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. 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. 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.

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

Methods

Animals and Exercising
Eight-week-old male C57/B16 (Charles River Laboratories, Wilmington, Mass), endothelial nitric oxide (NO) synthase–deficient (eNOS−/−; B6.129P2-Nos3, Charles River) mice, TERT−/− (B6.129S-Terttm1Yjc/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 ± 760 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). 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.

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/B16 mice but induced a 2.9-fold increase in aortic telomerase activity as determined by the telomere repeat amplification protocol...
(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/B16 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 down-regulation 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.
Effects of Voluntary Exercise Are Mediated by eNOS

Increased availability of endothelial NO is a hallmark of physical exercise. 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 vasodilation 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; 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).
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 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.

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.
To confirm these results, a real-time PCR method for measuring telomere length was used (online-only Data Supplement) 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 protecting telomere-stabilizing proteins, and senescence markers compared with untrained individuals.

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. 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 C57Bl6 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 programmed cell death. As a consequence, these mice show a hypertensive phenotype. 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. In agreement with the literature, it seems likely that TRF2 serves as a regulator of cellular aging and function beyond independently of telomere length in endothelial progenitor cells. In progenitor cells, TRF2 was identified as a regulator of clonogenic potential and migratory capacity. In agreement with the data, it seems likely that TRF2 mediates proapoptotic signaling in cardiomyocytes and was shown to signal independently of telomere length in endothelial progenitor cells. 

| Table. Baseline Characteristics of Young Athletes, Middle-Aged Athletes, and Control Subjects Without Regular Physical Training |
|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | Young Control    | 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 (6.9)       | <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) | 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.
upregulation of p16- and p53-dependent signaling pathways.\textsuperscript{15,20} In cultured endothelial cells, TERT gene transfer reduces replicative senescence and apoptosis.\textsuperscript{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.\textsuperscript{22} Reduced NO bioavailability has been proposed as a major component of the endothelial aging process.\textsuperscript{21} Recent evidence shows that active eNOS regulates TERT expression in the endothelium.\textsuperscript{23} Our experiments show that the observed protective effects were completely absent in eNOS\textsuperscript{−/−} animals. Functional assays showed a normal endothelium-dependent vasodilation and upregulation of phospho-eNOS in TERT\textsuperscript{−/−} 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\textsuperscript{−/−} was incomplete but fully absent in eNOS\textsuperscript{−/−} mice, it seems likely that eNOS exerts additional beneficial effects in this situation independently of TERT regulation, eg, benefits related to antioxidant effects.\textsuperscript{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.\textsuperscript{2,15} After 6 months of exercise, telomere length in the aorta, leukocytes, and myocardium was not changed.\textsuperscript{8} A
The 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. Thus, the functional improvement seen in murine endothelial progenitor cells after running wheel exercise 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. Recently, Ornish et al. reported a positive effect of lifestyle changes on MNC telomerase activity. Importantly, Wilson et al. 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 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 anti-senescent 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. Another small study in 16 obese middle-aged women showed no differences in leukocyte telomere length after 6 months of aerobic exercise training. 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, 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.

References

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.
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SUPPLEMENTAL MATERIAL

A – Expanded Materials and Methods

Animals

Animal experiments were approved by the animal ethics committee of the Universität des Saarlandes and conformed with NIH Pub. No. 85-23, revised 1996.

Telomerase activity

Telomerase activity was quantified using the Telomerase Repeat Amplification Protocol.\(^1\)-\(^4\)

Protein extracts (\(1\mu\)g) of mouse thoracic aorta or 10,000 mononuclear cells in 1 x CHAPS lysis buffer (Trapeze, MP Biochemicals, Germany), 0.1\(\mu\)g of Primer TS (template), 0.05\(\mu\)g Primer ACX in 20\(\mu\)l Lightcycler Fast Start SYBR Green PCR Master Mix (Roche, Mannheim, Germany) containing 1.5mM MgCl\(_2\) were incubated at 30\(^\circ\)C for 30 minutes to allow template elongation by telomerase activity. After immediate transfer to the Lightcycler instrument (Roche, Mannheim, Germany), telomerase activity was terminated and hotstart DNA polymerase activated by incubation at 95\(^\circ\)C for 10 minutes. 40 cycles of amplification were carried out with 20s at 95\(^\circ\)C, 30s at 60\(^\circ\)C and 50s at 72\(^\circ\)C. Protein extracts from human embryonic kidney (HEK 293, Gibco, Karlsruhe, Germany) cells were measured as positive controls in each assay (intra-assay variability 5.2%). A standard titration curve of HEK 293 cells was established from 0 to 10,000 cells to ensure linearity of the assay (\(R^2=0.99\), Suppl. Fig. 3A). Furthermore, serial dilutions of aortic protein extracts, Suppl. Fig. 3B) and human MNCs (5,000 to 20,000 cells, Suppl. Fig. 3C) were tested to ensure linearity of the PCR reaction. A positive result of the telomerase assay was considered if the quantity of telomerase activity was 3 times above the standard deviation of the mean negative control’s background level (1 x CHAPS lysis buffer). To reduce the risk of systematic errors in the human study samples from each of the four experimental groups were present in each run.

Telomere length analysis by Flow-FISH assays

Telomere length was determined by the Flow-FISH method.\(^1\), \(^2\), \(^5\) In brief, 600,000 peripheral blood leukocytes from athletes and controls were washed once (5% dextrose, 0.1% BSA, 10mM HEPES) and resuspended in hybridisation buffer (75% deionised formamide, 20mM Tris (pH 7.1), 20mM NaCl, 1% BSA) for 10 minutes. After denaturing cells, 90 minutes incubation with
either no probe (unstained control) or 0.18\(\mu\)g FITC labelled, telomere-specific \((C_T A)_3\) DNA probe (Cambridge Research Biochemicals, Cleveland, UK) was performed. After 3 rounds of washing (75% deionised formamide, 0.1% BSA, 10 mM Tris, 0.1% Tween 20), cells were resuspended in PBS, 0.1% BSA, RNase A at 10\(\mu\)g/ml and 0.1\(\mu\)g/ml LDS 751 (Exciton, Ohio, USA) for DNA counterstaining. Proper gating and DNA counterstaining with LDS751 ensured that only single, mononuclear and euploidic cells were analyzed (Online Suppl. figure 2). Flow cytometric analysis was carried out on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Telomere length was expressed as mean fluorescent signal intensity. To control for interday variation, FITC-labelled beads (Quantum-FITC low level, Polysciences, Eppelheim, Germany) with defined amounts of fluorescence (MESF = molecules of equivalent soluble fluorochrome) were run in parallel each day. To further reduce the risk of systematic errors all study samples were measured on only two experimental days. In addition, a reference MNC sample isolated from a donor not included in the study was run in parallel for each experiment and yielded identical median fluorescence intensities in all runs. A previously established standard curve was used for the conversion of the median telomere fluorescence intensities into kilobase pairs (kbp).

**Histological telomere length analysis by quantitative fluorescence in-situ hybridization**

Telomere length is directly related to its integrated fluorescence intensity when marked with a specific telomere peptide nucleic acid (PNA) probe using quantitative fluorescence in-situ hybridization as described.\(^6\) 5\(\mu\)M cryosections of the thoracic aorta were fixed in 4% paraformaldehyde for 30 min at room temperature followed by treatment with 0.1% Pepsine solution (Sigma-Aldrich, Germany) for 10 min at 37°C. After washing and dehydration, 25\(\mu\)l of the hybridisation mix (70% formamide, 0.13\(\mu\)g Cy3-conjugated telomere-specific PNA probe \((C_T A)_3\) (Applied Biosystems, Germany), blocking reagent (Roche, Germany), MgCl2 buffer and 1mM Tris pH 7.2) was added to the sections. Denaturation was carried out at 80°C for 3 min and preceded incubation for 2 hours in a humid chamber and washing with wash buffer (70% formamide, 10mM Tris pH 7.2, 0.1% bovine serum albumin), tris-buffered saline (+ 1% Tween) and phosphate-buffered saline for a total of 1 hour. Nuclei were then counterstained with DAPI. All sections were analyzed using a Nikon E600 epifluorescence microscope (Nikon, Germany) by an investigator blinded to the study. At least 3 representative images of the Cy3 and DAPI channel from each section were recorded at 1000 X magnification with Lucia G software with
identical exposure times. Telomere fluorescence intensity was calculated with TFL-Telo freeware version 2.2.07.0418 – 2002 (URL: http://www.bccrc.ca/tfl/research_lansdorp/Applications.htm).

Measurement of telomere length by real-time PCR

Genomic DNA was extracted from the mononuclear cells of athletes and controls with the QIamp DNA blood mini kit (Qiagen, Germany) according to the manufacturer’s protocol. Mononuclear cell telomere length was measured with a real-time PCR-based assay that compares telomere repeat sequence copy number to the single-copy gene 36b4. During optimisation of the assays, a six-point standard curve (two-fold dilution from 40 ng - 1.25 ng) was run from a pool of 10 control DNAs for both the telomere and 36b4 PCRs to ensure linearity of the reaction ($R^2 > 0.99$). PCR efficiencies for target and reference gene were approximately equal (data not shown). In each run 20ng of sample DNA was measured in duplicate. The pooled control DNA (20ng) was tested in all assays to allow comparability of the results. The mastermix for each 25µl PCR reaction was prepared with 12.5 µl Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Germany), 0.5 µM/l ROX reference dye, 150 nmol/L of telomere-specific primers (Forward: CGGTTTGTGGGTGTTGTTGTTGTTGTTGTT; Reverse: GGGTTCCTTACCCTACCCCTACCCCTACCCT) or 100 nmol/L 36b4 primers (Forward: CAGCAAGTGGAAGGTGAAT; Reverse: CCCATTCTATCATCAACGGGTTACAA). All Real-Time PCR reactions were carried out on a 7900 HT Sequence Detector (Applied Biosystems, Germany). The thermal cycling profile for both reactions consisted of a 95°C activation step, followed by 40 cycles of 95°C for 15 seconds and 58°C for 60 seconds. The PCR data were analyzed as age- and gender-matched pairs with the comparative Ct method ($2^{-\Delta\Delta Ct}$) to calculate the relative differences in the amount of telomere repeat DNA between the athletes and their corresponding controls.

Western blot analysis

Aortic tissue from mice or mononuclear cells from mice and humans were homogenized with 200µl lysis buffer (100mM Tris pH 6.8, 4% SDS, 20 Glycerol) containing the protease inhibitors PMSF 0.1mM, leupeptin 0.5µl and Aprotonin 0.5µl. 50µg protein were separated on 10% SDS–PAGE. Proteins were transferred to nitrocellulose membranes (Biorad 162-0112), blocked with 5% dry milk for 30 minutes and exposed to rabbit polyclonal IgG TRF2 (H-300 Santa Cruz, sc-9143, dilution 1:200), mouse monoclonal IgG P16 (F-12 Santa Cruz, sc-1661, dilution 1:250),
rabbit polyclonal IgG anti-P53 (FL-393 Santa Cruz, sc-6243, dilution 1:500 in dry milk 1%), mouse monoclonal IgG Chk2 (A-11 Santa Cruz, sc-17747, dilution 1:1000 in dry milk 1%) and mouse monoclonal IgG GAPDH (6C5 Santa Cruz, sc-32233, dilution 1:1000), rabbit polyclonal IgG eNOS (Acris Antibodies, Germany) and mouse monoclonal IgG p-eNOS S1177 (BD1250 Acris Antibodies, Germany). For analysis of TERT, immunoprecipitation was performed on 150µg total protein in HNTG buffer (20mM HEPES, 150mM NaCl, 0.1% Triton-X-100, 10% Glycerol) using polyclonal IgG anti-TERT (H-231 Santa Cruz, sc-7212) and Agarose-A Protein goat anti-rabbit IgG (Sigma, Germany). Immunodetection was accomplished using goat anti-rabbit IgG (1:4000 dilution) (Sigma, Germany) and goat anti-mouse IgG (1:5000 dilution) (Biorad, Germany) secondary antibodies, and an enhanced chemiluminescence kit (ECL, Amersham).

cGMP Measurement by ELISA
For cGMP measurements 2 mm² pieces from murine aortas were frozen in liquid nitrogen and homogenized. The obtained powder was dissolved in 5% trichloroacetic acid. After centrifugation, trichloroacetic acid was removed from the supernatant by ether. Equal amounts of the supernatant were used in a commercially available cGMP ELISA according to the manufacturer’s instructions (IBL Immuno-Biological Laboratories, Hamburg), and cGMP levels were obtained using a standard included in the assay.

Aortic Ring Preparations and Tension Recording
After excision, the descending thoracic aorta was immersed in Tyrode's solution (118 mM NaCl, 2.5 mM CaCl2, 4.73 mM KCl, 1.2 mM MgCl2, 1.2 mM KH2PO4, 2.5 mM NaHCO3, 0.026 mM Na EDTA, and 5.5 mM D-(+)-glucose, pH 7.4). Adventitial tissue was carefully removed. Per animal, four 3mm rings were mounted in organ bath chambers filled with Tyrode's solution (37°C, continuously aerated with 95% O2 and 5% CO2) and were attached to a force transducer recording isometric tension. Aortic rings were gradually stretched to a resting tension of 10 mN, which was maintained throughout the experiment, and were allowed to equilibrate for further 30 min.. Pharmacologically induced contraction of aortic rings was performed with a β-agonist, Phenylephrine-HCl (5 µM). Drugs were added in increasing concentrations to obtain cumulative concentration-response curves for carbachol (carbamylcholine-chloride, 1 nM–100 µM) as an endothelium-dependent relaxing agent, and glycercyl trinitrate (1 nM–10 µM) as an NO donor.
The drugs were washed out before adding the next substance. The relaxing effect of carbachol was abolished by adding N-nitro-L-arginine methyl ester (1 µM).

**RT-PCR**

Reverse transcription-polymerase chain reaction standardized to GAPDH was performed using the following primers (designed to match mouse cDNA unless otherwise specified):

- **TRF1**-for 5´-CAT GGA CTA CAC AGA CTT AC-3´  55°C, 27 cycles
- **TRF1**-rev 5´-ATC TGG CCT ATC CTT AGA CG-3´
- **TRF2**-for 5´- TGT CTG TCG CGC ATT GAA GA-3´  55 °C, 26 cycles
- **TRF2**-rev 5´-GCT GGA AGA CCT CAT AGG AA-3´
- **TRF2-human**-for 5´-TGT CTG TCG CGG ATT GAA GA-3´  53°C, 35 cycles
- **TRF2-human**-rev 5´-GCT GGA AGG TCT CAT ATG AA -3´
- **Ku70**-for 5´-GAG CAT CCA GTG TAT CCA GA-3´  55 °C, 26 cycles
- **Ku70**-rev 5´-CAG CAT GAT CCT CTG TG-3´
- **Ku80**-for 5´-TCA CAG TGC ACA GAC ATC TG-3´  56 °C, 26 cycles
- **Ku80**-rev 5´-AAC TGC AGA GAG ATG TCA CA-3´
- **Ku70-human**-for 5´-GAG CAT CCA GTG TAT CCA AA -3´  52 °C, 35 cycles
- **Ku70-human**-rev 5´-CAG CAT GAT CCT CTG TTG ACT AC-3´
- **Ku80-human**-for 5´-TCA CAG TGC ACA GAC ATC TG -3´  56 °C, 40 cycles
- **Ku80-human**-rev 5´-AAT TGC AGG GAG ATG TCA CA-3´
- **Chk2-human**-for 5´-GCA GCA GTG CCTGT T CAC A-3´  55°C, 35 cycles
- **Chk2-human**-rev 5´-TGG ATA TGC CCT GGG ACT GT-3´
- **GADPH**-for 5´-ACC ACA GTC CAT GCC ATC AC-3´  60°C, 30 cycles
- **GADPH**-rev 5´-TCC ACC ACC CTG TTG CTG TA-3´

**Quantification of apoptosis by hairpin oligonucleotide assay**

To detect apoptosis, 3 µm thick paraffin sections of formalin-fixed murine thoracic aortae were stained using the ApopTag Peroxidase In Situ Oligo Ligation Kit (Millipore). This in-situ oligoligation assay specifically detects apoptosis by staining only cells that contain double-stranded breaks that are blunt-ended or have a one base 3’ overhang (apoptosis). Cells containing nicked, gapped, 3’-recessed, 3’-overhanging ends longer than one base and single-stranded ends are not detected. Thus, unlike conventional terminal transferase-based labelling (TUNEL), the hairpin assay specifically stains apoptotic but not necrotic or transiently damaged cells.
References for Materials and Methods


B – Supplementary figures

Supplementary Figure 1 –

No effects of exercise on aortic telomere biology and apoptosis regulators in eNOS\(^{-/-}\) mice

**Suppl. Figure 1**
Quantification of the aortic effects of voluntary exercise for 3 weeks in B6.129/P2-Nos3 mice on (A) TRF1 mRNA (p=0.377), (B) Ku70 mRNA (p=0.774), (C) Chk2 (p=0.711) and (D) p16 protein (p=0.748) expression. Standardization for GAPDH. N=8 per group.
Supplementary figure 2 – Standard gating procedure for Flow-FISH assays

A - FACS gates for the fluorescent reference beads

B - FACS gates for the negative control (no telomere probe)

C - All FACS gates for a representative aged control (lymphocytes)

D - All FACS gates for a representative aged athlete (lymphocytes)

Suppl. figure 2

(A) Beads: Microbeads of defined size and fluorescence are depicted in scattergraphs. Median intensity values are measured for each bead (M1-M5) and settings are adjusted to allow comparable measurements between runs. Bead fluorescence intensity correlates with molecules of soluble fluorochrome (MESF, $R^2>0.99$); (B) Neg. controls: MNCs with LDS751 but no PNA probe are present in each run; lymphocytes / granulocytes are gated, marked by a weak FITC autofluorescence and DNA staining. The histogram shows a minor FL1-intensity below 10 arbitrary units. (C and D) Gating for an aged control and an aged athlete: R1 – lymphocytes / granulocytes; R2 – cells with DNA stain are gated; R3 – cells with high LDS fluorescence are excluded (cell clusters); R4 – LDS751-stained cells with sufficient PNA hybridization are selected. Analysis histograms show mean fluorescence intensity values of cells in R1-R4 (ranging between 40 and 120 arbitrary units).
Supplementary figure 3 – Standard curves for Telomere-Repeat Amplification Protocols

Suppl. figure 3

Telomerase activity was measured by TRAP assay in serial dilutions of (A) 0-10,000 HEK 293 cells, (B) 0.31-2.5µg aortic CHAPS protein lysates and (C) 1,000-20,000 human MNCs. Correlation coefficients (R$^2$) were > 0.99 for all experiments.