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Measurement of Endothelium-Dependent Vasodilation in Mice—Brief Report

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Objective—Endothelium-dependent, flow-mediated vasodilation after an increase in shear stress at the endothelial lining of conduit arteries during reactive hyperemia after ischemia is a fundamental principle of vascular physiology adapting blood flow to demand of supplied tissue. Flow-mediated vasodilation measurements have been performed in human studies and are of diagnostic and prognostic importance, but have been impossible because of technical limitations in transgenic mice to date, although these represent the most frequently used animal model in cardiovascular research.

Approach and Results—Using high-frequency ultrasound, we visualized, quantified, and characterized for the first time endothelium-dependent dilation of the femoral artery after temporal ischemia of the lower part of the hindlimb and demonstrated that the signaling was almost exclusively dependent on stimulation of endothelial nitric oxide synthase, similar to acetylcholine, completely abolished after pharmacological or genetic inhibition of endothelial nitric oxide synthase and endothelial denudation, substantially impaired in mice of increasing age and cholesterol-fed ApoE knock outs and increased by the dietary polyphenol (−)-epicatechin. Intra- and interindividual variability were similar to the human methodology.

Conclusions—The physiology of flow-mediated vasodilation in mice resembles that in humans underscoring the significance of this novel technology to noninvasively, serially, and reliably quantify flow-mediated vasodilation in transgenic mice. (***Arterioscler Thromb Vasc Biol*. 2014;34:2651-2657.**)

Key Words: aging ■ blood flow velocity ■ Doppler duplex ultrasonography ■ endothelial nitric oxide synthase
■ femoral artery ■ flavonoids ■ vasodilation

The vascular endothelium is crucially involved in the fundamental regulation of blood flow matching demand and supply of tissue. After transient ischemia, arterial inflow increases. As a response to increased shear forces during reactive hyperemia, healthy arteries dilate via release of NO or other endothelium-derived vasoactive substances. This endothelium-dependent flow-mediated vasodilation (FMD) is impaired in atherosclerosis.[1] As the presence of endothelial dysfunction is closely associated with cardiovascular risk and outcome, the measurement of FMD in the brachial artery has become a standard method for the assessment of endothelial function in patients and to evaluate therapeutic interventions targeting atherosclerosis. To date, this has been impossible in mice, although highly desirable to study mechanisms affecting endothelial function and in particular femoral artery dilation in transgenic mouse models.[2] Therefore, we here characterize the physiology of FMD in living mice using a methodology analogous to humans and present important examples of vascular pathologies.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

In healthy humans, the relative increase in brachial artery diameter at 45 to 60 sec after resolution of forearm ischemia is typically in the 5% to 10% range. To be able to detect such small diameter changes expected in mice (10–30 μm change at a baseline diameter of ≈200–300 μm) with a high heart rate, we used a high-resolution, high-frequency digital imaging platform and a 30 to 70 MHz linear array microscan transducer

Nonstandard Abbreviations and Acronyms	
eNOS	endothelial nitric oxide synthase
FMD	flow-mediated vasodilation

(Vevo 2100, VisualSonics) specifically designed for small animal vascular ultrasound. As FMD is temperature-dependent, the body core temperature in anesthetized mice was kept at 37±1°C by using a heated examination table that was also equipped with electrocardiogram electrodes (Figure 1A). The fur was removed from the hindlimbs and prewarmed ultrasound gel was applied in excess to the proximal inner thigh. The ultrasound probe was attached to a stereotactic holder and was manually aligned with the femoral vein visible at the upper inner thigh as the vein runs parallel with the artery, which in turn is not easy to visibly identify. As an initial proof of concept, we used a protocol with a 5 minutes period of total occlusion of the femoral artery to induce reactive hyperemia and monitor changes in vascular hemodynamics (Figure 1B), including flow velocity and diameter after distal hindlimb ischemia as compared with parameters before occlusion essentially as established in the human forearm. A vascular occluder (5 mm diameter, Harvard Apparatus) was placed around the lower limb to induce occlusion of the distal hindlimb as an ischemic trigger. Because of the small diameter of vessels and the anatomic proximity of femoral artery (Figure 1C) and vein, it was key to identify the artery for later semiautomated analysis offline (Figure 1D). Using colored duplex ultrasound mode, the artery could be easily identified by the typical pulsatile blood flow pattern as opposed to the adjacent vein with continuous blood flow and compressibility. Arterial blood flow was confirmed by pulsed wave Doppler (Figure 1E and F). After 5 minutes of ischemia, deflation of the cuff led to an immediate increase in flow velocity, which in turn went along with an increase in wall shear stress of the upstream conduit artery. This was followed by a significant dilation with a maximum at 60 to 90 sec. Our first experiments showed a similar response pattern of FMD in mice (Figure 1G, femoral artery) as compared with humans (Figure 1H, brachial artery), yet with a greater effect size (human FMD 7% versus mouse FMD 20%).

As depicted in Figure 2A, 2D, 2G, and 2K (black symbols), we observed that increasing times of ischemia lead to a linear increase in the maximum of flow velocity at the onset of reperfusion, indicating dilation of downstream resistance arteries. This in turn led to parallel increases in wall shear stress and consecutively dilation of upstream femoral artery. Interestingly, 1 to 5 minutes ischemia was followed by a temporal increase in diameter with maximal values at 60 to 90 sec, whereas 10 minutes occlusion led to significantly greater dilatory response that was sustained for ≥180 sec. In humans, the rational for using a 5 minutes forearm occlusion protocol is that FMD is almost entirely mediated by endothelial nitric oxide synthase (eNOS),[3] and FMD can be seen as an in vivo read-out of eNOS activity depending on the methodology used to measure it.[4] As the physiology of FMD in mice was not established, we induced hindlimb ischemia by 1, 3, 5, and 10 minutes lower limb occlusion again after infusion of L-NAME, a competitive NOS inhibitor (Figure 2D–2F), in the

same mice. Our results show that the flow velocity and immediate postocclusive wall shear stress increase was not affected by L-NAME. However, the vasodilator response of the upstream femoral artery of ≤5 minutes of lower limb occlusion led to an almost entirely NOS-dependent vasodilation of the femoral artery ≤60 sec after cuff release. The lack of femoral artery vasodilation was accompanied by significantly slower return of wall shear stress to baseline. Furthermore, we observed that with increasing ischemia times beyond 5 minutes and in the late reperfusion phase, beyond 60 sec after cuff release, NOS-independent vasodilating mechanisms were recruited again, resembling a flow response as observed in human beings (data not shown).

Using a 5 minutes occlusion protocol with 60 sec post occlusion measurement of FMD (Figure 2M and 2L), we further evaluated the signaling pathways involved in FMD. Apart from NO, several other endothelium-derived mediators, such as cytochrome P450 epoxygenase (CYP 2C9), generated endothelium-derived hyperpolarizing factor[5] and prostacyclin,[6] but also arginase may contribute to maintenance of vascular tone.[3] To evaluate these potential other mechanisms in the context of our protocol, we measured FMD before and after injection of inhibitors with a washout phase of 7 days between measurements (Figure 2M). Furthermore, endothelium-independent vasodilation was also measured at 2 minutes after nitroglycerin (Figure 2N). The injection of neither the COX inhibitor indomethacin, the CYP 2C9/endothelium-derived hyperpolarizing factor inhibitor sulfaphenazole, nor the arginase inhibitor nor-NOHA (N^{ω}-hydroxy-L-arginine) affected FMD as measured at 60 sec after ischemia in wild-type mice. FMD was practically abolished in eNOS-knockout (KO) mice, and this was not significantly further decreased by additional NOS inhibition with L-NAME. This implies that eNOS is the predominant NOS isoform responsible for FMD and that in eNOS KO animals compensatorily increased nNOS does not compensate for eNOS KO. Systemic injection of the endothelium-independent vasodilator nitroglycerin, on the contrary, led to a similar significant vasodilation of the femoral artery in both wild-type mice and eNOS-KO mice (Figure 2M), and this response was not altered by any of the inhibitors (data not shown). To further validate our approach, we also performed experiments comparing FMD with a classical endothelium-dependent vasodilator acetylcholine (Figure 2O). Our results showed that intra-aortic injection of acetylcholine led to a dose-dependent, almost instantaneous vasodilation of the femoral artery at a similar magnitude as observed during FMD. These data are in line with the proposition of eNOS being exclusively responsible for femoral artery FMD in the early phase of reactive hyperemia and can therefore be used as an in vivo read-out of eNOS activity in mice.

To provide biochemical insights that eNOS is indeed phosphorylated in this model, we harvested the thigh muscles at the time of peak FMD values (60 sec after 5 minutes of lower limb occlusion) and from the nonischemic contralateral leg as a control (Figure 3A and 3B). Western blot analysis followed by semiquantitative densitometric analysis of X-ray films demonstrate significantly increased phosphorylation on eNOS serine 1178 in ischemic as compared with nonischemic
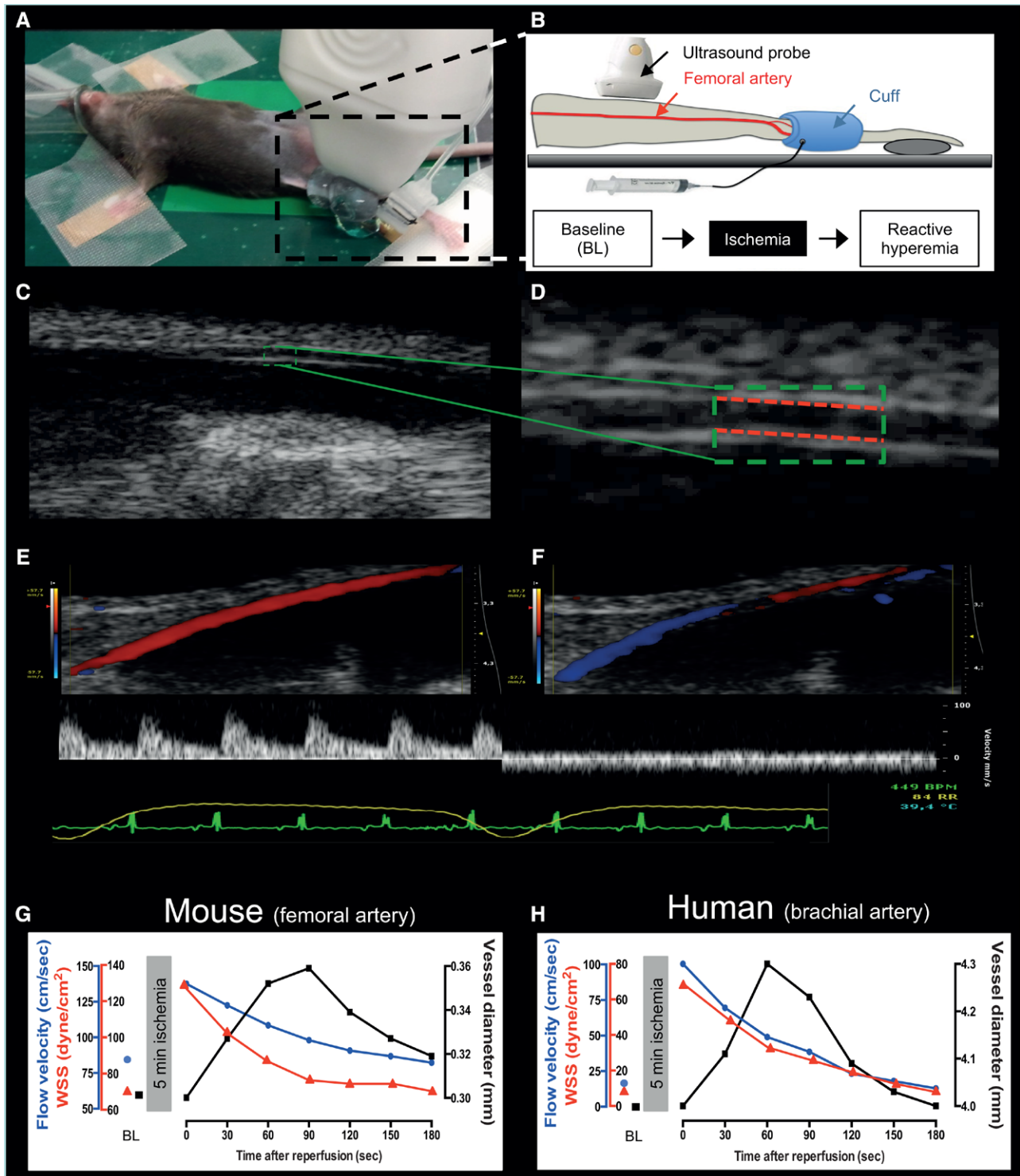
Figure 1. Principle of flow-mediated vasodilation (FMD) in mice. **A**, Set up with anesthetized mouse positioned on a temperature-controlled and electrocardiogram-equipped investigation table. **B**, The basic experimental protocol includes measurements of femoral artery diameter and blood flow velocity at baseline (BL) and over 3 minutes during reactive hyperemia after a 5 minutes distal ischemia induced by an inflatable cuff placed around the lower limb distal to the site of the ultrasound probe. The femoral artery (**C**) can be easily detected by Duplex ultrasound and a characteristic pulsatile blood flow pattern away from the body (toward the probe [**red**]). Importantly, the artery needs to be distinguished from the femoral vein (**D**), which is characterized by a continuous flow pattern directed toward the body (away from the ultrasound probe [**blue**]). **E**, Because of the small size of the mouse femoral artery (300 μm) and high heart rate, a linear array high-frequency probe with sufficient temporal and spatial resolution along with semiautomated edge detection software (**F**) is applied. **G**, After 5 minutes occlusion of the lower limb, reactive hyperemia starts with increased blood flow velocity (blue line) and wall shear stress (WSS, **red line**) in the femoral artery, which is because of ischemic vasodilation of the downstream resistance arteries. This leads to a delayed conduit artery vasodilation peaking at 60 to 90 sec (**black line**) that is qualitatively similar to human FMD physiology as measured in the brachial artery (**H**). **G**+**H** representative examples of n=1 each.
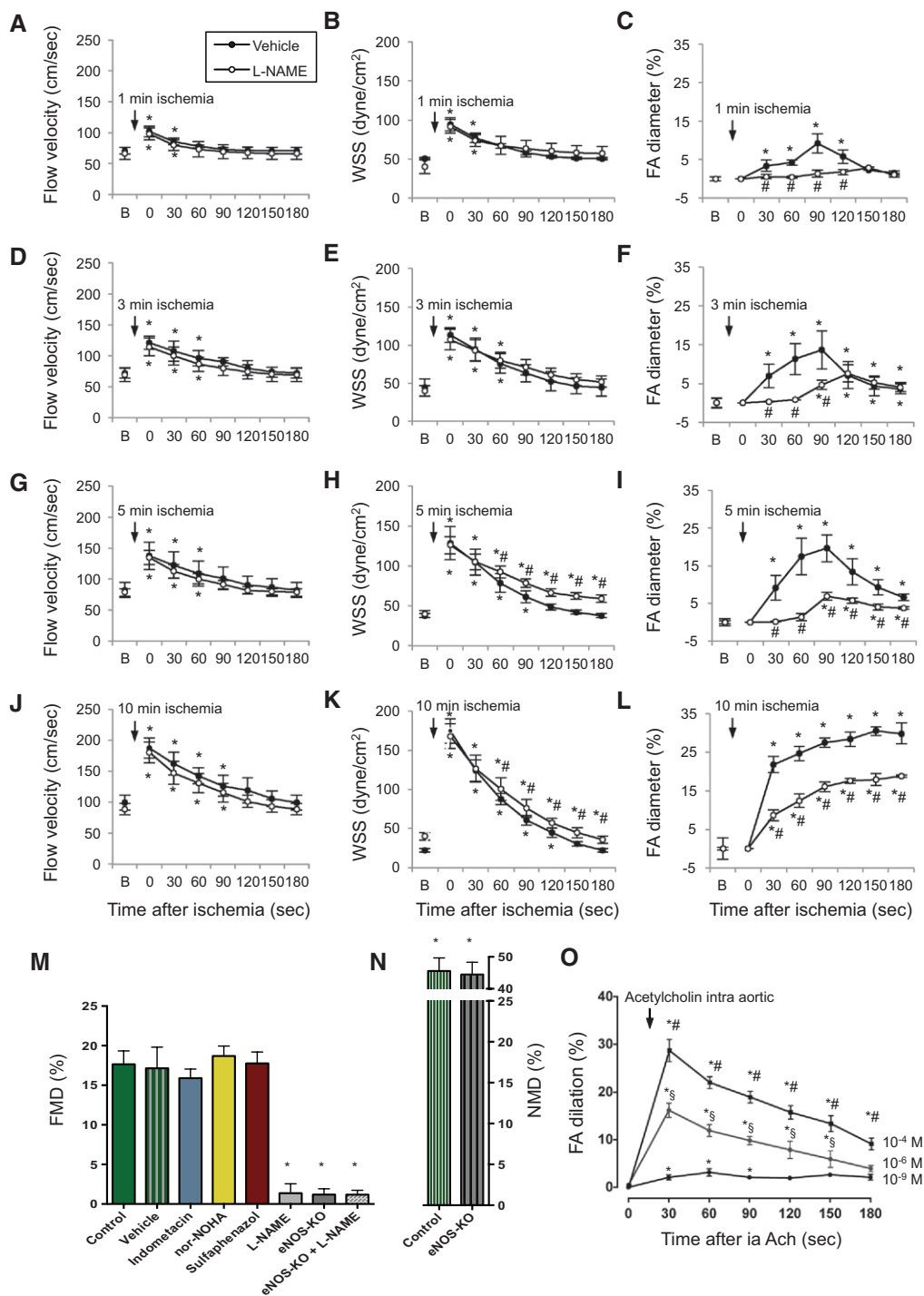
Figure 2. Flow-mediated vasodilation (FMD) signaling and physiology. **A–L**, Stimulus dependence of reactive hyperemia because of 1 (**A–C**), 3 (**D–F**), 5 (**G–I**), and 10 minutes (**J–L**) of hindlimb ischemia leads to increasing degrees of flow velocity (**A,D,G,J**), wall shear stress (WSS; **B,E,H,K**) and is followed by femoral artery vasodilation (**C,F,I,L**). Nitric oxide synthase (NOS) inhibition by N^ω-nitro-L-arginine methyl ester (L-NAME) did not significantly alter the increase in flow velocity and WSS in the early phase of reactive hyperemia. However, NOS inhibition altered the vasodilator response of the femoral artery and WSS remained significantly higher. After 1 to 5 minutes ischemia, the initial vasodilator response at ≤60 sec after reperfusion was completely abolished by L-NAME. At later time points after reperfusion (>60 sec) and when greater degrees of ischemia, that is, 10 minutes, were applied, vasodilation occurred which was not inhibited by L-NAME and was, therefore, NOS-independent. *$P<0.05$ vs baseline before ischemia (**B**) and #$P<0.05$ vs L-NAME (RM ANOVA, n=16). **M**, Although FMD after 5 minutes of hindlimb ischemia was not changed by vehicle, inhibition of cyclooxygenase (COX) with indomethacin, arginase by nor-NOAH, and EDHF by sulfaphenazole, it was largely abolished by the NOS inhibitor L-NAME and in endothelial NOS (eNOS) knockout (KO) mice. **N**, Nitroglycerin-mediated vasodilation (NMD) was similar in wild-type (WT) and eNOS KO mice. *$P<0.05$ vs vehicle (RM ANOVA, n=4/group). **O**, Vasodilation of the femoral artery after sequential intra-aortic injections of acetylcholine (Ach) boli at 10^{-9}, 10^{-6}, and 10^{-4} mol/L. *$P<0.05$ vs before Ach injection (0 sec), §$P<0.05$ vs 10^{-9} mol/L Ach, #$P<0.05$ vs 10^{-6} mol/L ACh (RM ANOVA, n=4).
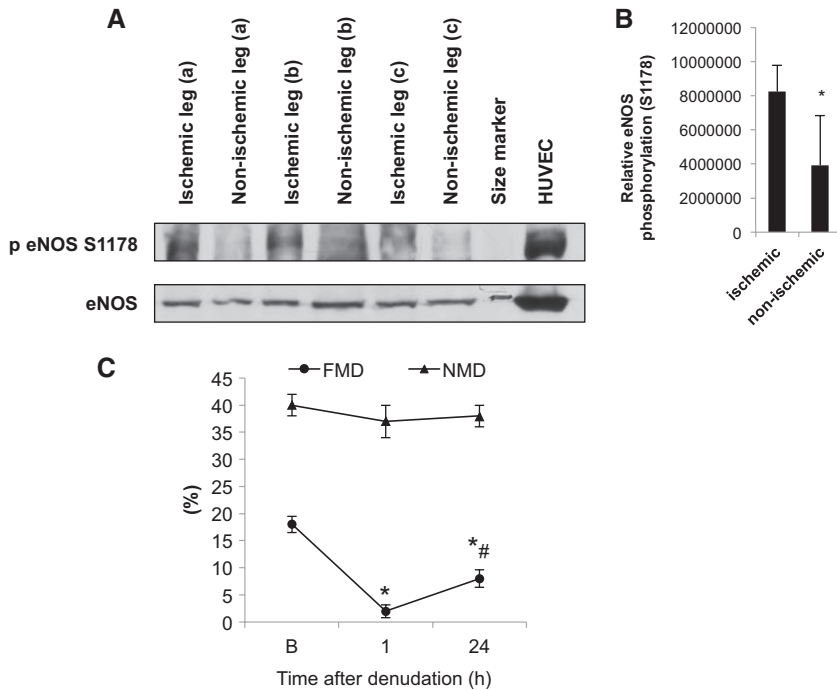
Figure 3. Phosphorylation of endothelial nitric oxide synthase (eNOS) and endothelium dependence. Thigh muscles were harvested at the time of peak flow-mediated vasodilation (FMD) values (60 sec after 5 minutes of lower limb occlusion) and from the nonischemic contralateral leg. **A**, Representative western blots (**upper panel**, phosphorylated eNOS on serine 1178; **lower panel**, total eNOS protein); **B**, semiquantitative densitometric analysis of X-ray films (n=5; *$P<0.05$ vs ischemic leg, paired t test); and **C**, immunohistochemistry of parallel legs demonstrate increased phosphorylated eNOS on serine 1178 in ischemic as compared with nonischemic control leg. **C**, After baseline measurements, mice were subjected to denudation of the femoral artery by insertion of a wire. This led to an almost complete abolition of FMD at 1 hour with partial recovery at 24 h. Nitroglycerin-mediated vasodilation (NMD) remained unaffected. *$P<0.05$ vs baseline before denudation (**B**) and #$P<0.05$ vs 1 hour (RM ANOVA, n=5).

control leg (Figure 3A and 3B). As expected, eNOS protein levels were not altered in the 5 minutes protocol (Figure 3A). To further confirm that the observed FMD responses indeed depend on the arterial endothelium, we performed femoral artery denudation experiments using a wire (Figure 3C). This led to an almost complete abolition of FMD at 1 hour.
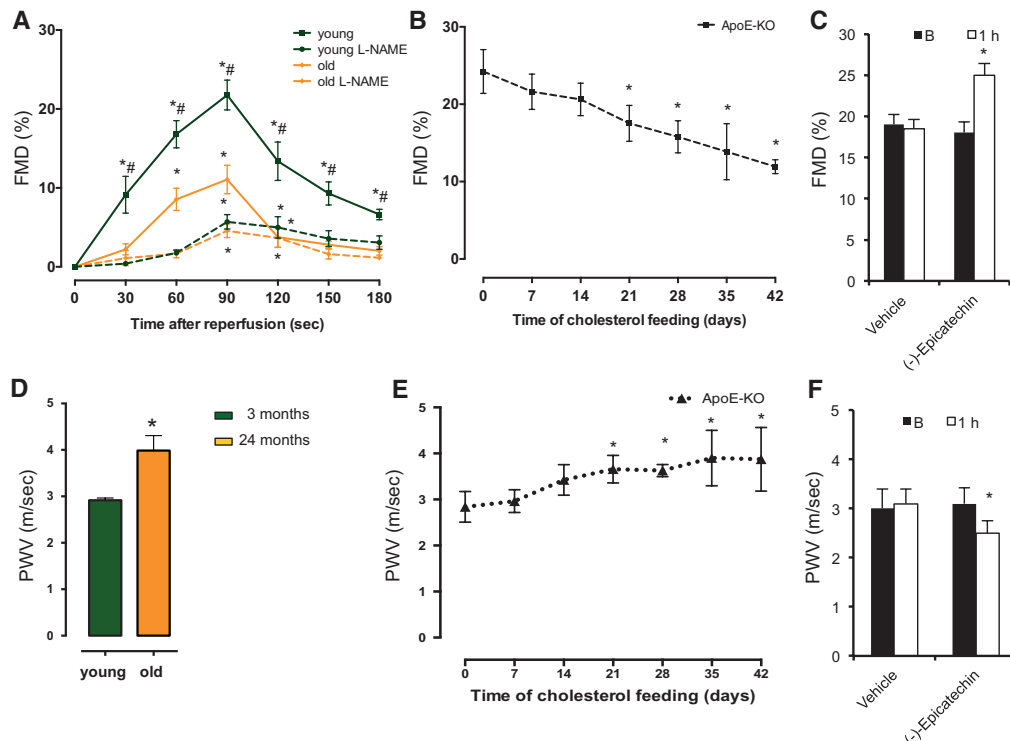
Figure 4. Effect of age, diet-induced accelerated atherosclerosis in transgenic ApoE knock out mice and polyphenol (−)-epicatechin. **A**, 24 months–aged mice (**orange lines**, n=6) exhibited significantly impaired NOS-dependent vasodilation as compared with young 3-month-old WT mice (**green line**, n=6), whereas the late (>60 sec, **dotted lines**) NOS-independent vasodilator responses as determined after N^{ω}-nitro-L-arginine methyl ester (L-NAME) application was not different between young and old mice. **B**, Flow-mediated vasodilation (FMD) response gradually decreased in ApoE KO mice on initiation of cholesterol feeding, approaching values of 24-months-old wild-type (WT) mice already after 1 month (n=8). **C**, FMD improved at 1 hour after intragastric (−)-epicatechin at 2 mg/kg body weight as compared with vehicle (n=6). Inverse changes in vascular stiffness as measured by pulse wave velocity in (**D**) young and old WT mice, (**E**) ApoE knockout (KO) mice during cholesterol feeding, and (**F**) after (−)-epicatechin. *$P<0.05$ vs B and #$P<0.05$ vs old (RM ANOVA).

Together with the fact that the endothelium-independent nitroglycerin mediated vasodilation remained unaffected confirms that the FMD response requires the presence of an arterial endothelium. Interestingly, we observed a partial recovery of FMD at 24 hours, suggesting that this model may also be useful to study endothelial functional recovery during vascular regeneration and reendothelialization.

It is well established that endothelial dysfunction progresses with age.[7,8] We compared FMD in young, 3-month-old mice and old methuselah mice (>24 months of age). The aged mice exhibited a markedly decreased FMD by almost 50% as compared with young animals (Figure 4A). Furthermore, our data suggested that after injection of L-NAME, FMD decreased in both young and aged mice to similar low values, suggesting that the main reason for impaired FMD in old mice could be attributed to impaired NO-bioavailability.[9]

We moved on to study FMD longitudinally during diet-induced accelerated atherosclerosis. Chow-fed apolipoprotein E KO (ApoE KO) mice are known to develop foam cell lesions at 10 weeks of age. This process can be accelerated by Western diet[10] and mimics the evolution of atherosclerotic lesions in humans that usually develop over several decades. We followed FMD in 10-weeks-old ApoE KO mice before and during accelerated development of atherosclerosis via Western diet. FMD was measured weekly for a time period of 6 weeks (Figure 4B). Our results showed that in ApoE KO mice, the initiation of Western diet leads to a progressive decrease in FMD. Initial FMD of 24% gradually decreased over time to 12% at week 6. It was previously shown in humans that dietary bioactives can positively affect endothelial function. Flavanols, a subgroup of dietary plant–derived bioactives, have gained increasing attention, as clinical studies have shown that a higher intake of flavanol-containing foods can increase arterial function in individuals at risk for cardiovascular disease and with established cardiovascular disease.[11] Flavanols are one of a few bioactives known today, for which causality between the intake and an improvement in arterial function has been demonstrated.[12] We demonstrate in our model that intragastric application of (−)-epicatechin (2 mg/kg body weight) but not vehicle acutely increased FMD at 1 hour. Endothelial function is a major determinant of arterial stiffness. Because of the recorded electrocardiogram and Doppler flow recording, it was possible to simultaneously calculate pulse wave velocity. Aged mice exhibited a marked increase in pulse wave velocity compared with young mice (Figure 4D). Furthermore, pulse wave velocity increased significantly in ApoE KO on initiation of the Western diet, indicating progressive arterial stiffness during accelerated atherogenesis, indicating that arteriosclerosis developed in parallel (Figure 4E). Epicatechin, however, led to a significant decrease in pulse wave velocity along with improved FMD values (Figure 4F).To our knowledge, this is the first time that a model is presented, which enables the longitudinal in vivo analysis of the atherosclerotic process over a relatively short time period, allowing to put changes, both positive and negative, in endothelial function and vascular physicomechanics in direct relation with morphological changes in conduit arteries.
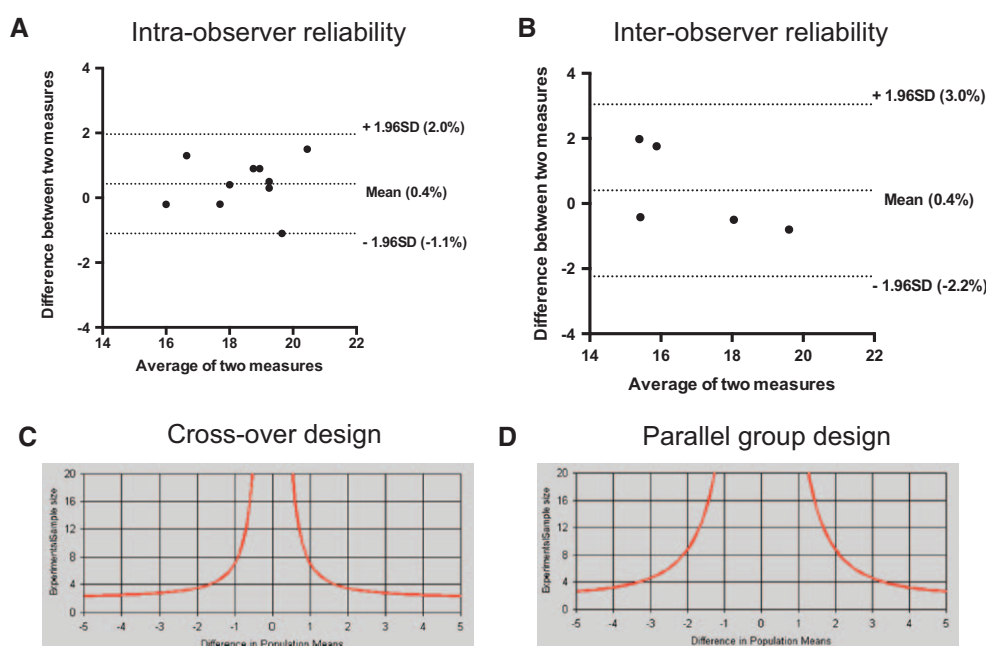

Figure 5. Intra- and interobserver analysis and corresponding sample size calculation. Bland–Altman plots showing (**A**) intraobserver and (**B**) interobserver reliability: mice were scanned twice by the same observer (n=10) or 2 different observers (n=5). *X*-axis are average flow-mediated vasodilation (FMD) values of both measurements and *Y*-axis represent individual differences in femoral artery FMD readings of the second measurement as compared with results of the first measurement. Dashed lines represent 1.96×standard deviation (ie, 95% limits of agreement) and mean deviation of repeated measurements (bias). **C**, Sample size analyses for FMD in mice showing necessary sample size as a function of minimally detectable difference (*P*=0.05, power 0.8) for paired samples in crossover design and (**D**) for independent samples in parallel group design based on the standard deviation of mean differences on repeated measurements (0.8%; intra individual variation) and standard deviation of population means (1.4%; inter individual variation), respectively, (PS Power and Samples Size Calculations Software V3.0).

To establish the reproducibility of our FMD model, intra- and interobserver variability was assessed by taking repeated measurements in mice.

Mice were either measured twice by the same sonographer or by 2 different sonographers. For intraindividual variability, the mean difference between measurements was 0.4% with a standard deviation of 0.8% and 95% limits of agreement of −1.1% to 2.0% (Figure 5A). The interobserver differences were 0.4% with a standard deviation of 1.3% and 95% limits of agreement of −2.2% to 3.0% (Figure 5B). Thus, this study shows that, presuming optimal sonographer training and constant environmental factors, FMD in mice can be assessed serially in a way that provides good intra- and inter-observer reliability with a low bias. Based on the standard deviation of differences between repeated measurements (intra-individual variation 0.8%) and standard deviation of FMD measurements in wild-type mice (1.4%), we performed a sample size analysis demonstrating the necessary n-value to detect a significant (power 0.8, α-error probability of 0.05) change in FMD in future cross-over and parallel group studies (Figure 5C and 5D).

Conclusions

Taken together, we present the fundamental physiology of FMD in the murine hindlimb using a new technique in mice that is analogous to human FMD measurements, the most widely used prognostically validated measure of endothelial function in humans mainly reflecting eNOS activity. The methodology is reproducible, enables to visualize changes in endothelial function, and to simultaneously investigate involved signaling cascades. Using young and aged wild-type, eNOS KO, and ApoE KO mice, we demonstrate that this approach can be used to assess in vivo vascular dysfunction in a range of genetically altered strains, allowing to noninvasively and longitudinally study pathophysiological processes and may be useful to investigate the potential and mechanisms of new therapeutic interventions.

Sources of Funding

Disclosures

None.

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Significance

Studying endothelium-dependent flow-mediated vasodilation has been impossible in transgenic mice to date. Using high frequency ultrasound, we reliably measured diameter changes of the femoral artery after ischemia in vivo. This was dependent on stimulation of endothelial nitric oxide synthase, completely abolished after pharmacological or genetic inhibition of endothelial nitric oxide synthase, similar to classical acetylcholine-mediated vasodilation, is endothelium-dependent, substantially impaired in aged mice and in ApoE knockout mice fed a high-fat Western-type diet, and improved by the polyphenol (−)-epicatechin. The flow-mediated vasodilation response in mice resembles that in humans. This novel technique allows for the first time to noninvasively, serially, and reliably quantify flow-mediated vasodilation in mice.

ONLINE METHODS

FMD protocol mice

The animal procedures were approved by the local authorities. The experimental setup is illustrated in figures 1 A+B. Male 10 to 12-wks old C57BL/6J mice were anesthetized with isoflurane (5 vol.-% induction and 2 vol.-% maintenance). Body core temperature was kept at 37°C or within ±1°C by using a heated examination table that was also equipped with EKG electrodes. The fur was removed from the hindlimbs and pre-warmed ultrasound gel was applied in access to the proximal inner thigh. The ultrasound probe was attached to a stereotactic holder and was manually aligned with the femoral vein visible at the upper inner thigh. We used the high-frequency, high-resolution digital VisualSonics imaging platform Vevo 2100 and a 30-70 MHz linear array microscan transducer (VisualSonics) to image the femoral arteries (FA) in mice. Using Duplex ultrasound mode, the artery could be easily identified by the typical pulsatile blood flow pattern as opposed to the adjacent vein with continuous blood flow. Arterial blood flow is confirmed by pulsed wave (PW) Doppler (Figures 1 C+D). In correspondence to sphygmomanometric blood pressure cuff during FMD-assessment in the brachial artery in human subjects, a vascular occluder (5 mm diameter, Harvard Apparatus) was placed around the lower limb to induce occlusion of the distal hindlimb as an ischemic trigger. The approach of placing the cuff around the upper thigh, proximal to the probe position, was for several reasons not pursued. The reasons included insufficient image quality due to displacement of FA during cuff inflation and the likelihood of formation of ischemic mediators other than NO at the site of FMD measurements.[1, 2]

Once the clearest image of the vessel wall was obtained and baseline readings recorded, the experiment was started by inflation of the vascular occluder. Inflation was carried out manually with an air-filled syringe. Vessel occlusion was confirmed by abrogation of directional arterial blood flow in Duplex Mode. Following hindlimb ischemia, the cuff was deflated and FA diameter measurements were continuously recorded for 3 min at 30 sec intervals. Online monitoring of FA blood flow velocity and heart rate by pulsed-wave (PW) Doppler was displayed as spectral Doppler curves. Off-line analysis of acquired images was performed from recorded loops using a semi-automated system (Brachial Analyzer, MIA, Iowa City) commonly used for human FMD analyses as described by Heiss *et al.*.[3] Briefly, the observer selects a region of interest in the longitudinal image of the FA. The software automatically traces the vessel lumen with accurate edge detection algorithms that can be manually edited – wall boundaries of the near and the far wall (M-line) of the selected vessel image – and quantifies the arterial lumen diameter [4] (Figures 1C+D). FMD was determined as $\Delta\%$ in average diameter change during reactive hyperemia as compared to baseline pre-ischemic values: [(Diameter$_{post-ischemic}$ – Diameter$_{baseline}$)∕Diameter$_{baseline}$] * 100. All diameter readings were taken at end diastole to limit the influence of potential differences in vascular compliance on diameter measurements. Wall shear stress (WSS) was calculated as 8 * µ * mean flow

velocity (V) / mean diameter, where blood viscosity (μ) was assumed to be constant at 0.035 dyn / sec * cm^2.

Human FMD

Brachial artery FMD and nitroglycerin-mediated vasodilation (NMD) using 400 μg of nitroglycerin sublingually were measured by ultrasound (Vivid I, GE) in combination with the automated analysis system (Brachial Analyzer, MIA, Iowa City) as described above.[5] Figure 1H shows a representative graph of flow velocity, WSS, and brachial artery diastolic diameter during reactive hyperemia following 5 min of forearm ischemia (representative example n=1).[6]

Mechanisms/signaling

To study the mechanisms underlying FMD in mice (10-12 wks old male), we measured FMD before and after injection of inhibitors for cyclooxygenase (COX), endothelium hyperpolarizing factor (EDHF) generated by cytochrome P450 epoxygenase (CYP2C9), arginase, and NOS in WT mice (C57BL/6J, n=8). In addition, we performed FMD measurements in eNOS-knockout mice (C57BL/6J, n=5) with and without NOS inhibitor. FMD was measured before and at 15 min after blinded intravenous injection of vehicle (0.3 ml saline/100 g body wheight (bw) or equal volumes of vehicle containing respective inhibitors: Indomethacin (0.2 mg/kg, Sigma), sulfaphenazole (Sigma), nor-NOHA (100 mg/kg bw Millipore) L-NAME (8 mg/kg bw, Sigma) on 3 different days.

We measured vasodilation in response to endothelium-independent vasodilator nitroglycerin and intra-arterial acetylcholine as an endothelium-dependent vasodilator. FA diameter and flow velocity were measured over 3 min following intravenous injection of nitroglycerin (0.4 µg/kg bw, Pohl) in WT (n=8) and eNOS (n=5) knockout mice. In order to further validate the model against a classic vasodilator, we intra arterially administered acetylcholine in the aortic arch through a catheter placed in the common carotid artery and measured the vasodilator response in the downstream femoral artery using ultrasound (n=5). After induction of isoflurane anesthesia, a catheter was inserted into the left common carotid artery and the tip advance to the aortic arch. Femoral artery diameter and flow velocity were measured at baseline and after consecutive injections of acetylcholine boli (100 μl) with acetylcholine at 10^{-9}, 10^{-6}, and 10^{-4} M. Measurements were taken at 30 sec intervals over 3 min following injections with 10 min breaks between injections. Measurements were performed with a washout phase of 7 days between measurements.

Measurement of eNOS phosphorylation

Thigh muscles were harvested at the time of peak FMD values (60 sec following 5 min of lower limb occlusion) and from the non-ischemic contralateral leg (n=5).

Preparation of protein lysates - Murine thighs were ground in liquid nitrogen and the resulting powder was lysed in lysis-buffer (20 mM TRIS-HCl pH 7,4, 1mM EGTA, 1 mM EDTA, 150 mM NaCl, 1% Triton100 f.c. + 1 tablet PhosphoSTOP + 1 tablet Protease Inhibitor cocktail (both Roche, Mannheim, Germany), for 30 min on ice. After removing cellular debris by centrifugation (16.000 x g, 15 min, 4°C), protein concentrations were measured using the Bradford reagent (BioRad, Munich, Germany).

Immunoblotting - Immunoblotting was performed with antibodies directed against eNOS (1:500, Abcam, Cambridge, UK) and phospho-eNOS (S1178, mouse) (1:500, Becton & Dickinson, Karlsruhe, Germany). Blotting membranes were incubated with primary antibodies overnight at 4°C before they were washed and incubated with secondary antibodies according to standard procedures. Detection was performed by enhanced chemiluminescence using the ECL reagent (GE Healthcare, Freiburg, Germany) and standard X-ray films. Semi-quantitative analyses were performed on scanned X-ray films using ImageJ 1.42q.[7]

Denudation experiment

Wire injury of the femoral artery *in vivo* was performed as follows. After induction of isoflurane anesthesia, baseline FMD measurements were performed in WT C57BL/6J (n=5) mice. The femoral artery was exposed distal to the inguinal ligament through an incision. This was followed by the dissection of the superficial epigastric artery (SEA) and its ligation approximately 1 cm distal to branching off the femoral artery. A second temporary ligation was placed at the proximal common femoral artery. After puncture of the SEA, a wire (0.05 mm, Conrad, Germany) was inserted and advanced to the proximal ligature and immediately removed. The SEA was ligated close to the femoral artery and the skin closed. FMD measurements were performed at 1 h after the wire pass with the animals still being under anesthesia and at 24 h under a second separate anesthesia.

Longitudinal atherosclerosis model

Chow-fed apolipoprotein E knockout (ApoE KO) mice are known to develop foam cell lesions at 10 wks of age. This process can be accelerated by high cholesterol Western diet [8] and mimics the evolution of atherosclerotic lesions in humans that usually develop over several decades. We followed FMD in 10-wks old ApoE KO mice (n=8) before and during accelerated development of

atherosclerosis via induction of Western diet. In parallel, we non-invasively determined pulse wave velocity, as a marker of progressive arteriosclerosis. FMD and PWV were measured weekly for a period of 6 weeks. Picrosirius Red staining for collagen deposition verified the presence of progressive arteriosclerosis (data not shown).[9]

Therapeutic (-)-Epicatechin application

FMD and PWV were measured in n=6 10 week old male WT C57BL/6J animals before and at 1 h after blinded intragastric application of vehicle or (-)-epicatechin (Sigma, 2 mg/kg body weight) a dose known in humans to increase FMD acutely.[10]

Measurement of vascular stiffness by PWV

Pulse wave velocity (PWV) is the most robust index of arterial wall stiffness and therefore regarded as the *in vivo* "gold standard".[11] The velocity of conduction of the pulse wave was accomplished by recording the temporal pulse profile at the ascending aorta and the femoral artery, providing an averaged PWV value over the distance of the two measurement points. PWV was computed by dividing the distance (Δd) between the suprasternal notch and femoral artery by the time delay (Δt) taken for the pulse wave to cover this distance.

Assessment of variability and statistical analyses

Assessment of inter- and intra-observer variability was based on FMD measurements performed by the some sonographer or by two different sonographers. Intra individual and inter individual variation of FMD in WT mice were based on repeated and group measurements by one investigator. Analyses were performed by one blinded operator. The Bland-Altman method for comparing paired FMD measurements was used to determine intra- and interobserver agreement (mean difference [bias], standard deviation of bias, 95% limits of agreement) using Prism 6.0 for Mac OS X, GraphPad Software Inc.. Sample size analyses were performed with PS Power and Sample Size Calculations Software Version 3.0 and graphs generated to show the necessary sample size (y-Axis) to detect a statistically significant difference (alpha=0.05, 1-beta=0.8) of FMD values (x-axis) for repeated measurements in the same animal (cross-over design) or between animals groups (parallel group designs) using the standard deviation of mean differences obtained after repeated measurements in one animal and standard deviation of average FMD values in untreated wildtype mice. All data are presented as mean ± standard deviation. Group differences were calculated with repeated measurements ANOVA and consecutive post hoc test when more than 2 groups were compared.

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Statistical analyses were performed with Prism 6.0 for Mac OS X, GraphPad Software Inc. Within Group differences were calculated by repeated measurements ANOVA or t-test if only 2 groups were compared. P-values of less than 0.05 were regarded as significant.

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