ONLINE SUPPLEMENT

Fenofibrate Improves Vascular Endothelial Function by Reducing Oxidative Stress While Increasing eNOS in Healthy Normolipidemic Older Adults

Ashley E Walker[1], Rachelle E Kaplon[1], Sara Marian S Lucking[1], Molly J Russell-Nowlan[1], Robert H Eckel[2], and Douglas R Seals[1]

[1] Department of Integrative Physiology, University of Colorado, Boulder, CO, 80309

[2] Division of Endocrinology, Metabolism and Diabetes, University of Colorado Anschutz Medical Campus, Aurora, CO, 80045

Correspondence:
Ashley E Walker, PhD
Division of Geriatrics
University of Utah
30 North 1900 East
Room AB193 SOM
Salt Lake City, UT 84132-0001
Phone: (801) 582-1565 x4334
Fax: (801) 584-5640
e-mail: Ashley.Walker@utah.edu

Supplemental Methods.

Subject Characteristics. Waist and hip circumferences and BMI were measured by anthropometry. Arterial blood pressure was measured in triplicate over the brachial artery during seated (for baseline subject characteristics) or supine (for experimental visits) rest using a semi-automated device (Dinamap Pro 100, GE Health Care, Waukesha, WI).

Blood Analyses. Fasting plasma metabolic factors were determined by the Clinical and Translational Research Center core laboratory using standard assays. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: [fasting glucose (mg/dL) x fasting insulin (μU/L)]/405. Serum concentrations of oxidized LDL (oxLDL) were measured by ELISA (Alpco Diagnostics, Salem, NH) and C-reactive protein (CRP) was measured using a high-sensitivity Chemistry Immuno Analyzer (AU400e, Olympus America, Center Valley, PA).

EDD and endothelium-independent dilation. Briefly, an ultrasound probe was clamped 3-6 cm proximal to the antecubital crease. After obtaining a baseline image, reactive hyperemia was produced by inflation of a blood pressure cuff placed on the upper forearm distal to the antecubital fossa for 5 min at 250 mmHg followed by a rapid deflation. Pulsed doppler signals were recorded at an angle of insonation of 68 degrees with a sample volume the entire width of the artery as previously described. [1] Time-averaged peak velocity was obtained from recording the first 10 velocity envelopes. Brachial artery peak hyperemic shear rate was calculated as 8 times (due to wide sample volume) the peak velocity immediately following 5 minutes of forearm occlusion, divided by occlusion diameter. Flow-mediated dilation (FMD) responses were expressed as relative (%) and absolute (mm) change from baseline diameter. FMD was measured first during saline infusion (control) and then during supraphysiological intravenous infusion of vitamin C as previously described.[2] 0.06 g/kg fat-free mass of vitamin C was infused for 20 minutes, immediately followed by FMD measurements during a 0.02 g/kg fat-free mass vitamin C maintenance drip infusion. Endothelium-independent dilation was assessed by measurement of maximal brachial artery dilation in response to sublingual glyceryl trinitrate (GTN, 0.4 mg). GTN was only administered to subjects with resting blood pressure sufficient to safely tolerate the vasodilation, thus only 11 individuals in the fenofibrate group and 7 individuals in the placebo group received GTN.

Endothelial cell protein expression. Two sterile J wires (Daig Corp, Minnetonka, Minn) were advanced into an antecubital vein (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter. The wires were then transferred to a dissociation buffer solution and cells were recovered after a washing and centrifugation protocol. Collected cells were fixed with 3.7% formaldehyde and plated on poly-L-lysine–coated slides (Sigma Chemical, St. Louis, Mo) and then frozen at –80°C until analysis. Cells were rehydrated and nonspecific binding sites were blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, Pa). Cells were incubated with monoclonal antibodies for endothelial nitric oxide synthase (eNOS; Transduction Laboratories, San Jose, Calif., USA). Cells were then incubated with an Alexaflour 555 fluorescent secondary antibody (Invitrogen Corp, Carlsbad, Calif). For analysis, slides were viewed with a fluorescence microscope (Eclipse 600, Nikon, Melville, NY), and cell images were captured digitally by a Photometrics CoolSNAPfx digital camera (Roper

Scientific, Inc, Tucson, Ariz). Endothelial cells were identified by staining for von Willebrand factor and nuclear integrity was confirmed with DAPI (4',6'-diamidino-2-phenylindole hydrochloride). Once endothelial cells with intact nuclei were identified, they were analyzed with Metamorph Software (Universal Imaging Corp, Downingtown, Pa). Values for eNOS are reported as a ratio of endothelial cell to human umbilical vein endothelial cell average pixel intensity. The technician was blinded to the identity of the subject and the experimental condition during the staining and analysis procedures. Endothelial cells were not successfully collected for all subjects at all time points; therefore, the data presented are for 9 subjects in the fenofibrate group and 7 subjects in the placebo group.

Supplemental References

1. Pierce GL, Lesniewski LA, Lawson BR, Beske SD, Seals DR. Nuclear factor-κb activation contributes to vascular endothelial dysfunction via oxidative stress in overweight/obese middle-aged and older humans. *Circulation*. 2009;119:1284-1292.
2. Eskurza I, Monahan KD, Robinson JA, Seals DR. Effect of acute and chronic ascorbic acid on flow-mediated dilatation with sedentary and physically active human ageing. *J Physiol*. 2004;556:315-324.

Supplemental Results

Table S1: Brachial artery characteristics in response to vitamin C in fenofibrate treatment group

Variable	Baseline		Day 2		Day 7	
	Saline	Vitamin C	Saline	Vitamin C	Saline	Vitamin C
BA diameter, mm	3.65 ± 0.23	3.65 ± 0.24	3.66 ± 0.23	3.65 ± 0.24	3.65 ± 0.23	3.67 ± 0.24
FMD, mm	0.17 ± 0.02	0.23 ± 0.03 *	0.21 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02
Peak SR, 1/s	904 ± 101	902 ± 127	979 ± 138 *	964 ± 127	955 ± 123	898 ± 100

Data are mean±SE. BA, brachial artery; FMD, flow-mediated dilation; SR, shear rate. *P<0.05 vs. saline within day.

Table S2: Brachial artery characteristics in response to vitamin C in placebo treatment group

Variable	Baseline		Day 2		Day 7	
	Saline	Vitamin C	Saline	Vitamin C	Saline	Vitamin C
BA diameter, mm	3.74 ± 0.18	3.65 ± 0.18	3.66 ± 0.18	3.63 ± 0.18	3.66 ± 0.17	3.66 ± 0.20
FMD, mm	0.22 ± 0.02	0.25 ± 0.03 *	0.22 ± 0.02	0.27 ± 0.04 *	0.22 ± 0.03	0.26 ± 0.03 *
Peak SR, 1/s	981 ± 115	954 ± 92	969 ± 150	914 ± 141	977 ± 95	957 ± 118

Data are mean±SE. BA, brachial artery; FMD, flow-mediated dilation; SR, shear rate. *P<0.05 vs. saline within day.