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Physical Exercise Prevents Cellular Senescence in Circulating Leukocytes and in the Vessel Wall

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Background—The underlying molecular mechanisms of the vasculoprotective effects of physical exercise are incompletely understood. Telomere erosion is a central component of aging, and telomere-associated proteins regulate cellular senescence and survival. This study examines the effects of exercising on vascular telomere biology and endothelial apoptosis in mice and the effects of long-term endurance training on telomere biology in humans.

Methods and Results—C57/Bl6 mice were randomized to voluntary running or no running wheel conditions for 3 weeks. Exercise upregulated telomerase activity in the thoracic aorta and in circulating mononuclear cells compared with sedentary controls, increased vascular expression of telomere repeat-binding factor 2 and Ku70, and reduced the expression of vascular apoptosis regulators such as cell-cycle–checkpoint kinase 2, p16, and p53. Mice preconditioned by voluntary running exhibited a marked reduction in lipopolysaccharide-induced aortic endothelial apoptosis. Transgenic mouse studies showed that endothelial nitric oxide synthase and telomerase reverse transcriptase synergize to confer endothelial stress resistance after physical activity. To test the significance of these data in humans, telomere biology in circulating leukocytes of young and middle-aged track and field athletes was analyzed. Peripheral blood leukocytes isolated from endurance athletes showed increased telomerase activity, expression of telomere-stabilizing proteins, and downregulation of cell-cycle inhibitors compared with untrained individuals. Long-term endurance training was associated with reduced leukocyte telomere erosion compared with untrained controls.

Conclusions—Physical activity regulates telomere-stabilizing proteins in mice and in humans and thereby protects from stress-induced vascular apoptosis. **(*Circulation*. 2009;120:2438-2447.)**

Key Words: aging ■ exercise ■ nitric oxide synthase ■ prevention ■ telomeres

Physical training is associated with improvements in exercise capacity, blood pressure regulation, insulin sensitivity, abdominal fat reduction, lipid profile, and psychosocial, hemodynamic, and inflammatory parameters. These effects contribute to an augmentation of endothelial function, delayed atherosclerotic lesion progression, and enhanced vascular collateralization in patients with diabetes mellitus, coronary artery disease, and chronic heart failure. However, despite the wealth of evidence, our understanding of the underlying molecular mechanisms, especially with regard to cellular survival and senescence, is limited.

Clinical Perspective on p 2447

Aging is a predominant and independent risk factor for the development of atherosclerotic diseases. Vascular aging is characterized by impaired endothelial function and arterial stiffening.[1] On the cellular level, telomere biology is a central regulator of the aging process. Telomeres and their regulatory proteins compose t-loop structures at both ends of eukaryotic chromosomes and protect the genome from degradation during repetitive cellular divisions.[2] The enzyme telomerase with its catalytic protein subunit telomerase reverse transcriptase (TERT) is the main component of the telomere complex. Other important proteins in the t loop include the telomere repeat-binding factors (TRFs), which interact with telomere-associated proteins and serve as binding platforms.[3]

Recent clinical data suggest that parameters of telomere biology in circulating mononuclear cells (MNCs) are associated with cardiovascular morbidity and can be used as indicators for the effect of therapeutic interventions.[4–6] Fur-

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ther studies revealed a close correlation of blood and vascular leukocyte telomere DNA content.[7] Our previous work demonstrated beneficial effects of physical exercise on myocardial telomere-regulating proteins.[8] However, the effects of physical exercise on vascular telomere biology and aging are unknown.

Methods

Animals and Exercising

Eight-week-old male C57/Bl6 (Charles River Laboratories, Wilmington, Mass), endothelial nitric oxide (NO) synthase–deficient (eNOS$^{-/-}$; B6.129/P2-Nos3, Charles River) mice, TERT$^{-/-}$ (B6.129S-Tert^{tm1Yjc}/J, The Jackson Laboratory, Bar Harbor, Me; mutant generation 2) mice, and strain-matched controls were studied. Exercising mice were kept in individual cages equipped with a running wheel and a mileage counter. The mean voluntary running distance of wild-type mice was 4280±670 m/24 h and did not differ significantly between TERT$^{-/-}$ mice and eNOS$^{-/-}$ mice. Indicated mice were treated with paraquat or lipopolysaccharide (Sigma-Aldrich, Munich, Germany).

Track and Field Athletes and Untrained Control Subjects

Blood MNCs were studied in professional young middle- and long-distance runners (German National track and field team; n=32; mean age, 20.4±0.6 years; 25 male, 7 female subjects; average running distance, 73±4.8 km/wk). In addition, we studied middle-aged athletes (marathon runners, triathletes) performing regular endurance training and competitions (n=25; mean age, 51.1±1.6 years; 19 male, 6 female subjects; average running distance, 80±7.5 km/wk; 35±2.7 years of training history). Two groups of nonsmoking healthy volunteers (26 young control subjects: mean age, 21.8±0.5 years; 15 male, 11 female subjects; 21 middle-aged control subjects: mean age, 50.9±1.6 years; 14 male, 7 female subjects) who reported <1 hour of exercise per week in the last year served as controls (the Table). Endurance capacity was assessed in all subjects by standardized ECG stress test. Blood samples were taken in the morning in the fasting state before training. Leukocytes were isolated from sodium citrate blood by Ficoll density gradient centrifugation. All subjects gave informed consent, and the study was approved by the ethics committee of the Ärztekammer des Saarlandes (No. 116/07).

Telomerase Activity and Telomere Length Analysis

Telomerase activity was assessed with the quantitative telomerase repeat amplification protocol. Telomere length was determined through the use of flow–fluorescence in situ hybridization (FISH), quantitative FISH, and real-time polymerase chain reaction (PCR).[8] Details are given in the online-only Data Supplement.

Western Blot Analysis, ELISA, Real-Time PCR, Aortic Ring Preparation and Tension Recording, and Quantification of Apoptosis by Hairpin Oligonucleotide Assay

These protocols are described in detail in the online-only Data Supplement.

Statistical Analysis

Band intensities were analyzed by densitometry. In mouse experiments, median values between 2 independent groups were compared by use of the Mann–Whitney test. The Kruskal-Wallis test was used to compare median values across ≥3 groups, and Bonferroni posthoc analyses were performed to account for multiple testing. In the figures, boxes represent medians and 25% and 75% percentiles; whiskers represent ranges. Means of the human data were compared by use of ANOVA and the Bonferroni test for posthoc analyses. Endothelial function data were subjected to regression analysis with a general linear model for repeated measures and Bonferroni posthoc tests. SPSS software version 17.0 (SPSS Inc, Chicago, Ill) was used. Differences were considered significant at $P<0.05$. Absolute values are shown for $P>0.001$.

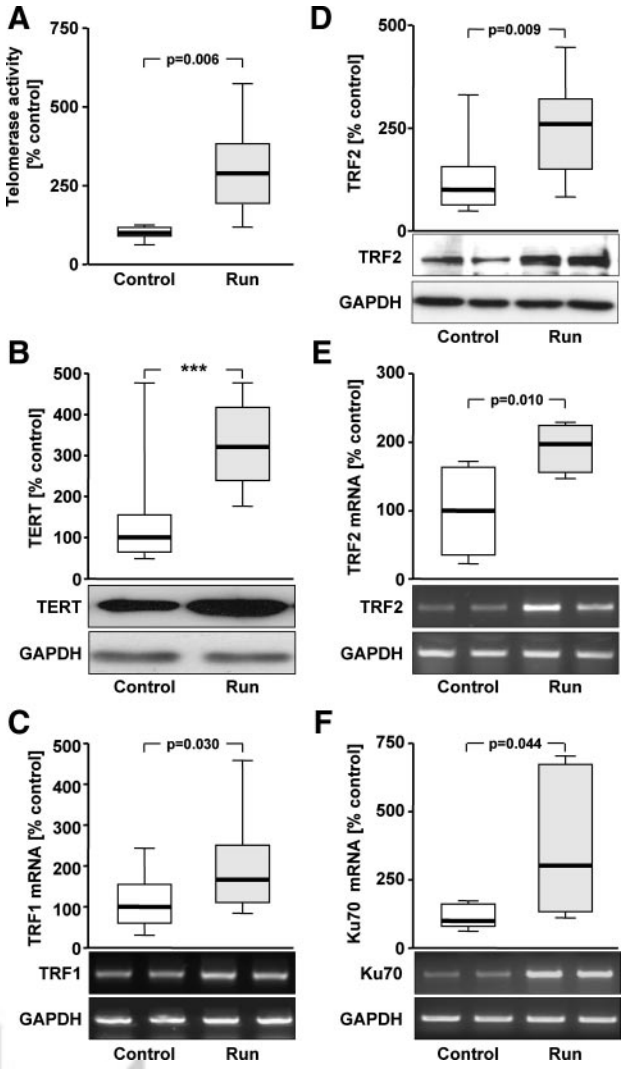

Figure 1. Voluntary physical exercise increases telomere-regulating proteins in the aorta. Effects of voluntary running exercise in C57/Bl6 mice for 21 days vs sedentary controls on aortic (A) telomerase activity determined by the telomerase repeat amplification protocol, (B) protein expression of the murine TERT, (C) mRNA expression of TRF1, (D) TRF2 protein, (E) mRNA expression, and (F) mRNA expression of the 70-kDa subunit of the DNA repair protein Ku, each shown with representative Western blots/PCR images and standardized for the housekeeping gene GAPDH. Box plots represent median and 25% to 75% percentiles; whiskers represent range; n=8 per group. ***$P<0.001$.

Results

Exercise Increases Aortic Telomerase Activity and Telomere-Stabilizing Proteins in Mice

Voluntary running for 3 weeks had no effect on lipid levels, body weight, blood pressure (controls, 116/91 mm Hg; exercise, 119/92 mm Hg; n=10 per group), or resting heart rate (controls, 478 bpm; exercise, 474 bpm) in C57/Bl6 mice but induced a 2.9-fold increase in aortic telomerase activity as determined by the telomere repeat amplification protocol

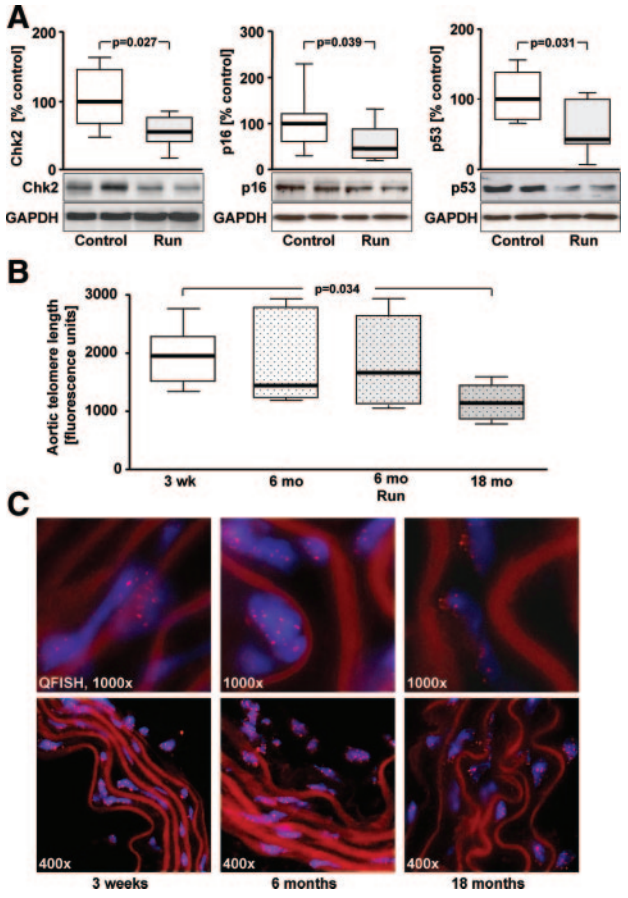

Figure 2. Running exercise decreases aortic expression of cell-cycle inhibitors independently of changes in telomere length. A, Effects of 21 days of running exercise in C57/Bl6 mice on aortic protein expression of Chk2, p16, and p53 standardized for GAPDH; n=8 per group. B, Effects of 6 months of running wheel exercise vs 3 weeks, 6 months, and 18 months of sedentary condition on aortic telomere length as determined by quantitative FISH (QFISH) and displayed as box plots and median telomere fluorescence units per high-powered field; n=4, with each n consisting of 4 aortic sections and 2 high-powered fields captured from each section. C, Exemplary images of aortic telomeres (QFISH, red dots) and the corresponding nuclei (DAPI, blue). Top row, ×1000 magnification; bottom row, ×400 magnification.

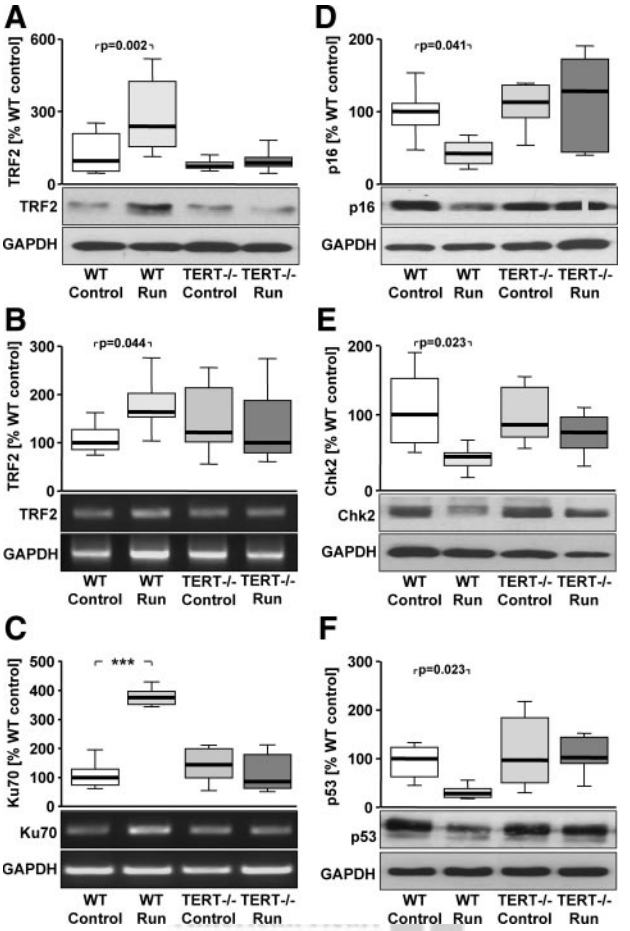

Figure 3. Effects of exercise on aortic telomere biology and cell cycle are blunted in TERT$^{-/-}$ mice. Representative Western blot analysis and quantification of the aortic effects of voluntary exercise for 21 days in B6.129S-Tert^{tm1Yjc}/J and their corresponding wild types (WT) on the expression of (A) TRF2 protein, (B) TRF2 mRNA, (C) Ku70 mRNA, (D) p16 protein, (E) Chk2 protein, and (F) p53 protein. Standardization for GAPDH. *P* values are calculated vs median of wild-type control; n=6 to 8 per group. ***$P<0.001$.

(Figure 1A). Physical training upregulated the protein expression of the murine TERT by 3.2-fold (Figure 1B). TRF1 mRNA expression was upregulated by 1.5-fold (Figure 1C). TRF2 protein expression increased by 2.6-fold, and TRF2 mRNA increased by 2.0-fold (Figures 1D and 1E). Exercise also upregulated mRNA expression of the 70-kDa subunit (3.0-fold; Figure 1F) but not of the 80-kDa subunit of the DNA repair protein Ku (data not shown).

Exercise Downregulates Vascular Cell-Cycle Inhibitors and Apoptosis Regulators

Mice supplied with a running wheel for 3 weeks were characterized by decreased aortic expression of cell-cycle inhibitors compared with animals without a running wheel (Figure 2A). Protein expression of p16 was reduced to 45%, of cell-cycle–checkpoint kinase 2 (Chk2) to 57%, and of p53 to 42%.

Long-Term Exercise for 6 Months Does Not Alter Aortic Telomere Length

Telomere length in aortic sections was examined by FISH (Figure 2B and 2C). There was no significant difference in aortic telomere length between mice after 3 weeks and 6 months of exercise and those in the sedentary condition. As a positive control, we studied a group of 18-month-old mice, which showed shorter telomeres.

Effects of Exercise Are Absent in TERT$^{-/-}$ Mice

TERT$^{-/-}$ mice and their strain-matched wild types were subjected to 21 days of voluntary running. Similar to the C57/Bl6 mice, B6.129S wild-type mice exhibited a marked upregulation of aortic TRF2 or Ku70 expression (Figure 3). However, running had no effect on TRF2 or Ku70 expression in TERT$^{-/-}$ mice. Importantly, the exercise-induced downregulation of p16, Chk2, and p53 expression in B6.129S wild types was completely absent in TERT$^{-/-}$ mice (Figure 3). These data identify TERT as a central mediator of the effects of exercising on telomere regulation and on survival proteins.

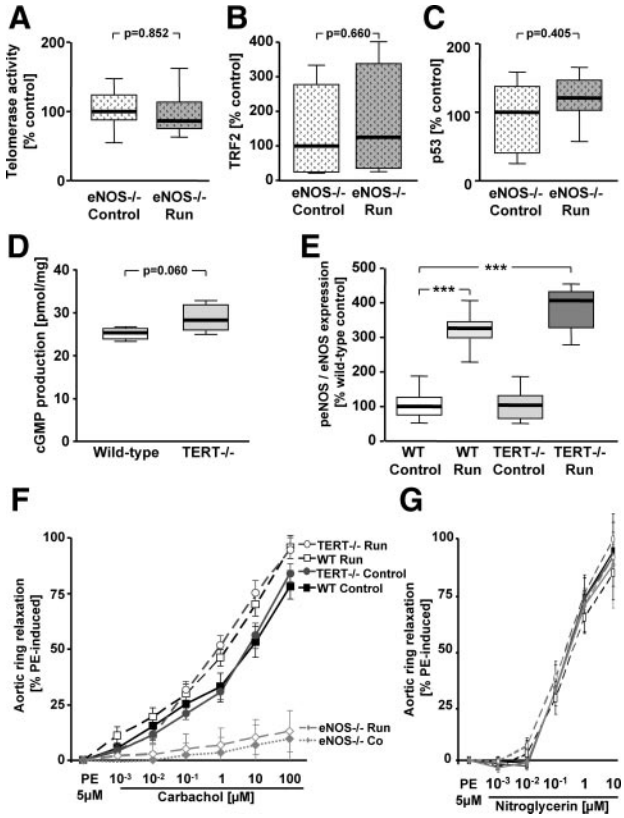

Figure 4. Exercise has no effects in eNOS$^{-/-}$ mice, and eNOS function is not impaired in TERT$^{-/-}$ mice. A through C, Training effects on aortic murine TERT activity and protein expression of TRF2 and p53 in eNOS$^{-/-}$ (B6.129/P2-Nos3) mice. Standardization for GAPDH; n=8. D, cGMP in TERT$^{-/-}$ mice and wild-type controls; n=5. E, Effects of 3 weeks of running exercise on protein expression of phospho-eNOS (peNOS)/eNOS in TERT$^{-/-}$ mice and wild-type controls (WT) standardized for GAPDH; n=6. ***P<0.001. F, Endothelium-dependent (carbachol-induced) vasodilation of isolated aortic rings; regression analysis: P=0.037, wild-type runner vs wild-type control; P=0.028, TERT runner vs TERT control. G, Endothelium-independent (nitroglycerin-induced) vasorelaxation in sedentary and exercised wild-type, TERT$^{-/-}$, and eNOS$^{-/-}$ mice expressed as percent of maximal phenylephrine (PE) -induced vasoconstriction (P=NS for all groups); n=5.

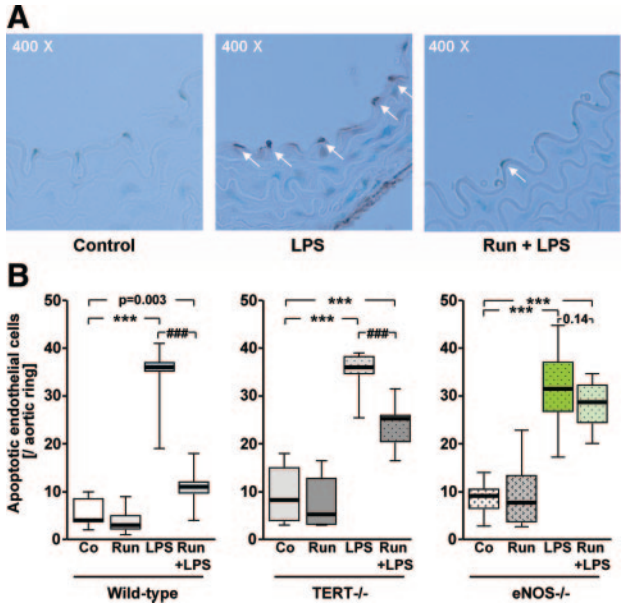

Figure 5. Exercise protects mice against stress-induced aortic endothelial apoptosis. Endothelial apoptosis of the thoracic aorta was measured by hairpin oligonucleotide assays in C57/Bl6, TERT$^{-/-}$, and eNOS$^{-/-}$ mice. A, Representative images showing dark brown peroxidase staining of apoptotic nuclei in the aorta of sedentary+lipopolysaccharide (LPS) and running+LPS mice (×400 magnification). B, Effect of LPS treatment (120 mg IP for 48 hours) in sedentary vs running wild-type, TERT$^{-/-}$, or eNOS$^{-/-}$ mice vs vehicle-treated controls (Co). Box plots represent median and 25% to 75% percentiles; whiskers represent range; n=4 to 5 per group. ***P<0.001 vs untreated control mice; ###P<0.001 vs LPS-treated sedentary control mice.

Effects of Voluntary Exercise Are Mediated by eNOS

Increased availability of endothelial NO is a hallmark of physical exercise.[9] Therefore, the experiments were repeated in eNOS$^{-/-}$ mice. Figure 4A through 4C and Figure I of the online-only Data Supplement show that there was no modification of aortic telomerase activity (telomere repeat amplification protocol) by exercise in eNOS$^{-/-}$ mice. PCR analysis showed no changes in TRF1, TRF2, Ku70, Ku80, or p53 mRNA expression. Similarly, Western blots found no significant differences in aortic TRF2, p16, Chk2, and p53 protein levels between running and sedentary eNOS$^{-/-}$ mice. Therefore, eNOS is a necessary mediator of the exercise-induced regulation of telomere proteins. Aortic cGMP content was not different in TERT$^{-/-}$ compared with wild-type mice (Figure 4D). Both B6.129S and TERT$^{-/-}$ mice showed an increase in the ratio of phospho-eNOS to total eNOS in the thoracic aorta after 3 weeks of exercise (Figure 4E). Endothelium-dependent vasodilation improved markedly in both wild-type and TERT$^{-/-}$ mice after 3 weeks of exercising but was completely absent in eNOS$^{-/-}$ animals (Figure 4F), whereas endothelium-independent vasodilatation was comparable in all groups (Figure 4G).

Exercise Reduces Endothelial Apoptosis

To test whether the exercise-induced regulation of telomere regulators and survival proteins would be physiologically relevant, endothelial cell apoptosis (hairpin oligonucleotide assays) was induced by treatment with lipopolysaccharide (120 mg IP for 48 hours; Figure 5A) or paraquat (25 mg/kg IP for 24 hours). Both agents strongly induced apoptosis (lipopolysaccharide by 7-fold, paraquat by 5-fold). Preconditioning with 3 weeks of voluntary exercise potently decreased the number of apoptotic aortic endothelial cells in the lipopolysaccharide experiments (2.1-fold; Figure 5B) and to a similar degree in the paraquat experiments (2.9-fold; P<0.05; data not shown). Importantly, in C57/B16 mice exercise training conferred potent protection from oxidative stress-induced endothelial apoptosis. When these experiments were repeated in TERT$^{-/-}$ mice, exercising partially abolished the exercise-induced antiapoptotic effect (Figure 5B). In eNOS$^{-/-}$ mice, a complete loss of the protective effect of exercise was observed (Figure 5B).

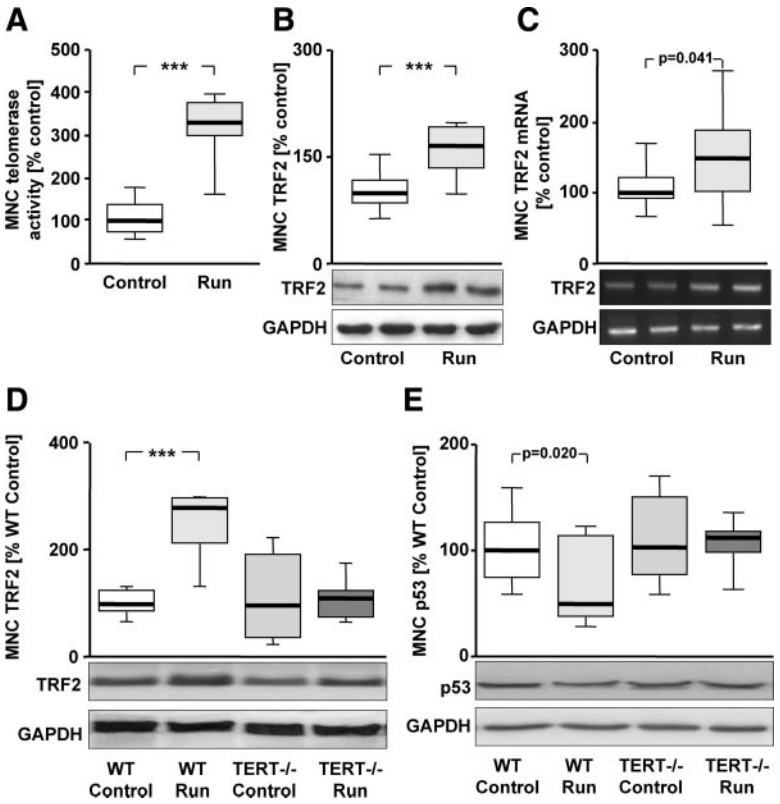

Figure 6. Running exercise has beneficial effects on telomere biology of MNCs in mice. MNCs were isolated by Ficoll gradient centrifugation from the spleens of C57/Bl6 mice after 3 weeks of running wheel exercise or sedentary condition. Effect of voluntary training on MNC (A) telomerase activity, (B) TRF2 protein, and (C) TRF2 mRNA expression; n=8. TRF2 (D) and p53 protein (E) levels are regulated in spleen-derived MNCs of exercising B6.129S mice. This effect is abolished in B6.129S-Tert^{tm1Yjc}/J mice; n=6. ***$P<0.001$.

Exercise Decreases Senescence Proteins in MNCs via Upregulation of Telomere Proteins

Exercise potently activated telomerase in MNCs isolated from the spleen by 3.3-fold (Figure 6A). This activation was also observed in circulating blood MNCs and MNCs isolated from bone marrow. Running led to an upregulation of TRF2 protein and mRNA expression (Figure 6B and 6C). In parallel to aorta, TRF2 protein levels were increased in the spleen-derived MNCs of exercised B6.129S wild-type animals (1.7-fold) but not in trained TERT knockout animals (Figure 6D). The exercise-induced downregulation of p53 was abolished in MNCs of TERT-deficient mice (Figure 6E). These data suggest that physical training positively influences telomere biology to similar degrees in MNCs and the vascular wall.

Comparison of Telomere Biology in Athletes With Untrained Individuals

To evaluate the effects of physical activity on telomere biology in humans, we compared young professional athletes from track and field disciplines and middle-aged athletes with a history of continuous intense endurance exercise since their youth with untrained control subjects of similar ages. All study participants were healthy nonsmokers. The fitness level of the athletes was superior; they were characterized by a lower resting heart rate, lower blood pressure, lower body mass index, and more favorable lipid profile.

Western blot analysis of circulating MNCs revealed an upregulation of TRF2 protein in young (1.8-fold) and middle-aged (1.7-fold) athletes compared with the untrained control groups (Figure 7A). TRF2 mRNA was upregulated in the young athletes (1.7-fold) and the middle-aged athletes (2.2-fold) (Figure 7B). Chk2 mRNA was downregulated in both the young and middle-aged athletes to less than half that of the controls and did not significantly differ between the age groups (Figure 7C). p53 Protein was not influenced by sports in the MNCs of young subjects, but its expression was upregulated in the untrained middle-aged individuals by 2.1-fold (Figure 7D). Interestingly, this upregulation was not observed in MNCs from middle-aged athletes. In young individuals, training did not change the expression of p16 or Ku proteins. However, in older individuals, training was associated with a marked downregulation of p16 and an increase in Ku 70 and 80 mRNA (Figure 7E through 7G).

Long-Term Exercise Training Activates Telomerase and Attenuates Telomere Attrition in Human Leukocytes

Exercise was associated with a marked increase in telomerase activity in the MNCs of both the young (2.5-fold) and middle-aged (1.8-fold; Figure 8A) athletes. Telomere length was measured in blood leukocytes cells through the use of 2 independent protocols. The Flow-FISH method allows differentiation between granulocytes and lymphocytes in the scatterplot (Figure 8C and Figure II of the online-only Data Supplement). In lymphocytes and granulocytes, telomere length was not different between professional athletes and untrained young subjects. However, older untrained individuals exhibited shorter MNC telomeres ($P<0.001$ versus all other groups; Figure 8B). This age-dependent telomere loss was attenuated in lymphocytes ($P<0.001$; Figure 8B) and granulocytes ($P<0.001$; Figure 8D) from individuals who had performed endurance exercise for several decades.

Table. Baseline Characteristics of Young Athletes, Middle-Aged Athletes, and Control Subjects Without Regular Physical Training

	Young Control Subjects	Young Athletes	*P*	Young Controls	Young Athletes	*P*
n	26	32		21	25	
Gender, M/F	15/11	25/7		14/7	19/6	
Age, y	21.8 (2.8)	20.4 (3.3)	0.097	50.9 (7.6)	51.1 (7.8)	0.953
Body mass index, kg/m^2	22.1 (3.3)	20.3 (1.3)	0.010	27.0 (3.6)	22.8 (2.4)	<0.001
Systolic BP, mm Hg	125.8 (6.8)	117.6 (10.6)	0.147	135.8 (21.1)	127.5 (17.0)	0.162
Diastolic BP, mm Hg	72.5 (9.6)	65.2 (7.5)	0.081	87.5 (7.6)	79.8 (17.7)	0.310
Heart rate, bpm	82.8 (5.1)	59.5 (8.9)	<0.001	78.5 (5.7)	51.8 (6.5)	<0.001
Training duration, h/wk	0.4 (0.4)	13.9 (4.1)	<0.001	0.4 (0.49)	9.6 (3.3)	<0.001
Training load, km/wk	NA	72.9 (27.1)		NA	80.5 (15.7)	*0.06
Maximum workload, W	206 (83)	340 (56)	<0.001	203 (45)	271 (71)	0.032
Relative maximum workload, W/kg	2.8 (1.1)	5.2 (0.8)	<0.001	2.2 (0.3)	3.8 (0.7)	<0.001
Fasting glucose, mg/dL	89.6 (12.1)	82.5 (11.8)	0.087	90.9 (14.3)	87.5 (10.0)	0.214
Total cholesterol, mg/dL	188.3 (43.5)	170.4 (28.8)	0.041	225.2 (36.6)	197.2 (29.5)	<0.001
HDL cholesterol, mg/dL	56.2 (16.4)	64.3 (20.2)	0.138	60.6 (13.7)	65.9 (20.0)	0.185
LDL cholesterol, mg/dL	115.3 (36.2)	87.6 (30.7)	0.005	143.0 (29.4)	108.2 (25.9)	<0.001
Triglycerides, mg/dL	112.8 (48.6)	78.8 (27.1)	0.005	140.3 (97.3)	82.9 (39.1)	<0.001

BP indicates blood pressure; HDL, high-density lipoprotein; and LDL, low-density lipoprotein. Data are presented as mean (SD). Training load (running distance [km/wk]) was not recordable in the control groups because <1 h/wk was spent on sports activities (mostly other than running).

*Young versus aged athletes.

To confirm these results, a real-time PCR method for measuring telomere length was used (online-only Data Supplement)[4] and showed a reduction in leukocyte telomere length in middle-aged control subjects ($P=0.006$ versus young control subjects). This telomere erosion was reduced in middle-aged athletes (Figure 8E).

Discussion

Our animal data show that physical exercise upregulates telomere-stabilizing proteins in the vascular wall and in MNCs. The underlying mechanism is the increase in endothelial NO, which synergizes with activation of telomerase to protect against cellular senescent and apoptotic signaling events. In mice, these effects are observed after only 3 weeks of voluntary running. Circulating leukocytes of track and field athletes show similar changes in telomerase activity, telomere-stabilizing proteins, and senescence markers compared with untrained individuals.

Improvement in endothelial NO availability is central to the vascular protection observed in the mouse model of voluntary running by mediating antioxidant effects and increasing circulating endothelial progenitor cells.[9] These data are now extended by the observed effects on the regulation of telomere proteins. One of the key findings of the present study is the marked upregulation of aortic and MNC telomerase activity. In accordance with this observation, protein expression of the catalytic subunit, the aortic TERT, is increased. Both telomerase and TERT have been shown to regulate endothelial cell growth and survival, and they act as antiapoptotic factors.[10] Defects in mice lacking the RNA component of telomerase involve apoptosis, and telomerase directly protects cells against programmed cell death.[2,11,12] As a consequence, these mice show a hypertensive phenotype.[13] In addition to telomerase, exercise upregulated the expression of TRF1 and TRF2. Interestingly, TRF2 has been suggested to serve as a binding platform for additional telomere-associated proteins, mediating signal transduction to DNA damage checkpoint controls.[3,14,15] TRF2 mediates proapoptotic signaling in cardiomyocytes and was shown to signal independently of telomere length in endothelial progenitor cells.[15,16] In progenitor cells, TRF2 was identified as a regulator of clonogenic potential and migratory capacity.[16,17] In agreement with the literature, it seems likely that TRF2 serves as a regulator of cellular aging and function beyond and potentially independently of protecting telomere length.[10–12]

The regulation of aortic telomere regulating proteins by exercise was paralleled by an inhibition of the expression of the DNA damage checkpoint kinase, Chk2, and the regulators of cell-cycle progression and survival, p16 and p53. The data agree with reports suggesting a role of these transformation-related proteins downstream of the telomere complex.[11,14,15,18,19] Our data identify voluntary running as a novel and potent inhibitor of Chk2, p16, and p53 in the vessel wall. To test whether the exercise-induced regulation of the telomere complex and the regulation of p53 represent a coincidence or may be causally related, the experiments were repeated in TERT-deficient mice. Running induced a very similar regulation of the transformation-related proteins in TERT$^{+/+}$ B6.129S and C57/Bl6 mice, but the exercise-mediated effects on these survival proteins were absent in the TERT$^{-/-}$ mice.

Stress-induced endothelial cell apoptosis has been suggested to cause endothelial dysfunction and is linked to the

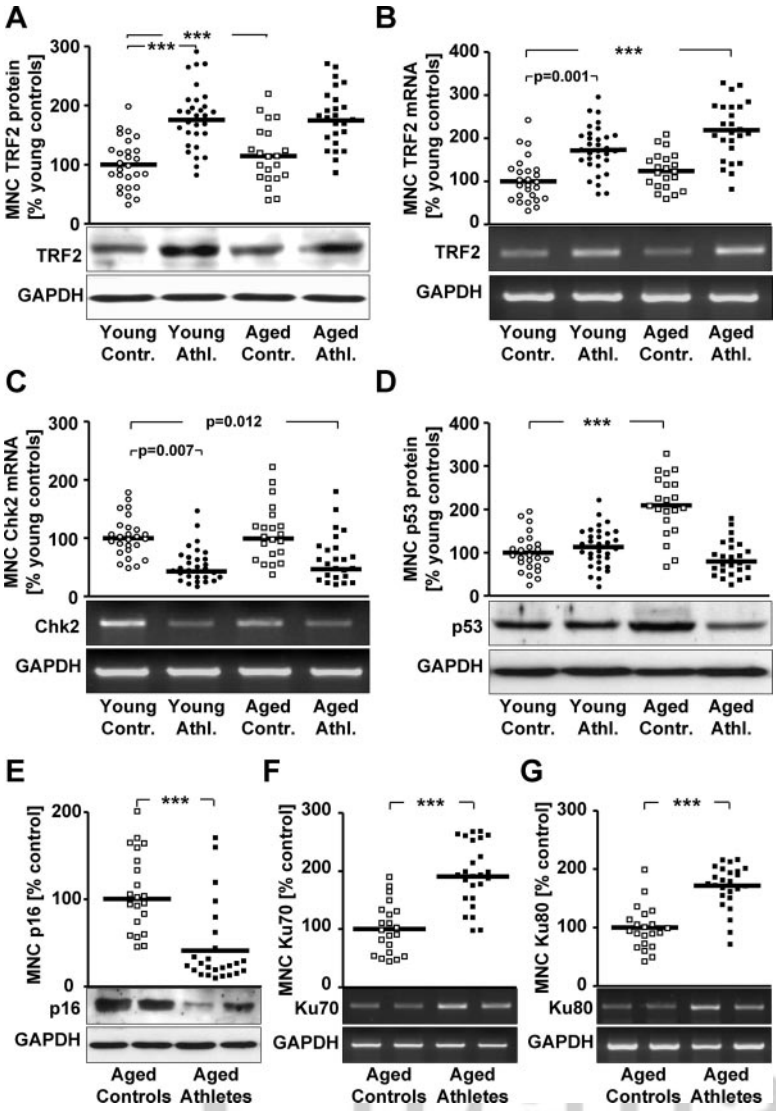

Figure 7. Telomere and cell-cycle regulation in athletes (Athl.) and control subjects (Contr.). Young (n=26; mean age, 21.9 years) and older (n=21; mean age, 50.9 years) untrained volunteers were compared with professional track and field athletes (n=32; mean age, 20.4 years) and older athletes (n=25; mean age, 51.1 years) with respect to telomere biology and cell-cycle regulators in circulating peripheral blood MNCs. Protein (A) and mRNA (B) expression of TRF2 and Chk2 (C), p53 protein (D) expression. Data are shown as scatterplots, with bars representing the means. p16 Protein (E), Ku70 (F), and Ku80 (G) mRNA expression of the middle-aged individuals. ***$P<0.001$.

upregulation of p16- and p53-dependent signaling pathways.[15,20] In cultured endothelial cells, TERT gene transfer reduces replicative senescence and apoptosis.[10,21] We therefore tested whether the observed upregulation of telomere-protecting proteins would be meaningful by protecting cellular survival in the presence of vascular injury. Two separate sets of experiments applying the bacterial endotoxin lipopolysaccharide and the herbicide paraquat, which induce endothelial cell apoptosis in vivo, revealed that running mice were potently protected from endothelial cell death. This protection may be mediated in part by NO-dependent telomerase activation during lipopolysaccharide stress in the mitochondria. Of note, the only intervention to achieve this marked effect was supplying mice with a running wheel in their cages for only 3 weeks before their intoxication.

One of the hallmarks of signaling induced by exercising is the increased bioavailability of endothelial NO.[22] Reduced NO bioavailability has been proposed as a major component of the endothelial aging process.[21] Recent evidence shows that active eNOS regulates TERT expression in the endothelium.[23] Our experiments show that the observed protective effects were completely absent in eNOS$^{-/-}$ animals. Functional assays showed a normal endothelium-dependent vasodilation and upregulation of phospho-eNOS in TERT$^{-/-}$ mice; however, they exhibit reduced protection against endothelial apoptosis by exercising. These data prove the importance of telomere proteins for exercise-mediated endothelial survival benefits. Because the protection by exercise in TERT$^{-/-}$ was incomplete but fully absent in eNOS$^{-/-}$ mice, it seems likely that eNOS exerts additional beneficial effects in this situation independently of TERT regulation, eg, benefits related to antioxidant effects.[9,21,22] The data are consistent with the concept that exercise-dependent improved vascular stress resistance reduces the need for reparative turnover and thus telomere attrition. Taken together, the data demonstrate the crucial role of eNOS for the exercise-mediated protection against endothelial apoptosis upstream of telomere-regulating proteins.

Relatively little is known about the physiological development of telomere length in healthy untreated mice over time.[2,15] After 6 months of exercise, telomere length in the aorta, leukocytes, and myocardium was not changed.[8] A

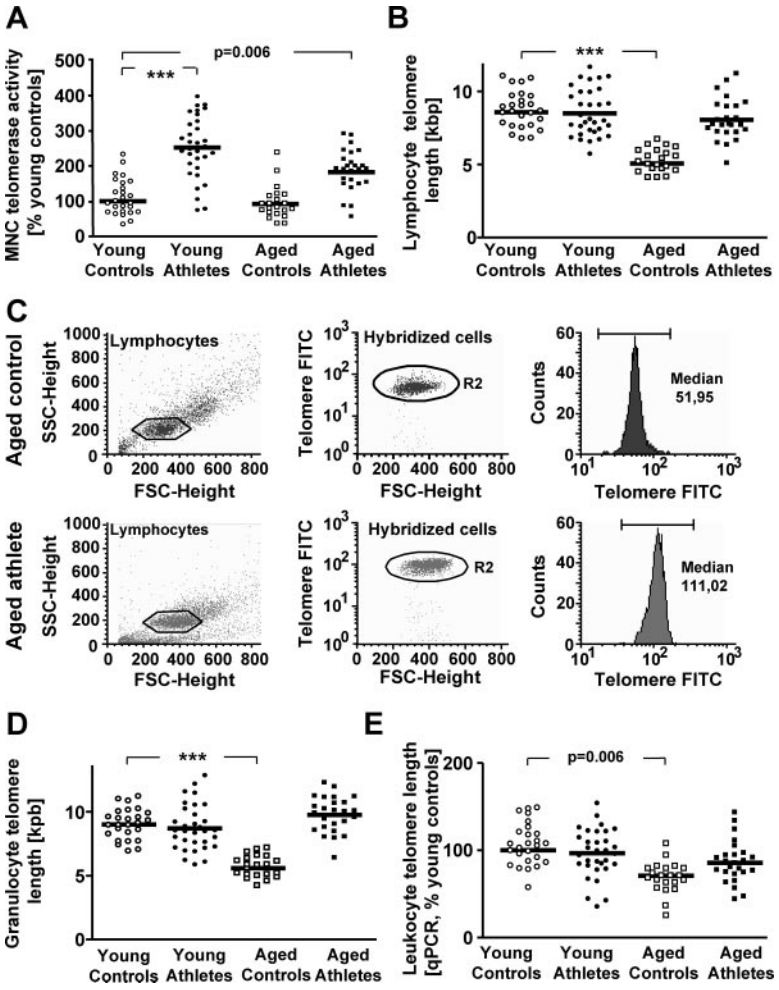

Figure 8. Long-term exercise training activates MNC telomerase and prevents telomere attrition. Telomere biology in young (n=26) and older (n=21) untrained volunteers vs young (n=32) and older (n=25) athletes. A, Human telomerase activity measured by telomere repeat amplification protocol. B, Lymphocyte telomere length measured by Flow-FISH assays. C, Representative fluorescence-activated cell sorter scan for an individual from the older control group (top row) and older athlete group (bottom row). Left column, Scatterplot with lymphocyte gate; middle column, cells gated by FSC vs intensity of telomere staining (FITC channel); right column, histogram analysis of telomere FITC intensity vs median intensity. D, Granulocyte telomere length measured by Flow-FISH assays. E, MNC telomere length determined by real-time PCR. FSC indicates forward scatter; SSC, sideways scatter. ***$P<0.001$.

control group of 18-month-old C57/Bl6 mice exhibited significantly shorter telomeres. Rodents have large telomeres, a fact that impedes the measurement of this parameter. Nevertheless, the regulation of telomerase activity, telomere-regulating factors, and downstream survival signaling may be independent of telomere length.

The functional capacity of bone marrow–derived MNCs and endothelial progenitor cells depends on age-related changes.[24–26] Thus, the functional improvement seen in murine endothelial progenitor cells after running wheel exercise[9] may be linked to changes in telomere biology. Our experiments show a robust regulation of telomerase activity, telomere-associated proteins, and senescence markers in leukocytes of running mice. The results were comparable in MNCs isolated from blood, bone marrow, and spleen, suggesting a systemic effect on these cells. Parameters of telomere biology in circulating MNCs are associated with the incidence and severity of cardiovascular disease in humans.[4–6] Recently, Ornish et al[27] showed a positive effect of lifestyle changes on MNC telomerase activity. Importantly, Wilson et al[7] reported that leukocyte telomere length correlates with aortic telomere length. Circulating MNCs from our young athletes and from middle-aged athletes with a long history of endurance exercise were characterized by a profound upregulation of telomerase activity and telomere proteins and downregulation of proapoptotic proteins compared with untrained individuals. In agreement with the animal data, this regulation occurred in the absence of a detectable change in telomere length in the young athletes. Recently, Cherkas and colleagues[28] reported a positive correlation between leukocyte telomere length and physical activity in 2401 twin volunteers. Our study population may have been too small and too young to detect subtle differences in telomere length, but the data show that beneficial antisenescent effects of physical activity are observed more rapidly than effects on telomere length itself. Indeed, long-term vigorous physical exercise in the older athletes is associated with a conservation of telomere length, as shown by the use of 2 independent methods of telomere length measurement, namely Flow-FISH assays and quantitative real-time PCRs. The published data on the effects of exercise on vascular telomere length in older subjects are limited. One study in a cohort of Chinese individuals >65 years of age reported no differences in leukocyte telomere length between physically active and inactive participants.[29] Another small study in 16 obese middle-aged women showed no differences in leukocyte telomere length after 6 months of aerobic exercise training.[30] However, these studies may have been limited by the use of questionnaires to detect physical activity or by the late onset of exercise. Presumably, the continuity, cumulative

duration, and intensity of endurance training were significantly higher in our study population.

Our study has limitations. We cannot rule out that the extent of telomere shortening in the older control group is due in part to an unknown selection bias. However, the key novel finding is the protection against telomere shortening by intensive exercise that is independent of the absolute telomere length of the control group. As expected, the athletes were characterized by a lower resting heart rate, blood pressure, and body mass index and a more favorable lipid profile. In the mouse studies, the regulation of telomere proteins was independent of blood pressure, heart rate, or lipid profile; however, in our clinical study, we cannot determine whether or to what extent the exercise-induced beneficial effects on metabolism, heart rate, and blood pressure affect the telomere proteins compared with exercising itself. It is conceivable that long-term exercise training has protected their cardiovascular system by increasing stress resistance and their maintenance systems in a way that vascular damage and subsequent reparative endothelial cell turnover over the years have been reduced and thus telomere attrition has been minimized. On the other hand, because mice lacking the RNA component of telomerase develop hypertension,[13] one could speculate that exercise-induced long-term upregulation of telomere-stabilizing proteins may exert direct beneficial vascular effects in humans.

Conclusions

The data identify voluntary physical exercise as a powerful intervention to upregulate telomere-stabilizing proteins in circulating cells and the vasculature, thereby protecting against endothelial apoptosis in mice. In agreement with the animal data, long-term continuous exercising leads to an attenuation of telomere erosion in the leukocytes of middle-aged athletes. Our data improve the molecular understanding of the vasculoprotective effects of exercise and underline the potency of physical training in reducing the impact of age-related diseases.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Telomere erosion is a central component of aging, and telomere-associated proteins regulate cellular senescence and survival. To elucidate the cellular mechanisms of the vasculoprotective effects of physical exercise, mice were randomized to voluntary running or no running wheel conditions. Exercise upregulated telomerase activity and telomere regulating proteins in the thoracic aorta and in circulating mononuclear cells compared with sedentary controls and reduced the expression of vascular apoptosis regulators such as cell-cycle–checkpoint kinase 2, p16, and p53. Mice preconditioned by voluntary running exhibited a marked reduction in lipopolysaccharide-induced endothelial apoptosis. Transgenic mouse studies showed that endothelial nitric oxide synthase and telomerase reverse transcriptase synergize to confer endothelial stress resistance after physical activity. To test the significance of these data in humans, telomere biology in circulating leukocytes of young and middle-aged track and field athletes was analyzed. Peripheral blood leukocytes isolated from endurance athletes showed increased telomerase activity, expression of telomere-stabilizing proteins, and downregulation of cell-cycle inhibitors compared with untrained individuals. Older athletes were characterized by reduced leukocyte telomere erosion compared with untrained controls. We therefore conclude that physical activity represents an “antiaging” intervention mediating cellular antisenescent and antiapoptotic vascular effects.