Antioxidants Inhibit Nuclear Export of Telomerase Reverse Transcriptase and Delay Replicative Senescence of Endothelial Cells

Judith Haendeler, Jörg Hoffmann, J. Florian Diehl, Mariuca Vasa, Ioakim Spyridopoulos, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—Aging is associated with a rise in intracellular reactive oxygen species (ROS) and a loss of telomerase reverse transcriptase activity. Incubation with \( \text{H}_2\text{O}_2 \) induced the nuclear export of telomerase reverse transcriptase (TERT) into the cytosol in a Src-family kinase–dependent manner. Therefore, we investigated the hypothesis that age-related increase in reactive oxygen species (ROS) may induce the nuclear export of TERT and contribute to endothelial cell senescence. Continuous cultivation of endothelial cells resulted in an increased endogenous formation of ROS starting after 29 population doublings (PDL). This increase was accompanied by mitochondrial DNA damage and preceded the onset of replicative senescence at PDL 37. Along with the enhanced formation of ROS, we detected an export of nuclear TERT protein from the nucleus into the cytoplasm and an activation of the Src-kinase. Moreover, the induction of premature senescence by low concentrations of \( \text{H}_2\text{O}_2 \) was completely blocked with the Src-family kinase inhibitor PP2, suggesting a crucial role for Src-family kinases in the induction of endothelial cell aging. Incubation with the antioxidant \( \text{N}^-\text{acetylcysteine} \), from PDL 26, reduced the intracellular ROS formation and prevented mitochondrial DNA damage. Likewise, nuclear export of TERT protein, loss in the overall TERT activity, and the onset of replicative senescence were delayed by incubation with \( \text{N}^-\text{acetylcysteine} \). Low doses of the statin, atorvastatin (0.1 \( \mu \text{mol/L} \)), had also effects similar to those of \( \text{N}^-\text{acetylcysteine} \). We conclude that both antioxidants and statins can delay the onset of replicative senescence by counteracting the increased ROS production linked to aging of endothelial cells. (Circ Res. 2004;94:768-775.)

Key Words: aging ■ reactive oxygen species ■ Src-family kinases ■ statins ■ TERT

Accumulating evidence suggests that telomeres and telomerase play a role in cellular senescence in vitro and in vivo.\(^1,2\) Telomeres, the physical ends of the chromosomes, are involved in control of chromosome stability, genetic integrity, and cell viability in a variety of different species.\(^3,4\) Primary mammalian cells have a finite life span in tissue culture. It has been implied that the induction of senescence in cultured cells could result from two sources of signals. Cell culturing can induce stress signals initiated by a “culture shock,” which results in the expression of different cell cycle inhibitors leading to a senescent phenotype. Secondly, intrinsic signals can be induced by a critical telomere shortening, which is a consequence of telomere length reduction during each cell division ("mitotic clock"), or by a disturbance of the telomere-associated proteins (telomere “uncapping”).\(^5\) Telomere shortening forces human primary cells including endothelial cells to stop dividing, when a critical minimum telomere length is reached.\(^2,6\) The enzyme telomerase, a ribonucleoprotein, counteracts the shortening of telomeres. Telomerase contains a catalytic subunit, the telomerase reverse transcriptase (TERT).\(^7\) Introduction of TERT into human cells extends both their lifespan and their telomeres to lengths typical of young cells.\(^8–10\) The regulation of TERT involves transcriptional and posttranscriptional mechanisms. Transcriptional regulation of TERT is predominantly implicated in the regulation of TERT activity in cancer cells.\(^11\) Different transcription factors can activate TERT expression.\(^12\) There is also growing evidence for posttranscriptional regulation of TERT. Thus, TERT activity can be posttranscriptionally regulated by the kinases c-Abl, PKC, ERK1/2, and Akt (see review\(^13\)). An additional regulatory mechanism is the import of TERT into the nucleus from the cytoplasm in T-cells and smooth muscle cells on stimulation with growth factors.\(^14,15\) Recently, we have demonstrated that increased generation of reactive oxygen species (ROS) stimulates export of TERT from the nucleus into the cytosol via the nuclear pores.\(^16\) However, the cellular circumstances, where transcriptional and posttranscriptional regulation of TERT takes place, are still not clear.
Reactive oxygen species (ROS) have been implicated in aging and numerous diseases. In aging, increased production of ROS may primarily derive from the NADPH oxidase activity and from the mitochondria. This is supported by observations that the life span of most organisms is roughly proportional to the rate of mitochondrial ROS generation. In line with these observations, mimetics of the antioxidant enzymes, superoxide dismutase and catalase, can extend worms life span. Nevertheless, a direct link between aged-induced ROS and the regulation of TERT during endothelial cell aging is still missing. In view of our previous findings that oxidants stimulate export of TERT from nucleus and that statins inhibit senescence of endothelial progenitor cells, we decided to investigate whether antioxidants and statins counteract the adverse effects of increased ROS production on the aging of endothelial cells in culture. Our data show that endothelial cell aging is linked to an increase in ROS formation which in turn affects TERT localization and activity. Preincubation of endothelial cells with low doses of N-acetylcysteine and atorvastatin significantly reduced ROS formation, and prevented TERT translocation and endothelial cell aging.

Materials and Methods

Cell Culture
Human endothelial cells (Clonetics, Cologne, Germany) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/mL), bovine serum albumin (10 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 μg/mL), epidermal growth factor (10 ng/mL), and 10% fetal calf serum until 15th passage as described. In brief, experiments were performed on multiple primary cultures. Starting from three different primary cultures (purchased from Clonetics), human endothelial cells were seeded at a cell number of 6×10^4 per 75 cm² flask and trypsinized on confluence (between 6 to 7 days). Population doublings (PDL) were calculated using the following formula: PDL = (log₁₀[F/F₀])/0.301 (F indicates number of cells of the end of the passage; F₀, number of cells when seeded). Endothelial cells were stained with von Willebrand factor to exclude dedifferentiation. After detachment with 0.2% trypsin, cells were pelleted by centrifugation. Cells were incubated with anti-von Willebrand factor (1:75; abcam, Germany) and with a Rhodamine RedX-conjugated secondary antibody (1:300, Molecular Probes) and analyzed by confocal laser scanning microscopy.

Detection of BRDU-Positive Cells
Living cells were incubated with BRDU-labeling reagent for 60 minutes at 37°C. Cells were trypsinized for 2 minutes, reaction was stopped with PBS containing 10% FCS, and cells were pelleted by centrifugation. Cells were incubated with anti-BrdU-FITC for 30 minutes and thereafter with 1 μg/mL propidium iodide. Analysis was performed using FACS.

Detection of Oxidative Stress
Living cells were incubated for dye uptake with 20 μmol/L 2’,7’-dichlorodihydrofluorescein diacetate (H,DCF-DA) for 30 minutes (Molecular Probes). Cells were trypsinized for 2 minutes, reaction was stopped with PBS containing 10% FCS, and cells were pelleted by centrifugation. Cells were resuspended in PBS and measured using FACS analysis.

Separation of Nuclear and Cytosolic Fractions
Nuclear and cytosolic fractions were separated using a commercially available kit according to the manufacturer’s protocol (Pierce) as described previously. In brief, cells were scraped off the dish in PBS and centrifuged at 800g for 5 minutes at 4°C. Purity of the fractions was assessed by immunoblotting with tubulin.

Long-Term PCR for Detection of Intact Mitochondrial DNA
Genomic DNA was isolated from cells using the DNeasy kit from Qiagen. For PCR analysis, 250 ng of genomic DNA and the following primers were used: sense, 5’-ATACCCATGGCGACACTTACTCTACTCTATT-3’ and anti-sense, 5’-CTAGAAGTTGGAACACCTAGGCTTTGATTAAGGC-3’; resulting in a PCR product of 6.3 kb of mitochondrial DNA. As a control for intact nuclear DNA, 180 bp of the GAPDH were amplified using 250 ng genomic DNA and the following primers: sense, 5’-GTGTCCCATCTGGGCAAACGTA-3’ and anti-sense, 5’-GGAGTGCTGGTGCCTGGTGTG-3’. The absolute number of blue cells in relation to the total number was determined out of 1000 cells.

Acidic β-Galactosidase (β-Gal) Staining
Cells were fixed for 10 minutes in 2% formaldehyde, 0.2% glutaraldehyde in PBS, and incubated for 18 hours at 37°C with fresh β-Gal staining solution as described previously. The absolute number of blue cells in relation to the total number was determined out of 1000 cells.

Results
Increase in ROS Formation and Loss of Intact Mitochondrial DNA Precede the Onset of Senescence in Endothelial Cells
Endothelial cell aging was studied by subjecting endothelial cells to subsequent passages until passage 14 as described previously. Dedifferentiation was excluded by von Willebrand factor staining [passage 14 (PDL 42): 94±9% von Willebrand factor–positive cells; passage 15 (PDL 44): 35±21% von Willebrand factor–positive cells]. In this experimental model, we investigate the age-dependent increase in ROS formation, mitochondrial dysfunction, and replicative senescence.

Therefore, ROS formation was measured by detection of H₂DCF-DA during continuous passaging of endothelial cells. In fact, formation of endogenous ROS was increased, starting from population doubling 29 (Figure 1A and data not shown). Recent studies implied that a substantial source for the
formation of ROS were dysfunctional mitochondria, which can result in loss of intact mitochondrial DNA by direct damage by ROS.25,26 Coinciding with the increased ROS formation in aged endothelial cells, the fraction of intact mitochondrial DNA was reduced by 60% at population doubling 35 compared with population doubling 10, using a semiquantitative PCR analysis as shown in Figure 1B. This loss in intact mitochondrial DNA was independent of the mitochondrial mass as determined by the MitoTracker nonyl acridine orange.27 As shown in Figure 1C, the mitochondrial mass did not decrease significantly before population doubling 35 (Figure 1C).

In order to assess the onset of replicative senescence, we first measured the proliferative capacity by BrdU incorporation. BrdU incorporation significantly declined at PDL 37 (Figure 1D). Likewise, at PDL 37 acidic β-galactosidase staining as a marker for senescence increased (Figure 1E). Senescence was additionally confirmed by measuring telomere lengths using FLOW FISH analysis, which showed shorter telomeres in aged endothelial cells (15% reduction in telomere length compared with young endothelial cells). Thus, the increase in ROS formation and the loss of intact mitochondrial DNA preceded the onset of replicative senescence of endothelial cells.

**Increase in ROS Formation Leads to Loss of Nuclear TERT Activity Before Reduction of Overall TERT Activity**

A variety of studies has demonstrated that the activity of the catalytic subunit of telomerase, the telomerase reverse transcriptase (TERT), is essential to prevent cells from entering senescence by elongation of telomeres.8,10 Recently, we have shown that on short-term stimulation of human embryonic kidney cells (HEK 293) with H2O2, TERT is translocated from the nucleus into the cytosol.24 To assess TERT distribution in aging endothelial cells, whole cell lysate as well as nuclear and cytosolic fractions were prepared from cultures between population doubling 26 and 37. Concomitantly with the significant increase in formation of ROS in endothelial cells, nuclear TERT activity was reduced between population doubling 29 and 32 (Figures 2A and 2B), whereas cytosolic TERT protein increased in cytosolic fractions (Figure 2C). Immunocytochemical studies confirmed these findings and also demonstrated a reduction of predominantly nuclear TERT staining at population doubling 32 (Figure 2D). In contrast, TERT activity and TERT protein was not altered in whole cell lysate between population doubling 29 and 32 (Figure 2A and data not shown), demonstrating that the
translocation of TERT from the nucleus into the cytosol preceded the downregulation of overall TERT activity (Figure 2A).

**Role of Src-Family Kinases in Aging Endothelial Cells**

ROS-induced nuclear export of TERT in HEK293 was mediated by Src-family kinase–dependent tyrosine phosphorylation. Moreover, it is well established that incubation with ROS leads to activation of Src-family kinases in different cell types. Therefore, we investigated whether the Src-family kinases are activated during endothelial cell aging. Src-activation was monitored by detection of phosphorylation of Tyr 416. Tyr 416 phosphorylation was significantly increased in PDL 29 (Figure 3A). Interestingly, the phosphorylation of the inhibitory site Tyr 527 within Src was also significantly declined starting at PDL 32 (Figure 3A).

In order to test the involvement of Src kinases in endothelial cell senescence, we established a model of premature senescence. For that purpose, we used H2O2 at a concentration of 50 μmol/L, which did not induce apoptosis, and incubated endothelial cells for 1 week. As demonstrated in Figure 3B, incubation with H2O2 significantly increased acidic β-galactosidase–positive cells. Interestingly, coincubation with the Src kinase inhibitor PP2 (500 nmol/L) completely blocked
Percentage of acidic ROS formation was measured with H2DCF-DA using FACS analysis added every second day (A through E). Endogenous ROS formation indeed causally contributes to TERT translocation, and subsequently, endothelial cell senescence of endothelial cells (Figure 4E).

Atorvastatin Delays Endothelial Cell Aging

Recently, it has been shown that statins can exert antioxidative effects by inhibiting p23 expression in smooth muscle cells, thereby inhibiting NADPH oxidase activity and ROS formation. To examine whether atorvastatin would modulate age-associated increase in ROS formation, export of nuclear TERT protein, and finally, the onset of replicative senescence, endothelial cells were incubated with 0.01 μmol/L atorvastatin starting from population doubling 26. Increase in ROS formation was significantly reduced by atorvastatin (Figure 5A). Incubation with atorvastatin also inhibited the loss of intact mitochondrial DNA (Figure 5B) and abrogated the reduction of nuclear and overall TERT activity and protein (Figures 5C and 5D and data not shown). Furthermore, incubation with atorvastatin delayed the onset of senescence of endothelial cells (Figure 5E).

Taken together, atorvastatin reduced age-induced ROS formation, TERT translocation, and subsequently, endothelial cell senescence.

Discussion

The present study demonstrates that aging of endothelial cells lead to an increase in ROS formation, which is in line with findings from Carlisle et al. Moreover, increase in ROS formation and loss of intact mitochondrial DNA occurs before the onset of replicative senescence of endothelial cells. ROS, such as the superoxide radical, H2O2, the hydroxyl radical, and possibly singlet oxygen, which are formed during aerobic metabolism, are generally viewed as important regulators of aging processes. ROS are generated from different sources in cells. Among them are the NADPH oxidase and the mitochondria. Both appear to play a role in the aging process. Whereas the sources for ROS in our model are not clear, the findings that mitochondrial DNA is damaged before the onset of senescence strongly suggest that mitochondrial generated ROS are involved. Because many of the proteins encoded by the mitochondrial genome are components of the respiratory chain, it is conceivable that ROS-induced mitochondrial DNA damage could lead to defects in respiratory enzyme activities.

Telomerase is capable to counteract the onset of cellular senescence. A variety of studies have investigated the role of telomeres and telomerase in cellular senescence. Recent studies have demonstrated that introduction of TERT into human vascular cells can extend their life span and preserve a younger phenotype, underlying the important role of telomerase and of telomere stabilization for longevity and functional activity of endothelial cells. Moreover, overexpression of TERT prevented downregulation of eNOS, improved functional activity of endothelial progenitor cells for vascular...
regeneration, and extended the life span of smooth muscle cells to engineer mechanically robust human vessels.\textsuperscript{32–34} By contrast, endothelial cells with senescence-associated phenotypes are found in regenerated porcine arteries and in human atherosclerotic plaques,\textsuperscript{1,35} which suggests that endothelial cell aging may contribute to atherogenesis.\textsuperscript{36} Although ROS are known to be elevated during aging, a direct link between increased ROS formation and regulation of telomerase during cell aging may contribute to atherogenesis.\textsuperscript{36} Although endogenous TERT levels are low in nontransformed cells, the onset of proliferation was shown to increase the expression and activity of TERT in primary human cells including endothelial cells.\textsuperscript{14,15,39,40} The reduction of nuclear TERT protein and activity by ROS in proliferating cells may lead to a loss of the capacity of cells to prolong telomeres, which may result in progressive telomere shortening and subsequent onset of replicative senescence. In line with this concept, senescent endothelial cells were found in regenerated areas after vascular injury, where proliferation of the endothelial cells is required.\textsuperscript{41} In addition to the maintenance of telomere length and proliferative capacity, TERT exerts telomere-length-independent effects.\textsuperscript{42} Catalytically active human telomerase mutants failed to increase the life span of human primary fibroblasts.\textsuperscript{43} Moreover, TERT prevents apoptosis and increases levels of growth factors.\textsuperscript{44–46} Interestingly, nuclear-targeted TERT enhanced its antiapoptotic activity in human embryonic kidney cells.\textsuperscript{16} A recent study supports the concept that TERT suppresses a nuclear signal that is essential for apoptosis induction.\textsuperscript{47} Additionally, subnuclear shuttling of TERT has been shown between the nucleolus and the nucleoli in primary cells. Moreover, transformation and DNA damage have different effects on the shuttling affecting the access of TERT to both telomeric and nontelomeric substrates, further underscoring the concept that TERT has functions independent of the telomeres.\textsuperscript{48} Taken together, the reduction of nuclear TERT during endothelial cell aging, demonstrated in the present study, may additionally contribute to the increased sensitivity of aged endothelial cells toward apoptotic stimuli.\textsuperscript{23,49}

3-Hydroxy-3-methylglutaryl HMG-CoA reductase inhibitors, or statins, are effective lipid lowering agents that are widely described to lower cholesterol levels in patients at risk for cardiovascular disease.\textsuperscript{50} A variety of experimental studies underscored that the beneficial effects of statins are not only due to an improved lipid profile but also due to pleiotropic effects.\textsuperscript{51} Recently, statins have been shown to exert antiinflammatory and antioxidative effects. Statins act as direct inhibitors of induction of myosin heavy chain II (MHC II) expression by interferon-\(\gamma\) (IFN-\(\gamma\)),\textsuperscript{52} which is a process dependent on a rise in intracellular ROS. Statins also reduced expression of the \(p22^{phox}\) subunit of the NADPH oxidase.
oxidase in smooth muscle cells leading to a reduction in intracellular ROS formation. Thus, these antioxidative effects might contribute to the observed statin-mediated reduction of ROS during endothelial cell aging. However, statins have also been shown to increase eNOS expression and Akt-dependent phosphorylation, thereby, enhancing NO synthesis. In addition, we demonstrated that exogenous NO donors delay endothelial cell senescence. Therefore, it is tempting to speculate that statins exert their effects on endothelial cell senescence via increasing NO bioavailability, which then may reduce ROS generation and subsequently prevent nuclear export of TERT. However, the role of NO in regulating TERT has not been elucidated yet and further experiments are needed to explore the mechanism. Interestingly, statins may use distinct pathways to prevent senescence in different cell types. Whereas in mature endothelial cells, statins reduce ROS formation, as seen in this study, endothelial progenitor cells were shown to be protected against premature senescence independently of ROS and eNOS activity.

Notably, the prolonged incubation of endothelial cells with atorvastatin exerts a concentration-dependent effect. Whereas lower concentrations of atorvastatin (≤0.1 μmol/L) delay the onset of senescence, higher concentrations increase intracellular ROS formation in endothelial cells (data not shown). This is in line with findings demonstrating that statins reduce ROS formation, as seen in this study, endothelial progenitor cells were shown to be protected against premature senescence independently of ROS and eNOS activity.

Taken together, the present data provide first evidence elucidating a ROS-dependent mechanism for the onset of endothelial cell senescence. Thereby, age-associated increase in ROS induces the nuclear export of TERT protein into the cytosol, which is followed by the onset of endothelial cell senescence. Moreover, our data suggest that statins prevent endothelial cell senescence possibly via interfering with the redox balance of endothelial cells.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (Ha 2868/2-1 and SFB 553 project B6). We would like to thank Christine Goy and Carmen Schön for excellent technical assistance.

References

32. Murasawa S, Lleuvadot J, Silver M, Isner JM, Llosado DW, Asahara T. Constitutive human telomerase reverse transcriptase expression enhances


45. Oh T, Taffet GE, Youker KA, Entman ML, Overbeek PA, Michael LH, Schneider MD. Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. *Proc Natl Acad Sci U S A.* 2001;98:10308–10313.


Antioxidants Inhibit Nuclear Export of Telomerase Reverse Transcriptase and Delay Replicative Senescence of Endothelial Cells
Judith Haendeler, Jörg Hoffmann, J. Florian Diehl, Mariuca Vasa, Ioakim Spyridopoulos, Andreas M. Zeiher and Stefanie Dimmeler

Circ Res. 2004;94:768-775; originally published online February 12, 2004;
doi: 10.1161/01.RES.0000121104.05977.F3

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/94/6/768

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/