Critical Role for Telomerase in the Mechanism of Flow Mediated Dilation in the Human Microcirculation

Andreas M. Beyer\textsuperscript{1,2}, Julie K. Freed\textsuperscript{1}, Matthew J. Durand\textsuperscript{1,4}, Michael Riedel\textsuperscript{1}, Karima Ait-Aissa\textsuperscript{1,2}, Paula Green\textsuperscript{3}, Joseph C. Hockenberry\textsuperscript{1}, R. Garret Morgan\textsuperscript{6}, Anthony J. Donato\textsuperscript{6}, Refael Peleg\textsuperscript{7}, Mario Gasparri\textsuperscript{8}, Chris K. Rokkas\textsuperscript{8}, Janine H. Santos\textsuperscript{5}, Esther Priel\textsuperscript{7}, David D. Gutterman\textsuperscript{1,2}.

\textsuperscript{1}Department of Medicine, Cardiovascular Center, Medical College of Wisconsin, Milwaukee WI; \textsuperscript{2}Department of Physiology, Medical College of Wisconsin, Milwaukee WI; \textsuperscript{3}Department of Anesthesiology, Medical College of Wisconsin, Milwaukee WI; \textsuperscript{4}Department of Physical Medicine and Rehabilitation, Medical College of Wisconsin, Milwaukee, WI; \textsuperscript{5}Departments of Pharmacology and Physiology, New Jersey Medical School of Rutgers, Newark, NJ; \textsuperscript{6}Department of Internal Medicine, University of Utah School of Medicine, Salt Lake City, Utah; \textsuperscript{7}Shraga Segal Departments of Immunology and Microbiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel, and; \textsuperscript{8}Departments of Surgery, Cardiothoracic Surgery, Medical College of Wisconsin, Milwaukee WI.

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Address correspondence to:
Dr. Andreas Beyer
Medical College of Wisconsin
Medicine
8701 Watertown Plank Rd., HRC H4160
Milwaukee, Wisconsin 53266
United States
Tel: 414-456-7514
Fax: 414-955-6565
abeyer@mcw.edu

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ABSTRACT

**Rationale:** Telomerase is a nuclear regulator of telomere elongation with recent reports suggesting a role in regulation of mitochondrial reactive oxygen species (mtROS). Since mtROS (H$_2$O$_2$) mediate flow-mediated dilation (FMD) in the human microcirculation.

**Objective:** We examined the hypothesis that telomerase activity modulates microvascular FMD and loss of telomerase activity contributes to the change of mediator from nitric oxide (NO) to mtH$_2$O$_2$ in patients with coronary artery disease (CAD).

**Methods and Results:** Human coronary and adipose arterioles were isolated for videomicroscopy. FMD was measured in vessels pre-treated with the telomerase inhibitor BIBR-1532 or vehicle. Statistical differences between groups were determined using a two-way ANOVA (n≥4; P<0.05). L-NAME (NO synthase inhibitor) abolished FMD in arterioles from subjects without CAD, while PEG-Catalase (H$_2$O$_2$ scavenger) had no effect. Following exposure to BIBR-1532, arterioles from non-CAD subjects maintained the magnitude of dilation but changed the mediator from NO to mtH$_2$O$_2$ (% max diameter at 100 cm H$_2$O: Vehicle 74.6±4.1, L-NAME 37.0±2.0*, PEG-Catalase 82.1±2.8; BIBR-1532 69.9±4.0, L-NAME 84.7±2.2, PEG-Catalase 36.5±6.9*). Conversely, treatment of microvessels from CAD patients with the telomerase activator AGS-499 converted the PEG-catalase-inhibitable dilation to one mediated by NO (% max diameter at 100 cm H$_2$O: Adipose, AGS 499 78.5±3.9; L-NAME 10.9±17.5*; Peg-Catalase 79.2±4.9). Endothelial-independent dilation was not altered with either treatment.

**Conclusion:** We have identified a novel role for telomerase in re-establishing a physiological mechanism of vasodilation in arterioles from subjects with CAD. These findings suggest a new target for reducing the oxidative milieu in the microvasculature of patients with CAD.

**Keywords:** Telomerase activity, mitochondria, coronary artery disease, flow mediated dilation, reactive oxygen species, microvascular dysfunction, vascular biology

**Nonstandard Abbreviations and Acronyms:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
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<tr>
<td>FMD</td>
<td>Flow mediated dilation</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HF</td>
<td>Human fibroblasts</td>
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<tr>
<td>mt</td>
<td>Mitochondrial</td>
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<tr>
<td>MitoPY1</td>
<td>Mito Peroxy Yellow 1</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nuc</td>
<td>Nuclear</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>TERT</td>
<td>Catalytic subunit of telomerase complex</td>
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INTRODUCTION

Telomerase is classically known as an enzyme that maintains telomere length in nuclear DNA. Telomerase activity is inversely related to endothelial senescence\(^1\), especially in atherosclerotic prone vessels\(^2\), and may attenuate premature CAD and myocardial infarction\(^3\). Increased telomerase activity can also protect against ROS-induced endothelial dysfunction\(^4\),\(^5\). Not all of the effects of telomerase can be easily explained by its nuclear actions.

Growing evidence suggests TERT, the catalytic subunit of telomerase, can reversibly translocate from the nucleus to organelles (including the mitochondria), in a dose- and time-dependent manner, in response to stressors such as H\(_2\)O\(_2\) or prolonged hypoxia\(^6\),\(^7\),\(^8\). Yet no physiological effects of this translocation have been described in connection with cardiovascular phenotypes.

Once translocated to the mitochondria, TERT can suppress mitochondrial ROS (mtROS) production\(^6\),\(^7\),\(^9\). TERT-overexpressing cells demonstrate lower mitochondrial superoxide (O\(_2\).\(^-\)) production\(^6\), while in conditions of reduced telomerase activity mtROS increases, contributing to cellular oxidative stress\(^6\),\(^10\). Whether this system is active in the vascular endothelium and whether it contributes to redox regulation of vasodilation is not known.

We have shown previously that in atrial and adipose vessels from human subjects without CAD, NO is the primary mediator of endothelium-dependent dilation in response to increased shear stress (flow mediated dilation; FMD)\(^11\),\(^12\). In CAD, FMD persists but is mediated almost exclusively by H\(_2\)O\(_2\) released from the mitochondria, independent of NOS\(^13\),\(^14\). Telomerase can modulate the cellular redox state by upregulation of mitochondrial antioxidant enzymes\(^15\) and by directly reducing mtROS production\(^16\), making it ideally suited for contributing to the switch from NO to mitochondrial-derived H\(_2\)O\(_2\) as the mediator of FMD in subjects with CAD.

Eitan et al. have recently described a specific activator of telomerase activity that reduces mtROS. The small molecule AGS 499 was confirmed to act in a telomerase-dependent manner since in telomerase-depleted cells (by shRNA or knockout) AGS 499 did not elicit protection against increased oxidative stress\(^17\),\(^18\). These studies also showed that AGS 499 does not act nonspecifically as an antioxidant but specifically increases extra-nuclear levels of TERT, including mitochondrial TERT\(^1\). AGS 499 is beneficial in a variety of disease conditions characterized by elevations in mtROS\(^17\),\(^18\). We hypothesized that a reduction in telomerase activity, insufficient to reduce telomere length, evokes mtROS production and reduces NO synthesis, recapitulating the phenotype observed in CAD where h2o2 mediates FMD. Conversely, in vessels from subjects with CAD and elevated mtROS, we tested whether short term telomerase activation could restore NO mediated dilation.
METHODS

Tissue acquisition and general protocol.
All protocols were approved by the Institutional Review Board of the Medical College of Wisconsin and Froedtert Hospital. Sections of human atrial and adipose (visceral and subcutaneous) tissue were obtained in de-identified fashion as otherwise discarded tissue at the time of surgery and placed in cold 4°C HEPES (NaCl 275 mM, KCl 7.99 mM, MgSO₄ 4.9 mM, CaCl₂·2H₂O 3.2 mM, KH₂PO₄ 2.35 mM, EDTA 0.07 mM, Glucose 12 mM, HEPES acid 20 mM) (adipose) or cardioplegic (atrial) buffer solution. Tissue from subjects without known cardiovascular risk factors or clinical diagnosis of CAD was used for “non-CAD” groups.

Cannulated artery preparation.
Coronary and adipose arterioles (50-200 μm inner diameter) were cleaned of fat and connective tissue and prepared for continuous measurements of diameter. Briefly, in an organ chamber both ends of the vessel were cannulated with glass micropipettes filled with PSS and pressurized (60 mmHg) for videomicroscopy of diameter change as we have described previously. Data are reported as diameter at a given pressure gradient. Pressure gradients of 5-100 cm H₂O were generated, assessing steady-state diameter and flow after each change, representing estimated shear rates of 5-25 dynes/cm². Two flow-response curves were generated for each vessel comparing no treatment (vehicle) to effects of pharmacological inhibitors (L-NAME [100 μM]; PEG-catalase [500 U/ml]; rotenone [1 μM]; 2-(4-Carboxyphenyl)-4,5-dihydro-4,5,5-tetramethyl-1H-imidazol-1-yl-oxo-3-oxide (c-PTIO) [1 μM]), Actinomycin D (1.59 µM) and MitoTempol (10 μM). In some cases vessels were incubated for 15-20 hours in endothelial cell growth medium containing 5% serum (Lonza) containing either vehicle (DMSO 2% by volume), BIBR-1532 (10 μM) or AGS 499 (20 nM). After incubation, media was replaced with Krebs buffer, intraluminal pressure was raised from 30 mmHg to 60 mmHg, and vessels were constricted with ET-1 for assessment of FMD.

All pharmacological agents were added to the external bathing solution (volume added was <1% of the circulating external bath solution). Concentrations are stated as final concentrations in organ bath. Vessels were constricted with ET-1 (0.1 – 1 nM) to achieve a 20%-50% stable reduction in passive diameter. Dose response curves to the endothelial-dependent vasodilator acetylcholine (Ach; 1 nM-100 μM) and the endothelial-independent vasodilator papaverine (0.1 nM – 100 μM) were performed to evaluate specific endothelial and smooth muscle-dependent dilation. At the end of each experiment papaverine (100 μM) was used to determine the maximal (passive) diameter at 60 mmHg.

Quantitative real-time PCR.
Total mRNA was harvested from Human Umbilical Vein Endothelial Cells (HUVECs) using an Ambion PureLink RNA Kit. Approximately 1500 ng of RNA was used to synthesize cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit. Gene expression was quantified by RT-qPCR using primers (Hs_TERT_1_SG QuantiTeet Primer Assay QT00073409) and SYBR green from Qiagen in a BioRad CFX96 Touch™ Real-Time PCR Detection System. Expression levels were normalized to 18S rRNA (Hs_RRN18S_1_SG QT00199367).

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Cell culture experiments.
HUVECs were freshly isolated from umbilical cords collected from local hospitals, processed by the hybridoma core facility of the Blood Research Institute of Wisconsin, and supplied at passage 3-5. Cells were exposed to AGS 499 for identical time and conditions as isolated vessels. For stressing cells in culture, heat shock treatment was used wherein culture dishes were sealed with Parafilm and immersed in a water bath at 42°C for 30 min. Subsequently, the cells were put into a 37°C incubator for 16h of recovery. Control cells were sealed for 30 min but remained in the incubator.

Normal human fibroblasts (HF) were cultured under standard conditions as described previously9. In brief, cells were transiently transfected with either whole cell (WC) TERT or R3E/R6E TERT (nucTERT). Oxidative stress was generated by 30 minutes of 200 µM H2O2 treatment. Purity of isolated mitochondria preparation was tested using the standard subcellular markers tubulin (cytoplasmic), mHSP70 and Ku80 (nuclear isoform [higher molecular weight] and mitochondrial isoform). Mitochondria were labeled with Mitotracker Red using manufacturer’s specifications.

Measurement of mitochondrial reactive oxygen species.
In vessels Mito Peroxy Yellow 1 (MitoPY1)22 was used to evaluate microvessel generation of mH2O2. As previously described12, following cannulation in a warmed chamber (37°C) containing HEPES buffer at pH 7.4, arterioles were perfused intraluminally with MitoPY1 (5 µM, 1hr) at low levels of flow, below the threshold for dilation, until the luminal surface was bathed in MitoPY1 containing buffer. Next the transvascular pressure gradient was changed from zero to 100 cm H2O and fluorescence measured after 5 min. Experiments were performed in the presence or absence of PEG-catalase (500 U/mL). Fluorescence was evaluated with a Nikon Eclipse TE200 microscope using a krypton/argon laser at excitation wavelength of 488 nm and measured emission between 530-590 nm. All comparisons were made using vessels studied at the same session with constant microscope image display settings. In cultured cells mitoSox was used to measure superoxide generation. Cells were incubated for 30 min with 10 µM mitoSox. Relative average fluorescence intensity was normalized to background fluorescence and presented as percent change from baseline.

Telomere length.
Genomic DNA from left ventricle tissue or isolated atrial arterioles was prepared by the MCW pathology tissue bank using Qiagen spin columns according to manufacturer’s specifications. PCR reaction was carried out under following conditions 0.75 SYBR Green I (Invitrogen), 10mM Tris–HCl pH8.3, 50mM KCl , 3mM MgCl2 , 0.2mM each dNTP, 1mM DTT and 1M betaine (U.S. Biochemicals). For 25 ml reaction 0.625U AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.) was used. Multiplex QPCR primer pair final concentrations: 900 nM each. Telomere primers telg; ACACATAAGGT TGTTGGGTTG GTTGGTTGGTTGGTTAGTG TGTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA human albumin primers albu; CCGCGCGGCGCGGGCGCGGGCGCTGGGCGGAAATGTCAGAAGATACTTG; albd: GCCCGGC CGCGGTCCCGGCGGCGGCGGCGGCGGAAATGTCAGAAGATACTTG. PCR reaction was carried out as follows Step 1 (1X): 95°C for 15 min Step 2 (36X): 98°C for 2s 48°C for 1min 74°C for 15s (data acquisition) 84°C for 30s 85°C for 15s (data acquisition) as previously described by Morgan et al.23

Materials.
The telomerase modulators BIBR-1532 (Tocris Bioscience) and AGS 499 (Ester Priel, PhD, Israel), and the mitochondrial ROS-detecting dyes MitoPY1 (Cayman chemicals) and mitoSox (life technologies) were prepared in DMSO. ET-1 (Peninsula Laboratories) was prepared in saline with 1% bovine serum albumin. All other chemicals were obtained from Sigma-Aldrich and prepared in distilled water or physiological salt solution. Other agents were prepared in distilled water or physiological salt solution. All concentrations represent the final concentrations in the organ bath.

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Statistical Methods.

Data are presented as mean ± SEM. For all flow response curves, differences between groups at each concentration were determined using a two-way, repeated-measures analysis of variance (ANOVA). A post hoc Tukey’s test was used for individual comparisons following ANOVA. A probability value of p<0.05 was considered to be statistically significant. For fluorescence studies, telomere length, Western blot or expression analysis, either a paired t-test or one-way ANOVA with post hoc Tukey’s test was used to identify individual differences within groups. Values outside of 2 standard deviations of the mean were considered statistical outliers and excluded from analysis.

RESULTS

Tissue was collected from a total of 58 subjects. Results are derived from arterioles of 36 patients without CAD and 18 patients with CAD. Detailed patient demographic information is shown in Table 1.

Effect on FMD of inhibiting telomerase.

After incubating atrial or adipose arterioles for 15-20 hours in the specific telomerase inhibitor BIBR-1532, the magnitude of FMD was not changed (Fig. 1A). However, the mediator of FMD shifted from NO to \( \text{H}_2\text{O}_2 \) (PEG-catalase inhibitable and L-NAME-insensitive) in both vessel types after treatment with BIBR1532 for 15-20h (Figure 1B + C). Endothelial-dependent dilation to \( \text{ACh} \) was also reduced after telomerase inhibition (Figure 1D). Maximal endothelium-independent dilation (assessed with papaverine) was not altered by BIBR-532 (Figure 1E).

Subjects with CAD have reduced cardiac and vascular TERT levels but normal telomere length.

To establish whether the difference in vasodilator mechanism between CAD and non-CAD subjects correlates with telomere length, we measured telomere length in heart and microvessels from individuals with and without CAD. No difference in telomere length was observed between vessels from patients with or those without CAD. TERT protein expression was significantly higher in non-CAD subjects vs. those with CAD (Figure 2), although CAD subjects tended to be older (66±4 vs. 48±7 years). Immunohistochemistry of coronary vessels from subjects with and without CAD revealed expression of TERT in both smooth muscle and endothelial cells (Online Figure I).

Role of mitochondria as the source of \( \text{H}_2\text{O}_2 \) and the site of TERT action in flow mediated dilation.

Using MitoPY1, a mitochondrial specific fluorescent indicator for hydrogen peroxide, we observed that for 15-20h BIBR-1532 treatment in vessels from subjects without CAD caused stimulated mitochondrial release of \( \text{H}_2\text{O}_2 \) in response to flow (Fig. 3 a+b). Addition of the complex I inhibitor rotenone abolished FMD in vessels treated with BIBR-1532 (atrial and adipose) but had no effect on vehicle-treated vessels (Figure 3). Scavenging mitochondrial \( \text{O}_2^- \) with the mitochondrial-specific antioxidant mitoTEMPOL (10 µM) reduced FMD in vessels treated with BIBR-1532 (Figure 3 c). Taken together, these data suggest that the source of ROS modulated by the telomerase inhibitor BIBR-1532 is from the mitochondrial electron transport chain.

Due to the small tissue volume of human arterioles, we were not able to quantify protein content. Therefore we sought direct evidence for TERT translocation to the mitochondria using cultured human cells transfected with TERT-eGFP-expression vectors as previously published9.
In cells expressing wild type (WT) TERT protein fused to eGFP nuclear localization of TERT was reduced following stress (application of H$_2$O$_2$). A corresponding increase in mitochondrial localization of TERT-GFP was seen by co-localization with the mitochondrial probe mitotracker red (Figure 4a).

To test whether the mitochondrial localization of TERT limits mtROS production, we used a mutant version of TERT (nucTERT) that we have previously shown to be functional (elongation of telomeres) but excluded from the mitochondria$^9$. Western blots confirmed the mitochondrial localization of the WT protein and the lack of mitochondrial localization of nucTERT (Figure 4b). Whole cell (WC) TERT levels were comparable among WT and nucTERT groups but only WT TERT is detected in highly purified mitochondria (Figure 4b). Generation of mtROS (mitoSOX) depended upon the subcellular localization of TERT, with nucTERT cells showing consistently higher mitoSox signal (Figure 4c). To evaluate the effects of TERT localization on the response to stress, wtTERT or nucTERT-expressing cells were plated for 16 hours then treated with 200 µM H$_2$O$_2$ for 60 min. Cells expressing nucTERT (aka absence of mtTERT) show a significant decrease in the steady state amount of ATP produced compared to WT (Figure 4d), indicating that absence of mtTERT has direct effects on mitochondrial function by impairing ATP production capacity following stress.

Effects of telomerase inhibition are independent of transcription.

It is possible that the switch in mechanism of FMD based on TERT modulation is transcriptionally-induced since TERT regulates gene expression (reviewed by Zhou et al$^{24}$). To test this possibility, the transcription inhibitor Actinomycin D (2 µg/mL$^{25}$ was used. Actinomycin D had no effect on the mediator of FMD in vessels from patients without CAD treated 15-20h with vehicle or with the telomerase inhibitor BIBR-1532 (Figure 5). Efficacy of the dose of Actinomycin D was demonstrated in endothelial cells treated for 2 hours with Actinomycin D or vehicle and exposed for 1 hour to heat shock (42°C). 15-20 hours later, cells in the vehicle group showed an increase in HSP 27 and 70 compared to unstressed cells. Pretreatment with Actinomycin D eliminated this effect (Figure 5 c + d). These data indicate that nuclear transcriptional properties of telomerase are not involved in the functional and redox changes observed in this study.

Activation of telomerase restores NOS-mediated FMD in vessels from subjects with CAD.

Adipose and atrial vessels from subjects with CAD were exposed to AGS 499 (15-20h; 22 nM), an activator of telomerase. AGS 499 restored the mediator of FMD from H$_2$O$_2$ to a NOS-derived product. Maximal dilation to flow (100 cm H$_2$O) in vessels from subjects with CAD treated with AGS 499 was of similar magnitude to vehicle treated vessels and was inhibited by L-NNAME but not by PEG-catalase (% max diameter at 100 cm H$_2$O in atrial vessels (n=3-4): Vehicle 64.5±8.7, L-NNAME 12.6±15.3*, PEG-catalase 65.2±7.8, c-PTIO 22.9±17.3*; adipose vessels (n=5-8): AGS 499 + Vehicle 79.5±3.9, L-NNAME 10.9±17.2*, PEG-catalase 79.2±4.9; *p<0.05 two way ANOVA RM) (Figure 6). Inhibition of telomerase activity in vessels from subjects with CAD had no effect on the magnitude or mechanism of dilation (Figure 6c). The dose of AGS 499 used was transcriptionally active since HUVECs treated with the same dose demonstrated a ~4 fold increase of total TERT mRNA (Figure 6d).

To establish the effect of telomerase on eNOS we used q-PCR and Western blotting. In HUVECs AGS 499 increased eNOS message while BIBR-1532 had no effect. (Online Figure II a). Levels of p-eNOS (ser 1117) increase after treatment with AGS 499 Online Figure II b. Similarly, Daf-2A (HPLC; supplemental methods) significantly increased with AGS 499 treatment in HUVECs while BIBR-1532 had no effect (Online Figure II c).
DISCUSSION

There are three primary novel findings of this study. First, we establish that inhibition of telomerase in vessels from subjects without CAD recapitulates the vascular phenotype seen in patients with CAD by converting the mediator of FMD from NO to H2O2. Activation of telomerase can restore a NOS-mediated FMD in vessels from patients with CAD. TERT protein levels are reduced in patients with CAD compared to those without CAD and mtROS production is elevated when telomerase activity is reduced (mitoPY1). Second, the intracellular site for these actions of telomerase is the mitochondria and not the nucleus as demonstrated with novel mutant telomerase constructs that target either the whole cell (wild type) or the nucleus alone (nucTERT). Third, the effects of telomerase inhibition are not dependent on changes in gene expression. Collectively these data for the first time provide evidence for an extra-nuclear role for telomerase in modulating vascular function. Figure 7 illustrates our findings.

An extranuclear role for telomerase suggests non-canonical sites of actions. Based on our subcellular localization experiments and prior studies in other cell types, we postulate an effect on mitochondria. The short timeframe of the observed changes (hours) supports such a telomere-independent mechanism. Our study provides a physiological role for the mitochondrial ROS-suppressing effects of TERT. Since TERT suppresses mtROS production in vascular endothelial cells, reduced TERT, as seen in CAD, would support mtROS production necessary for FMD in human arterioles. TERT as a regulator of mtROS is important in other diseases as well. Transcriptional activation of TERT via AGS 499 delayed the onset of amyotrophic lateral sclerosis in mice by increasing cell survival after oxidative stress. The same mechanism of action was shown in human bone marrow mesenchymal stem cells which were protected from oxidative stress by TERT.

The role of telomerase in chronic disease is multifactorial. Traditionally TERT protects tissues including the vasculature from chromosomal rearrangements by maintaining telomere length over time. For example, shortened telomeres and decreased telomerase activity are associated with atherosclerosis and activation of senescence pathways. Of recent it has been show that telomere uncapping without change in telomere length which can also invoke cellular aging and inflammation, or novel data show a role of TERT as a regulator of gene expression by modulating chromatin. In addition some of the pathological effects of reduced telomerase related to loss of telomerase outside the nucleus, including the increase of mitochondria free radical formation have been established. mtROS production changes inversely with expression of TERT in the mitochondria, an action that involves extranuclear TERT.

As observed previously, the magnitude of FMD in human microvessels is maintained but the mechanism changes in response to stress while agonist induced dilation to ACh is simply lost. The reason for this difference is not known, but it has been speculated that ACh is but one of many agonists which can stimulate NO release, while shear stress-induced increases in NO involves multiple signaling pathways (i.e. ACh binds to muscarinic receptors to induce dilation while redundant pathways may exist for FMD).

We have not determined the precise mechanism by which reduced telomerase activity modulates mtROS. However this modulations independent of nuclear transcriptional events and from telomere elongation suggesting a novel non telomeric site of action. However we cannot exclude a role for telomere uncapping, a focus for future investigations.

In this study we used a physiological stimulus for endothelium-dependent dilation, shear stress. The mechano-chemical signal transduction during shear that leads to dilation has been studied extensively. Depending on the tissue and species, the signaling pathway involves glycocalyx, TRPV4 or other mechano-sensitive ion channels, and/or focal adhesions tethering endothelial cells to the underlying basement membrane by cytoskeletal elements. Secondary pathways include tyrosine kinases or calcium signaling
to activate eNOS and other vasoactive mediators. In human vessels from subjects with CAD, a different signaling pathway is invoked with stimulation of mitochondrial H2O2 production. The present study provides novel evidence that telomerase lies at the intersection of these pathways and regulates the switch between shear-induced mediators. We speculate that augmentation of telomerase activity may improve the coronary and peripheral vascular phenotypes in patients with CAD. However the potential adverse effects associated with increasing telomerase activity, including accelerated tumor growth, have to be carefully considered.

Both NO and ROS are signaling pathways for mitochondrial - nuclear communication (reviewed by 41, 42) and TERT contains both mitochondrial and nuclear targeting sequences9, 43-45. Site-specific phosphorylation of TERT initiates either mitochondrial or nuclear translocation. This puts telomerase in a position to serve as a critical mediator of nuclear-mitochondrial cross-talk, and implicates involvement of AKT, which phosphorylates TERT at its nuclear localization site, increasing binding affinity for the nuclear transporter importin-α, promoting translocation43. AKT also targets mitochondrial biogenesis, and NO signaling (reviewed by 46, 47). A critical role for non-nuclear telomerase activity in regulating this interplay represents a new field of study with implications for diseases in which mROS play a prominent role such as diabetes48, 49 and neurodegenerative17, 18 diseases and cardiovascular responses to chemotherapies such as doxorubicin and PK inhibitors50, 51.

It is well established that telomerase activity and telomere length decrease during the natural aging process. A parallel reduction in NO bioavailability is seen after the 4th decade of life, reducing vasodilator capacity52, 53 54, 55. The shift toward a more oxidative environment could explain the reduction in NO bioavailability and increase in ROS production observed with CAD and represents a common feature that contributes to the development of other disease phenotypes17, 18. The telomerase activator AGS 499, which normalized FMD and ROS production in microvessels from subjects with CAD, also delays onset of ALS in rodent models18. Thus the ability of TERT to regulate NO production could be important beyond vasodilation.

Compared to age matched controls, subjects with CAD show a similar degree of FMD. Importantly, the underlying mechanism of FMD changes from NO to H2O2. Inhibition of telomerase can initiate this “pathological” phenotype (H2O2-mediated FMD) in vessels from subjects without CAD in less than 24 hours. Conversely, in subjects with chronic CAD, stimulation of TERT activity is sufficient to subacutely restore NOS as critical for FMD, likely via generation of NO (L-NAME and c-PTIO inhibitable). Induced upregulation of telomerase activity may be beneficial in CAD since NO is vasoprotective, while H2O2 is pro-inflammatory in the vasculature56, 57. With growing evidence that the microcirculatory dynamics influence cardiovascular outcomes (reviewed by Lockhart et al.58), understanding mechanisms of arteriolar endothelium-dependent dilation is of both physiological and clinical importance.

We have previously demonstrated that in a matter of minutes, an acute increase in intraluminal pressure can evoke the same change in mediator of FMD following telomerase inhibition or as seen in CAD subjects35. Using ceramide as a physiological stressor, Freed et. al 12 showed a similar shift in vasodilator mechanism as we see with telomerase inhibition. Ceramide is known to suppress TERT expression59, thus it is intriguing to speculate that ceramide activation of mROS is TERT-dependent. Future studies will evaluate if ceramide and TERT are part of the same pathway modulating mitochondrial redox function.

**Study limitations.**

Our functional studies point toward a critical role of NOS and NO in arteriolar FMD under normal conditions. We cannot rule out a role of prostacyclin’s, EETs or other EDHFs, however our prior studies argue against a role of EETs, since the enzyme responsible for their production, CYP450 epoxygenase, is directly inhibited by H2O2 60. Although it is not technically feasible to directly assess NO bioavailability in
the small tissue samples used, we used DAF-FM as a surrogate marker for NO production in cultured human endothelial cells. We observed an elevation in eNOS message and p-eNOS (ser 1177) following activation of TERT with AGS 499. NO production showed a statistically significant increase in cultured HUVECs after treatment with AGS 499. Our combined data in cultured human endothelial cells and isolated vessels strongly suggests that NOS-derived NO is responsible for FMD in vessels from patients without CAD and in vessels from patients with CAD treated with AGS 499 15-20h (dilation changes form Peg-Catalase inhibitable to L-NAME and c-PTIO inhibitable). Future studies will define the underlying differences between the acute vs. chronic effects of telomerase deficiency in vivo and in isolated vessel studies.

We used primary human fibroblasts to provide proof of principle data for translocation of TERT in response to oxidative stress. Due to the fact that endothelial cells are difficult to use for transient transfections normal human fibroblasts where chosen as a surrogate. In addition fibroblasts have higher levels of mitochondrial number enabling us to perform ATP production assays, which are extremely difficult to perform in primary human endothelial cells.

We cannot eliminate a contribution of vascular smooth muscle cells (VSMC) to the observed phenotype. However we believe this is unlikely since inhibition of telomerase did not alter VSMC function (Figures 1), while activation of telomerase restored physiological endothelial dependent NOS-mediated (NO) dilation to flow. As telomerase activity is known to increase cellular proliferation, activation of telomerase in VSMC would be counterproductive to vascular health and would be expected to impair vasoactive function. In mice with systemic deletion of telomerase a decrease in atherosclerosis-like phenotype has been reported. Similarly published evidence supports that the well-established anti-inflammatory and anti-atherosclerosis properties of PPAR γ are at least in part due to suppression of telomerase activity in the VSMC.

Conclusions.

In summary, we demonstrate direct functional evidence that decreased telomerase activity is sufficient to initiate the transition from NO to H2O2 as the primary mediator of FMD in the human resistance vasculature, recapitulating the microvascular phenotype found in patients with CAD. Furthermore we show that increasing telomerase activity reverses the pathological phenotype seen in CAD. This pathological switch has clinical implications especially in the coronary circulation where beyond sharing vasodilator properties, the local influences are opposing with H2O2 exerting pro- and NO producing anti-proliferative effects. Identifying a central and extranuclear role for telomerase in this switch suggests novel pharmacological approaches for maintaining or restoring normal endothelial function, by limiting endothelial activation inflammation, and vascular ROS production. This study provides support for the idea that mitochondrial telomerase activity regulates ROS production with direct physiological consequences.
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DISCLOSURES
EP filed a patent on AGS 499 and other derivatives as telomerase increasing compounds.

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FIGURE LEGENDS

Table 1: Patient characteristics for microvessels used in study.

| # total of 7 subjects with no information on height and weight (3 non CAD, 4 CAD) |
| * P<0.05 t-test. Compared to non CAD |

Figure 1: Telomerase inhibition replicates the CAD vascular phenotype in human adipose and atrial vessels from non-CAD subjects. Flow mediated dilation was evaluated in isolated microvessels treated with A) vehicle (adipose), B) BIBR-1532 (adipose), and C) BIBR-1532 (atrial). In BIBR-treated vessels, the mechanism of FMD changed from NO to H2O2. D) ACh induced dilation was virtually eliminated after telomerase inhibition. E) No change in endothelium-independent dilation to papaverine was observed. N = 4-12 Adipose, N= 5-9 Atrial * p<0.05 two way ANOVA RM Tukey post hoc analysis.

Figure 2: CAD does not cause telomere shortening in microvessels or left ventricular tissue but decreases expression of the catalytic subunit TERT. A) Total average telomere length was evaluated in genomic DNA from microvessels (adipose) and left ventricular tissue from subjects with and without CAD. B) Expression of the catalytic subunit of telomerase (TERT) was evaluated in left ventricular tissue from subjects with and without CAD by Western blot. Values are normalized to beta actin loading control and expressed as fold change compared to non-CAD control. N = 4-7 * p<0.05 t-test.

Figure 3: Mitochondrial H2O2 mediated FMD after Inhibition of Telomerase. H2O2 levels where analyzed using the H2O2 specific fluorescent probe PYI targeted to mitochondria (MitoPYI) in vessels form non CAD subjects. A) representative image; numbers represent florescent intensity above background B) summary of florescence intensity at 5 min after initiation of flow. Specificity of the probe for H2O2 was confirmed using Peg-Catalase. C) An inhibitor of electron transport chain complex I (rotenone) or a mitochondrial targeted ROS scavenger (MitoTempol) inhibited FMD after telomerase inhibition. N = 4 * p<0.05 two way ANOVA RM (dose response curve) or t-test (fluorescence data) with Tukey post hoc.

Figure 4: Mitochondrial Translocation of TERT after Exposure to Acute Oxidative Stress. A) Treatment of normal HF with H2O2 resulted in translocation of TERT-GFP from the nucleus to the mitochondria (co-localized with Mitotracker Red) Cells were imaged using a 63X oil immersion objective. B) To confirm purity of isolated mitochondria of cells either transfected with whole cell (WC) TERT or R3E/R6E TERT (nucTERT) western blots for tubulin (cytoplasmic marker), HSP70 and Ku80 (nuclear isoform [higher molecular weight] and mitochondrial isoform) were performed. C) Expression of nucTERT increased mitochondrial superoxide production as measured with MitoSox. D) Expression of nucTERT decreased cellular ATP production after external stress. n = 3-5 * p<0.05 t-Test.

Figure 5: Change in Vascular Phenotype is Independent of Changes in Gene Expression. Flow mediated dilation was evaluated in isolated adipose microvessels from subjects without CAD treated with Actinomycin D, a transcriptional inhibitor. A) Act D + vehicle B) Act D + BIBR-1532. Mechanism of FMD changed from NO (Act D)→ H2O2 (Act D + BIBR-1532). Act D was sufficient to prevent transcriptional activation of heat shock proteins 27 and 70 after acute exposure to 42°C. Sample blot shows CO= control; HS = Heat Shock. *p <0.05 two way ANOVA -RM Tukey post hoc analysis (functional studies). N = 4-5 * p<0.05 vs. untreated control t-Test Tukey post hoc analysis (protein levels).

Figure 6: Pharmacological Activation of Telomerase Restores NO Mediated Dilation in Response to Flow. Flow mediated dilation was evaluated in isolated adipose and atrial microvessels from subjects with CAD treated with the selective transcriptional telomerase activator AGS 499 (22 nM). Vehicle treated vessels are historic controls. A) CAD Adipose + AGS 499, B) CAD Atrial + AGS 499. The mechanism of FMD changed from Peg catalase sensitive (H2O2 in CAD) to L-NAME and c-PTIO sensitive (NO mediated dilation)
in CAD + AGS 499). C) Inhibition of telomerase activity in vessels from subjects with CAD did not alter mechanism or overall dilator capacity in response to flow. D) AGS 499 resulted in transcriptional increase of TERT mRNA * p <0.05 vs. AGS 499 control two way ANOVA RM Tukey post hoc analysis. N = 5-8 adipose, N = 3-4 atrial * p>0.05 vs. AGS 499 only treated t-Test Tukey post hoc analysis (RNA levels). #AGS 499+c-PTIO N=3 (2 adipose one atrial).

**Figure 7: Proposed Mechanism of TERT in Regulating Balance of Endothelial Mitochondrial ROS and NO to Maintain FMD in Human Microvessels.** Abbreviations in alphabetical order: AGS499 = Telomeres activator; ATP = Adenosine triphosphate; CAD = coronary artery disease; FMD = Flow mediated dilation; H2O2 = Hydrogen peroxide; mt = mitochondrial; nuc = nuclear; NAD+ = Nicotinamide adenine dinucleotide; NO = Nitric oxide; O2− = Superoxide; ROS = reactive oxygen species; TERT = catalytic subunit of human telomerase complex.
Novelty and Significance

What Is Known?

- Endothelial dysfunction predicts future cardiovascular events.
- Under physiological conditions the primary mediator of flow-mediated dilation (FMD), is nitric oxide (NO), whereas in pathology such as coronary artery disease (CAD), the FMD is mediated by the mitochondria-derived, pro-inflammatory hydrogen peroxide (H$_2$O$_2$).
- The catalytic subunit of telomerase, TERT, elongates telomeres in the nucleus to prevent cellular aging and promote proliferation.
- A non-nuclear role for TERT in regulating levels of mitochondrial derived reactive oxygen species (mtROS) is established in other cell types but its role in vascular cells has not been examined.

What New Information Does This Article Contribute?

- Telomerase activity is decreased in subjects with CAD without measurable telomere shortening.
- Pharmacological inhibition of telomerase activity in arterioles from patients without CAD causes a transition in mechanism of FMD from NO to H$_2$O$_2$, similar to the mechanism observed in vessels from patients with CAD.
- Transcriptional activation of TERT restores NO as the dominant mediator of FMD in subjects with CAD.
- Telomerase activity regulates endothelial production of either NO (health) or H$_2$O$_2$ (disease) in response to endothelial shear stress in the microcirculation.

Endothelial release of NO mediates flow-mediated dilation (FMD) under physiological conditions and serves to prevent vascular smooth muscle proliferation and inflammation. In subjects with coronary artery disease (CAD), however, arteriolar FMD is mediated by mtROS, specifically hydrogen peroxide (H$_2$O$_2$), a pro-inflammatory and pro-atherosclerotic dilator. The present study, identifies telomerase activity as a regulator of the underlying mechanism of FMD. Telomerase activity represents a novel target to modulate FMD and thereby, vascular health.
**TABLE 1**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Adipose non CAD (n=38)</th>
<th>CAD (n=8)</th>
<th>Atrial non CAD (n=10)</th>
<th>CAD (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>14/24</td>
<td>7/1</td>
<td>4/6</td>
<td>5/5</td>
</tr>
<tr>
<td>Age, years (average ± SEM)</td>
<td>49.1±2.6</td>
<td>65.6±2.6*</td>
<td>56.2±4.1</td>
<td>62.6±2.8*</td>
</tr>
<tr>
<td>BMI (average ± SEM)</td>
<td>28.9±1.3</td>
<td>31.8±1.8</td>
<td>27.6±1.4</td>
<td>29.4±1.5</td>
</tr>
<tr>
<td>BMI ≥ 30</td>
<td>8 of 38</td>
<td>3 of 8</td>
<td>2 of 10</td>
<td>3 of 5</td>
</tr>
</tbody>
</table>

**Underlying diseases/risk factors**

- **Coronary Artery Disease**: 0/8/0/10
- **Hypertension**: 1/7/5/7
- **Hypercholesterolemia**: 1/4/1/4
- **Diabetes Mellitus**: 0/3/1/2
- **Congestive Heart Failure**: 0/1/0/0
- **None of the Above**: 37/0/5/0

# total of 7 subjects with no information on height and weight (3 non CAD, 4 CAD)

*p<0.05 vs. non-CAD by t-test
Figure 2

(A) Telomere Length (base pairs) compared between Microvessels and Left Ventricle.

(B) TERT Protein (Fold Change from non CAD) in the Left Ventricle, showing a significant difference (*).
Figure 3

A vehicle  BIBR 1532  BIBR 1532 + Peg-Ca
Baseline

Flow

% increase in fluorescence

Pressure gradient (cmH2O)

% Max. Diameter

Vehicle + Rotenone
BIBR 1532 + Rotenone
Vehicle + mtTempol
BIBR1532 + mtTempol

Control
BIBR 1532
BIBR 1532 + Peg-Cat
Figure 5

(A) Adipose non CAD

(B) Adipose non CAD

(C) HSP70

HSP27

GAPDH

(Co Co HS HS Co Co HS HS + Act D)

(D) Fold Change from control

Vehicle Act D

HSP 27

HSP 70

Co HS Co HS
Figure 6
Figure 7

CAD

AGS 499

ROS

mt TERT

Mitochondrial DNA Damage

↓ ATP  ↓ NAD⁺  ↑ mtROS

↑ O₂⁻  ↑ H₂O₂

Physiological FMD

NO

Pathological FMD

Telomere length

nuc TERT
Critical Role for Telomerase in the Mechanism of Flow Mediated Dilation in the Human Microcirculation
Andreas M Beyer, Julie K Freed, Matthew J Durand, Michael Riedel, Karima Ait-Aissa, Paula Green, Joseph C Hockenberry, R G Morgan, Anthony J Donato, Refael Peleg, Mario Gasparri, Chris K Rokkas, Janine H Santos, Ester Priel and David D Gutterman

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Supplemental Material

Nitric Oxide production was estimated by following the conversion of DAF2 to DAF2-triazole (DAF2-T) by HPLC as published previously\textsuperscript{1}. Briefly DAF2-DA (5 µM) was added to cells for 1 hour. Cells were harvested and lysed and supernatants were filtered through a 5Kd cut-off microcon filter. Eluent (80 µl) was injected onto a C18 reverse-phase HPLC column and separated using a mobile phase of sodium phosphate (10 mM, pH 7.5) and acetonitrile (5%) with a flow-rate of 1 ml/mn. DAF2-T was detected by fluorescence (ex 490 nm, em 515 nm) and quantified by area under the curve. Data was normalized to control for any given experimental date. Outliers were identified using Thompson tau method.

Protein expression was evaluated using standard Western blot techniques. Cells were lysed centrifuged and separated from debris in cold lysis buffer supplemented with a protease inhibitor cocktail (Roche)\textsuperscript{2-4}. Isolated protein was quantified using a Bradford micro assay. Protein (10-50 µg) was subjected to 10% SDS-PAGE and transferred to PVDF membranes prior to blotting with polyclonal antibodies against p-eNOS/ eNOS (1:1000 BD Biosciences) or GABDH.

Immunohistochemistry: was performed at Children’s Hospital of Wisconsin pathology core. In brief, optimal immunostaining protocol was developed with the Leica Bond Max Immunostainer platform. Tissues were fixed in 10% neutral-buffered formalin, processed and embedded into paraffin blocks. Sections were cut at 4µm, mounted on positively charged glass slides and incubated at overnight at 45°C. All paraffin sections were de-waxed prior to staining on the instrument. The TERT antibody required antigen retrieval using Leica H1 antigen retrieval reagent for 10 minutes. The antibody was detected and visualized using Bond Polymer Refine Detection System (DS9800) with the addition of a DAB enhancer (AR9432), using the MOD F protocol/software installed by Leica field service technicians. An optimal concentration of 1:100 was determined for TERT. All slides were counter-stained with hematoxylin and cover slipped using a synthetic mounting media. Omission of the primary antibody served as negative control.

Supplemental Study limitations

Influence of Clinical Parameters and Tissue Origin

We used adipose tissue from several body sites, both visceral and subcutaneous, potentially increasing the variability of our data since vessels from these sites can demonstrate unique responses to selective vasodilators\textsuperscript{5}. However our experience indicates that in responses to flow and mechanisms of FMD in health and disease are similar in arterioles across fat depots, and similar to responses observed in human coronary arterioles. Thus the same change in dilator mechanism to flow that we observe in adipose vessels exposed to an inhibitor of telomerase, occurs in coronary arterioles in response to an elevation in arterial pressure \textsuperscript{6} or in vessels taken from patients with CAD \textsuperscript{7}. Thus the switch from NO to H\textsubscript{2}O\textsubscript{2} as a mediator of FMD is a common
response to acute or chronic vascular stressors and appears conserved across a limited number of tissues.

Since discarded de-identified specimens were used, it was not possible to control for all clinical variables such as duration and extent of CAD, effect of medications, and presence of risk factors such as hypertension, diabetes, obesity or high cholesterol. No heart tissue and only limited amounts of adipose tissue from truly healthy subjects were included in this study since all underwent surgery, limiting our conclusions to pathophysiological conditions. Diseases such as obesity, hypertension, and hyperlipidemia may be confounders in contributing to \( \cdot \)ROS production. The high prevalence of diabetes in our cohort makes it difficult to exclude an effect of diabetes on our observed phenotypes. Importantly when stratified by presence or absence of CAD, each group had similarly elevated overall BMI (Table 1). Heterogeneity among subjects enhances the strength of our findings that decreased telomerase activity contributes to the development of CAD by enabling a rise in mitochondrial free radical production.

While we cannot completely exclude effects of medications on the observed changes we believe it is not likely that there were residual drug effects since vessels were incubated and repetitively washed in buffer and/or media for several hours prior to experimentation. We have previously shown that this is sufficient for washout of some medications with long half-lives. In addition, the effects of applied pharmacological dilator agents are reversed by washing with fresh buffer, suggesting that any residual influence is minimized. Future studies will need to address the influence of specific cardiovascular risk factors and other phenotypical characteristics.

Due to the limitation in tissue volume (each microvessel weighs less than 10 µg wet weight) we cannot reliably evaluate telomerase protein levels in the microvessels and instead presented data from whole heart tissue. We did measure telomere length in isolated arterioles and found it to be significantly shorter in heart tissue compared to microvascular samples, with no difference between CAD and non CAD in either tissue. Both measurements are consistent with the idea that the effects of TERT are telomere-independent and can be normalized by stimulating TERT transcription (AGS 499) in CAD tissue.

**Online Figure I: Immunohistochemistry of isolated human coronary vessels from subjects with and without CAD.** TERT expression was observed in both SMC and vascular endothelial cells. No quantification was performed. Slides were scanned and imaged using Hamamatsu digital imaging software NDPview2. Indicated Scale bar = 100µM for each individual image.

**Online Figure II: Telomerase Activation Increased NOS levels and NO Production in Cultured Endothelial Cells.** Cultured HUVECS were treated with the TERT activator or inhibitor of telomerase activity (AGS 499 or BIBR 1532 respectively). All values displayed as fold change to Vehicle. A) Telomerase activation resulted in transcriptional increase eNOS mRNA while inhibition of telomerase activity had no effect; \( N \geq 5 \). B) Telomerase activation increased p-eNOS S1117 without effecting total protein (both normalized to GABDH) \( N \geq 5 \), C) Telomerase activation increased NO producing while telomerase inhibition had no effect. No measured via DAF-2A/HPLC. * \( p < 0.05 \) vs. Vehicle t-Test Tukey post hoc analysis.


Online Figure II

A) eNOS mRNA

B) Protein

C) DAF-2A