetric) were from Abcam, Inc. Senescence associated β-galactosidase staining kit from Cell Signaling Technology Inc. and the Nitrate/Nitrite fluorometric assay kit from Cayman Chemicals Inc. Primary antibodies against phospho-eNOS at ser-1177, eNOS, β-tubulin, β-galactosidase, phospho-sirtuin, transcriptional factor mitochondria (TFAM), acetylated-lysine, and anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were from Cell Signaling, Inc. Sirtuin-1 was from Santa Cruz Biotechnology, Inc. Primary antibodies against complex I, ATP synthase and mitofillin were from MitoSciences, Inc.

Cell culture

Cells were grown in complete medium supplemented with 10% serum, 1% antibiotic/antimycotic solution, and 1% nonessential amino acids. Cells were maintained in a humidified atmosphere at

37°C with 5% CO_2 and 95% O_2. Passages 8-13 were used as a model for young endothelial cells while passages 31-35 were used as a model for aged endothelial cells. Cells were used for experiments at 80% confluence.

Cell Treatment

For all experiments 24 h before treatment, growth medium was replaced with media 1% serum, phenol red free, 1% antibiotic/antimycotic solution and 1% nonessential amino acids (starving media). Epi treatment was applied to confluent cells using 1 μM of Epi (diluted in water) for 48 h. Only vehicle was applied to cells in the control group. Fresh starving media and Epi was reapplied every 24 h. In select experiments, the NOS inhibitor L-NAME (300 μM), was added to the cell medium 4 h previous the addition of Epi and during the 48 h treatment with Epi.

Total protein extraction

Cells were washed three times with cold buffer (4 ml/plate) and lysed in 80 μl of ice cold lysis buffer with protease and phosphatase inhibitor cocktails. Homogenates were sonicated for 15 min at 4°C, and centrifuged at 13,000 g for 15 min to remove cell debris. Total protein concentration was measured in the supernatant using the Bradford method.

NO measurements

After 48 h treatment with Epi, 100 μl of media was collected to test NO levels using a fluorescent kit according to manufacturer instructions. Briefly, as the final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-), the best estimation of total NO production is the sum of both NO_2^- and NO_3^-. We used nitrate/nitrite fluorometric assay as an accurate method for measurement of total nitrate/nitrite concentration. In the assay, the conversion of nitrate to nitrite utilizes nitrate reductase followed by the addition of acidic solution diaminonaphthalene (DAN) and NaOH, which enhances the detection of the fluorescent product, 1(H)-naphthotriazole.

Measurement of the fluorescence generated is quantified using a fluorometer (FLx800 Bio-Tek Instruments). Nitrate/nitrite values were normalized by total protein after the cells were scrapped from the plate and lysed as described previously. Plasma (50 µl) was collected from treated animals (see below) and NO levels measured as above.

Senescence associated β-galactosidase activity

Young and aged cells were grown in 12 and 96 well-plates. At 80% confluence, cells were treated for 48 h (control, Epi, Epi-L-NAME, and L-NAME). After the 48 h treatment, β-galactosidase activity was determined using a senescence associated β-galactosidase staining kit. The cells in the 12 well plates were used for visualizing the characteristic blue staining of β-galactosidase activity under the microscope and images were taken. The cells in the 96 well plate were used for measuring β-galactosidase activity via colometric analysis in a spectrophotometer (µQuant, Bio-Tek Instruments, Inc.).

Western Blot

In order to examine aging markers, eNOS/NO pathway, and mitochondrial related proteins, Western blotting was performed. Cells were treated as described above. After treatment, cells were lysed and total protein was extracted. A total of 30 µg of protein was loaded onto a 4-15% gel, electrotransferred, incubated for 1 h and followed by either 1-3 h incubation at room temperature or overnight incubation at 4°C with primary antibodies. Primary antibodies were typically diluted 1:1,000 or 1:2,000 in buffer plus 5% bovine serum albumin or 2 % nonfat milk. Membranes were washed (3 X for 5 min) in buffer and incubated 1 h at room temperature in the presence of conjugated secondary antibodies diluted 1:5,000 in blocking solution. Membranes were again washed three times in buffer and immunoblots were developed. Band intensities were digitally quantified and normalized by β-tubulin.

Protein acetylation measurements

Immunoprecipitation was used to selectively isolate eNOS and quantify its acetylation status. Treated cells were lysed, total protein extracted, and protein content measured in the supernatant by Bradford method. A total of 0.5 mg protein was precleared by adding 1 μg of normal rabbit IgG control and 20 μL protein agarose and mixed for 30 min (4°C) with subsequent centrifugation at 12,000 g for 10 min at 4°C. The supernatant was recovered and incubated at 4°C under mild agitation for 3 h with 10 μL of eNOS immunoprecipitation antibody. Twenty μl of protein A/G-sepharose was added, and the mixture was incubated overnight at 4 °C with shaking. The immunoprecipitation mixture was centrifuged at 12,000 g for 15 min at 4°C and the supernatant recovered and stored at 4°C for later analysis. The pellet was washed 3 X with extraction buffer under shaking 15 min and centrifuged at 12,000 g for 15 min at 4°C. The immunoprecipitation proteins in the pellet and those remaining in the supernatant were applied to a precast 4-15% sodium dodecyl sulfate–polyacrylamide gradient gel electrophoresis for Westerns against acetylated-lysine and sirtuin-1.

Mitochondrial complex II and IV

Complex II and IV protein content was evaluated in cells using a MitoBiogenesis kit. Cells were seeded onto 96 well plates (50,000/well) and cultured using standard media. Cells were incubated with vehicle, Epi, Epi and L-NAME, and L-NAME for 48 h. Cells were washed with buffer and fixed with 4% formaldehyde followed by washes with buffer. Cells were exposed to 0.5% acetic acid for 5 min to block endogenous alkaline phosphatase activity followed by washes with buffer and exposure to a permeabilization buffer for 30 min. Cells were exposed to a blocking solution and incubated with primary antibodies overnight at 4°C, followed by rinsing with wash buffer and incubation with secondary antibodies labeled with alkaline phosphatase

and horseradish peroxidase for 2 h. Developing solutions (alkaline phosphatase for complex II and horseradish peroxidase for complex IV) were added and kinetic colorimetric signals measured in a spectrophotometer (µQuant, Bio-Tek Instruments, Inc.) using 405 nm for complex II and 600 nm for IV. Total absorbance values were normalized against cell number as derived from the use of Green Janus staining.

Citrate synthase activity (CS)

After 48 h of treatment, cells were lysed and total protein was extracted. An amount of 20 µg of protein was used for the assay. CS activity was determined by immunocapture using a citrate synthase kit in a 96 well plate according to manufacturer's instructions. The activity of the enzyme captured in the wells was determinated by color development at 412 nm. All samples were tested in duplicates and activity measured at room temperature.

Mitotracker Green Assay

Mitochondria were labeled using the cell-permeable probe Mitotracker Green FM dye. Cells were plated in 96-well plates (1×10^4 cells/well). After 24 h of incubation with growth media, cells were incubated with Epi, Epi and L-NAME, and L-NAME for 48 h. Cells were washed with 100 µL serum-free DMEM and stained with 200 nM Mitotracker Green FM dissolved in Hank's Buffered Saline Solution for 30 min. Unstained control cells were incubated with serum-free media containing no dye but treatment or vehicle as the stained samples. After staining, cells were washed three times with 100 µL serum-free media and thereafter 100 µL of buffer was added to each well. For the microplate assay, staining was detected on a fluorescence microplate reader (µQuant from Bio-Tek instruments) (excitation 485 nm, emission 520 nm), and fluorescence was subtracted from the unstained control and expressed relative to cell number.

Animals

Aortas from young (3 month old, 250-300 g) or aged (18 month old, 500-600 g) male Wistar rats were employed for *ex vivo* evaluations of Epi vascular effects. Rats were allocated in 4 groups (8 aminals/group): 1) young control (vehicle); 2) young Epi; 3) aged control; 4) aged Epi. Animals were provided either with vehicle (water) or Epi (1 mg/kg/day) for 15 days. Animals were maintained at room temperature (18–25∘C) and on a 12 h light/dark cycle, with food and water provided ad libitum. Animals were handled following guidelines and in full compliance with Mexican federal regulations for animal experimentation and care and the good practices of the Comité Interno para el Cuidado y Uso de los Animales de Laboratorio with regards to research using animals. The Institutional committee on animal research of the Escuela de Medicina del Instituto Politecnico Nacional approved the experiments.

Blood pressure

A non-invasive method to measure systolic blood pressure was employed by using a pressure transducer placed on the tail of the animals with values digitally recorded.

Vascular reactivity

Under anesthesia (pentobarbital 60 mg/kg) animals were decapitated and the thoracic aorta from the diaphragm to the aortic arch isolated. Aortas were immediately submerged in cold Krebs solution to remove all adjacent connective tissue, then cut into ring segments (4-5 mm long), which were then mounted on two stainless steel hooks within an isolated organ chamber. One of the hooks was fixed to the bottom of the chamber and the other to a transducer linked to a Biopac System apparatus for registering changes in tension (force). The isolated organ chamber contained 10 ml of Krebs bicarbonate solution. The chamber was maintained at a constant temperature of 37˚C, pH of 7.4 and a continuous bubbling with a mixture of 95% O^2 and 5% CO^2. Aorta rings were pre-contracted using phenylephrine [1×10^{-11}]), and standard

concentration-response curves of acetylcholine [$1 \times 10^{-11} - 1 \times 10^{-4}$ M]) were constructed to

analyze the effect of 15 days of Epi treatment on the vascular reactivity of the pre-contracted

aortas.

Immunofluorescence

In order to detect post-translational modifications (phosphorylation and acetylation) on eNOS in

aging, we used immunofluorescence staining of dissected aortas from young and aged rats.

Aortas were cut into 0.5 cm fragments. Each vessel fragment was filled with tissue freezing

medium and frozen in liquid nitrogen-isopentane. Cryosections (7 μm in thickness) from each

vessel were permeabilized for 3 min with 0.3% Triton X-100. After incubation with blocking

solution (5% bovine serum albumin in phosphate-buffered solution [PBS]) for 1 h at room

temperature, cryosections were incubated for 3 h with one primary antibody (single staining) or

two primary antibodies (double staining) at room temperature. After several washes, sections

were incubated with a fluorescent secondary antibody for 1 h at room temperature. After rinsing

with buffer, sections were mounted using Vectashield medium, counterstained to visualize nuclei

(Vector Laboratories, Burlingame, Calif., USA) and observed using a fluorescence microscope.

Data Analysis

For cell culture experiments, at least three independent experiments each in triplicate were

performed. For animal studies, six rats per group were included. Results are expressed as a mean

± standard error of the mean (SEM). Data analysis was performed by one-way analysis of

variance (ANOVA) followed by Tukey's post hoc test using GraphPad Prism (GraphPad

Software Inc., San Diego, USA). A *p* value of <0.05 was considered to be statistically

significant.

Results

Figure 1A denotes β-galactosidase staining observed in young and aged cells while figure 1B reports on their quantification using 96 well stained plates. At baseline, increased staining levels are observed in aged vs. young cells, which were reduced by Epi treatment. The use of L-NAME in untreated aged cells further increased staining and was able to block the effects of Epi. In young cells, Epi and/or L-NAME treatments did not apparently alter β-galactosidase staining. As illustrated in figure 1C, a similar pattern of changes was observed in β-galactosidase protein levels measured by Western blot whereby young cells evidence low levels of staining regardless of the treatment applied. Aged cells demonstrated significantly higher protein levels vs. young and Epi treatment suppressed these increases. Figure 2A and B report on the effects triggered by aging and treatments on NO and p-eNOS levels respectively. Treatment of young cells with Epi significantly increased NO levels. With aging, cells evidenced significantly reduced (~40%) NO levels and Epi was able to restore NO levels to those of young cells. p-eNOS results essentially paralleled those noted for NO levels.

Figure 3A and B shows the effects of aging on sirtuin-1. In young cells, Epi stimulated the phosphorylation of sirtuin-1 by ~40%. Aging led to a decrease of ~50% in p-sirtuin-1 levels which were restored to those of young cells with Epi. Figure 4A reports on the total protein acetylation levels of young vs. aged cells. While no differences were noted in young cells, aging led to ~3 fold increase in total protein acetylation which was essentially normalized with Epi treatment. Figure 4B and 4C plots eNOS acetylation levels in young and aged cells as well as the levels of sirtuin-1that were associated with eNOS following immunoprecipitation. While no effects were noted in young cells, eNOS acetylation essentially doubled in aged cells and was partially restored to young cell levels by Epi treatment. Sirtuin-1 levels associated with eNOS

decreased by ~60% in aged cells and were essentially restored to those of young cells by Epi

treatment.

Figure 5A and 5B illustrates the effects of aging and treatments on markers of mitochondrial

biogenesis, cristae and oxidative phosphorylation (TFAM, mitofilin, and complex V). In young

cells, an increase of ~25% was noted with Epi treatment in all proteins. Aging led to ~50%

reduction in the protein levels and treatment with Epi, fully restored levels to those of young

cells. Figure 6A/B reports on the effects of aging and treatment on oxidative phosphorylation

complexes II and IV. In young cells, a stimulation was induced with Epi. Aged cells have a

decrease of ~30% in protein levels that were fully restored by Epi. Figure 6C reports on

mitochondrial function as assessed by citrate synthase activity. A stimulatory effect was noted

with Epi in young cells. In aged cells, there was a ~25% decrease in citrate synthase activity vs.

young. With Epi treatment, levels were comparable to those of young. To quantify the effects of

aging and Epi treatment on mitochondrial mass (density), Mitotracker green staining of cells was

used and quantified using a fluorescence plate reader. As shown in figure 6D, aged cells

evidence less Mitotracker staining density vs. young cells. Epi treatment of young cells

stimulated staining levels while in aged cells it restored their intensity to those of young cells. As

noted in figures 2 through 6, in all cases, pre-treatment of cells with L-NAME was able to block

Epi induced stimulation thus, evidencing the critical role played by eNOS.

Figure 7A reports on systolic blood pressure levels in young and aged rats and 7B on

corresponding plasma NO levels detected. Aged animals evidenced higher systolic pressures

(~130 vs. 100 mmHg, aged vs. young) and Epi treatment was able to normalize their levels to

those of young rats. In aged animals, plasma NO levels were reduced by ~50% and Epi was able

to restore them to those of young rats (while also significantly stimulating them in this group).

Figure 7C and D shows the effect of Epi treatment in excised aortas. In aortic rings from young

(C) and aged (D) animals, a vasodilatory effect of acetylcholine was evidenced in concentrations

ranging from 10^{-11} to 10^{-4} mol/L. The calculated ED_{50} values in young (2.1^{-7} mol/L) vs. aged

(5.8^{-7} mol/L) indicate a mild impairment in aged animal aorta vasorelaxation response. An

enhanced response was triggered by Epi treatment in both groups of animals, ED_{50} in young

animals (6.6^{-10} M) vs. aged (1.6^{-8} mol/L) suggest an increased effects in aortic rings from young

animals reaching a maximal level of relaxation of ~1.5 g vs 1.2 g in aortas from aged rats. Figure

8A and B denote p-eNOS levels as detected from immunofluorescent staining of aortic

endothelium in the same animals evidencing parallel results to those noted for NO levels. Panel

8C illustrates eNOS and total protein acetylation staining levels in the endothelium of aortas of

young and aged rats. With aging, a reduction in total eNOS levels were noted while total protein

acetylation increased. Epi treatment was able to increase eNOS levels while reducing total

protein acetylation in endothelial cells.

Discussion

Results indicate that in aged endothelium, a reduction in NO production occurs which is associated with decreased eNOS phosphorylation at activation sites coupled to higher levels of amino acid residue acetylation. Aged endothelium also evidences decreased levels for regulators of mitochondria biogenesis including sirtuin-1 and organelle function. Epi restored aged endothelium and rat NO production to levels comparable to those of young cells/animals. Effects were associated with increased levels of eNOS phosphorylation, reduced acetylation, and improved mitochondrial function. These results follow the ability of Epi to restore sirtuin-1 protein levels and deacetylase activity and thus, mitigate aging induced loss of vascular function. The restoration of physiological levels of NO to those comparable of a younger phenotype is seen as an appealing strategy to mitigate CVD development. Recommended strategies include aerobic exercise and intake of foods rich in dietary nitrates such as beetroot juice. (15-18). However, exercise activity may be difficult to maintain in particular, later in life. Natural supplements such as resveratrol have been considered and tested with modest to null results. (19-21). Interestingly, there is a greater level of recognition for the pleiotropic effects of statins, which include positive effects on the eNOS regulatory system (22). However, statin use (beyond cholesterol reducing effects) remains controversial in particular, since a large percentage of the population is opposed to consuming drugs for possibly decades where the adverse effects of such compounds may also become evident (myopathy and type 2 diabetes)(23,24). The use of androgen precursors and estrogen supplementation has also been considered to mitigate vascular inflammation and/or restoring function (25,26). However, the use of hormone supplementation remains controversial (27).

Epidemiological studies associate cocoa product consumption with reduced cardiometabolic risk

(28,29). Clinical studies using cocoa products document positive effects on vascular structure

and/or function in menopausal women, smokers and those with ischemic heart disease (30,31).

Studies also demonstrate that cocoa consumption can mobilize angiogenic cells (32) and improve

memory following 3 months of supplementation in normal 60-year-old subjects while increasing

brain blood volume (33). Evidence has been generated regarding the mechanisms that may

underlie the vascular effects of Epi. We demonstrated that human coronary artery endothelial

cells treated with 1 µM Epi, stimulates serine 633 and 1177 eNOS phosphorylation and the

dephosphorylation at threonine 495 thus, activating the enzyme. Epi also promotes eNOS

uncoupling from caveolin-1 via its interaction with heat shock protein 90 and binding to

calmodulin-1 leading to its activation (12). Furthermore, we reported on Epi's unique ability to

induce eNOS activation in a calcium independent manner (13). We also established that eNOS

amino acid residue glycosylation levels are higher (yielding enzyme inactivation) in obese mice

and human coronary artery endothelial cells exposed to high glucose and that Epi is able to

reduce these to those of control samples (34). These results demonstrate the versatility of this

compound in its ability to activate eNOS and thus, restore age-related NO decline via non-

redundant actions.

An accepted marker of cell aging is β-galactosidase activity (35). In this study, β-galactosidase

activity increased with cell passage number and Epi treatment mitigated the effect. The use of L-

NAME further aggravated β-galactosidase activity differences noting the dependence of cell

aging on NO. Aging of cells led to a diminished capacity to produce NO, which correlated

closely with reduced levels of eNOS phosphorylation. Epi treatment was able to restore NO and

eNOS phosphorylation levels to those similar of young cells. These effects, are comparable to

those reported for statins in human umbilical cord endothelial cells aged with hydrogen peroxide

treatment (36).

Results indicate that loss of sirtuin-1 mediated deacetlyase activity underlies at least in part,

aging induced loss of eNOS function. A reduced level of sirtuin-1 activity may be secondary to

decreases in protein levels and/or its phosphorylation. Here, we report on the capacity of Epi to

restore sirtuin-1 protein and phosphorylation levels effects, that can also be triggered by agents

such as statins and resveratrol (36,37). Aging is also associated with higher eNOS acetylation, an

effect that was also suppressed by Epi treatment and was linked to the enhanced protein-protein

interaction of eNOS with sirtuin-1. As reported by Ota et al, statins also increase sirtuin-1

expression and its physical coupling to eNOS (36).

In close association to the health status of the endothelial cells, are the indicators of

mitochondrial structural integrity and function. In our study, we assessed protein levels for a

recognized regulator of mitochondrial biogenesis (TFAM), cristae structure (mitofilin), oxidative

phosphorylation (complex V) and function (CS activity) including indicators of mitochondrial

biogenesis markers (complex II/IV) and abundance (mitotracker green). In all cases, aging

depleted the levels of these endpoints, which were fully restored by Epi. Thus, treatment with

Epi appears to reinstate the metabolic oxidative capacity of the cells to that comparable to young

ones. The coupling of increases in mitochondria abundance, structure and function in

endothelial cells to enhanced NO levels has also been demonstrated using resveratrol and statins

(36,38). As demonstrated by Nisoli et al, NO effects appear partly dependent on increases in

cyclic guanosine monophosphate and induction of peroxisome proliferator-activated receptor γ

coactivator 1α as mitochondrial biogenesis induced by exposure to cold was markedly reduced

in brown adipose tissue of eNOS null-mutant mice, which had a reduced metabolic rate and accelerated weight gain vs. wild-type mice (39).

Crucial to support our *in vitro* were responses triggered by Epi *in vivo*. In aged rats, an increase in blood pressure was noted suggesting an increased stiffness and/or endothelial dysfunction, which was corroborated with decreased acethylcholine-induced vasodilation. Epi partly restored the functional responses (blood pressure and vasodilation) to those noted in young animals and these changes correlated with enhanced endothelial eNOS phosphorylation and reduced acetylation. A limitation to this study relates to the fact that when Epi is provided orally it is readily metabolized and Epi metabolites can reach "high" levels in blood and trigger similar effects on endothelium to the native molecule (40). Ultimately, in the in vivo setting, the additive role of each chemical entity (including native Epi) likely accounts for the "final" response noted after the flavanol is provided orally. This scenario is not distinct from that given by the use of drugs such as losartan in cell culture where the metabolites that are generated in vivo are as or more potent than the native compound (41).

Beyond endothelium effects, our group has published a series of studies documenting the positive impact that either cocoa or Epi can provide. In rodent models of myocardial infarction, obesity/insulin resistance and aging we have documented the protective effects that 1 mg/kg/day of Epi triggers on multiple endpoints and in many organs. Treatment reduces infarct size, stimulates angiogenesis, ameliorates obesity-induced indicators of cardiometabolic dysregulation, improves multiple indicators of oxidative stress, mitochondrial, skeletal and cardiac structure function in normal and aged animals (14,34,42-45). In proof of concept studies, cocoa supplementation (that yields blood levels similar to those of 1 mg/kg/day of Epi ~ 300 nM) improves multiple indicators of skeletal muscle structure and function (including NOS and

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NO levels) in patients with heart failure and type 2 diabetes (46). In normal, sedentary subjects cocoa supplementation enhances multiple indicators of muscle metabolism while improving organ function (47). Epi can also increase muscle strength in normal subjects and significantly reduce triglyceride levels in those with hypertriglyceridemia (48,49). A common central theme underlying Epi effects, are improvements in NO levels and stimulation of mitochondria.

In conclusion, we demonstrate that a compound found in high concentrations in cocoa reverses aging associated loss of vascular function. The consumption of products enriched with Epi is likely to promote the preservation of vascular health and positively impact societies well being. Greater introspect as to the promise of flavanols to exert a positive impact on CVD risk is likely to be gained from the ongoing COcoa Supplement and Multivitamin Outcomes Study (COSMOS) trial whereby, 18,000 volunteers are to be provided a high flavanol cocoa capsules for 5 years. If outcomes are positive, this trial may pave the way for a greater appreciation of the positive impact that cocoa flavanol supplementation may have in a range of diseases.

Conflict of Interest

Dr. Villarreal is a co-founder and stockholder (Dr. Ceballos) of Cardero Therapeutics, Inc. The

co-authors Ramirez-Sanchez, Mansour, Navarrete, Ayala, Guevara, Castillo, Loredo and

Bustamante have no disclosures.

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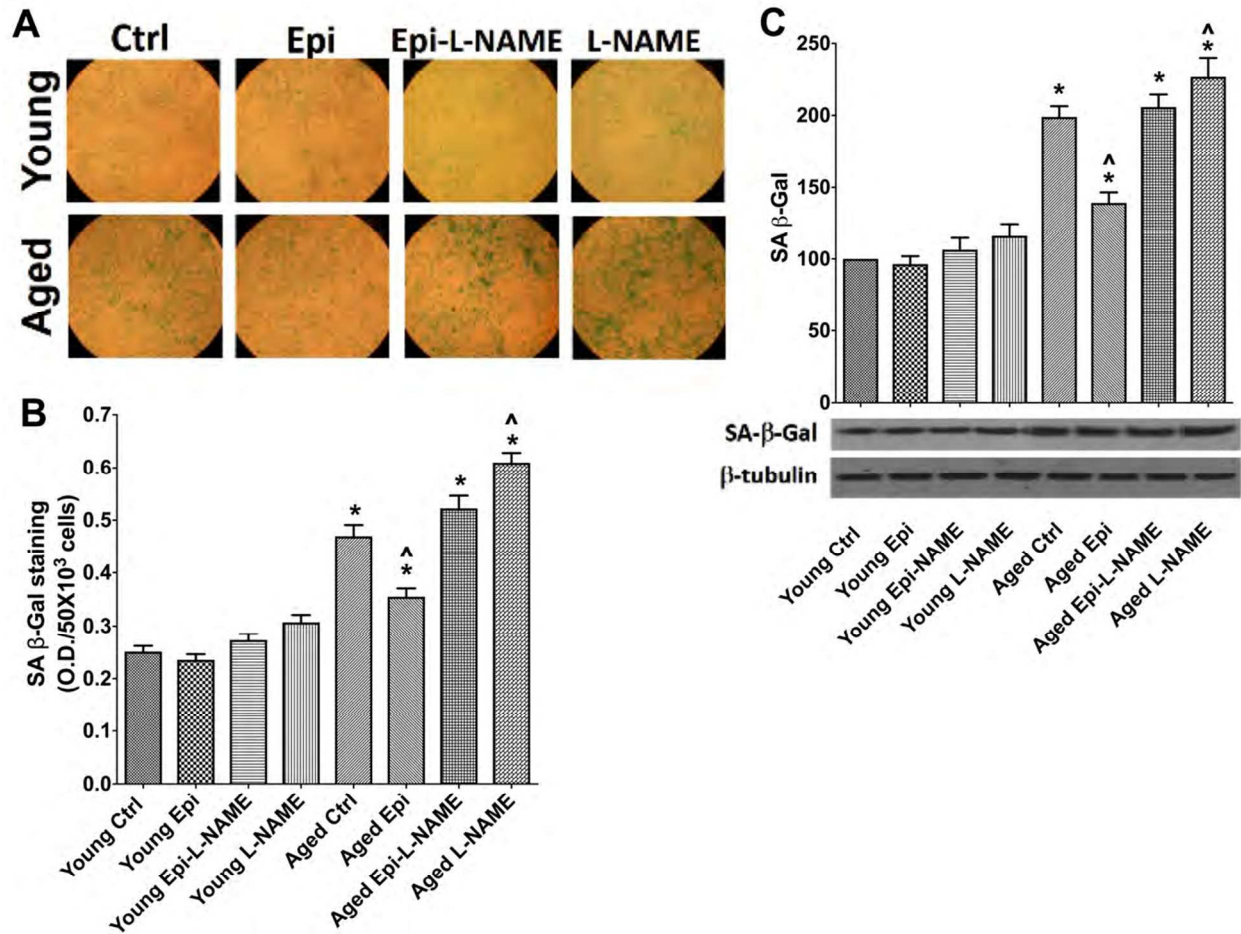
Figure 1. β-Galactosidase staining, activity, and protein levels using young and aged control (Ctrl), (-)-epicatechin and L-NAME treated cells. (A) Staining assay for visualizing β-galactosidase activity in 6 well cell culture plates. (B) β-galactosidase activity in 96 well plate staining. Absorbance values were normalized by number of cells. (C) β-galactosidase and β-tubulin (loading control) protein levels evaluated by Western Blot. (Values are expressed as mean ± SEM n=4/group, *$p<0.05$ vs. young control, ^ vs. aged control). Senescence associated β-Galactosidase = SA-β-Gal, Control = Ctrl, (-)-epicatechin = Epi, optical density = O.D.
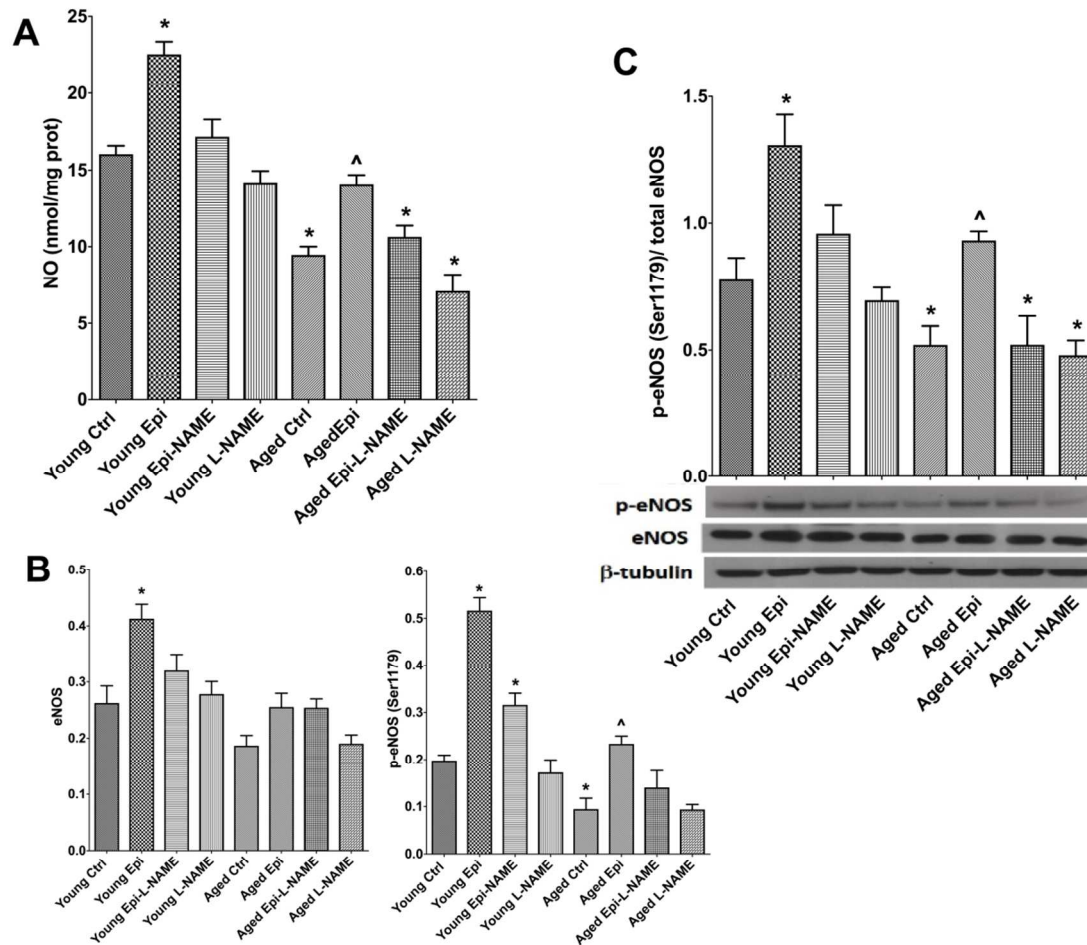
Figure 2. Effects of aging and (-)-epicatechin treatment on nitric oxide and eNOS. (A) Young

and aged cells were treated with Epi, L-NAME, or Epi and L-NAME and nitric oxide (NO)

levels were measured after 48 h. NO (nitrite/nitrate, nmol/mg protein) levels were normalized by

protein content. (B) Under the same conditions, total eNOS, p-eNOS (Ser1179) expression levels

was evaluated by Western blots. (C) p-eNOS/eNOS ratio was calculated. eNOS and p-eNOS

protein levels were normalized against β-tubulin. (Values are expressed as mean ± SEM

n=3/group, *p<0.05 vs. young control, ^ vs. aged control). (-)-epicatechin = Epi, nitric oxide =

NO.
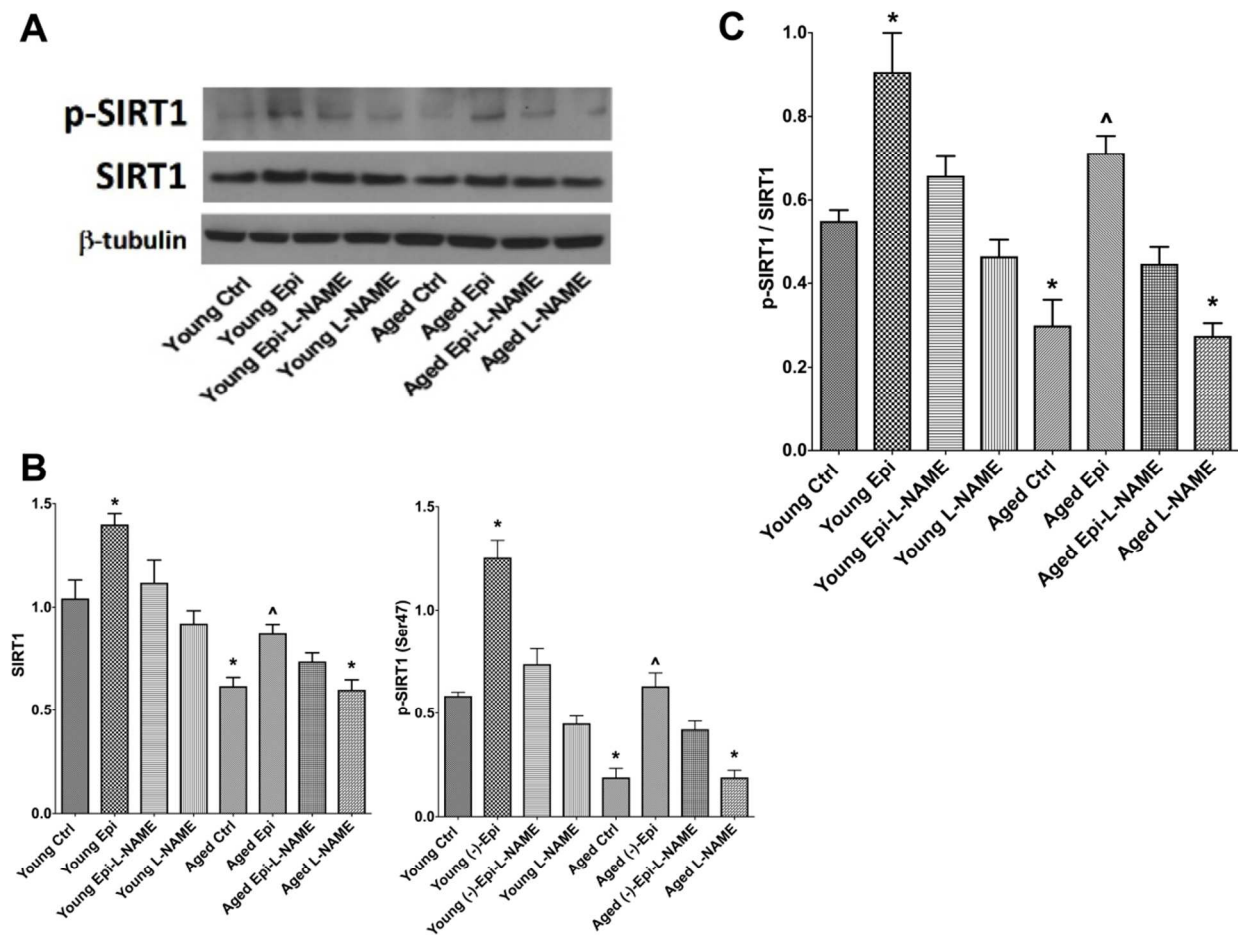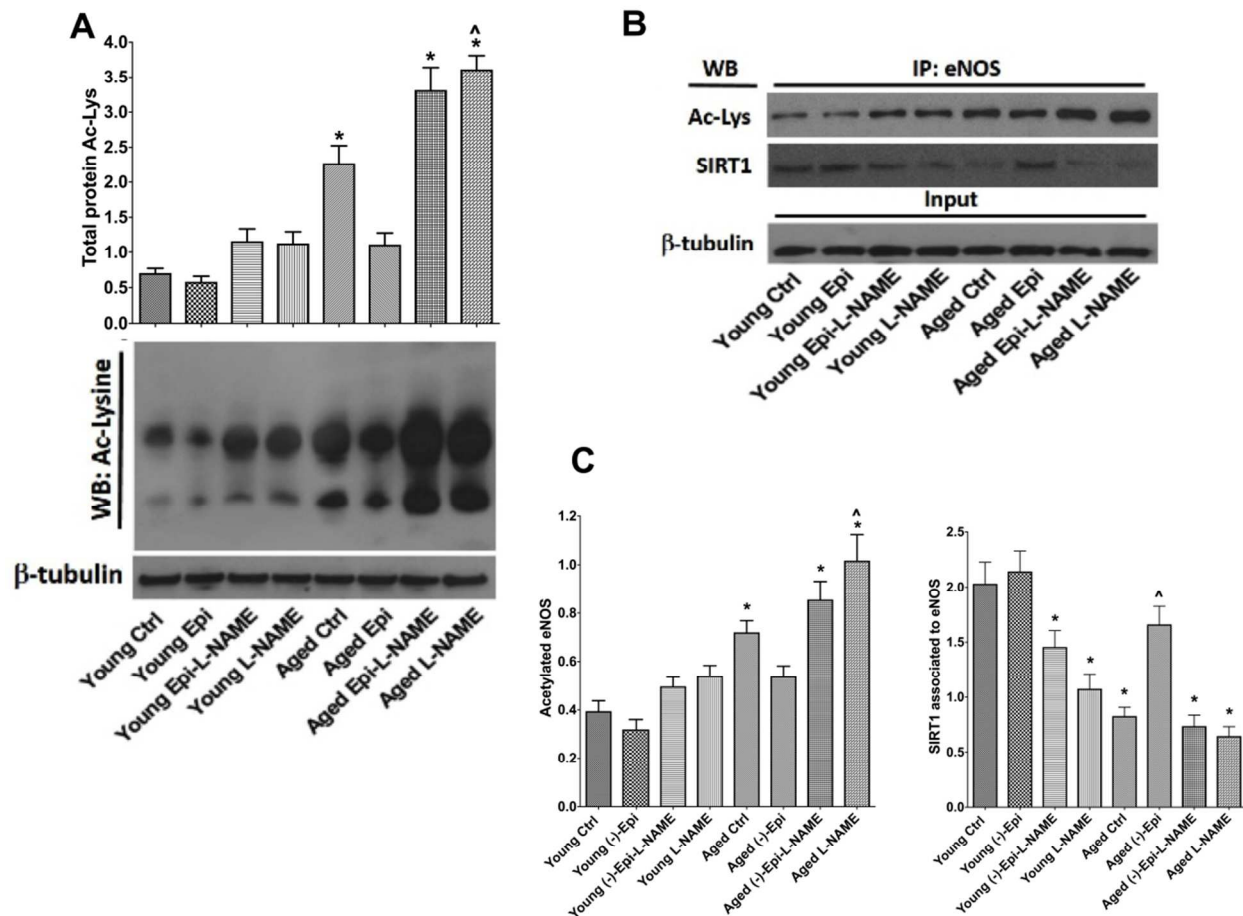
Figure 3. Effects of aging and (-)-epicatechin treatment on sirtuin-1 and p-sirtuin-1 protein

levels. Young and aged cells were treated with Epi, L-NAME, or Epi and L-NAME and Western

blots were used to determine relative protein levels. (A) p-sirtuin-1, sirtuin-1, and β-tubulin

protein content (B) Total sirtuin-1 and p-sirtuin-1 levels (C) p-sirtuin-1/sirtuin-1 ratio. Protein

levels were normalized against β-tubulin. (Values are expressed as mean ± SEM n=3/group,

*p<0.05 vs. young control, ^ vs. aged control). (-)-epicatechin = Epi, SIRT1 = sirtuin-1.

Figure 4. Effects of aging and (-)-epicatechin treatment on protein acetylation. Young and aged cells were treated with Epi, L-NAME, or Epi and L-NAME and Western blots were used to determine relative protein levels. (A) Total protein acetylation in yong and aged cells using anti acetylated-lysine antibody and normalized against β-tubulin. (B) immunoprecipitation using an antibody against eNOS to evaluate acetylation levels and its association with sirtuin-1. (C) Acetylated and sirtuin-1 associated to eNOS levels were normalized against β-tubulin. (Values are expressed as mean ± SEM n=3/group, *p<0.05 vs. young control, ^ vs. aged control). Acetylated lysine = Ac-Lys, (-)-epicatechin = Epi, sirtuin-1 = SIRT1.
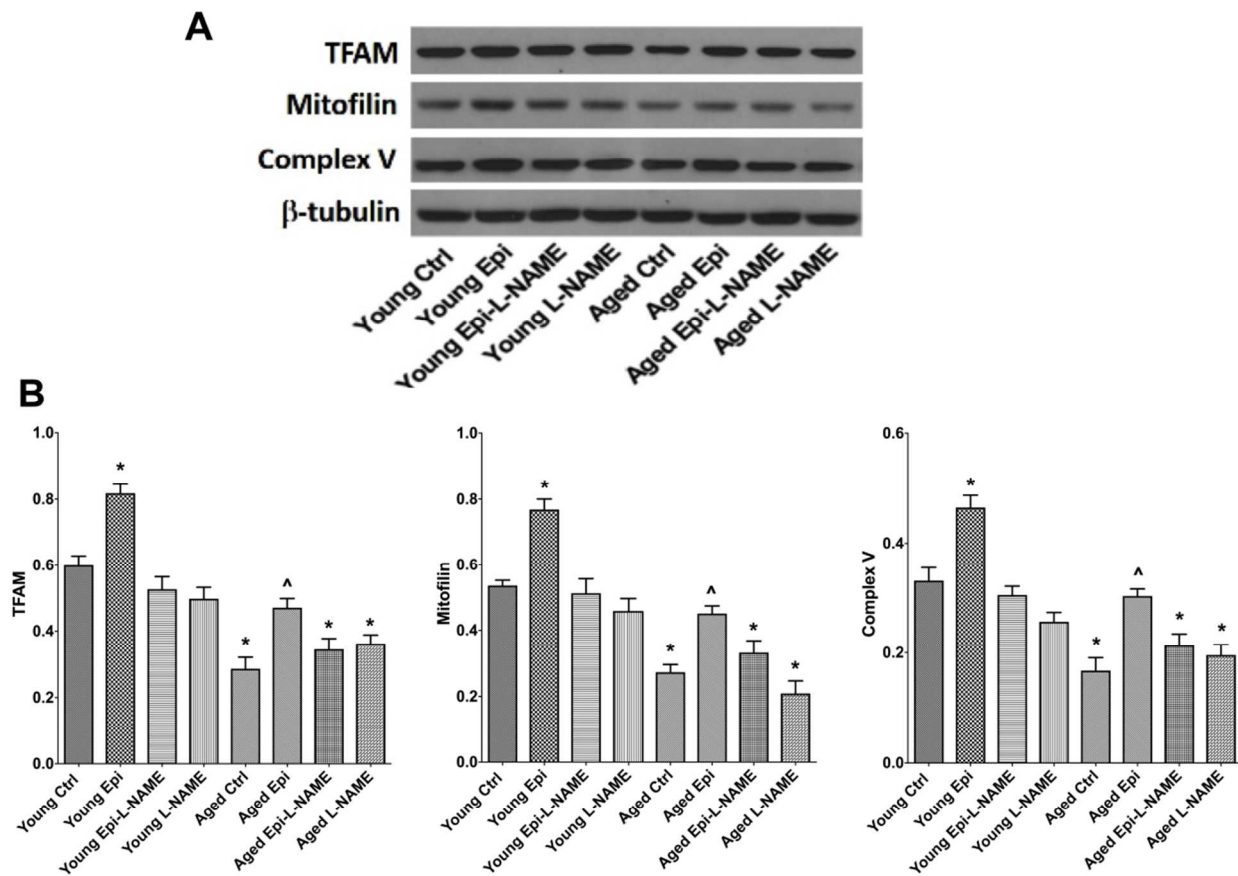
Figure 5. Effects of aging and (-)-epicatechin treatment on mitochondrial related protein levels (mitofilin, complex V, and TFAM). Young and aged cells were treated with Epi, L-NAME, or Epi and L-NAME and Western blots were used to determine relative protein levels (A) Representative protein levels of TFAM, mitofilin, and complex V. (B) Quantification of TFAM, mitofilin, and complex V. (Values are expressed as mean ± SEM n=3/group, *$p<0.05$ vs. young control, ^ vs. aged control). (-)-epicatechin = Epi.
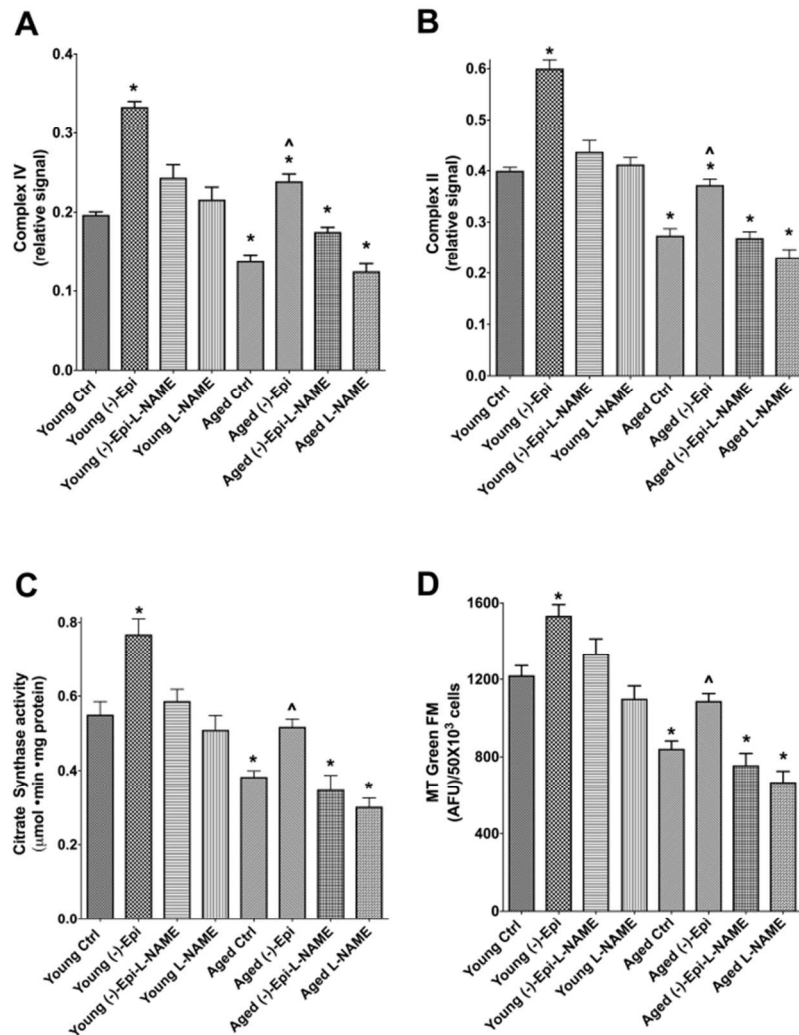
Figure 6. Effects of aging and (-)-epicatechin on mitochondrial density and function. Young and aged cells were treated with Epi, L-NAME, or Epi and L-NAME. Quantification of complex IV (A) and II (B) protein levels determined by ELISA. Citrate synthase activity levels in young and aged treated cells (C). Changes in activity were normalized by protein content. Quantification of mitotracker green fluorescence measured in a microplate system (D). Fluorescence levels are expressed in arbitrary fluorescence units and were normalized by cell density. (Values are expressed as mean ± SEM n=5/group, *p<0.05 vs. young control, ^ vs. aged control). Arbitrary fluorescent units = AFU, (-)-epicatechin = Epi, mitotracker = MT.
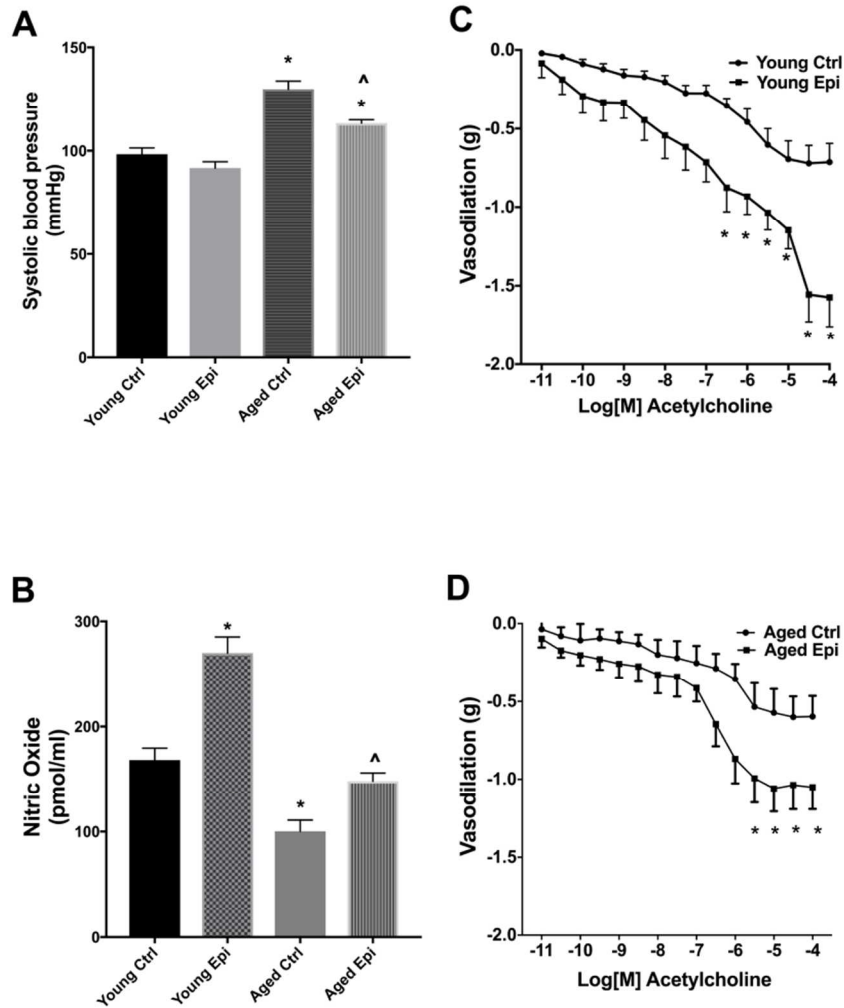
Figure 7. Effects of aging and (-)-epicatechin treatment on nitric oxide (NO) levels, systemic blood pressure and vasodilatory capacity of aortic rings. (A) Systolic blood pressure as measured by tail cuff. (B) NO levels in control and treated young and aged rats. Comparison of acetylcholine induced vasodilatory effects in young (C) and aged (D) aortic rings and responses obtained by 15 days of pretreatment with (-)-epicatechin. (Values are expressed as mean ± SEM n=6/group, *p<0.05 vs. young control, ^ vs. aged control). Control = Ctrl, (-)-epicatechin = Epi.
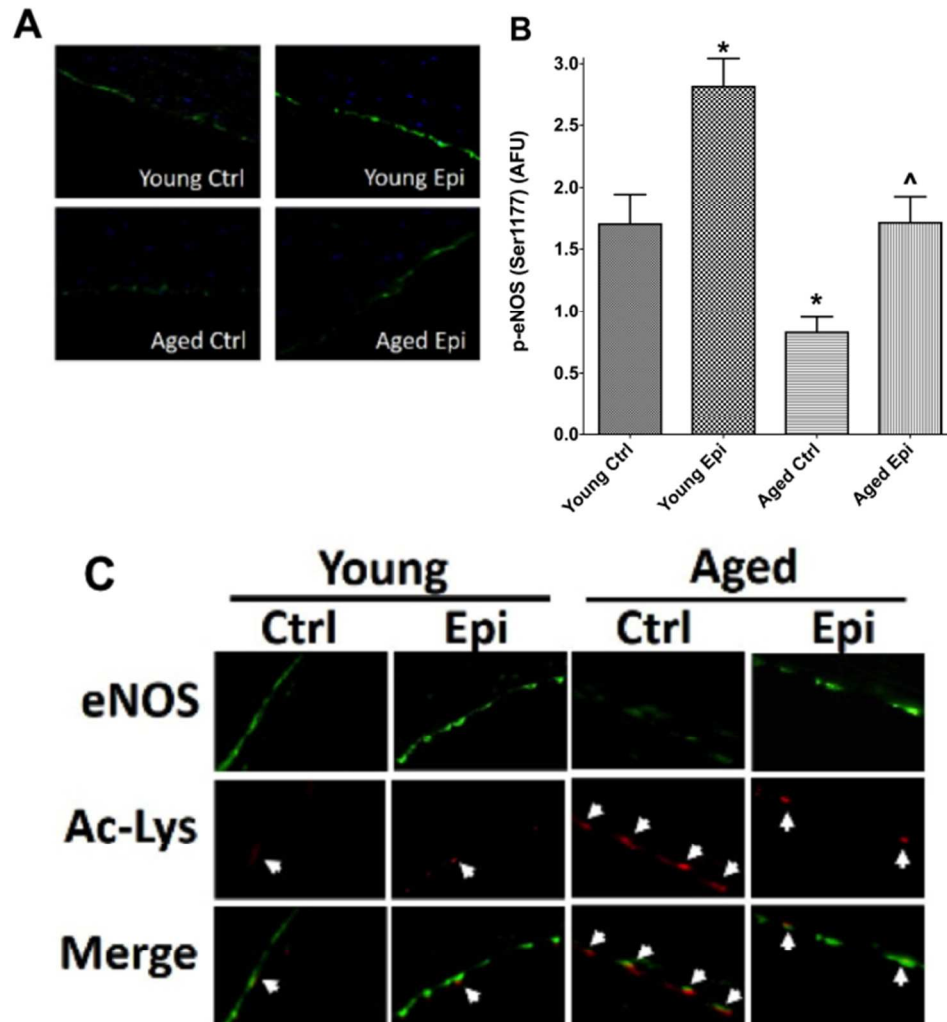
Figure 8. Effects of aging and (-)-epicatechin treatment on p-eNOS levels and eNOS acetylation in young and aged rats as detected by immunofluorescence. (A) Immunofluorescence of p-eNOS (green staining) in endothelium of aortic rings as induced by 15 days pretreatment of animals with Epi or vehicle (control animals). Cell nuclei are stained blue. (B) Quantification of p-eNOS immunofluorescence levels detected. (C) Immunofluorescence of eNOS (green) and acetylated-lysine (red) in endothelium of aortic rings of vehicle and Epi treated young and aged rats. (n=6/group, *p<0.05 vs. young control, ^ vs. aged control). Arbitrary fluorescence units (AFU), control = Ctrl, (-)-epicatechin = Epi.