

## Materials and Methods

**Tissue acquisition.** All tissue acquisition procedures were approved by the Institutional Review Board of the Medical College of Wisconsin. Discarded atrial tissue from cardiac surgery or adipose tissue from plastic or general surgery was obtained from the operating room and immediately placed in cold HEPES buffer (adipose) or cardiopulmonary solution (atrial) and transferred to the laboratory for study. All tissues were de-identified; but a brief medical history including the patient's age, height, weight, sex and clinical diagnosis of cardiovascular risk factors was completed by the operating room staff. The presence of CAD was determined based on a positive diagnosis through standard clinical diagnostics, which would most likely be made based on results of coronary arteriography, occurrence of an acute coronary syndrome event, or indirect evidence from cardiac stress imaging. A positive diagnosis of CAD was recorded from the subjects' medical records by the operating room staff. Based on our tissue collection procedures, subjects with CAD generally tend to be older than subjects without CAD. To eliminate the effects of age bias on our findings, only non-CAD tissue samples from subjects aged 50 years or more were used.

**Fluorescent detection of mitochondrial ROS.** The small molecule fluorescent probe mitochondria peroxy yellow 1 (MitoPY1; Tocris Bioscience, Bristol, UK)<sup>1</sup> was used to detect flow-induced mitochondrial H<sub>2</sub>O<sub>2</sub> generation in atrial microvessels as previously described<sup>2, 3</sup>. All vessels were from subjects with CAD and had been incubated  $\pm$  ANG 1-7 ( $10^{-9}$  mol/L) overnight. A separate group of vehicle-treated vessels were incubated with the H<sub>2</sub>O<sub>2</sub> scavenger PEG catalase (500 U/mL) 30 minutes prior to assessing flow-induced changes in fluorescence. Vessels were cannulated in the same manner as described above, except HEPES buffer (275 mM NaCl, 7.99 mM KCl, 4.9 mM MgSO<sub>4</sub>, 3.2 mM CaCl<sub>2</sub>, 2.35 mM KH<sub>2</sub>PO<sub>4</sub>, 0.07 mM EDTA, 12 mM glucose, and 20 mM HEPES acid; pH 7.4 at 37°C) was used. All vessels were perfused intraluminally with MitoPY1 (10  $\mu$ M) and equilibrated in the dark for 60 minutes at a pressure of 60 mmHg. After equilibration, baseline MitoPY1 fluorescence was assessed with fluorescent microscopy using a Nikon TE200 microscope equipped with a 10X objective and a krypton/argon laser with an excitation wavelength of 488 nm coupled with emission detection at 540 nm. After capturing a baseline image, intraluminal flow was generated by simultaneously raising one perfusing reservoir while lowering the other to create a pressure gradient of 100 cm H<sub>2</sub>O. After 1 minute of intraluminal flow, a second image was captured. Separate adipose arterioles had either BIBR-1532 (10  $\mu$ M) or antimycin A (1  $\mu$ M) added to the tissue bath for 30 minutes.

Images were analyzed using Metamorph (Universal Imaging Corporation, Downingtown, PA). Vessel segments were freehand selected and fluorescence was measured in arbitrary units. Background fluorescence was subtracted from the fluorescence intensity of each vessel. All data is presented as a percent change in vessel fluorescence after 1 minute of intraluminal flow.

**Cannulated vessel studies.** All arterioles were studied within 48 hours of tissue acquisition. Based on our experience, vasodilator sensitivity and the mechanism of dilation do not change whether arterioles are studied immediately upon acquisition or 48 hours post-acquisition. The cannulated vessel preparation and assessment of vasodilation by videomicroscopy of human arterioles has been described in detail elsewhere.<sup>4</sup> Briefly, arterioles 100-200  $\mu$ m in diameter were dissected from the tissue, cleaned of connective tissue and cannulated onto glass micropipettes of equal impedance in a heated organ chamber filled with Krebs solution and aerated with a 74% N<sub>2</sub>, 21% O<sub>2</sub> and 5% CO<sub>2</sub> gas mixture to maintain pH. After 60 minutes of equilibration the vessels were constricted to 30-50% of their maximal diameter with endothelin-1. Once stable constriction was achieved, the vasodilator response to cumulative additions of ANG 1-7 ( $10^{-12}$  –  $10^{-6}$  mol/L) or graded increases in intraluminal flow (5-

100 cm H<sub>2</sub>O pressure gradients) were assessed. For all vessels, a control vasodilator response was assessed to ensure vessel viability, followed by a washout period and 30 minute incubation with the following pharmacological agents: A779 (Mas receptor antagonist; 10<sup>-5</sup> mol/L); N<sup>w</sup>-nitro-L-arginine methyl ester (L-NAME; nitric oxide synthase inhibitor; 10<sup>-4</sup> mol/L); or polyethylene glycol–catalase (PEG–catalase; H<sub>2</sub>O<sub>2</sub> scavenger; 500 U/mL). Two vasodilator curves were assessed per vessel. At the end of the experiment, the direct smooth muscle activator papaverine was added to the tissue bath to assess maximal dilation and vascular smooth muscle cell viability. Percent vasodilation was calculated as follows:  $(D_m - D_c)/(D_{max} - D_c) \times 100$ , where  $D_m$  is the measured diameter,  $D_c$  is the initial constricted diameter, and  $D_{max}$  is the maximum diameter of the vessel following papaverine addition. ANG 1-7, A779, L-NAME and PEG catalase were all purchased from Sigma Aldrich, St. Louis, MO.

Separate groups of vessels were incubated overnight (16-20 hours) at 37°C in cell culture media (EGM™-2MV; Lonza; Walkersville, MD) with either ANG 1-7 (10<sup>-9</sup> mol/L), ANG 1-7 + a specific inhibitor of telomerase activity (BIBR-1532<sup>5</sup>; 10<sup>-5</sup> mol/L; Tocris Bioscience), BIBR-1532 alone, or cell culture media alone (vehicle). A separate group of CAD atrial vessels were incubated overnight with the PPAR-γ activator rosiglitazone (10<sup>-5</sup> mol/L) ± BIBR-1532. The EC<sub>50</sub> of BIBR-1532 has been shown to be in the nM range,<sup>5</sup> therefore the dosage used in our experiments would be expected to significantly decrease telomerase activity.

**Cell culture of Human Coronary Artery Endothelial Cells (HCAECs).** Commercially available HCAECs (Lonza) were grown to approximately 80-90% confluency and used at passage five. Cells were grown in EGM™-2MV media (Lonza) containing 5% fetal bovine serum in 100 mm dishes in a sterile cell culture incubator in a 5% CO<sub>2</sub>, 21% O<sub>2</sub>, 74% N<sub>2</sub> environment. ANG 1-7 (10<sup>-9</sup> mol/L) was added to half of the culture dishes 24 hours prior to harvesting the cells. To harvest, cells were trypsinized, centrifuged to form a loose pellet (1,000 RCF, 5 minutes), and lysed for 30 minutes using ice cold MOPS buffer containing protease and phosphatase inhibitors. After lysing, the cells were centrifuged at 12,000 RCF and the supernatant was collected for assessment of telomerase activity using the telomeric repeat amplification protocol (TRAP) assay (see below).

**Telomerase activity.** A TRAP assay was used to measure telomerase activity of the cell lysate and product was quantified using droplet digital polymerase chain reaction (ddPCR) as previously described.<sup>6</sup> For each sample, 8 µg of protein was added to a 50 µl telomerase extension reaction that contained 1x TRAP reaction buffer (10x concentration: 200 mmol/L Tris-HCl, pH 8.3, 15 mmol/L MgCl<sub>2</sub>), 0.4 mg/ml BSA, TS (telomerase extension substrate) primer (200 nmol/L, HPLC purified, 5-AATCCGTCTGAGCAGAGTT), and dNTPs (2.5 mmol/L each). The reactions were incubated at 25°C for 40 minutes followed by heat inactivation at 95°C for 5 minutes. Next, 8 µl of the extension reaction was added to a 20 µl ddPCR reaction which consisted of 1x EvaGreen ddPCR Supermix (Bio-Rad, Hercules, CA, USA), 50 nmol/L TS primer, 50 nmol/L ACX primer (HPLC purified, 5'-GCGCGG[CTTACC]<sub>3</sub>CTAACC-3). RNase A controls (1 ug/ul RNase A added to cell lyase and incubated 20 min at 37° C) were also included in the extension reaction. Droplets were formed using the BioRad QX100 droplet generator (Hercules, CA) and transferred to a 96 well plate (Eppendorf AG, Hamburg, Germany), foil sealed and put into a thermocycler (Eppendorff Mastercycler) for amplification (95°C for 5 min, 40 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, hold at 10°C). The plate was then loaded into the QX200 droplet reader (Biorad) where the total number of positive and negative droplets was read using both the EvaGreen and HEX channels on the instrument. The positive droplet population was identified using 2D amplitude analysis on the QuantaSoft software (Version 1.7.4.0917, BioRad) where the positive droplets clustered at a higher fluorescent intensity than negative droplets. The droplet populations were compared to a no primer control that only contained the extension reaction and EvaGreen reaction mix. The

concentration tab in the software calculated the extension products per microliter for the EvaGreen channel.

**PCR assessment of TERT expression.** HCAEC RNA was collected using Ambion's PureLink RNA Mini Kit according to the product insert (Thermo Fisher Scientific, Waltham, MA). 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 Qiagen QuantiTect Primer Assay primers for human telomerase reverse transcriptase (Hs\_TERT\_1\_SG QuantiTect Primer Assay QT00073409) and SYBR green from Qiagen in a BioRad CFX96 Touch™ Real-Time PCR Detection System. Expression levels were normalized to human 18S rRNA (Hs\_RRN18S\_1\_SG QT00199367).

**Western blot analysis of TERT expression.** The expression of TERT was determined in HCAECs treated with ANG 1-7 ( $10^{-9}$  M for 24 hours) or PBS vehicle using a commercially available antibody for TERT (Bioss, Boston, MA) and the Criterion Cell and Blotter System (Biorad). Briefly, cells were lysed in ice cold MOPS buffer containing protease and phosphatase inhibitors, centrifuged at 12,000 RCF, and 20 µg of protein was loaded per lane in a Criterion pre-cast gel for separation by SDS PAGE. After separation and transfer to a nitrocellulose membrane, 5% non-fat dry milk was applied to the membrane for 1 hour, then primary antibodies for TERT and  $\beta$ -Actin were applied to the membrane overnight in a 2% bovine serum albumin solution in a cold room. Secondary antibodies were applied to the membrane the following day for two hours, washed, and protein bands were detected using Clarity Western ECL Substrate. Intensity of the protein bands was measured using the Analyze Gels function in ImageJ. TERT expression was normalized to  $\beta$ -Actin.

**Immunohistochemical analysis of Mas receptor expression.** An optimal immunostaining protocol was developed with the Leica Bond Max Immunostainer platform. Fresh arterioles or whole atrial tissue were fixed in zinc formalin, embedded into paraffin blocks, sectioned into 4 µm thick slices, mounted on positively charged glass slides, and incubated overnight at 45°C with a primary antibody for the Mas receptor (Novus Biologicals, Littleton, CO, USA). 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. All slides were counter-stained with hematoxylin, dehydrated, cleared and coverslipped using a synthetic mounting media. Omission of the primary antibody served as negative control.

**Statistical Analysis.** All statistical analysis was performed using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA) and normality was determined using a Shapiro-Wilk test. All data are presented as mean value  $\pm$  SEM. Differences in the vasodilator responses to ANG 1-7 or increased intraluminal flow between groups were assessed using a two-way repeated measures analysis of variance (ANOVA). A Student-Newman-Keuls post-hoc test was used to test for differences between individual means. Differences in TERT mRNA expression and telomerase activity were assessed using a parametric unpaired Student's t-test and a non-parametric Mann-Whitney Rank Sum test, respectively. For Western Blot analysis, a single value was excluded using a Modified Thompson Tau test for statistical outliers based on a  $\tau$  value of 1.7110 and an  $n=7$ . After exclusion of a single value, differences between control and ANG 1-7 treated values were determined using a non-parametric Mann-Whitney rank sum test. Differences in MitoPY1 fluorescence were assessed using one-way ANOVA with a post hoc Tukey test. A single outlying value that was more than two standard deviations from the mean was excluded from the MitoPY1 analysis. A P value  $<0.05$  was considered statistically significant for all analysis.

## References

1. Dickinson BC, Lin VS, Chang CJ. Preparation and use of mitopy1 for imaging hydrogen peroxide in mitochondria of live cells. *Nat. Protoc.* 2013;8:1249-1259
2. Beyer AM, Durand MJ, Hockenberry J, Gamblin TC, Phillips SA, Gutterman DD. An acute rise in intraluminal pressure shifts the mediator of flow-mediated dilation from nitric oxide to hydrogen peroxide in human arterioles. *Am. J. Physiol. Heart Circ. Physiol.* 2014;307:H1587-1593
3. Freed JK, Beyer AM, LoGiudice JA, Hockenberry JC, Gutterman DD. Ceramide changes the mediator of flow-induced vasodilation from nitric oxide to hydrogen peroxide in the human microcirculation. *Circ. Res.* 2014;115:525-532
4. Durand MJ, Phillips SA, Widlansky ME, Otterson MF, Gutterman DD. The vascular renin-angiotensin system contributes to blunted vasodilation induced by transient high pressure in human adipose microvessels. *Am. J. Physiol. Heart Circ. Physiol.* 2014;307:H25-32
5. Pascolo E, Wenz C, Lingner J, Huel N, Priepke H, Kauffmann I, Garin-Chesa P, Rettig WJ, Damm K, Schnapp A. Mechanism of human telomerase inhibition by bibr1532, a synthetic, non-nucleosidic drug candidate. *J. Biol. Chem.* 2002;277:15566-15572
6. Ludlow AT, Robin JD, Sayed M, Litterst CM, Shelton DN, Shay JW, Wright WE. Quantitative telomerase enzyme activity determination using droplet digital pcr with single cell resolution. *Nucleic Acids Res.* 2014;42:e104