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Title: Assessment of tissue perfusion and vascular function in mice by scanning laser Doppler perfusion imaging.

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Keywords: Laser Doppler perfusion imaging; hindlimb; reactive hyperemia; nitric oxide synthase; age-dependent endothelial dysfunction.

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Abstract: Post-occlusive reactive hyperemia (PORH) is a key feature of physiological vasomotion to appropriately match the supply/demand ratio of tissues. This adaptive mechanism is severely disturbed in endothelial dysfunction with a reduced flow-mediated dilation (FMD). Reduced PORH and FMD are powerful prognostic risk factors in cardiovascular disease. While these parameters are frequently determined in human beings, comparable methods applicable to mouse models are sparse. We aimed to evaluate the applicability and accuracy of scanning laser Doppler perfusion imaging (LDPI) to measure PORH in the mouse hindlimb. Changes in mean perfusion in response to vasoactive drugs and PORH (assessed by scanning LDPI) were compared with changes in diameter and blood flow in the femoral artery, as assessed by high-resolution ultrasound. We found that the measured LDPI signal significantly correlated with changes of inflow into the femoral artery. Vasodilation induced by administration of nitroglycerine and acetylcholine increased vessel diameter, blood flow and mean perfusion, while vasoconstriction following administration of epinephrine decreased all three parameters. PORH was induced by temporal occlusion of the femoral artery with an external cuff. During occlusion, mean perfusion decreased to a condition of zero-perfusion and release of the cuff induced an immediate increase in blood flow that was followed by femoral artery dilation driving PORH/ perfusion. Surgical removal of the femoral artery decreased mean perfusion to a zero-perfusion level and fully abolished PORH. Importantly, the measurement of the PORH response by scanning LDPI is highly reproducible as determined by repeated measurements and intra/interobserver variation analysis. Last, we found that the PORH response was dependent on nitric oxide synthase and cyclooxygenase and declined with age. Thus, we here provide novel and robust non-invasive methods to serially measure tissue perfusion at baseline and during physiological and pharmacological modulation of vasomotor tone in the hindlimb of mice. The application of these LDPI

scanning and ultrasound-based methods may be useful for testing the effects of drugs affecting vasomotor function or future elucidation of mechanisms leading to vasomotor dysfunction in mice in vivo.

POINT BY POINT REPLY

Editor comments:

1. Please confirm that the suppliers and their location are provided in the manuscript text for every chemical used in this study.

Response. We confirm that suppliers and their location are provided.

2. Format your text as an outline (1. Introduction, 2. Materials and Methods, 3. Results, 4. Discussion). Remove the "Summary and Conclusions" header and integrate that text into "4. Discussion".

Response. The text has been now formatted as requested.

3. Collate the figure and table legends in the text file on one or two pages and upload the figures and tables as separate files. Be sure to provide your figures in the above recommended formats.

Response. We formatted figure legends as requested and checked that figures were in the recommended format.

Reviewers' comments:

Reviewer #1:

This manuscript entitled "Assessment of tissue perfusion and vascular function in mice by scanning laser Doppler perfusion imaging" by Leo et al. describes the non-invasive methods using laser Doppler perfusion imaging (LDPI) and high- resolution ultrasound to measure post-occlusive reactive hyperemia (PORH) in mouse hindlimb. This study provides novel method to examine vascular function and tissue perfusion in vivo. However, there are several issues need to be addressed before it can be published.

Response. Thank you for the careful evaluation of our manuscript!

The major points are the following:

1) In P. 9 Lines 16-29: The authors mentioned the changes in blood pressure and heart rate during the treatment with vasoactive drugs. It is recommended to show the cardiovascular parameters in additional Table.

Response. Data are now reported in Table 1. Thank you for pointing this out.

2) In Figure legend 2 (P. 20 Lines 54-55): saline and pharmacological controls were run on the same mouse. It is recommended to describe how many injections on the same mouse and the interval for each injection?

Response. Each mouse received two injections, the first being saline/vehicle and the second being the vasodilator/vasoconstrictor after a time interval of 5 minutes. This information was added to the text. Please see page 22, Line 22-25

3) The response in wild type C57Bl/6 mice after administration of NOS inhibitor (L-NNA) in baseline, Max, and PORH are increase in Table 2, but the effects after administration of L-NNA in wild type mice or effects in eNOS-/- mice in baseline, Max, and PORH are decrease in Table 3. Please input some comment in discussion about this point.

Response. The difference in the averaged data listed in the table noticed by this reviewer is not statistically significant and should not be considered (please compare the SD). The only parameters that are statistically different in both show changes in the same directions in table 2 and table 3, which were obtained by analyzing independent cohorts.

Minor comments:

1) The origin of eNOS-/- mice? and, the age of eNOS-/- mice used?

Response. This information was added in the text. Please see page 4, Line 7-11

2) In P.5 Line 17, the abbreviation of Nitroglycerin should be NTG.

Response. Corrected. Thank you.

3) The description in P. 8-9: Administration of adenosine (Fig. 2B), epinephrine (Fig. 2C), and acetylcholine (Fig. 2D) did not match to Fig. 2.

Response. Corrected. Thank you. There was a mistake in panel labeling!

4) The font size in Fig. 1C and Fig. 2 is too small.

Response. Corrected. Thank you.

5) Table 2 in P.27 should be Table 3.

Response. Corrected. Thank you.

Reviewer #2:

Based on the data presented in the submitted manuscript, the authors propose that the scanning LDPI is a robust and reproducible non-invasive method, which may be applied to evaluate pharmacological response to vasodilators/vasoconstrictors and PORH in mice models of cardiovascular disease. Thus, the authors conclude, that scanning LDPI of the PORH response in the mouse hindlimb can be applied for assessing vascular function in mice *in vivo*. However, some points need to be clarified.

Response. Thank you for your careful reading of the manuscript and the expert evaluation.

Major points

1. A number of past studies have focused on blood flow to a single limb (arm or leg) and extrapolated these findings to all vascular beds to determine systemic vascular function. However, there are clear limb differences in vascular control mechanisms (i.e., arm versus leg), and thus any conclusions drawn are likely specific to the vascular bed studied. If this difference does not exist in rodents, it still needs to be clarified by the authors. (See studies: Newcomer SC, Leuenberger UA, Hogeman CS, Handly BD, and Proctor DN. Different vasodilator responses of human arms and legs. *J Physiol* 556: 1001-1011, 2004 and Proctor DN, and Newcomer SC. Is there a difference in vascular reactivity of the arms and legs? *Med Sci Sports Exerc* 38: 1819-1828, 2006.

Response. Thank you for mentioning this important point. We indeed expect that there will be a difference between mouse anterior and posterior limb. There is a technical limitation of assessment of blood flow in the anterior limb, mainly due to the dimensions of the limb. Nevertheless, we now mentioned this important point in the discussion and cited the suggested references for completeness. Please refer to page 15 of the Discussion and to the reference therein.

2. As the author himself suggests, the LPDI technique cannot discriminate feeding arteries and their ramifications up to 3th and 4th order arteries or even microcirculation in ROI. Although the author reflects on the depth of the microcirculation technique in the skin in humans in comparison to animal experiments, it not shed light on the application of the LPDI technique in older animals that have noticeably increased body mass compared to young animals that keep the same ROI of interest.

Response. Thank you for making this point. Although the increase in body mass of the mice due to aging may appear big it is referred to as the overall animals. Differently from humans, the overall length/diameter of the limbs does not change as much that affect the ROI. What is commonly referred to in the literature is that the limbs are commonly subjected to modifications after an intense exercise of the mice (Experimental Evolution and Phenotypic Plasticity of Hindlimb Bones in High-Activity House Mice, Kelly et al., *Journal of Morphology*, 2006). Both forced and voluntary chronic exercise cause increased hindlimb bone diameter, mass, and strength. The changes affecting the limbs in mice are associated with an elastic adaptation of the animal that occurred following forced physical exercise, in which access to the wheel causes an increase in exercise with a consequent decrease in body mass and strengthening/thickening of the limbs. However, this change is not observed in animals of different ages (12-24 months) indicating a lack of correlation, in situations of stability, between length/diameter of limbs and body mass. In general, the body mass does not influence the size of the limbs in the mouse. Specific "longshanks" mice have recently been developed to identify landmarks for 3D geometric morphometric analyses of mouse limbs (Changes in shape and cross-sectional geometry in the tibia of mice selectively bred for increases in relative bone length, Cosman et al., *J Anat*, 2016). It would be interesting to evaluate those with LDPI.

In addition, nothing is commented about the microcirculation in the skeletal muscle, which surrounds the femoral artery and is most likely part of the LPDI analysis and this should be commented in the discussion section. An experimental pharmacological alternative could be modulate the participation of β 2R adrenergic that seems to mediate vasodilation primarily in skeletal muscle.

Response. Thank you for making this point, which allows us to clarify matters. We now rephrased the discussions referring to the fact that LDPI in mouse detects both macro- and microcirculation to make

this point clearer (Please refer to make this point clear on page 13 and page 15). We added your suggestion to use beta-2 adrenergic agonists in the skeletal muscle to determine that.

--- Although there is a relationship between vasodilation and increased velocity flow in feed artery it is still very uncertain if a close relationship occurs between this two phenomena. For example, in humans the velocity peak occurs within the first 15 seconds and the vasodilation peak can be expected to occur 45-80 seconds following cuff release which may mean difference between species. Please, see Mark A. Black, N. Timothy Cable, Dick H.J. Thijssen, Daniel J. Green. Importance of Measuring the Time Course of Flow-Mediated Dilatation in Humans, 2008.

Response. Thank you! We now mentioned this comparison with the human data and the references suggested at the end of the discussion at page 15.

---In LPDI analyses, interesting results show that peak time is dependent on prostaglandin and NO, however, does not affect the magnitude of peak perfusion. Results of Heiss et al., 2008 show that the inhibition of NOS (L-NMMA) almost exclusively abolished femoral artery (FA) vasodilation without affecting flow velocity and WSS (Shear Stress) see: Am J Physiol Heart Circ Physiol 294: H1086-H1093, 2008. (ex. How could peak time delay be related to fail of dilation? These differences between species and vascular parameters among the techniques can be better explored by the authors in discussion session.

Response. Thank you! We now mentioned this point on page 15.

In addition, a correlation analysis between different vascular parameters could be useful and show more strength to the results (eg. magnitude of the flow velocity and the magnitude of the dilation, and the relationship with the time at which these two phenomena occur).

Response. We agree with the reviewer that a direct correlation between each parameter measured by FMD and LDPI would give powerful data. This kind of correlation would need data obtained in the same mouse and in the same conditions and ideally even repeated measurements to be meaningful. The data set we have does not allow this correlation, but we will take this into account in future studies! Thank you!

---The authors provide little evidence on how diameter analysis was performed. The authors cited the work of Heiss et al., (2008). Studies which have assessed the diameter at multiple time points after cuff deflation may fail to identify the true peak diameter if this falls between, or outside, the selected discrete measurement periods. In addition, despite reviews have been published regarding the methodology used to assess FMD these do not describe the optimal method for assessing time-to-peak diameter. The post-occlusion diameter analysis can serve as a tool for calculating blood flow during all reactive hyperemia. Undoubtedly, the recommended practice is to measure diameter and velocity simultaneously (frame to frame) during the preferred approach to capture reactive hyperemia appropriately.

Response. Diameter analysis was performed on discrete frames by using the software "brachial analyzer" (MIA). Unfortunately, in this set of experiments we did not save the movies, but just discrete frames; so as correctly pointed out by this reviewer we cannot determine time-to-peak diameter correctly. These suggestions will be taken into account for further development and optimization of these methods for future studies.

---The vascular function and the local blood flow regulation is a result of multiple physiological inputs and may be influenced by systemic responses. In the Methods section, the authors described that they have made some blood pressure measurements. However, this data has been omitted from the manuscript and I recommend that this data should be included.

Response. Data are now reported in Table 1. Thank you for pointing this out.

Furthermore, the results clearly show that vasoactive substances could modify perfusion in the ultrasound and LPDI technique. In fig 2 (left) please indicate the time point in which the drugs were administered. Apparently, the curves of the percentage diameter (left) and perfusion (middle) after the infusion of vasoactive substances are not corresponding.

Response. In figure 2 left panels we overlapped two independent experiments, done in the same mouse with a least 2 hours of recovery in between. The overlapping was done to demonstrate what is the relationship between the responses. However, the curves represent the mean of all curves obtained in different individuals. For a perfect overlapping of the curves, we should have carried out the curves that were overlapped by the time point of the injection.

---Repeated pressure induced by cuff occlusion usually potentiate the dilation response (see review: Skeletal muscle vasodilatation at the onset of exercise; Clifford 2006). As the occlusion experiments were performed with the same animal, it is strongly suggested to clarify the time interval between each evaluation in the methods section.

Response. We now added the time interval as requested. The time interval between treatment with L-NNA or indomethacin was 1 week, which should not affect the PORH response.

---Interesting knockout animals for eNOS enzyme results suggest in general, sources of NO production other than eNOS-induced are important for reactive hyperemia or that compensatory mechanisms are occurring in eNOS knockout animals to produce reactive hyperemia. However, one limitation of the present study is that the number of animals per group is very different from one set of experiments to another which can make it difficult to track the results. For example: After 6 days of L-NNA administration (n = 19), AUC was reduced (Table 2). However, 6 days of L-NNA (n = 5) AUC administration was unchanged.

Response. Thank you! The reviewer is right. Please consider that n = 5 indicates intraindividual measurements and n = 19 correspond to all individuals measured in the study and show statistical significance because of the increase in the number of individuals measured.

Minor points:

The manuscript has numerous editing errors and therefore a complete revision of it is strongly recommended (example...).

- P14 Line 29 (...of endothelial ... in vivo: PROH vs. FMD)
- P15 Line 1 (... variable connecting ...)
- P19 Line 43 (... will comprise both ... in the mouse) → page 20 line 11-14

Also, the unit of measurement for femoral artery depth is different (P12 Line 54 and P19 line 38).

Response. Thank you! We corrected these mistakes! Please see:

(..of endothelial ..) – page 14 line 28;

(..variables connecting ..) – page 15 line 8

(..will comprise both ..) – page 20 line 15

The unit of measurement for femoral artery depth has been corrected into 4mm – please refer to page 8 line 15.

Figures and legends:

-- Please, check Fig. 2B (left). The graph does not match the description of the results. Also, in the legends only panel (A) is indicated. This is repeated in other subtitles. Please, be consistent.

Response. Thank you! We corrected these mistakes! These were due to wrong labeling of the panels/panel exchange and we apologize for this mistake!

--In Fig 4 A and B it is not clear the MPU (axis Y) values for reactive hyperemia at different occlusion times.

Response. We sincerely apologize, but we do not understand this comment.

Reviewer #3:

This is an interesting study demonstrating in the living mouse that the authors' measurement of perfusion in the hindlimb by scanning laser Doppler perfusion imaging can provide sensitive physiological and pharmacological changes in vessel diameter. While the experimental design is well executed with novel methods being used, the data obtained in this study are well presented, and the paper is carefully written, I have a number of concerns for the authors to consider.

Response: Thank you for thi encouraging and very positive comments.

1) Page 6, paragraph 3: "To control for femoral artery perfusion signal chronic hindlimb ischemia was induced by surgical excision of the femoral artery, according to the protocol described by Limbourg et al. (18)." A brief but more detailed explanation for this should be provided.

Response. A more detailed description was provided. Please refer to the methods section on page 6.

2) Page 6, paragraph 4: "Indomethacin (but not indomethacine) was administered in drinking water for 6 days at a dose (but not concentration) of 30 microg/mL." How did the authors dissolve indomethacin? Indomethacin is a lipophilic drug that is poorly soluble in water. Its dissolution method should be mentioned.

Response. The reviewer is correct. Indomethacin was dissolved in ethanol and diluted into drinking water to a concentration of 30 $\mu\text{g/mL}$ (final ethanol concentration was 1%). The resulting dose for 1 mouse is 150 μg / day / (or 5 mg/Kg/day) .

3) The legend of Figure 2: (B), (C), and (D) are lacking.

Response. Thank you. We added the numbering (B-D) in the legend to figure 2.

4) Figure 2: Perfusion responses were measured before and after the treatment? If so, what is the reason why multiple comparison test was performed? On the other hand, in Figure 5A, statistical analysis for PORH responses before and after L-NNA treatment was made by paired t-test.

Response. To compare the responses of different treatments before/after the treatment we applied repeated measurement (RM) 2-way ANOVA (2 variables for before/after treatment). Paired T-Test (adjusted for multiple comparisons) was chosen for comparison of selected pairs, which are clearly identified in the picture by using specific symbols. Please refer to GraphPad Prism 8 guide on repeated measurements 2-way ANOVA for these analyses. Please refer also to H.J. Motulsky "Essential Biostatistics", ISBN: 978-0199365067

5) Figure 5A: It seems that PORH responses before and after L-NNA or indomethacin were compared using the same animals. However, L-NNA and indomethacin were administered in drinking water for 6 days. The authors should show no changes in PORH responses when mice were given drinking water with their vehicles for 6 days.

Response. The results were provided in table 3.

6) Finally, no biochemical and molecular biological data are found in the text. The main experiments stem from novel physiological/functional techniques. What is the reason why the authors chose Biochemical Pharmacology as the submission of this paper?

Response. Thank you. This paper is focused on the physiological and pharmacological characterization of vascular response in genetically modified mice, which may help to identify biochemical pharmacological targets. This paper was submitted after consultation with the editors for a special issue on nitric oxide signaling. The editors judged this paper as appropriate for biochemical pharmacology.

Reviewer #4:

The MS Leo et al. addresses the possibility of using scanning laser Doppler perfusion imaging (LDPI) to measure post occlusive reactive hyperthermia (PORH). The study was conducted in preclinical model of non immunodepressed mice.

The study is very interesting, rigorously performed and clearly demonstrates the possibility to evaluate pharmacological response to classical vasodilators/vasoconstrictors. The study should be of interest for "Biochem Pharmacol" readers.

Response. Thank you so much for these encouraging comments. We are glad to see our paper under consideration for publication in Biochemical Pharmacology.

Comments

Different ages of mice were used in this study. No differences in weight were noted between 48- and 96-weeks ages mice groups, probably indicating slowing down of metabolic processes.

Response. We kindly request to refer to the table published by Jackson laboratories on this mouse strain (C57Bl6) <https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-aged-b6>. This table lists how the weight of C57BL/6J mice changes weekly until 78 weeks. There is only a slight increase, which is probably not significant because of the limited number of mice/group.

Could this aging induce blood flow changes beyond authors expectations related to less sustained hyperemia?

Response. Thank you for this comment. The purpose of the experiment was indeed to evaluate how aging processes would affect reactive hyperemia. A metabolic decline and increase in endothelial dysfunction in aging vessels would also reduce hyperemia. As the reviewer pointed out in the previous comment, aging is a complex process involving also metabolic changes. With the methods presented here coupled with metabolic profiling, it will be possible to determine the effects of metabolism on endothelial function during aging.

Can the authors comment on the advantages/disadvantages of LDPI compared with OxyFlow system. The latter is widely used for preclinical studies.

Response. The ideal method of measuring skin blood flow would be both non-invasive and accurate. OxyFlow devices are laser Doppler flowmetry (LDF) monitors intended for monitoring of relative microvascular blood flow in living tissues. Most applications are concerned with monitoring the competence of regional microvascular blood supply in peripheral vascular disorders, models of stroke and brain injury, angiogenesis and more. Laser Doppler Pressure Imaging (LDPI) can be used to measure superficial blood flow in the skin and other vascularized tissues.

The LDF method has been widely used, but one of the main disadvantages of the technique is its low spatial resolution (~1 mm²) and the need to apply the probe directly to the tissue that needs to be studied, thus interfering with the flow if undue pressure is used. In addition, movements during the measurements may result in artifacts that make interpretation of the results difficult. However, in the new devices an artifact rejection filter has been introduced, able to eliminate transient motion artifacts in the blood flow signal. On the other hand, tissue perfusion frequently shows a spatial variation resulting in a significant difference in perfusion values, even at adjacent measurement sites. To overcome these most studies have relied on taking multiple readings with mean values then being calculated.

The disadvantages of LDF are largely overcome if the mapping of the spatial tissue perfusion by LDPI is used. With this technique, there is no contact with the skin and the scanner cannot itself influence the blood flow as can happen with the probe-based LDF. Technically there are also advantages including the possibilities to further image processing such as spatial averaging subtraction of images, contrast manipulation and quantitative measurement of areas and flow. The new technique of LDPI shows, therefore, advantages over the more established techniques of LDF for assessment of microvascular perfusion skin and other organs in a variety of clinical and research contexts.

Assessment of tissue perfusion and vascular function in mice by scanning laser Doppler perfusion imaging

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Running title: Hyperemia measurement in mice

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Abstract

Post-occlusive reactive hyperemia (PORH) is a key feature of physiological vasomotion to appropriately match the supply/demand ratio of tissues. This adaptive mechanism is severely disturbed in endothelial dysfunction with a reduced flow-mediated dilation (FMD). Reduced PORH and FMD are powerful prognostic risk factors in cardiovascular disease. While these parameters are frequently determined in human beings, comparable methods applicable to mouse models are sparse. We aimed to evaluate the applicability and accuracy of scanning laser Doppler perfusion imaging (LDPI) to measure PORH in the mouse hindlimb. Changes in mean perfusion in response to vasoactive drugs and PORH (assessed by scanning LDPI) were compared with changes in diameter and blood flow in the femoral artery, as assessed by high-resolution ultrasound. We found that the measured LDPI signal significantly correlated with changes of inflow into the femoral artery. Vasodilation induced by administration of nitroglycerine and acetylcholine increased vessel diameter, blood flow and mean perfusion, while vasoconstriction following administration of epinephrine decreased all three parameters. PORH was induced by temporal occlusion of the femoral artery with an external cuff. During occlusion, mean perfusion decreased to a condition of zero-perfusion and release of the cuff induced an immediate increase in blood flow that was followed by femoral artery dilation driving PORH/ perfusion. Surgical removal of the femoral artery decreased mean perfusion to a zero-perfusion level and fully abolished PORH. Importantly, the measurement of the PORH response by scanning LDPI is highly reproducible as determined by repeated measurements and intra/interobserver variation analysis. Last, we found that the PORH response was dependent on nitric oxide synthase and cyclooxygenase and declined with age. Thus, we here provide novel and robust non-invasive methods to serially measure tissue perfusion at baseline and during physiological and pharmacological modulation of vasomotor tone in the hindlimb of mice. The application of these LDPI scanning and ultrasound-based methods may be useful for testing the effects of drugs affecting vasomotor function or future elucidation of mechanisms leading to vasomotor dysfunction in mice *in vivo*.

Keywords: Laser Doppler perfusion imaging; hindlimb; reactive hyperemia; nitric oxide synthase; age-dependent endothelial dysfunction.

1. Introduction

A wide range of animal models to study endothelial dysfunction are available, including hyperlipidemic mice and rats, spontaneously hypertensive rats, genetically modified mice lacking/overexpressing specific apolipoproteins (e.g. apoE or apoB), hypertensive mice lacking the endothelial nitric oxide synthase (eNOS) and surgical models of vessel injury (1). In these mice models, endothelial and vascular function is frequently studied *ex vivo*, which has provided valuable new insight into vessel wall function. However, vascular function and blood flow **are** a result of multiple physiological inputs. Here, we propose a new method to examine the vascular function and tissue perfusion *in vivo*.

Post occlusive reactive hyperemia (PORH) is the physiological increase of blood flow to the tissue following ischemia after transient arterial occlusion. PORH has been applied for testing the endothelial function of **conductance** vessels in humans, e.g. by measurement of flow-mediated dilation (FMD) (2, 3), venous occlusion plethysmography (VOP), or skin microcirculation by laser Doppler flowmetry or imaging (4, 5). Scanning laser Doppler perfusion imaging (LDPI) has been applied in clinical settings to assess functional responses of the cutaneous microcirculation to vasoactive molecules or post occlusive reactive hyperemia (PORH) in patients with endothelial dysfunction due to hypertension (6), peripheral arterial disease (7), coronary artery disease (5), diabetes (8), smoking (9) (10) or age (11). In swine and dog animal models scanning LDPI was applied for measuring revascularization in wound healing (12, 13), mucosal microcirculation (14), or retinal capillary blood flow (15). In rodents and especially in mice scanning LDPI is classically used to study angiogenesis and neovascularization after hindlimb ischemia (16-19), but it has never been applied to serially study vasomotor function in mice.

In this study, we aimed to evaluate the applicability of scanning LDPI to measure PORH in mice and hypothesized that it would be suitable to characterize tissue perfusion and changes in vascular function. We found that scanning LDPI is a robust and reproducible **non-invasive** method, which may be applied to evaluate pharmacological response to vasodilators/vasoconstrictors and PORH in mice models of cardiovascular disease.

2. Materials and Methods

Animals

All procedures were conducted in conformity with the American Physiological Society's "Guiding principles for research involving animals and human beings" and were approved by the LANUV Nordrhein-Westfalen. Mice were fed standard laboratory chow ad libitum. Male C57/BL6 mice were purchased from Janvier SAS (Le Genest, France). Global eNOS KO mice(20) were kindly provided by A. Gödecke. Department of Physiology, Medical Faculty, Heinrich Heine University of Düsseldorf. Mice of the following ages were randomly assigned to groups and used for experiments: 8 ± 2 weeks (wk)-old (22 - 25 g), 24 wk-old (30 - 35 g), 48 wk-old (37 - 47 g) and 96 wk-old (34 - 45 g) mice.

Scanning LDPI

Scanning LDPI measurements were performed by using a PeriScan PIM 3 System and proprietary acquisition software (LDPIwin) (both by Perimed-Instruments, Järfälla, Sweden). The experimental setup is presented in Fig. 1. Mice were anesthetized with isoflurane (Piramal Critical Care, USA) (3% induction and 2% maintenance) and fixed on a heated platform (set to 39°C, body temperature approx. 36°C) in a supine position. Body hair was removed from the inner thigh using a depilation cream (Veet, Reckitt Benckiser, Germany). A measuring distance of 10 cm, a scanning area of 5 x 3 cm and a scanning velocity of 1 image/second were chosen and kept constant throughout the measurements. The signal was quantified as mean perfusion units (MPU). Baseline values were taken for 15 minutes (min) before starting the interventions. Data analysis was performed off-line using LDPIwin software (Perimed-Instruments).

High-resolution ultrasound

The femoral artery was visualized by using a VEVO2100 high-resolution ultrasound device equipped with ultra-high frequency transducers (MS400: 18-38MHz, MS700: 30-70MHz) and proprietary software (VisualSonics, Toronto, Canada), as previously described (21). Mice were anesthetized and prepared as described for LDPI. The femoral artery was visualized by its characteristic flow pattern in color doppler mode, PW-Mode, and B-Mode. Flow velocity, heart rate (HR), respiration rate (RR) and body

temperature were determined off-line with the dedicated software. Vessel diameter (d) was evaluated by using the Brachial Analyzer 5 software (Medical Imaging Applications, Coralville, IA).

Treatment with vasoactive drugs

All stock solutions were freshly prepared on the experimental day with warm (37°C) sterile saline (NaCl 0.9%) as a vehicle. The effects of injection of the vehicle control were tested in the same groups of animals before injecting the vasoactive substances. The doses applied were chosen according to preliminary tests carried out by measuring changes in vessel diameter by ultrasound (22, 23).

Nitroglycerin (NTG). NO-dependent and endothelium-independent vasodilation were induced by bolus **intraperitoneal** (i.p.) administration of 12 mg/kg NTG in 100 µl vehicle (0.9% NaCl) (stock 1 mg/ml, G.Pohl-Boskamp, Hohenlockstedt, Germany) or vehicle only. Readings were taken immediately after injection as described above.

Adenosine (Ado). To pharmacologically mimic the blood flow increase during reactive hyperemia, we administered 0.1 mg/kg bolus dose of adenosine in 100 µl vehicle (stock 10^{-4} mol/l; Sigma-Aldrich, St. Louis, USA) into the tail vein.

Epinephrine (Epi). To induce **vasoconstriction**, we used a local subcutaneous injection of 0.7 mg/kg epinephrine in 100 µl vehicle (stock 10^{-3} mol/l; Sigma-Aldrich) into the upper thigh.

Acetylcholine (ACh). To induce endothelium-dependent vasodilation, 0.07 mg/kg acetylcholine in 100 µl vehicle (stock 10^{-4} mol/L; Sigma-Aldrich) was injected into the tail vein. Acetylcholine stock solutions were prepared freshly with the vehicle on each day.

Induction of post-occlusive reactive hyperemia (PORH)

PORH was induced by using an air inflatable vascular occluder with a diameter of 10 mm (KentScientific, Connecticut, USA) as described (22, 23). Briefly, the hindlimb of a mouse was pulled through the cuff (see Fig. 1A). To induce PORH the cuff was inflated at a pressure of ~300 mmHg. Occlusion was confirmed by measuring distal blood flow (mean perfusion < 30 MPU) in preliminary experiments. After occlusion, the cuff was rapidly deflated. The following experimental protocol was used for both LDPI and ultrasound measurements: 15 min. equilibration period, 7 min. baseline

measurement (stabilization of perfusion), 5 min. ischemia (zero perfusion) and 8 min. of reperfusion (hyperemia and recovery of perfusion).

Parameters used for quantifying the PORH measured by scanning LDPI are described in Fig. 3A: 1) baseline perfusion; 2) maximal perfusion; 3) perfusion reserve as $\Delta\% = (\text{max. reperfusion} - \text{baseline}) / \text{baseline} \times 100$; 4) ratio = max. reperfusion / baseline; time to peak = time to maximal perfusion after releasing the cuff; and area under the curve (AUC) = integral of the reperfusion curve.

Changes in the diameter of the femoral artery and flow after PORH were assessed by high-resolution ultrasound. The dilation of the femoral artery is analyzed as % flow-mediated dilation (FMD) by comparing the diameter change after occlusion in relation to the basal diameter, as described (21).

Effects of physiological and pharmacological modulation of the PORH response.

To control for femoral artery perfusion signal chronic hindlimb ischemia was induced by surgical excision of the femoral artery, according to the protocol described by Limbourg et al. (18). Briefly, mice anesthetized i.p with ketamine (100 mg/kg) and xylazine (5 mg/kg) were positioned in dorsal recumbency. The skin was incised over the femoral artery. The femoral artery was isolated ligated and excised in its entirety.

The effects of inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX) on the PORH response were analyzed by the administration of the NOS inhibitor nitro-L-arginine (L-NNA, Sigma-Aldrich), the COX inhibitor indomethacin (Sigma-Aldrich) and in mice lacking the endothelial NOS (eNOS^{-/-})(20). L-NNA or indomethacin was administrated in drinking water for 6 days. L-NNA was dissolved in 1 M HCl (stock 160 mg/ml) and diluted to a final concentration of 1.6 mg /ml (and 0.01 HCl) in drinking water, resulting in a daily dose of 8 mg L-NNA / day / 1 mouse. Indomethacin was dissolved in ethanol and diluted to a concentration of 30 µg / ml, resulting in a daily dose of 150 µg/day (and 1% ethanol). Corresponding vehicle controls were used in separate animal groups. In a subgroup measurements were conducted longitudinally before and 6 days after administration of the drugs.

Blood pressure measurements in mice

Mean arterial blood pressure in mice was measured in the aorta ascendens using a 1.4F SPR-839 pressure catheter from *Millar Instruments* (Houston, TX, USA) placed into the aorta through the right carotid artery. Pressure data were analyzed with dedicated software (IOX, EMKA, Paris, France) to determine peak systolic (SBP) and diastolic pressure (DBP).

Statistical Analyses

Values reported are means $\pm$ SD or as box plots according to Tuckey. Comparisons between groups were made using either a 2-sided student's t-test or 1- way or 2-way ANOVA, followed by an appropriate post-hoc test as indicated in the figure legends and the tables. Differences were deemed significant when $p < 0.05$. Statistical analyses were performed using GraphPad Prism 8.00 (GraphPad, San Diego, CA, USA) or SPSS 18.0 statistics (IBM).

3. Results

Visualization and quantification of blood flow and mean perfusion in the hindlimb by scanning LDPI and high-resolution ultrasound.

In this study, we aimed to validate the measurement of hindlimb perfusion by LDPI in the scope to evaluate physiological and pharmacological changes of vascular function in vivo. The experimental setting is depicted and described in Figure 1. Mice are anesthetized with isoflurane and fixed in a supine position and the hindlimb cleared of hair. A vessel occluder was applied for inducing post-occlusive reactive hyperemia (Fig. 1A). The scanning head is adjusted to a standardized distance of 10 cm from the measuring surface (Fig. 1B). When laser light penetrates the tissue (Fig. 1C) it is partly absorbed or scattered and light particles, which hit moving blood cells, undergo a change in wavelength (Doppler shift). As depicted in Fig. 1C, the scanning LDPI signal is mainly dependent on the penetration depth of the laser (estimated to be between 0.5 and 1 mm) and on the blood flow in the vessels encountered by the laser within the tissue. The femoral artery was found at 0.4 mm penetration depth, as visualized by high-resolution ultrasound (Fig. 1D, left panel). In its proximity, the perfusion map obtained by scanning a 4 x 8 mm area showed a discrete region with a very high signal (Fig. 1D, right panel), which was quantified as mean perfusion in a gated area and indicated in mean perfusion units (MPU).

Modulation of blood flow and mean perfusion by vasoactive molecules.

To analyze how changes in mean perfusion correlate with changes in blood flow in the femoral artery, we applied different well-characterized pharmacological agents inducing vasoconstriction or -dilation and measured both mean perfusion by scanning LDPI and blood flow by high-resolution ultrasound (Fig. 2). Systemic i.p. injection of a 12 mg/kg NTG bolus rapidly increased vessel diameter and flow within the femoral artery as assessed by ultrasound (Fig. 2A, left panel) and increased mean perfusion as compared to baseline, reaching a maximum within approximately 100 s (Fig. 2A, middle), whereas the vehicle control had no effect. The maximal increase in mean perfusion after administration of NTG was 128% as compared to vehicle control (435 ± 23 MPU after NTG vs. 191 ± 36 MPU $n = 5$, Fig. 2A, right panel). Administration of the adrenergic receptor agonist epinephrine (adrenaline) s.c. caused a rapid vasoconstriction as demonstrated by decrease in vessel diameter and flow (Fig 2B,

left panel), and a rapid 57% drop in mean perfusion (90 ± 18 MPU vs. 209 ± 59 , $n=4$, Fig. 2B, right panel), which lasted throughout the entire measurement interval (Fig. 2B, middle panel). Intravenous injection of the endothelium-dependent vasodilator acetylcholine caused a significant increase in vessel diameter and flow (Fig. 2C, left), and mean perfusion (Fig. 2C, middle and right), reaching a maximum of 132% within 300 sec ($n = 5$, 537 ± 35 MPU vs. 406 ± 48 MPU baseline). Stimulation of purinergic receptors by i.v. bolus administration of adenosine significantly increased vessel diameter and later on peak flow velocity (Fig. 2D, left panel), as well as mean perfusion within approximately 150 s (Fig. 2D, middle), with a maximal increase of 41% as compared to baseline values (316 ± 43 vs. 224 ± 34 MPU baseline) (Fig. 2D, right panel). The LDPI signal remained unaffected after vehicle injection. During the ultrasound measurements, there was no significant change in heart rate (HR), respiration rate and body temperature (Table 1). As expected, systolic and diastolic blood pressure measured in the aorta by Millar catheterization were significantly transiently decreased by all vasodilators applied and increased by epinephrine (Table 1). Taken together, our results show that changes in blood flow in the femoral artery as a result of the administration of well-characterized vasodilators and vasoconstrictors directly correlate to changes in mean perfusion, as assessed by LDPI.

Measurement of the PORH response by scanning LDPI

PORH was induced by inflating a cuff at a pressure of ~300 mmHg until reaching the point of zero perfusion (mean perfusion < 30 MPU), and then rapidly deflating it (Fig 3). Five minutes of occlusion time led to a rapid (< 1 min) FMD of the femoral artery accompanied by an increase in mean perfusion up to 200% as compared to baseline values, which then returned to baseline after 5-6 minutes (Fig 3). We found that the peak of maximum perfusion corresponded to a maximum dilation of the femoral artery (Fig. 3C); however, the increase in perfusion lasted longer than the vasodilatory response of the femoral artery. The peak of mean perfusion after PORH ($150 \pm 15\%$) was similar to the maximum NTG-induced perfusion increase ($149 \pm 42\%$) and significantly higher than the Ach ($38 \pm 12\%$) and adenosine ($43 \pm 8\%$) induced responses. The parameters used to quantify PORH are described in Figure 3A (also see Methods for details).

PORH in chronic hindlimb ischemia

If the femoral artery was surgically excised, mean perfusion was as low as zero perfusion after inducing transient ischemia in control animals (Fig.3A grey curve), and the PORH response was absent (Fig. 3A, gray curve).

Effects of occlusion time and reproducibility of measurements of PORH by scanning LDPI

To test the dependency of the PORH response on the duration of ischemia as assessed by LDPI, we occluded the hindlimb for 1, 3, and 5 min (Fig. 4 A-C). By increasing the occlusion time, we measured an increase in maximal perfusion, ratio, delta percent of the response and time-to-peak, while the AUC © was only significantly increased after 5 minutes of ischemia (Fig. 4 A-D; * $p < 0.05$, $n = 5$ independent measurements carried out on the same mouse). These data were also further analyzed to compare intra-individual variability with inter-individual variability of the different parameters by comparing their coefficient of variation (Table 1). We found the smallest variation for time to peak and perfusion reserve, while AUC measurements showed the highest variability.

The dependency of the PORH response on the availability of endogenous vasodilators.

To investigate the role of NOS-dependent NO synthesis, mice were treated with NOS-inhibitor L-NNA (1.6 mg/ml = 270 mg/kg / d) in drinking water for 6 days. NOS inhibition significantly decreased the rapidity of the PORH response with a greater time to peak determined both in intraindividual (before –after) measurements. (Fig. 5 A, Table 2) and in different groups (Fig. 5B and Table 3). Other parameters were unchanged (Tables 2 and 3). Mice with a genetic deletion of eNOS (eNOS^{-/-}) show increased time-to-peak (Fig. 5B and Table 3), but no changes in other parameters. To determine the role of COX-dependent prostaglandin production, the cyclooxygenase inhibitor indomethacin (30 µg/ml = 5 mg/kg/d) was administered to mice in drinking water for 6 days (Fig. 5C), which resulted in an increase in time-to-peak, but had no effect on the other parameters (Table 2).

1 **The dependency of the PORH response on mice age.**

2 As further proof of **concept**, we additionally determined PORH in wildtype mice cohorts
3 of different **ages** (Fig. 6). The rapidity of hyperemic reperfusion is altered with a
4 prolonged time to peak (Fig. 6A) and a faster perfusion drop to baseline values in older
5 mice (Fig. 6B). **Furthermore**, we found that 12 months and 24 months old mice have
6 a significantly diminished AUC (Fig. 6D) and % perfusion increase of PORH (Fig. 6C),
7 as compared to younger mice.

8 Taken together, these data show that the measurement of perfusion in the mouse
9 hindlimb by LDPI is sensitive physiological and pharmacological changes in vessel
10 diameter and is valid for evaluating intraindividual changes for significant in vivo
11 investigations with limited numbers of animals.

4. Discussion

In this study, we evaluated the potential of measuring the PORH response by scanning LDPI. We show that 1) changes in flow in the femoral artery due to administration of vasoactive drugs correlate to changes in mean perfusion in the hindlimb; 2) the PORH response (changes in mean perfusion by LDPI) correlate to changes in diameter (as FMD% by ultrasound) 3) measurements of the PORH response by scanning LDPI are reproducible, are modulated by intervention with NOS and COX inhibitors, and decrease with increasing animal age. Thus, we propose that **measurements** of PORH by scanning LDPI might be applied for the assessment of changes in vascular function in mice models of cardiovascular disease.

What is scanning LDPI measuring?

Scanning LDPI is based on the physical phenomenon defined as the Doppler shift or Doppler effect, which is a change in the observed frequency of a wave occurring when the source and observer are in motion relative to each other. When laser light penetrates a tissue, it is backscattered and returns to its source without change of frequency. But if the laser light is backscattered by moving objects (i.e. blood cells), it undergoes a shift in frequency directly proportional to their concentration and velocity. The *measuring depth* of the laser, i.e. how deep the laser light penetrates the tissue, depends (a) on the energy and wavelength of the laser, (b) on the measuring distance and, most importantly, (c) on biophysical and anatomical characteristics of the tissue, which can influence its backscattering properties; these include (but they are not limited to) density and pigmentation of the skin and presence of hair, as well as density and diameter of the vessels (microcirculation, resistance vessels, macrocirculation), amount of blood cells.

The perfusion map obtained by scanning a defined area of the hindlimb shows a discrete region of very high signal intensity, which corresponds to the area of tissue with and around the femoral artery. According to the manufacturer's information, the measurement depth in our system in human skin has been calculated to be around 1 mm. **In the mouse, the** femoral artery can be visualized at 0.4 mm penetration depth with high-resolution ultrasound. Thus, a major part of the measured perfusion in the mouse is dependent on both blood flow in the femoral artery (macrocirculation) and microcirculatory tissue perfusion; however, **because** of the velocity of the blood cell in

the conduit vessels, the blood flow in the femoral artery will predominate the overall signal. Therefore, the use of scanning LDPI of the mouse hindlimb is not comparable to its use in human skin, where – considering the penetration depth of the laser – it is applied to assess microcirculatory function only.

Measurements of the PORH response by scanning LDPI: reproducibility, and dependency on physiological stimuli.

Measurement of PORH with different methods is routinely applied in clinical settings to evaluate the endothelial functionality of different vessel beds, and it is known to be dependent on occlusion time and on endogenous vasodilators.

As the first step of scanning LDPI validation for assessment of hyperemic responses, we administered vasoactive drugs and compared the LDPI signal to ultrasound-assessed changes in femoral artery hemodynamics. We found that the LDPI signal (mean perfusion assessed in MPU) is directly correlated to changes in diameter and blood flow in the femoral artery. For example, administration of both endothelial independent and endothelial-dependent vasodilators (NTG, acetylcholine, adenosine) caused an increase in vessel diameter and blood flow as compared to baseline, which corresponded to a comparable increase in mean perfusion as assessed by LDPI. Conversely, the vasoconstrictor epinephrine caused a strong and rapid decrease in vessel diameter, blood flow and mean perfusion. Therefore, scanning LDPI can be used for measuring pharmacologically modulated hyperemic responses in the mouse hindlimb.

In addition, we verified that PORH-induced changes in mean perfusion corresponded to FMD, which is a widely accepted parameter for determining endothelial function in vivo. We found that the peak of maximum perfusion corresponds to the point of maximum flow-mediated dilation of the femoral artery and that the elevation of perfusion (MPU) lasts longer than the vasodilatory response of the femoral artery, indicating a microvascular component of the LDPI signal. Interestingly, maximal perfusion (MPU) after PORH was comparable with the maximum NTG-induced perfusion increase and significantly higher than the acetylcholine or adenosine-induced response. Importantly, PORH was absent in chronic hindlimb ischemia and PORH-induced changes in mean perfusion were dependent on the duration of the

1 occlusion and increased with longer occlusion times (1-5 minutes), further validating
2 this measurement approach.

3 Last, we analyzed the reproducibility and variability of the PORH response assessed
4 by LDPI by repeated intra-individual and inter-individual measurements. The most
5 robust parameter was the *time-to-peak* (which is an indication of the rapidity of the
6 response) and the *ratio of maximum and baseline perfusion* (which is an indicator of
7 the magnitude of the response). Interestingly, we found that the rapidity of the
8 hyperemic response was dependent on the enzymatic synthesis of both NO and
9 prostaglandins, as the NOS inhibitor L-NNA as well as the COX inhibitor indomethacin
10 increased time to peak.

11 Vascular aging is known to induce endothelial dysfunction in humans (24) and rats
12 (21) and is associated with impaired angiogenesis in mice (19). Here, we found that
13 12- and 24-months old mice have slower vascular responsiveness (time to peak),
14 significantly decreased magnitude of response (perfusion reserve and AUC) and a
15 less sustained hyperemia (Fig. 6B) as compared to younger mice (Fig. 6D). This data
16 is in agreement with other reports (21-23) again demonstrating the robustness of our
17 technique.

18 **Assessment of endothelial function in vivo: PROH vs. FMD**

19 Although the use of in vivo models of cardiovascular disease is widely spread,
20 methods for evaluating endothelial function in rodents and particularly in mice are
21 mainly restricted to ex vivo techniques or measurement of high-resolution ultrasound
22 of FMD after PORH (21-23). It is very important to consider the fundamental
23 differences in the physiological responses assessed by these two methods.

24 Measurement of PORH by scanning LDPI of PORH in the mouse hindlimb¹ allows
25 measurement of both microcirculatory and microcirculatory tissue perfusion in a
26 defined region of interest, while high-resolution ultrasound allow determination of FMD
27 of a specific conduit vessel. Therefore, measurement of PORH by scanning LDPI is
28 less specific for the determination of endothelial function (i.e. it does not measure the
29 diameter and flow velocity of a specific vessel) and it cannot distinguish between

¹ As discussed, due to the penetration depth of the laser, the measurement of PORH in the mouse hindlimb by scanning LDPI is a measurement of both macrocirculation and microcirculation, while measurements of PORH by scanning LDPI in humans is only a measurement of microcirculation

1 **macrocirculatory and microcirculatory blood flow.** A way to distinguish the
2 microcirculatory component from the microcirculatory component could be the
3 application of a b2 receptor antagonist to skeletal **muscle and** should be investigated
4 in the future.

5 On the other hand, FMD assessed by high-resolution ultrasound only provides very
6 local blood flow velocity and estimation of perfusion of a specific conduit vessel. **Only**
7 **a combination of the two techniques may allow understanding all physiological**
8 **variables connecting endothelial function, microcirculation and global tissue perfusion,**
9 **and their physiological changes in age or disease conditions.**

10 Moreover, scanning LDPI is more robust and less sensitive than ultrasound to tiny
11 changes in hindlimb position during inflation and deflation of the cuff, temperature and
12 small changes in cardiac and circulatory parameters; these characteristics make
13 scanning LDPI easier to use, requires less training time to achieve reproducibility, and
14 is more robust. A LDPI scanner is also more affordable than a high-resolution
15 ultrasound, which ensures greater access to the method.

16 **In the comparison of the two techniques it is important to point out that although there**
17 **is a relationship between vasodilation and increased velocity flow in the feed artery, it**
18 **is very uncertain if a direct relationship occurs between the two phenomena. In**
19 **humans, the velocity peak occurs within the first 15 seconds and the vasodilation peak**
20 **can be expected to occur 40-80 seconds following cuff release (25). These responses**
21 **are clearly different from rat (21) and mouse (assessed here and elsewhere (23)).**

22 **Other species-specific and technique-dependent differences were also observed by**
23 **comparing the effects of NOS and COX inhibition. We found here that the**
24 **administration of L-NNA and indomethacin decreased *time-to-peak*, but did not affect**
25 **the magnitude of peak perfusion. Results by Heiss et al. (21) in rats show that NOS**
26 **inhibition (by L-NMMA) almost exclusively abolished femoral artery vasodilation**
27 **without affecting flow velocity and wall shear stress as assessed by ultrasound.**
28 **Whether this is due to species specificity or because LDPI is measuring a mixed**
29 **macro/microcirculatory signal need to be determined experimentally by **carrying** out**
30 **these experiments side-by-side.**

31 **A further consideration is that at least in humans there are clear limb differences in**
32 **vascular control mechanisms, i.e. vascular responses of the arm are different from**

1 vascular responses in the legs (26). these potential differences need to be taken into
2 consideration in rodents and – premise the availability of a system allowing the
3 measurements – should be tested.

4 To conclude, scanning LDPI of the PORH response in the mouse hindlimb can be
5 applied for assessing vascular function in mice in vivo. This method allows testing the
6 effects of pathophysiological changes of vascular function in correlation to genetic or
7 pharmacological manipulation, or physiological processes like aging both in a cross-
8 sectional or longitudinal experimental setting in the living animal, thus permitting an
9 easier translation of the findings to the clinical application in humans.

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5. Figure legends

Fig.1: Principles and application of laser Doppler perfusion imaging (LDPI) for measurement of perfusion of the mouse hindlimb. (A) Picture of a live mouse anesthetized with isoflurane, positioned on a 37°C heated surface; the picture shows the cuff/vascular occlude used for PORH measurements and the laser light illuminating the area of measurement on the hindlimb. (B) Schematic representation of the experimental setup, showing the optimized measuring distance of the LDPI scanner. (C) In normal human skin, the 670-690 nm wavelength laser of the PeriScan PIM 3 System is partly absorbed or scattered, and the measuring depth is in the order of 0.5 - 1 mm. (D) The left panel shows an ultrasound image of the mouse hindlimb with visualization of the femoral artery around 0.4 mm underneath the skin. The laser is systematically making 1-2 measurements/sec producing a colored image where red represents areas of high perfusion and black represents areas of low perfusion (right panel). Due to the presence of the femoral artery and its branching (middle panel), the LDPI signal (right panel) will comprise both microcirculation and microcirculation in the mouse.

Fig.2: Effects of pharmacological modulation of vessel diameter and hindlimb perfusion by the administration of vasodilators and vasoconstrictors. Changes in femoral artery diameter and peak/mean flow velocity by ultrasound (left panels) and changes in tissue perfusion by LDPI (middle and right panels) in response to vasodilators and vasoconstrictors were assessed by (A) acute application of nitroglycerin (12 mg/Kg n = 4), (B) s. c. application of the α/β -agonist epinephrine (0.7 mg / Kg, n=4), (C) i.v. application of the endothelium-dependent vasodilator acetylcholine (0.07 mg/Kg, n=5) and (D) i.v. application of the purinergic mediator adenosine (0.1 mg/Kg, n = 4). Warm saline served as control and had no effect on the perfusion values. Data are reported as means $\pm$ SD. RM 2-way ANOVA $p < 0.001$; Sidak's multiple comparison test * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$ vs. baseline; saline and pharmacological controls were run on the same mouse.)

Fig.3: Effects of physiological modulation of hindlimb perfusion by inducing reactive hyperemia. Representative images (A) and curves (B) showing changes in tissue perfusion during baseline, ischemia and reactive hyperemia (black line) assessed by LDPI. In B the analysis parameters used to quantify the hyperemic response are represented: basal perfusion, zero perfusion, maximum perfusion, time-to-peak, amplitude (max - basal perfusion), perfusion reserve (amplitude / basal perfusion*100), ratio (max / basal perfusion) and AUC (area-under-the-curve). The grey line represents zero-perfusion obtained after surgical excision of the femoral artery (one representative curve of 3 independent measurements from different mice). (C) The changes in perfusion (red curve, n = 10) after occlusion correspond to changes in femoral artery diameter assessed as flow-mediated dilation (FMD) by high-resolution ultrasound (blue curve, n = 10). All data are expressed as mean $\pm$ SD.

Fig.4: Effects of occlusion time on PORH response assessed by LDPI. The degree of maximal hyperemic blood flow depends on the duration of occlusion. Data represent mean repeated measurement carried out on the same mouse at 5 different

days. Lines represent mean $\pm$ SD. RM 1-way ANOVA $p < 0.05$ for all panels (except baseline 1-way ANOVA n.s.). Tuckey's * $p < 0.05$ vs. time 1 min.

Fig.5: NOS and COX inhibition prolong the PORH response. Effects of chronic inhibition of NO synthase (NOS) by administration of L-NG-Nitroarginine (L-NNA; 7 mM in drinking water, corresponding to 270 mg / Kg/day) or **indomethacin** (Indo, 30 μ g/ ml in drinking water, corresponding to a dose of 5 mg/Kg/day) for 6. (A) Measurements of PORH response were carried **out** before and after the treatment. RM 2-way ANOVA $p < 0.05$; Sidack's ** $p < 0.01$; Paired T-test # $p < 0.05$. (B) Measurements of PORH response were carried out in independent groups of wild type (WT) mice left untreated or treated with L-NNA or mice lacking eNOS (eNOS^{-/-}). 1-way ANOVA $p < 0.001$; Tuckey's * $p < 0.05$; ** $p < 0.01$

Fig. 6: The degree of reactive **hyperemic blood flow decreases with age.** In aged mice, we measured a significant decrease in the rapidity of reperfusion in the first phase (time to peak) (A) and a **faster** recovery (time to base) (B). Moreover, PORH after ischemia is significantly lower in adult and aging mice (6, 12, 24 months) vs. young mice (2-3 months) as assessed by a significant decrease in overall perfusion (AUC) shown in (C) and in PORH response (D). 1-way ANOVA* $P < 0.01$ for all the panels. Tuckey's * $p < 0.05$ *** $p < 0.001$ vs. 2-3 months old mice; T-Test # $p < 0.05$ vs. 2-3 months old mice.

6. Table legends

Table 1. Effects of administration of vasodilators/constrictors on hemodynamics, body temperature and respiratory rate. Physiological parameters were determined before and after administration of nitroglycerine (NTG), epinephrine (Epi), acetylcholine (ACh) and adenosine (Ado). Data are mean $\pm$ SEM. * $p < 0.05$ ** $p < 0.01$ $n = 4$ per group.

Table 2: Intra and interindividual variability of parameters assessed to quantify the PORH response in wild type mice.

Table 3: Effects of NOS and COX inhibition on PORH response. Measurement of PORH response in wild type C57Bl/6 mice before and after administration of the NOS inhibitor L-NNA (270 mg/Kg/day) or the COX inhibitor indomethacin (5 mg/Kg/day) for 6 days in drinking water.

Table 4: PORH response in mice lacking eNOS expression. Measurement of PORH response in untreated wild type (WT) C57Bl/6 mice, or after administration of the NOS inhibitor L-NNA (270 mg / Kg / day) for 6 days or in eNOS knockout (eNOS^{-/-}) mice. 1-way ANOVA, Tuckey's * = $P < 0.05$; ** = $P < 0.01$.

Assessment of tissue perfusion and vascular function in mice by scanning laser Doppler perfusion imaging

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Running title: Hyperemia measurement in mice

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Abstract

Post-occlusive reactive hyperemia (PORH) is a key feature of physiological vasomotion to appropriately match the supply/demand ratio of tissues. This adaptive mechanism is severely disturbed in endothelial dysfunction with a reduced flow-mediated dilation (FMD). Reduced PORH and FMD are powerful prognostic risk factors in cardiovascular disease. While these parameters are frequently determined in human beings, comparable methods applicable to mouse models are sparse. We aimed to evaluate the applicability and accuracy of scanning laser Doppler perfusion imaging (LDPI) to measure PORH in the mouse hindlimb. Changes in mean perfusion in response to vasoactive drugs and PORH (assessed by scanning LDPI) were compared with changes in diameter and blood flow in the femoral artery, as assessed by high-resolution ultrasound. We found that the measured LDPI signal significantly correlated with changes of inflow into the femoral artery. Vasodilation induced by administration of nitroglycerine and acetylcholine increased vessel diameter, blood flow and mean perfusion, while vasoconstriction following administration of epinephrine decreased all three parameters. PORH was induced by temporal occlusion of the femoral artery with an external cuff. During occlusion, mean perfusion decreased to a condition of zero-perfusion and release of the cuff induced an immediate increase in blood flow that was followed by femoral artery dilation driving PORH/ perfusion. Surgical removal of the femoral artery decreased mean perfusion to a zero-perfusion level and fully abolished PORH. Importantly, the measurement of the PORH response by scanning LDPI is highly reproducible as determined by repeated measurements and intra/interobserver variation analysis. Last, we found that the PORH response was dependent on nitric oxide synthase and cyclooxygenase and declined with age. Thus, we here provide novel and robust non-invasive methods to serially measure tissue perfusion at baseline and during physiological and pharmacological modulation of vasomotor tone in the hindlimb of mice. The application of these LDPI scanning and ultrasound-based methods may be useful for testing the effects of drugs affecting vasomotor function or future elucidation of mechanisms leading to vasomotor dysfunction in mice *in vivo*.

Keywords: Laser Doppler perfusion imaging; hindlimb; reactive hyperemia; nitric oxide synthase; age-dependent endothelial dysfunction.

1. Introduction

A wide range of animal models to study endothelial dysfunction are available, including hyperlipidemic mice and rats, spontaneously hypertensive rats, genetically modified mice lacking/overexpressing specific apolipoproteins (e.g. apoE or apoB), hypertensive mice lacking the endothelial nitric oxide synthase (eNOS) and surgical models of vessel injury (1). In these mice models, endothelial and vascular function is frequently studied *ex vivo*, which has provided valuable new insight into vessel wall function. However, vascular function and blood flow are a result of multiple physiological inputs. Here, we propose a new method to examine the vascular function and tissue perfusion *in vivo*.

Post occlusive reactive hyperemia (PORH) is the physiological increase of blood flow to the tissue following ischemia after transient arterial occlusion. PORH has been applied for testing the endothelial function of conductance vessels in humans, e.g. by measurement of flow-mediated dilation (FMD) (2, 3), venous occlusion plethysmography (VOP), or skin microcirculation by laser Doppler flowmetry or imaging (4, 5). Scanning laser Doppler perfusion imaging (LDPI) has been applied in clinical settings to assess functional responses of the cutaneous microcirculation to vasoactive molecules or post occlusive reactive hyperemia (PORH) in patients with endothelial dysfunction due to hypertension (6), peripheral arterial disease (7), coronary artery disease (5), diabetes (8), smoking (9) (10) or age (11). In swine and dog animal models scanning LDPI was applied for measuring revascularization in wound healing (12, 13), mucosal microcirculation (14), or retinal capillary blood flow (15). In rodents and especially in mice scanning LDPI is classically used to study angiogenesis and neovascularization after hindlimb ischemia (16-19), but it has never been applied to serially study vasomotor function in mice.

In this study, we aimed to evaluate the applicability of scanning LDPI to measure PORH in mice and hypothesized that it would be suitable to characterize tissue perfusion and changes in vascular function. We found that scanning LDPI is a robust and reproducible non-invasive method, which may be applied to evaluate pharmacological response to vasodilators/vasoconstrictors and PORH in mice models of cardiovascular disease.

2. Materials and Methods

Animals

All procedures were conducted in conformity with the American Physiological Society's "Guiding principles for research involving animals and human beings" and were approved by the LANUV Nordrhein-Westfalen. Mice were fed standard laboratory chow ad libitum. Male C57/BL6 mice were purchased from Janvier SAS (Le Genest, France). Global eNOS KO mice(20) were kindly provided by A. Gödecke. Department of Physiology, Medical Faculty, Heinrich Heine University of Düsseldorf. Mice of the following ages were randomly assigned to groups and used for experiments: 8 ± 2 weeks (wk)-old (22 - 25 g), 24 wk-old (30 - 35 g), 48 wk-old (37 - 47 g) and 96 wk-old (34 - 45 g) mice.

Scanning LDPI

Scanning LDPI measurements were performed by using a PeriScan PIM 3 System and proprietary acquisition software (LDPIwin) (both by Perimed-Instruments, Järfälla, Sweden). The experimental setup is presented in Fig. 1. Mice were anesthetized with isoflurane (Piramal Critical Care, USA) (3% induction and 2% maintenance) and fixed on a heated platform (set to 39°C, body temperature approx. 36°C) in a supine position. Body hair was removed from the inner thigh using a depilation cream (Veet, Reckitt Benckiser, Germany). A measuring distance of 10 cm, a scanning area of 5 x 3 cm and a scanning velocity of 1 image/second were chosen and kept constant throughout the measurements. The signal was quantified as mean perfusion units (MPU). Baseline values were taken for 15 minutes (min) before starting the interventions. Data analysis was performed off-line using LDPIwin software (Perimed-Instruments).

High-resolution ultrasound

The femoral artery was visualized by using a VEVO2100 high-resolution ultrasound device equipped with ultra-high frequency transducers (MS400: 18-38MHz, MS700: 30-70MHz) and proprietary software (VisualSonics, Toronto, Canada), as previously described (21). Mice were anesthetized and prepared as described for LDPI. The femoral artery was visualized by its characteristic flow pattern in color doppler mode, PW-Mode, and B-Mode. Flow velocity, heart rate (HR), respiration rate (RR) and

body temperature were determined off-line with the dedicated software. Vessel diameter (d) was evaluated by using the Brachial Analyzer 5 software (Medical Imaging Applications, Coralville, IA).

Treatment with vasoactive drugs

All stock solutions were freshly prepared on the experimental day with warm (37°C) sterile saline (NaCl 0.9%) as a vehicle. The effects of injection of the vehicle control were tested in the same groups of animals before injecting the vasoactive substances. The doses applied were chosen according to preliminary tests carried out by measuring changes in vessel diameter by ultrasound (22, 23).

Nitroglycerin (NTG). NO-dependent and endothelium-independent vasodilation were induced by bolus intraperitoneal (i.p.) administration of 12 mg/kg NTG in 100 µl vehicle (0.9% NaCl) (stock 1 mg/ml, G.Pohl-Boskamp, Hohenlockstedt, Germany) or vehicle only. Readings were taken immediately after injection as described above.

Adenosine (Ado). To pharmacologically mimic the blood flow increase during reactive hyperemia, we administered 0.1 mg/kg bolus dose of adenosine in 100 µl vehicle (stock 10^{-4} mol/l; Sigma-Aldrich, St. Louis, USA) into the tail vein.

Epinephrine (Epi). To induce vasoconstriction, we used a local subcutaneous injection of 0.7 mg/kg epinephrine in 100 µl vehicle (stock 10^{-3} mol/l; Sigma-Aldrich) into the upper thigh.

Acetylcholine (ACh). To induce endothelium-dependent vasodilation, 0.07 mg/kg acetylcholine in 100 µl vehicle (stock 10^{-4} mol/L; Sigma-Aldrich) was injected into the tail vein. Acetylcholine stock solutions were prepared freshly with the vehicle on each day.

Induction of post-occlusive reactive hyperemia (PORH)

PORH was induced by using an air inflatable vascular occluder with a diameter of 10 mm (KentScientific, Connecticut, USA) as described (22, 23). Briefly, the hindlimb of a mouse was pulled through the cuff (see Fig. 1A). To induce PORH the cuff was inflated at a pressure of ~300 mmHg. Occlusion was confirmed by measuring distal blood flow (mean perfusion < 30 MPU) in preliminary experiments. After occlusion, the cuff was rapidly deflated. The following experimental protocol was used for both LDPI and ultrasound measurements: 15 min. equilibration period, 7 min. baseline

measurement (stabilization of perfusion), 5 min. ischemia (zero perfusion) and 8 min. of reperfusion (hyperemia and recovery of perfusion).

Parameters used for quantifying the PORH measured by scanning LDPI are described in Fig. 3A: 1) baseline perfusion; 2) maximal perfusion; 3) perfusion reserve as $\Delta\% = (\text{max. reperfusion} - \text{baseline}) / \text{baseline} \times 100$; 4) ratio = max. reperfusion / baseline; time to peak = time to maximal perfusion after releasing the cuff; and area under the curve (AUC) = integral of the reperfusion curve.

Changes in the diameter of the femoral artery and flow after PORH were assessed by high-resolution ultrasound. The dilation of the femoral artery is analyzed as % flow-mediated dilation (FMD) by comparing the diameter change after occlusion in relation to the basal diameter, as described (21).

Effects of physiological and pharmacological modulation of the PORH response.

To control for femoral artery perfusion signal chronic hindlimb ischemia was induced by surgical excision of the femoral artery, according to the protocol described by Limbourg et al. (18). Briefly, mice anesthetized i.p with ketamine (100 mg/kg) and xylazine (5 mg/kg) were positioned in dorsal recumbency. The skin was incised over the femoral artery. The femoral artery was isolated ligated and excised in its entirety.

The effects of inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX) on the PORH response were analyzed by the administration of the NOS inhibitor nitro-L-arginine (L-NNA, Sigma-Aldrich), the COX inhibitor indomethacin (Sigma-Aldrich) and in mice lacking the endothelial NOS (eNOS^{-/-})(20). L-NNA or indomethacin was administrated in drinking water for 6 days. L-NNA was dissolved in 1 M HCl (stock 160 mg/ml) and diluted to a final concentration of 1.6 mg /ml (and (0.01 HCl) in drinking water, resulting in a daily dose of 8 mg L-NNA / day / 1 mouse. Indomethacin was dissolved in ethanol and diluted to a concentration of 30 µg / ml, resulting in a daily dose of 150 µg/day (and 1% ethanol). Corresponding vehicle controls were used in separate animal groups. In a subgroup measurements were conducted longitudinally before and 6 days after administration of the drugs.

Blood pressure measurements in mice

Mean arterial blood pressure in mice was measured in the aorta ascendens using a 1.4F SPR-839 pressure catheter from *Millar Instruments* (Houston, TX, USA) placed into the aorta through the right carotid artery. Pressure data were analyzed with dedicated software (IOX, EMKA, Paris, France) to determine peak systolic (SBP) and diastolic pressure (DBP).

Statistical Analyses

Values reported are means $\pm$ SD or as box plots according to Tuckey. Comparisons between groups were made using either a 2-sided student's t-test or 1- way or 2-way ANOVA, followed by an appropriate post-hoc test as indicated in the figure legends and the tables. Differences were deemed significant when $p < 0.05$. Statistical analyses were performed using GraphPad Prism 8.00 (GraphPad, San Diego, CA, USA) or SPSS 18.0 statistics (IBM).

3. Results

Visualization and quantification of blood flow and mean perfusion in the hindlimb by scanning LDPI and high-resolution ultrasound.

In this study, we aimed to validate the measurement of hindlimb perfusion by LDPI in the scope to evaluate physiological and pharmacological changes of vascular function in vivo. The experimental setting is depicted and described in Figure 1. Mice are anesthetized with isoflurane and fixed in a supine position and the hindlimb cleared of hair. A vessel occluder was applied for inducing post-occlusive reactive hyperemia (Fig. 1A). The scanning head is adjusted to a standardized distance of 10 cm from the measuring surface (Fig. 1B). When laser light penetrates the tissue (Fig. 1C) it is partly absorbed or scattered and light particles, which hit moving blood cells, undergo a change in wavelength (Doppler shift). As depicted in Fig. 1C, the scanning LDPI signal is mainly dependent on the penetration depth of the laser (estimated to be between 0.5 and 1 mm) and on the blood flow in the vessels encountered by the laser within the tissue. The femoral artery was found at 0.4 mm penetration depth, as visualized by high-resolution ultrasound (Fig. 1D, left panel). In its proximity, the perfusion map obtained by scanning a 4 x 8 mm area showed a discrete region with a very high signal (Fig. 1D, right panel), which was quantified as mean perfusion in a gated area and indicated in mean perfusion units (MPU).

Modulation of blood flow and mean perfusion by vasoactive molecules.

To analyze how changes in mean perfusion correlate with changes in blood flow in the femoral artery, we applied different well-characterized pharmacological agents inducing vasoconstriction or -dilation and measured both mean perfusion by scanning LDPI and blood flow by high-resolution ultrasound (Fig. 2). Systemic i.p. injection of a 12 mg/kg NTG bolus rapidly increased vessel diameter and flow within the femoral artery as assessed by ultrasound (Fig. 2A, left panel) and increased mean perfusion as compared to baseline, reaching a maximum within approximately 100 s (Fig. 2A, middle), whereas the vehicle control had no effect. The maximal increase in mean perfusion after administration of NTG was 128% as compared to vehicle control (435 ± 23 MPU after NTG vs. 191 ± 36 MPU $n = 5$, Fig. 2A, right panel). Administration of the adrenergic receptor agonist epinephrine (adrenaline) s.c. caused a rapid vasoconstriction as demonstrated by decrease in vessel

diameter and flow (Fig 2B, left panel), and a rapid 57% drop in mean perfusion (90 ± 18 MPU vs. 209 ± 59 , $n=4$, Fig. 2B, right panel), which lasted throughout the entire measurement interval (Fig. 2B, middle panel). Intravenous injection of the endothelium-dependent vasodilator acetylcholine caused a significant increase in vessel diameter and flow (Fig. 2C, left), and mean perfusion (Fig. 2C, middle and right), reaching a maximum of 132% within 300 sec ($n = 5$, 537 ± 35 MPU vs. 406 ± 48 MPU baseline). Stimulation of purinergic receptors by i.v. bolus administration of adenosine significantly increased vessel diameter and later on peak flow velocity (Fig. 2D, left panel), as well as mean perfusion within approximately 150 s (Fig. 2D, middle), with a maximal increase of 41% as compared to baseline values (316 ± 43 vs. 224 ± 34 MPU baseline) (Fig. 2D, right panel). The LDPI signal remained unaffected after vehicle injection. During the ultrasound measurements, there was no significant change in heart rate (HR), respiration rate and body temperature (Table 1). As expected, systolic and diastolic blood pressure measured in the aorta by Millar catheterization were significantly transiently decreased by all vasodilators applied and increased by epinephrine (Table 1). Taken together, our results show that changes in blood flow in the femoral artery as a result of the administration of well-characterized vasodilators and vasoconstrictors directly correlate to changes in mean perfusion, as assessed by LDPI.

Measurement of the PORH response by scanning LDPI

PORH was induced by inflating a cuff at a pressure of ~300 mmHg until reaching the point of zero perfusion (mean perfusion < 30 MPU), and then rapidly deflating it (Fig 3). Five minutes of occlusion time led to a rapid (< 1 min) FMD of the femoral artery accompanied by an increase in mean perfusion up to 200% as compared to baseline values, which then returned to baseline after 5-6 minutes (Fig 3). We found that the peak of maximum perfusion corresponded to a maximum dilation of the femoral artery (Fig. 3C); however, the increase in perfusion lasted longer than the vasodilatory response of the femoral artery. The peak of mean perfusion after PORH ($150 \pm 15\%$) was similar to the maximum NTG-induced perfusion increase ($149 \pm 42\%$) and significantly higher than the Ach ($38 \pm 12\%$) and adenosine ($43 \pm 8\%$) induced responses. The parameters used to quantify PORH are described in Figure 3A (also see Methods for details).

PORH in chronic hindlimb ischemia

If the femoral artery was surgically excised, mean perfusion was as low as zero perfusion after inducing transient ischemia in control animals (Fig.3A grey curve), and the PORH response was absent (Fig. 3A, gray curve).

Effects of occlusion time and reproducibility of measurements of PORH by scanning LDPI

To test the dependency of the PORH response on the duration of ischemia as assessed by LDPI, we occluded the hindlimb for 1, 3, and 5 min (Fig. 4 A-C). By increasing the occlusion time, we measured an increase in maximal perfusion, ratio, delta percent of the response and time-to-peak, while the AUC © was only significantly increased after 5 minutes of ischemia (Fig. 4 A-D; * $p < 0.05$, $n = 5$ independent measurements carried out on the same mouse). These data were also further analyzed to compare intra-individual variability with inter-individual variability of the different parameters by comparing their coefficient of variation (Table 1). We found the smallest variation for time to peak and perfusion reserve, while AUC measurements showed the highest variability.

The dependency of the PORH response on the availability of endogenous vasodilators.

To investigate the role of NOS-dependent NO synthesis, mice were treated with NOS-inhibitor L-NNA (1.6 mg/ml = 270 mg/kg / d) in drinking water for 6 days. NOS inhibition significantly decreased the rapidity of the PORH response with a greater time to peak determined both in intraindividual (before –after) measurements. (Fig. 5 A, Table 2) and in different groups (Fig. 5B and Table 3). Other parameters were unchanged (Tables 2 and 3). Mice with a genetic deletion of eNOS (eNOS^{-/-}) show increased time-to-peak (Fig. 5B and Table 3), but no changes in other parameters. To determine the role of COX-dependent prostaglandin production, the cyclooxygenase inhibitor indomethacin (30 µg/ml = 5 mg/kg/d) was administered to mice in drinking water for 6 days (Fig. 5C), which resulted in an increase in time-to-peak, but had no effect on the other parameters (Table 2).

1 The dependency of the PORH response on mice age.

2 As further proof of concept, we additionally determined PORH in wildtype mice
3 cohorts of different ages (Fig. 6). The rapidity of hyperemic reperfusion is altered
4 with a prolonged time to peak (Fig. 6A) and a faster perfusion drop to baseline
5 values in older mice (Fig. 6B). Furthermore, we found that 12 months and 24 months
6 old mice have a significantly diminished AUC (Fig. 6D) and % perfusion increase of
7 PORH (Fig. 6C), as compared to younger mice.

8 Taken together, these data show that the measurement of perfusion in the mouse
9 hindlimb by LDPI is sensitive physiological and pharmacological changes in vessel
10 diameter and is valid for evaluating intraindividual changes for significant in vivo
11 investigations with limited numbers of animals.

4. Discussion

In this study, we evaluated the potential of measuring the PORH response by scanning LDPI. We show that 1) changes in flow in the femoral artery due to administration of vasoactive drugs correlate to changes in mean perfusion in the hindlimb; 2) the PORH response (changes in mean perfusion by LDPI) correlate to changes in diameter (as FMD% by ultrasound) 3) measurements of the PORH response by scanning LDPI are reproducible, are modulated by intervention with NOS and COX inhibitors, and decrease with increasing animal age. Thus, we propose that measurements of PORH by scanning LDPI might be applied for the assessment of changes in vascular function in mice models of cardiovascular disease.

What is scanning LDPI measuring?

Scanning LDPI is based on the physical phenomenon defined as the Doppler shift or Doppler effect, which is a change in the observed frequency of a wave occurring when the source and observer are in motion relative to each other. When laser light penetrates a tissue, it is backscattered and returns to its source without change of frequency. But if the laser light is backscattered by moving objects (i.e. blood cells), it undergoes a shift in frequency directly proportional to their concentration and velocity. The *measuring depth* of the laser, i.e. how deep the laser light penetrates the tissue, depends (a) on the energy and wavelength of the laser, (b) on the measuring distance and, most importantly, (c) on biophysical and anatomical characteristics of the tissue, which can influence its backscattering properties; these include (but they are not limited to) density and pigmentation of the skin and presence of hair, as well as density and diameter of the vessels (microcirculation, resistance vessels, macrocirculation), amount of blood cells.

The perfusion map obtained by scanning a defined area of the hindlimb shows a discrete region of very high signal intensity, which corresponds to the area of tissue with and around the femoral artery. According to the manufacturer's information, the measurement depth in our system in human skin has been calculated to be around 1 mm. In the mouse, the femoral artery can be visualized at 0.4 mm penetration depth with high-resolution ultrasound. Thus, a major part of the measured perfusion in the mouse is dependent on both blood flow in the femoral artery (macrocirculation) and

microcirculatory tissue perfusion; however, because of the velocity of the blood cell in the conduit vessels, the blood flow in the femoral artery will predominate the overall signal. Therefore, the use of scanning LDPI of the mouse hindlimb is not comparable to its use in human skin, where – considering the penetration depth of the laser – it is applied to assess microcirculatory function only.

Measurements of the PORH response by scanning LDPI: reproducibility, and dependency on physiological stimuli.

Measurement of PORH with different methods is routinely applied in clinical settings to evaluate the endothelial functionality of different vessel beds, and it is known to be dependent on occlusion time and on endogenous vasodilators.

As the first step of scanning LDPI validation for assessment of hyperemic responses, we administered vasoactive drugs and compared the LDPI signal to ultrasound-assessed changes in femoral artery hemodynamics. We found that the LDPI signal (mean perfusion assessed in MPU) is directly correlated to changes in diameter and blood flow in the femoral artery. For example, administration of both endothelial independent and endothelial-dependent vasodilators (NTG, acetylcholine, adenosine) caused an increase in vessel diameter and blood flow as compared to baseline, which corresponded to a comparable increase in mean perfusion as assessed by LDPI. Conversely, the vasoconstrictor epinephrine caused a strong and rapid decrease in vessel diameter, blood flow and mean perfusion. Therefore, scanning LDPI can be used for measuring pharmacologically modulated hyperemic responses in the mouse hindlimb.

In addition, we verified that PORH-induced changes in mean perfusion corresponded to FMD, which is a widely accepted parameter for determining endothelial function in vivo. We found that the peak of maximum perfusion corresponds to the point of maximum flow-mediated dilation of the femoral artery and that the elevation of perfusion (MPU) lasts longer than the vasodilatory response of the femoral artery, indicating a microvascular component of the LDPI signal. Interestingly, maximal perfusion (MPU) after PORH was comparable with the maximum NTG-induced perfusion increase and significantly higher than the acetylcholine or adenosine-induced response. Importantly, PORH was absent in chronic hindlimb ischemia and PORH-induced changes in mean perfusion were dependent on the duration of the

1 occlusion and increased with longer occlusion times (1-5 minutes), further validating
2 this measurement approach.

3 Last, we analyzed the reproducibility and variability of the PORH response assessed
4 by LDPI by repeated intra-individual and inter-individual measurements. The most
5 robust parameter was the *time-to-peak* (which is an indication of the rapidity of the
6 response) and the *ratio of maximum and baseline perfusion* (which is an indicator of
7 the magnitude of the response). Interestingly, we found that the rapidity of the
8 hyperemic response was dependent on the enzymatic synthesis of both NO and
9 prostaglandins, as the NOS inhibitor L-NNA as well as the COX inhibitor
10 indomethacin increased time to peak.

11 Vascular aging is known to induce endothelial dysfunction in humans (24) and rats
12 (21) and is associated with impaired angiogenesis in mice (19). Here, we found that
13 12- and 24-months old mice have slower vascular responsiveness (time to peak),
14 significantly decreased magnitude of response (perfusion reserve and AUC) and a
15 less sustained hyperemia (Fig. 6B) as compared to younger mice (Fig. 6D). This
16 data is in agreement with other reports (21-23) again demonstrating the robustness
17 of our technique.

18 **Assessment of endothelial function in vivo: PROH vs. FMD**

19 Although the use of in vivo models of cardiovascular disease is widely spread,
20 methods for evaluating endothelial function in rodents and particularly in mice are
21 mainly restricted to ex vivo techniques or measurement of high-resolution ultrasound
22 of FMD after PORH (21-23). It is very important to consider the fundamental
23 differences in the physiological responses assessed by these two methods.

24 Measurement of PORH by scanning LDPI of PORH in the mouse hindlimb¹ allows
25 measurement of both microcirculatory and microcirculatory tissue perfusion in a
26 defined region of interest, while high-resolution ultrasound allow determination of
27 FMD of a specific conduit vessel. Therefore, measurement of PORH by scanning
28 LDPI is less specific for the determination of endothelial function (i.e. it does not
29 measure the diameter and flow velocity of a specific vessel) and it cannot distinguish

¹ As discussed, due to the penetration depth of the laser, the measurement of PORH in the mouse hindlimb by scanning LDPI is a measurement of both macrocirculation and microcirculation, while measurements of PORH by scanning LDPI in humans is only a measurement of microcirculation

1 between macrocirculatory and microcirculatory blood flow. A way to distinguish the
2 microcirculatory component from the microcirculatory component could be the
3 application of a b2 receptor antagonist to skeletal muscle and should be investigated
4 in the future.

5 On the other hand, FMD assessed by high-resolution ultrasound only provides very
6 local blood flow velocity and estimation of perfusion of a specific conduit vessel. Only
7 a combination of the two techniques may allow understanding all physiological
8 variables connecting endothelial function, microcirculation and global tissue
9 perfusion, and their physiological changes in age or disease conditions.

10 Moreover, scanning LDPI is more robust and less sensitive than ultrasound to tiny
11 changes in hindlimb position during inflation and deflation of the cuff, temperature
12 and small changes in cardiac and circulatory parameters; these characteristics make
13 scanning LDPI easier to use, requires less training time to achieve reproducibility,
14 and is more robust. A LDPI scanner is also more affordable than a high-resolution
15 ultrasound, which ensures greater access to the method.

16 In the comparison of the two techniques it is important to point out that although
17 there is a relationship between vasodilation and increased velocity flow in the feed
18 artery, it is very uncertain if a direct relationship occurs between the two phenomena.
19 In humans, the velocity peak occurs within the first 15 seconds and the vasodilation
20 peak can be expected to occur 40-80 seconds following cuff release (25). These
21 responses are clearly different from rat (21) and mouse (assessed here and
22 elsewhere (23)).

23 Other species-specific and technique-dependent differences were also observed by
24 comparing the effects of NOS and COX inhibition. We found here that the
25 administration of L-NNA and indomethacin decreased *time-to-peak*, but did not affect
26 the magnitude of peak perfusion. Results by Heiss et al. (21) in rats show that NOS
27 inhibition (by L-NMMA) almost exclusively abolished femoral artery vasodilation
28 without affecting flow velocity and wall shear stress as assessed by ultrasound.
29 Whether this is due to species specificity or because LDPI is measuring a mixed
30 macro/microcirculatory signal need to be determined experimentally by carrying out
31 these experiments side-by-side.

1 A further consideration is that at least in humans there are clear limb differences in
2 vascular control mechanisms, i.e. vascular responses of the arm are different from
3 vascular responses in the legs (26). these potential differences need to be taken into
4 consideration in rodents and – premise the availability of a system allowing the
5 measurements – should be tested.

6 To conclude, scanning LDPI of the PORH response in the mouse hindlimb can be
7 applied for assessing vascular function in mice in vivo. This method allows testing
8 the effects of pathophysiological changes of vascular function in correlation to
9 genetic or pharmacological manipulation, or physiological processes like aging both
10 in a cross-sectional or longitudinal experimental setting in the living animal, thus
11 permitting an easier translation of the findings to the clinical application in humans.

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5. Figure legends

Fig.1: Principles and application of laser Doppler perfusion imaging (LDPI) for measurement of perfusion of the mouse hindlimb. (A) Picture of a live mouse anesthetized with isoflurane, positioned on a 37°C heated surface; the picture shows the cuff/vascular occlude used for PORH measurements and the laser light illuminating the area of measurement on the hindlimb. (B) Schematic representation of the experimental setup, showing the optimized measuring distance of the LDPI scanner. (C) In normal human skin, the 670-690 nm wavelength laser of the PeriScan PIM 3 System is partly absorbed or scattered, and the measuring depth is in the order of 0.5 - 1 mm. **D)** The left panel shows an ultrasound image of the mouse hindlimb with visualization of the femoral artery around 0.4 mm underneath the skin. The laser is systematically making 1-2 measurements/sec producing a colored image where red represents areas of high perfusion and black represents areas of low perfusion (right panel). Due to the presence of the femoral artery and its branching (middle panel), the LDPI signal (right panel) will comprise both microcirculation and microcirculation in the mouse.

Fig.2: Effects of pharmacological modulation of vessel diameter and hindlimb perfusion by the administration of vasodilators and vasoconstrictors. Changes in femoral artery diameter and peak/mean flow velocity by ultrasound (left panels) and changes in tissue perfusion by LDPI (middle and right panels) in response to vasodilators and vasoconstrictors were assessed by **(A)** acute application of nitroglycerin (12 mg/Kg n = 4), **(B)** s. c. application of the α/β -agonist epinephrine (0.7 mg / Kg, n=4), **(C)** i.v. application of the endothelium-dependent vasodilator acetylcholine (0.07 mg/Kg, n=5) and **(D)** i.v. application of the purinergic mediator adenosine (0.1 mg/Kg, n = 4). Warm saline served as control and had no effect on the perfusion values. Data are reported as means $\pm$ SD. RM 2-way ANOVA $p < 0.001$; Sidak's multiple comparison test * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$ vs. baseline; saline and pharmacological controls were run on the same mouse.)

Fig.3: Effects of physiological modulation of hindlimb perfusion by inducing reactive hyperemia. Representative images **(A)** and curves **(B)** showing changes in tissue perfusion during baseline, ischemia and reactive hyperemia (black line) assessed by LDPI. In B the analysis parameters used to quantify the hyperemic response are represented: basal perfusion, zero perfusion, maximum perfusion, time-to-peak, amplitude (max - basal perfusion), perfusion reserve (amplitude / basal perfusion*100), ratio (max / basal perfusion) and AUC (area-under-the-curve). The grey line represents zero-perfusion obtained after surgical excision of the femoral artery (one representative curve of 3 independent measurements from different mice). **(C)** The changes in perfusion (red curve, n = 10) after occlusion correspond to changes in femoral artery diameter assessed as flow-mediated dilation (FMD) by high-resolution ultrasound (blue curve, n = 10). All data are expressed as mean $\pm$ SD.

Fig.4: Effects of occlusion time on PORH response assessed by LDPI. The degree of maximal hyperemic blood flow depends on the duration of occlusion. Data

represent mean repeated measurement carried out on the same mouse at 5 different days. Lines represent mean $\pm$ SD. RM 1-way ANOVA $p < 0.05$ for all panels (except baseline 1-way ANOVA n.s.). Tuckey's * $p < 0.05$ vs. time 1 min.

Fig.5: NOS and COX inhibition prolong the PORH response. Effects of chronic inhibition of NO synthase (NOS) by administration of L-NG-Nitroarginine (L-NNA; 7 mM in drinking water, corresponding to 270 mg / Kg/day) or indomethacin (Indo, 30 μ g/ ml in drinking water, corresponding to a dose of 5 mg/Kg/day) for 6. (A) Measurements of PORH response were carried out before and after the treatment. RM 2-way ANOVA $p < 0.05$; Sidack's ** $p < 0.01$; Paired T-test # $p < 0.05$. (B) Measurements of PORH response were carried out in independent groups of wild type (WT) mice left untreated or treated with L-NNA or mice lacking eNOS (eNOS^{-/-}). 1-way ANOVA $p < 0.001$; Tuckey's * $p < 0.05$; ** $p < 0.01$

Fig. 6: The degree of reactive hyperemic blood flow decreases with age. In aged mice, we measured a significant decrease in the rapidity of reperfusion in the first phase (time to peak) (A) and a faster recovery (time to base) (B). Moreover, PORH after ischemia is significantly lower in adult and aging mice (6, 12, 24 months) vs. young mice (2-3 months) as assessed by a significant decrease in overall perfusion (AUC) shown in (C) and in PORH response (D). 1-way ANOVA* $P < 0.01$ for all the panels. Tuckey's * $p < 0.05$ *** $p < 0.001$ vs. 2-3 months old mice; T-Test # $p < 0.05$ vs. 2-3 months old mice.

6. Table legends

Table 1. Effects of administration of vasodilators/constrictors on hemodynamics, body temperature and respiratory rate. Physiological parameters were determined before and after administration of nitroglycerine (NTG), epinephrine (Epi), acetylcholine (ACh) and adenosine (Ado). Data are mean $\pm$ SEM. * $p < 0.05$ ** $p < 0.01$ $n = 4$ per group.

Table 2: Intra and interindividual variability of parameters assessed to quantify the PORH response in wild type mice.

Table 3: Effects of NOS and COX inhibition on PORH response. Measurement of PORH response in wild type C57Bl/6 mice before and after administration of the NOS inhibitor L-NNA (270 mg/Kg/day) or the COX inhibitor indomethacin (5 mg/Kg/day) for 6 days in drinking water.

Table 4: PORH response in mice lacking eNOS expression. Measurement of PORH response in untreated wild type (WT) C57Bl/6 mice, or after administration of the NOS inhibitor L-NNA (270 mg / Kg / day) for 6 days or in eNOS knockout (eNOS^{-/-}) mice. 1-way ANOVA, Tuckey's * = $P < 0.05$; ** = $P < 0.01$.

Assessment of tissue perfusion and vascular function in mice by scanning laser Doppler perfusion imaging

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Running title: Hyperemia measurement in mice

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Abstract

Post-occlusive reactive hyperemia (PORH) is a key feature of physiological vasomotion to appropriately match the supply/demand ratio of tissues. This adaptive mechanism is severely disturbed in endothelial dysfunction with a reduced flow-mediated dilation (FMD). Reduced PORH and FMD are powerful prognostic risk factors in cardiovascular disease. While these parameters are frequently determined in human beings, comparable methods applicable to mouse models are sparse. We aimed to evaluate the applicability and accuracy of scanning laser Doppler perfusion imaging (LDPI) to measure PORH in the mouse hindlimb. Changes in mean perfusion in response to vasoactive drugs and PORH (assessed by scanning LDPI) were compared with changes in diameter and blood flow in the femoral artery, as assessed by high-resolution ultrasound. We found that the measured LDPI signal significantly correlated with changes of inflow into the femoral artery. Vasodilation induced by administration of nitroglycerine and acetylcholine increased vessel diameter, blood flow and mean perfusion, while vasoconstriction following administration of epinephrine decreased all three parameters. PORH was induced by temporal occlusion of the femoral artery with an external cuff. During occlusion, mean perfusion decreased to a condition of zero-perfusion and release of the cuff induced an immediate increase in blood flow that was followed by femoral artery dilation driving PORH/ perfusion. Surgical removal of the femoral artery decreased mean perfusion to a zero-perfusion level and fully abolished PORH. Importantly, the measurement of the PORH response by scanning LDPI is highly reproducible as determined by repeated measurements and intra/interobserver variation analysis. Last, we found that the PORH response was dependent on nitric oxide synthase and cyclooxygenase and declined with age. Thus, we here provide novel and robust non-invasive methods to serially measure tissue perfusion at baseline and during physiological and pharmacological modulation of vasomotor tone in the hindlimb of mice. The application of these LDPI scanning and ultrasound-based methods may be useful for testing the effects of drugs affecting vasomotor function or future elucidation of mechanisms leading to vasomotor dysfunction in mice *in vivo*.

Keywords: Laser Doppler perfusion imaging; hindlimb; reactive hyperemia; nitric oxide synthase; age-dependent endothelial dysfunction.

1. Introduction

A wide range of animal models to study endothelial dysfunction are available, including hyperlipidemic mice and rats, spontaneously hypertensive rats, genetically modified mice lacking/overexpressing specific apolipoproteins (e.g. apoE or apoB), hypertensive mice lacking the endothelial nitric oxide synthase (eNOS) and surgical models of vessel injury (1). In these mice models, endothelial and vascular function is frequently studied *ex vivo*, which has provided valuable new insight into vessel wall function. However, vascular function and blood flow are a result of multiple physiological inputs. Here, we propose a new method to examine the vascular function and tissue perfusion *in vivo*.

Post occlusive reactive hyperemia (PORH) is the physiological increase of blood flow to the tissue following ischemia after transient arterial occlusion. PORH has been applied for testing the endothelial function of conductance vessels in humans, e.g. by measurement of flow-mediated dilation (FMD) (2, 3), venous occlusion plethysmography (VOP), or skin microcirculation by laser Doppler flowmetry or imaging (4, 5). Scanning laser Doppler perfusion imaging (LDPI) has been applied in clinical settings to assess functional responses of the cutaneous microcirculation to vasoactive molecules or post occlusive reactive hyperemia (PORH) in patients with endothelial dysfunction due to hypertension (6), peripheral arterial disease (7), coronary artery disease (5), diabetes (8), smoking (9) (10) or age (11). In swine and dog animal models scanning LDPI was applied for measuring revascularization in wound healing (12, 13), mucosal microcirculation (14), or retinal capillary blood flow (15). In rodents and especially in mice scanning LDPI is classically used to study angiogenesis and neovascularization after hindlimb ischemia (16-19), but it has never been applied to serially study vasomotor function in mice.

In this study, we aimed to evaluate the applicability of scanning LDPI to measure PORH in mice and hypothesized that it would be suitable to characterize tissue perfusion and changes in vascular function. We found that scanning LDPI is a robust and reproducible non-invasive method, which may be applied to evaluate pharmacological response to vasodilators/vasoconstrictors and PORH in mice models of cardiovascular disease.

2. Materials and Methods

Animals

All procedures were conducted in conformity with the American Physiological Society's "Guiding principles for research involving animals and human beings" and were approved by the LANUV Nordrhein-Westfalen. Mice were fed standard laboratory chow ad libitum. Male C57/BL6 mice were purchased from Janvier SAS (Le Genest, France). Global eNOS KO mice(20) were kindly provided by A. Gödecke. Department of Physiology, Medical Faculty, Heinrich Heine University of Düsseldorf. Mice of the following ages were randomly assigned to groups and used for experiments: 8 ± 2 weeks (wk)-old (22 - 25 g), 24 wk-old (30 - 35 g), 48 wk-old (37 - 47 g) and 96 wk-old (34 - 45 g) mice.

Scanning LDPI

Scanning LDPI measurements were performed by using a PeriScan PIM 3 System and proprietary acquisition software (LDPIwin) (both by Perimed-Instruments, Järfälla, Sweden). The experimental setup is presented in Fig. 1. Mice were anesthetized with isoflurane (Piramal Critical Care, USA) (3% induction and 2% maintenance) and fixed on a heated platform (set to 39°C, body temperature approx. 36°C) in a supine position. Body hair was removed from the inner thigh using a depilation cream (Veet, Reckitt Benckiser, Germany). A measuring distance of 10 cm, a scanning area of 5 x 3 cm and a scanning velocity of 1 image/second were chosen and kept constant throughout the measurements. The signal was quantified as mean perfusion units (MPU). Baseline values were taken for 15 minutes (min) before starting the interventions. Data analysis was performed off-line using LDPIwin software (Perimed-Instruments).

High-resolution ultrasound

The femoral artery was visualized by using a VEVO2100 high-resolution ultrasound device equipped with ultra-high frequency transducers (MS400: 18-38MHz, MS700: 30-70MHz) and proprietary software (VisualSonics, Toronto, Canada), as previously described (21). Mice were anesthetized and prepared as described for LDPI. The femoral artery was visualized by its characteristic flow pattern in color doppler mode, PW-Mode, and B-Mode. Flow velocity, heart rate (HR), respiration rate (RR) and

body temperature were determined off-line with the dedicated software. Vessel diameter (d) was evaluated by using the Brachial Analyzer 5 software (Medical Imaging Applications, Coralville, IA).

Treatment with vasoactive drugs

All stock solutions were freshly prepared on the experimental day with warm (37°C) sterile saline (NaCl 0.9%) as a vehicle. The effects of injection of the vehicle control were tested in the same groups of animals before injecting the vasoactive substances. The doses applied were chosen according to preliminary tests carried out by measuring changes in vessel diameter by ultrasound (22, 23).

Nitroglycerin (NTG). NO-dependent and endothelium-independent vasodilation were induced by bolus intraperitoneal (i.p.) administration of 12 mg/kg NTG in 100 µl vehicle (0.9% NaCl) (stock 1 mg/ml, G.Pohl-Boskamp, Hohenlockstedt, Germany) or vehicle only. Readings were taken immediately after injection as described above.

Adenosine (Ado). To pharmacologically mimic the blood flow increase during reactive hyperemia, we administered 0.1 mg/kg bolus dose of adenosine in 100 µl vehicle (stock 10^{-4} mol/l; Sigma-Aldrich, St. Louis, USA) into the tail vein.

Epinephrine (Epi). To induce vasoconstriction, we used a local subcutaneous injection of 0.7 mg/kg epinephrine in 100 µl vehicle (stock 10^{-3} mol/l; Sigma-Aldrich) into the upper thigh.

Acetylcholine (ACh). To induce endothelium-dependent vasodilation, 0.07 mg/kg acetylcholine in 100 µl vehicle (stock 10^{-4} mol/L; Sigma-Aldrich) was injected into the tail vein. Acetylcholine stock solutions were prepared freshly with the vehicle on each day.

Induction of post-occlusive reactive hyperemia (PORH)

PORH was induced by using an air inflatable vascular occluder with a diameter of 10 mm (KentScientific, Connecticut, USA) as described (22, 23). Briefly, the hindlimb of a mouse was pulled through the cuff (see Fig. 1A). To induce PORH the cuff was inflated at a pressure of ~300 mmHg. Occlusion was confirmed by measuring distal blood flow (mean perfusion < 30 MPU) in preliminary experiments. After occlusion, the cuff was rapidly deflated. The following experimental protocol was used for both LDPI and ultrasound measurements: 15 min. equilibration period, 7 min. baseline

1 measurement (stabilization of perfusion), 5 min. ischemia (zero perfusion) and 8 min.
2 of reperfusion (hyperemia and recovery of perfusion).

3 Parameters used for quantifying the PORH measured by scanning LDPI are
4 described in Fig. 3A: 1) baseline perfusion; 2) maximal perfusion; 3) perfusion
5 reserve as $\Delta\% = (\text{max. reperfusion} - \text{baseline}) / \text{baseline} \times 100$; 4) ratio = max.
6 reperfusion / baseline; time to peak = time to maximal perfusion after releasing the
7 cuff; and area under the curve (AUC) = integral of the reperfusion curve.

8 Changes in the diameter of the femoral artery and flow after PORH were assessed
9 by high-resolution ultrasound. The dilation of the femoral artery is analyzed as %
10 flow-mediated dilation (FMD) by comparing the diameter change after occlusion in
11 relation to the basal diameter, as described (21).

12 **Effects of physiological and pharmacological modulation of the PORH** 13 **response.**

14 To control for femoral artery perfusion signal chronic hindlimb ischemia was induced
15 by surgical excision of the femoral artery, according to the protocol described by
16 Limbourg et al. (18). Briefly, mice anesthetized i.p with ketamine (100 mg/kg) and
17 xylazine (5 mg/kg) were positioned in dorsal recumbency. The skin was incised over
18 the femoral artery. The femoral artery was isolated ligated and excised in its entirety.

19 The effects of inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX)
20 on the PORH response were analyzed by the administration of the NOS inhibitor
21 nitro-L-arginine (L-NNA, Sigma-Aldrich), the COX inhibitor indomethacin (Sigma-
22 Aldrich) and in mice lacking the endothelial NOS (eNOS^{-/-})(20). L-NNA or
23 indomethacin was administrated in drinking water for 6 days. L-NNA was dissolved
24 in 1 M HCl (stock 160 mg/ml) and diluted to a final concentration of 1.6 mg /ml (and
25 (0.01 HCl) in drinking water, resulting in a daily dose of 8 mg L-NNA / day / 1 mouse.
26 Indomethacin was dissolved in ethanol and diluted to a concentration of 30 µg / ml,
27 resulting in a daily dose of 150 µg/day (and 1% ethanol). Corresponding vehicle
28 controls were used in separate animal groups. In a subgroup measurements were
29 conducted longitudinally before and 6 days after administration of the drugs.

1 **Blood pressure measurements in mice**

2 Mean arterial blood pressure in mice was measured in the aorta ascendens using a
3 1.4F SPR-839 pressure catheter from *Millar Instruments* (Houston, TX, USA) placed
4 into the aorta through the right carotid artery. Pressure data were analyzed with
5 dedicated software (IOX, EMKA, Paris, France) to determine peak systolic (SBP)
6 and diastolic pressure (DBP).

7 **Statistical Analyses**

8 Values reported are means $\pm$ SD or as box plots according to Tuckey. Comparisons
9 between groups were made using either a 2-sided student's t-test or 1- way or 2-way
10 ANOVA, followed by an appropriate post-hoc test as indicated in the figure legends
11 and the tables. Differences were deemed significant when $p < 0.05$. Statistical
12 analyses were performed using GraphPad Prism 8.00 (GraphPad, San Diego, CA,
13 USA) or SPSS 18.0 statistics (IBM).

3. Results

Visualization and quantification of blood flow and mean perfusion in the hindlimb by scanning LDPI and high-resolution ultrasound.

In this study, we aimed to validate the measurement of hindlimb perfusion by LDPI in the scope to evaluate physiological and pharmacological changes of vascular function in vivo. The experimental setting is depicted and described in Figure 1. Mice are anesthetized with isoflurane and fixed in a supine position and the hindlimb cleared of hair. A vessel occluder was applied for inducing post-occlusive reactive hyperemia (Fig. 1A). The scanning head is adjusted to a standardized distance of 10 cm from the measuring surface (Fig. 1B). When laser light penetrates the tissue (Fig. 1C) it is partly absorbed or scattered and light particles, which hit moving blood cells, undergo a change in wavelength (Doppler shift). As depicted in Fig. 1C, the scanning LDPI signal is mainly dependent on the penetration depth of the laser (estimated to be between 0.5 and 1 mm) and on the blood flow in the vessels encountered by the laser within the tissue. The femoral artery was found at 0.4 mm penetration depth, as visualized by high-resolution ultrasound (Fig. 1D, left panel). In its proximity, the perfusion map obtained by scanning a 4 x 8 mm area showed a discrete region with a very high signal (Fig. 1D, right panel), which was quantified as mean perfusion in a gated area and indicated in mean perfusion units (MPU).

Modulation of blood flow and mean perfusion by vasoactive molecules.

To analyze how changes in mean perfusion correlate with changes in blood flow in the femoral artery, we applied different well-characterized pharmacological agents inducing vasoconstriction or -dilation and measured both mean perfusion by scanning LDPI and blood flow by high-resolution ultrasound (Fig. 2). Systemic i.p. injection of a 12 mg/kg NTG bolus rapidly increased vessel diameter and flow within the femoral artery as assessed by ultrasound (Fig. 2A, left panel) and increased mean perfusion as compared to baseline, reaching a maximum within approximately 100 s (Fig. 2A, middle), whereas the vehicle control had no effect. The maximal increase in mean perfusion after administration of NTG was 128% as compared to vehicle control (435 ± 23 MPU after NTG vs. 191 ± 36 MPU $n = 5$, Fig. 2A, right panel). Administration of the adrenergic receptor agonist epinephrine (adrenaline) s.c. caused a rapid vasoconstriction as demonstrated by decrease in vessel

diameter and flow (Fig 2B, left panel), and a rapid 57% drop in mean perfusion (90 ± 18 MPU vs. 209 ± 59 , $n=4$, Fig. 2B, right panel), which lasted throughout the entire measurement interval (Fig. 2B, middle panel). Intravenous injection of the endothelium-dependent vasodilator acetylcholine caused a significant increase in vessel diameter and flow (Fig. 2C, left), and mean perfusion (Fig. 2C, middle and right), reaching a maximum of 132% within 300 sec ($n = 5$, 537 ± 35 MPU vs. 406 ± 48 MPU baseline). Stimulation of purinergic receptors by i.v. bolus administration of adenosine significantly increased vessel diameter and later on peak flow velocity (Fig. 2D, left panel), as well as mean perfusion within approximately 150 s (Fig. 2D, middle), with a maximal increase of 41% as compared to baseline values (316 ± 43 vs. 224 ± 34 MPU baseline) (Fig. 2D, right panel). The LDPI signal remained unaffected after vehicle injection. During the ultrasound measurements, there was no significant change in heart rate (HR), respiration rate and body temperature (Table 1). As expected, systolic and diastolic blood pressure measured in the aorta by Millar catheterization were significantly transiently decreased by all vasodilators applied and increased by epinephrine (Table 1). Taken together, our results show that changes in blood flow in the femoral artery as a result of the administration of well-characterized vasodilators and vasoconstrictors directly correlate to changes in mean perfusion, as assessed by LDPI.

Measurement of the PORH response by scanning LDPI

PORH was induced by inflating a cuff at a pressure of ~300 mmHg until reaching the point of zero perfusion (mean perfusion < 30 MPU), and then rapidly deflating it (Fig 3). Five minutes of occlusion time led to a rapid (< 1 min) FMD of the femoral artery accompanied by an increase in mean perfusion up to 200% as compared to baseline values, which then returned to baseline after 5-6 minutes (Fig 3). We found that the peak of maximum perfusion corresponded to a maximum dilation of the femoral artery (Fig. 3C); however, the increase in perfusion lasted longer than the vasodilatory response of the femoral artery. The peak of mean perfusion after PORH ($150 \pm 15\%$) was similar to the maximum NTG-induced perfusion increase ($149 \pm 42\%$) and significantly higher than the Ach ($38 \pm 12\%$) and adenosine ($43 \pm 8\%$) induced responses. The parameters used to quantify PORH are described in Figure 3A (also see Methods for details).

PORH in chronic hindlimb ischemia

If the femoral artery was surgically excised, mean perfusion was as low as zero perfusion after inducing transient ischemia in control animals (Fig.3A grey curve), and the PORH response was absent (Fig. 3A, gray curve).

Effects of occlusion time and reproducibility of measurements of PORH by scanning LDPI

To test the dependency of the PORH response on the duration of ischemia as assessed by LDPI, we occluded the hindlimb for 1, 3, and 5 min (Fig. 4 A-C). By increasing the occlusion time, we measured an increase in maximal perfusion, ratio, delta percent of the response and time-to-peak, while the AUC © was only significantly increased after 5 minutes of ischemia (Fig. 4 A-D; * $p < 0.05$, $n = 5$ independent measurements carried out on the same mouse). These data were also further analyzed to compare intra-individual variability with inter-individual variability of the different parameters by comparing their coefficient of variation (Table 1). We found the smallest variation for time to peak and perfusion reserve, while AUC measurements showed the highest variability.

The dependency of the PORH response on the availability of endogenous vasodilators.

To investigate the role of NOS-dependent NO synthesis, mice were treated with NOS-inhibitor L-NNA (1.6 mg/ml = 270 mg/kg / d) in drinking water for 6 days. NOS inhibition significantly decreased the rapidity of the PORH response with a greater time to peak determined both in intraindividual (before –after) measurements. (Fig. 5 A, Table 2) and in different groups (Fig. 5B and Table 3). Other parameters were unchanged (Tables 2 and 3). Mice with a genetic deletion of eNOS (eNOS^{-/-}) show increased time-to-peak (Fig. 5B and Table 3), but no changes in other parameters. To determine the role of COX-dependent prostaglandin production, the cyclooxygenase inhibitor indomethacin (30 µg/ml = 5 mg/kg/d) was administered to mice in drinking water for 6 days (Fig. 5C), which resulted in an increase in time-to-peak, but had no effect on the other parameters (Table 2).

1 **The dependency of the PORH response on mice age.**

2 As further proof of concept, we additionally determined PORH in wildtype mice
3 cohorts of different ages (Fig. 6). The rapidity of hyperemic reperfusion is altered
4 with a prolonged time to peak (Fig. 6A) and a faster perfusion drop to baseline
5 values in older mice (Fig. 6B). Furthermore, we found that 12 months and 24 months
6 old mice have a significantly diminished AUC (Fig. 6D) and % perfusion increase of
7 PORH (Fig. 6C), as compared to younger mice.

8 Taken together, these data show that the measurement of perfusion in the mouse
9 hindlimb by LDPI is sensitive physiological and pharmacological changes in vessel
10 diameter and is valid for evaluating intraindividual changes for significant in vivo
11 investigations with limited numbers of animals.

4. Discussion

In this study, we evaluated the potential of measuring the PORH response by scanning LDPI. We show that 1) changes in flow in the femoral artery due to administration of vasoactive drugs correlate to changes in mean perfusion in the hindlimb; 2) the PORH response (changes in mean perfusion by LDPI) correlate to changes in diameter (as FMD% by ultrasound) 3) measurements of the PORH response by scanning LDPI are reproducible, are modulated by intervention with NOS and COX inhibitors, and decrease with increasing animal age. Thus, we propose that measurements of PORH by scanning LDPI might be applied for the assessment of changes in vascular function in mice models of cardiovascular disease.

What is scanning LDPI measuring?

Scanning LDPI is based on the physical phenomenon defined as the Doppler shift or Doppler effect, which is a change in the observed frequency of a wave occurring when the source and observer are in motion relative to each other. When laser light penetrates a tissue, it is backscattered and returns to its source without change of frequency. But if the laser light is backscattered by moving objects (i.e. blood cells), it undergoes a shift in frequency directly proportional to their concentration and velocity. The *measuring depth* of the laser, i.e. how deep the laser light penetrates the tissue, depends (a) on the energy and wavelength of the laser, (b) on the measuring distance and, most importantly, (c) on biophysical and anatomical characteristics of the tissue, which can influence its backscattering properties; these include (but they are not limited to) density and pigmentation of the skin and presence of hair, as well as density and diameter of the vessels (microcirculation, resistance vessels, macrocirculation), amount of blood cells.

The perfusion map obtained by scanning a defined area of the hindlimb shows a discrete region of very high signal intensity, which corresponds to the area of tissue with and around the femoral artery. According to the manufacturer's information, the measurement depth in our system in human skin has been calculated to be around 1 mm. In the mouse, the femoral artery can be visualized at 0.4 cm penetration depth with high-resolution ultrasound. Thus, a major part of the measured perfusion in the mouse is dependent on both blood flow in the femoral artery (macrocirculation) and

microcirculatory tissue perfusion; however, because of the velocity of the blood cell in the conduit vessels, the blood flow in the femoral artery will predominate the overall signal. Therefore, the use of scanning LDPI of the mouse hindlimb is not comparable to its use in human skin, where – considering the penetration depth of the laser – it is applied to assess microcirculatory function only.

Measurements of the PORH response by scanning LDPI: reproducibility, and dependency on physiological stimuli.

Measurement of PORH with different methods is routinely applied in clinical settings to evaluate the endothelial functionality of different vessel beds, and it is known to be dependent on occlusion time and on endogenous vasodilators.

As the first step of scanning LDPI validation for assessment of hyperemic responses, we administered vasoactive drugs and compared the LDPI signal to ultrasound-assessed changes in femoral artery hemodynamics. We found that the LDPI signal (mean perfusion assessed in MPU) is directly correlated to changes in diameter and blood flow in the femoral artery. For example, administration of both endothelial independent and endothelial-dependent vasodilators (NTG, acetylcholine, adenosine) caused an increase in vessel diameter and blood flow as compared to baseline, which corresponded to a comparable increase in mean perfusion as assessed by LDPI. Conversely, the vasoconstrictor epinephrine caused a strong and rapid decrease in vessel diameter, blood flow and mean perfusion. Therefore, scanning LDPI can be used for measuring pharmacologically modulated hyperemic responses in the mouse hindlimb.

In addition, we verified that PORH-induced changes in mean perfusion corresponded to FMD, which is a widely accepted parameter for determining endothelial function in vivo. We found that the peak of maximum perfusion corresponds to the point of maximum flow-mediated dilation of the femoral artery and that the elevation of perfusion (MPU) lasts longer than the vasodilatory response of the femoral artery, indicating a microvascular component of the LDPI signal. Interestingly, maximal perfusion (MPU) after PORH was comparable with the maximum NTG-induced perfusion increase and significantly higher than the acetylcholine or adenosine-induced response. Importantly, PORH was absent in chronic hindlimb ischemia and PORH-induced changes in mean perfusion were dependent on the duration of the

1 occlusion and increased with longer occlusion times (1-5 minutes), further validating
2 this measurement approach.

3 Last, we analyzed the reproducibility and variability of the PORH response assessed
4 by LDPI by repeated intra-individual and inter-individual measurements. The most
5 robust parameter was the *time-to-peak* (which is an indication of the rapidity of the
6 response) and the *ratio of maximum and baseline perfusion* (which is an indicator of
7 the magnitude of the response). Interestingly, we found that the rapidity of the
8 hyperemic response was dependent on the enzymatic synthesis of both NO and
9 prostaglandins, as the NOS inhibitor L-NNA as well as the COX inhibitor
10 indomethacin increased time to peak.

11 Vascular aging is known to induce endothelial dysfunction in humans (24) and rats
12 (21) and is associated with impaired angiogenesis in mice (19). Here, we found that
13 12- and 24-months old mice have slower vascular responsiveness (time to peak),
14 significantly decreased magnitude of response (perfusion reserve and AUC) and a
15 less sustained hyperemia (Fig. 6B) as compared to younger mice (Fig. 6D). This
16 data is in agreement with other reports (21-23) again demonstrating the robustness
17 of our technique.

18 **Assessment of endothelial function in vivo: PROH vs. FMD**

19 Although the use of in vivo models of cardiovascular disease is widely spread,
20 methods for evaluating endothelial function in rodents and particularly in mice are
21 mainly restricted to ex vivo techniques or measurement of high-resolution ultrasound
22 of FMD after PORH (21-23). It is very important to consider the fundamental
23 differences in the physiological responses assessed by these two methods.

24 Measurement of PORH by scanning LDPI of PORH in the mouse hindlimb¹ allows
25 measurement of both microcirculatory and microcirculatory tissue perfusion in a
26 defined region of interest, while high-resolution ultrasound allow determination of
27 FMD of a specific conduit vessel. Therefore, measurement of PORH by scanning
28 LDPI is less specific for the determination of endothelial function (i.e. it does not
29 measure the diameter and flow velocity of a specific vessel) and it cannot distinguish

¹ As discussed, due to the penetration depth of the laser, the measurement of PORH in the mouse hindlimb by scanning LDPI is a measurement of both macrocirculation and microcirculation, while measurements of PORH by scanning LDPI in humans is only a measurement of microcirculation

1 between macrocirculatory and microcirculatory blood flow. A way to distinguish the
2 microcirculatory component from the microcirculatory component could be the
3 application of a β_2 receptor antagonist to skeletal muscle and should be investigated
4 in the future.

5 On the other hand, FMD assessed by high-resolution ultrasound only provides very
6 local blood flow velocity and estimation of perfusion of a specific conduit vessel. Only
7 a combination of the two techniques may allow understanding all physiological
8 variables connecting endothelial function, microcirculation and global tissue
9 perfusion, and their physiological changes in age or disease conditions.

10 Moreover, scanning LDPI is more robust and less sensitive than ultrasound to tiny
11 changes in hindlimb position during inflation and deflation of the cuff, temperature
12 and small changes in cardiac and circulatory parameters; these characteristics make
13 scanning LDPI easier to use, requires less training time to achieve reproducibility,
14 and is more robust. A LDPI scanner is also more affordable than a high-resolution
15 ultrasound, which ensures greater access to the method.

16 In the comparison of the two techniques it is important to point out that although
17 there is a relationship between vasodilation and increased velocity flow in the feed
18 artery, it is very uncertain if a direct relationship occurs between the two phenomena.
19 In humans, the velocity peak occurs within the first 15 seconds and the vasodilation
20 peak can be expected to occur 40-80 seconds following cuff release (25). These
21 responses are clearly different from rat (21) and mouse (assessed here and
22 elsewhere (23)).

23 Other species-specific and technique-dependent differences were also observed by
24 comparing the effects of NOS and COX inhibition. We found here that the
25 administration of L-NNA and indomethacin decreased *time-to-peak*, but did not affect
26 the magnitude of peak perfusion. Results by Heiss et al. (21) in rats show that NOS
27 inhibition (by L-NMMA) almost exclusively abolished femoral artery vasodilation
28 without affecting flow velocity and wall shear stress as assessed by ultrasound.
29 Whether this is due to species specificity or because LDPI is measuring a mixed
30 macro/microcirculatory signal need to be determined experimentally by carrying out
31 these experiments side-by-side.

1 A further consideration is that at least in humans there are clear limb differences in
2 vascular control mechanisms, i.e. vascular responses of the arm are different from
3 vascular responses in the legs (26). these potential differences need to be taken into
4 consideration in rodents and – premise the availability of a system allowing the
5 measurements – should be tested.

6 To conclude, scanning LDPI of the PORH response in the mouse hindlimb can be
7 applied for assessing vascular function in mice in vivo. This method allows testing
8 the effects of pathophysiological changes of vascular function in correlation to
9 genetic or pharmacological manipulation, or physiological processes like aging both
10 in a cross-sectional or longitudinal experimental setting in the living animal, thus
11 permitting an easier translation of the findings to the clinical application in humans.

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5. Figure legends

Fig.1: Principles and application of laser Doppler perfusion imaging (LDPI) for measurement of perfusion of the mouse hindlimb. (A) Picture of a live mouse anesthetized with isoflurane, positioned on a 37°C heated surface; the picture shows the cuff/vascular occlude used for PORH measurements and the laser light illuminating the area of measurement on the hindlimb. (B) Schematic representation of the experimental setup, showing the optimized measuring distance of the LDPI scanner. C) In normal human skin, the 670-690 nm wavelength laser of the PeriScan PIM 3 System is partly absorbed or scattered, and the measuring depth is in the order of 0.5 - 1 mm. **D)** The left panel shows an ultrasound image of the mouse hindlimb with visualization of the femoral artery around 0.4 mm underneath the skin. The laser is systematically making 1-2 measurements/sec producing a colored image where red represents areas of high perfusion and black represents areas of low perfusion (right panel). Due to the presence of the femoral artery and its branching (middle panel), the LDPI signal (right panel) will comprise both macrocirculation and microcirculation in the mouse.

Fig.2: Effects of pharmacological modulation of vessel diameter and hindlimb perfusion by the administration of vasodilators and vasoconstrictors. Changes in femoral artery diameter and peak/mean flow velocity by ultrasound (left panels) and changes in tissue perfusion by LDPI (middle and right panels) in response to vasodilators and vasoconstrictors were assessed by **(A)** acute application of nitroglycerin (12 mg/Kg n = 4), **(B)** s. c. application of the α/β -agonist epinephrine (0.7 mg / Kg, n=4), **(C)** i.v. application of the endothelium-dependent vasodilator acetylcholine (0.07 mg/Kg, n=5) and **(D)** i.v. application of the purinergic mediator adenosine (0.1 mg/Kg, n = 4). Warm saline served as control and had no effect on the perfusion values. Data are reported as means $\pm$ SD. RM 2-way ANOVA $p < 0.001$; Sidak's multiple comparison test * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$ vs. baseline; saline and pharmacological controls were run on the same mouse.)

Fig.3: Effects of physiological modulation of hindlimb perfusion by inducing reactive hyperemia. Representative images **(A)** and curves **(B)** showing changes in tissue perfusion during baseline, ischemia and reactive hyperemia (black line) assessed by LDPI. In B the analysis parameters used to quantify the hyperemic response are represented: basal perfusion, zero perfusion, maximum perfusion, time-to-peak, amplitude (max - basal perfusion), perfusion reserve (amplitude / basal perfusion*100), ratio (max / basal perfusion) and AUC (area-under-the-curve). The grey line represents zero-perfusion obtained after surgical excision of the femoral artery (one representative curve of 3 independent measurements from different mice). **(C)** The changes in perfusion (red curve, n = 10) after occlusion correspond to changes in femoral artery diameter assessed as flow-mediated dilation (FMD) by high-resolution ultrasound (blue curve, n = 10). All data are expressed as mean $\pm$ SD.

Fig.4: Effects of occlusion time on PORH response assessed by LDPI. The degree of maximal hyperemic blood flow depends on the duration of occlusion. Data

represent mean repeated measurement carried out on the same mouse at 5 different days. Lines represent mean $\pm$ SD. RM 1-way ANOVA $p < 0.05$ for all panels (except baseline 1-way ANOVA n.s.). Tuckey's * $p < 0.05$ vs. time 1 min.

Fig.5: NOS and COX inhibition prolong the PORH response. Effects of chronic inhibition of NO synthase (NOS) by administration of L-NG-Nitroarginine (L-NNA; 7 mM in drinking water, corresponding to 270 mg / Kg/day) or indomethacin (Indo, 30 μ g/ ml in drinking water, corresponding to a dose of 5 mg/Kg/day) for 6. (A) Measurements of PORH response were carried out before and after the treatment. RM 2-way ANOVA $p < 0.05$; Sidack's ** $p < 0.01$; Paired T-test # $p < 0.05$. (B) Measurements of PORH response were carried out in independent groups of wild type (WT) mice left untreated or treated with L-NNA or mice lacking eNOS (eNOS^{-/-}). 1-way ANOVA $p < 0.001$; Tuckey's * $p < 0.05$; ** $p < 0.01$

Fig. 6: The degree of reactive hyperemic blood flow decreases with age. In aged mice, we measured a significant decrease in the rapidity of reperfusion in the first phase (time to peak) (A) and a faster recovery (time to base) (B). Moreover, PORH after ischemia is significantly lower in adult and aging mice (6, 12, 24 months) vs. young mice (2-3 months) as assessed by a significant decrease in overall perfusion (AUC) shown in (C) and in PORH response (D). 1-way ANOVA* $P < 0.01$ for all the panels. Tuckey's * $p < 0.05$ *** $p < 0.001$ vs. 2-3 months old mice; T-Test # $p < 0.05$ vs. 2-3 months old mice.

6. Table legends

Table 1. Effects of administration of vasodilators/constrictors on hemodynamics, body temperature and respiratory rate. Physiological parameters were determined before and after administration of nitroglycerine (NTG), epinephrine (Epi), acetylcholine (ACh) and adenosine (Ado). Data are mean $\pm$ SEM. * $p < 0.05$ ** $p < 0.01$ $n = 4$ per group.

Table 2: Intra and interindividual variability of parameters assessed to quantify the PORH response in wild type mice.

Table 3: Effects of NOS and COX inhibition on PORH response. Measurement of PORH response in wild type C57Bl/6 mice before and after administration of the NOS inhibitor L-NNA (270 mg/Kg/day) or the COX inhibitor indomethacin (5 mg/Kg/day) for 6 days in drinking water.

Table 4: PORH response in mice lacking eNOS expression. Measurement of PORH response in untreated wild type (WT) C57Bl/6 mice, or after administration of the NOS inhibitor L-NNA (270 mg / Kg / day) for 6 days or in eNOS knockout (eNOS^{-/-}) mice. 1-way ANOVA, Tuckey's * = $P < 0.05$; ** = $P < 0.01$.

Tables

Table 1: Effects of administration of vasodilators/constrictors on hemodynamics, body temperature and respiratory rate. Physiological parameters were determined before and after administration of nitroglycerine (NTG), epinephrine (Epi), acetylcholine (ACh) and adenosine (Ado). Data are mean ± SEM. * p < 0.05 ** p< 0.01 n = 4 per group.

ECHOCARDIOGRAPHY					
a.	respiration rate (RR)		heart rate (HR)		
	baseline	treatment	baseline	treatment	
+ NTG	143 ± 26	133 ± 25	464 ± 56	505 ± 46	n=3
+ ACH	94 ± 18	93 ± 22	441 ± 38	491 ± 53	n=3
+ Adenosine	120 ± 17	120 ± 34	381 ± 26	398 ± 57	n=4
+ Epinephrine	132 ± 23	128 ± 37	537 ± 33	532 ± 56	n=3

BLOOD PRESSURE MEASUREMENTS					
b.	Blood pressure (BP) PS / PD (mmHg)		heart rate (HR)		
	baseline	treatment	baseline	treatment	
+ NTG	97 ± 7 / 61 ± 7	83 ± 7 / ** 59 ± 7	605 ± 21	596 ± 26	n=4
+ ACH	104 ± 1 / 72 ± 2	103 ± 4 / 72 ± 4	630 ± 42	591 ± 4	n=2
+ Adenosine	97 ± 8 / 63 ± 11	91 ± 8 / ** 55 ± 13 *	580 ± 37	540 ± 19	n=2
+ Epinephrine	95 ± 5 / 69 ± 4	104 ± 6 / ** 73 ± 3 *	542 ± 51	651 ± 17	n=4

Table 2: Intra and interindividual variability of parameters assessed to quantify the PORH response in wild type mice.

	<i>Baseline</i>	<i>Max</i>	<i>PORH</i>	<i>Time to peak</i>	<i>AUC</i>	<i>ratio</i>	<i>Time to base</i>	<i>n</i>
	<i>MPU</i>	<i>MPU</i>	<i>%.</i>	<i>s</i>	<i>MPU</i>	<i>a.u.</i>	<i>s</i>	
Intraindividual	11.7%	18.4%	29.5%	12.3%	46.4%	15.2%	21.9%	5
Interindividual	28.7%	25.2%	42.2%	29.3%	52.7%	18.3%	26.0%	18

Table 3: Effects of NOS and COX inhibition on PORH response. Measurement of PORH response in wild type C57Bl/6 mice before and after administration of the NOS inhibitor L-NNA (270 mg / Kg / day) or the COX inhibitor indomethacin (5 mg / Kg / day) for 6 days in drinking water.

	<i>Baseline</i>	<i>Max</i>	<i>PORH</i>	<i>Time to peak</i>	<i>AUC</i>	<i>Ratio</i>	<i>n</i>
	<i>MPU</i>	<i>MPU</i>	<i>%</i>	<i>s</i>	<i>MPU</i>	<i>a.u.</i>	
<i>Before</i>	206.5 ± 48.1	409.9 ± 124	100.8 ± 45	35.8 ± 11	71320 ± 52713	1.9 ± 0.4	5
<i>After L-NNA</i>	222.6 ± 31.3	542.3 ± 59.7	146.8 ± 35.9	77.5 ± 35.6 *	281125 ± 113296	2.6 ± 0.3 *	5
<i>Before</i>	236.3 ± 31.8	601.9 ± 101.7	155.5 ± 32.5	48.3 ± 9.5	63377 ± 5420	2.6 ± 0.3	4
<i>After Indo</i>	260.2 ± 119	601.8 ± 335.9	126.2 ± 49.8	64.5 ± 10.7 *	31747 ± 30221	0.5 ± 0.2	4

Table 4: PORH response in mice lacking eNOS expression. Measurement of PORH response in untreated wild type (WT) C57Bl/6 mice, or after administration of the NOS inhibitor L-NNA (270 mg / Kg / day) for 6 days or in eNOS knockout (eNOS^{-/-}) mice. 1-way ANOVA, Tuckey's * = P < 0.05; ** = P < 0.01.

	<i>Baseline</i>	<i>Max</i>	<i>PORH</i>	<i>Time to peak</i>	<i>AUC</i>	<i>Ratio</i>	<i>n</i>
	<i>MPU</i>	<i>MPU</i>	%	s	<i>MPU</i>	a.u.	
<i>WT</i>	317.9 ± 184.3	562.6 ± 255.3	106.5 ± 68.44	63.8 ± 20.0	34315 ± 24437	2 ± 0.74	18
<i>WT+L-NNA</i>	263.3 ± 53.7	481.7 ± 69.5	88.5 ± 40.9	101.3 ± 39.3 **	19511 ± 8701 *	1.9 ± 0.4	19
<i>eNOS KO</i>	230.6 ± 77.8	442.1 ± 140.1	95.3 ± 32.1	70.6 ± 23.9*	25547 ± 22401	2 ± 0.3	19

Figure 1

Figure 2
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Figure 3
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Figure 6
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