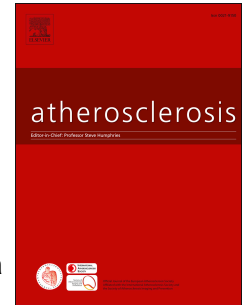


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Vasoactive enzymes and blood flow responses to passive and active exercise in peripheral arterial disease

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Abstract

Background: Peripheral arterial disease (PAD) is characterised by impaired leg blood flow, which contributes to claudication and reduced exercise capacity. This study investigated to what extent vasoactive enzymes might contribute to altered blood flow in PAD (Fontaine stage II).

Methods: We compared femoral artery blood flow during reactive hyperaemia, leg-extension exercise and passive leg movement, and determined the level of vasoactive enzymes in skeletal muscle samples from the vastus lateralis in PAD (n = 10, 68.5 ± 6.5 years) and healthy controls (CON, n = 9, 62.1 ± 12.3 years). Leg blood flow was measured with Doppler ultrasound and muscle protein levels of phosphorylated endothelial nitric oxide synthase, NADPH oxidase, cyclooxygenase 1 and 2, thromboxane synthase, and prostacyclin synthase were determined.

Results: Leg blood flow during the initial 90s of passive leg movement (242 ± 33 vs 441 ± 75 ml·min⁻¹, P = 0.03) and during reactive hyperaemia (423 ± 100 vs 1255 ± 175 ml·min⁻¹, P = 0.002) was lower in PAD than CON, whereas no significant difference was observed for leg blood flow during exercise (1490 ± 250 vs 1887 ± 349 ml·min⁻¹, P = 0.37). PAD had higher NADPH oxidase than CON (1.04 ± 0.19 vs 0.50 ± 0.06 AU, P = 0.02), with no differences for other enzymes. Leg blood flow during exercise was correlated with prostacyclin synthase (P = 0.001).

Conclusion: Elevated NADPH oxidase indicates that oxidative stress may be a primary cause of low nitric oxide availability and impaired blood flow in PAD.

Keywords: peripheral arterial disease; leg blood flow; vasoactive enzymes; NADPH oxidase

Abbreviations: AUC, area under the curve; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PAD, peripheral arterial disease; ROS, reactive oxygen species

Highlights:

- Passive movement hyperaemia was low in PAD suggesting low nitric oxide availability
- Leg blood flow during exercise correlated with leg muscle prostacyclin synthase
- NADPH oxidase is elevated and leg blood flow is reduced in PAD compared to control

Introduction

Peripheral arterial disease (PAD) is characterised by reduced blood flow to the legs attributed to atherosclerotic lesions leading to stenosis and/or occlusion of the conduit arteries [1]. Typically, PAD patients who experience intermittent claudication have impaired muscle function and reduced exercise tolerance that limits daily physical activities [2, 3]. While alterations in muscle morphology and metabolism are believed to contribute to these functional impairments, it is likely that the limb blood flow limitation is the primary cause of this impairment [4]. However, the extent to which limb blood flow is limited during leg exercise, and any contribution of endothelial dysfunction and the vasodilating systems, has not been established in PAD.

A recently developed test for the assessment of nitric oxide (NO) dependent vascular function is that of femoral blood flow response to passive movement of the lower leg [5]. Previous studies have shown that both aged individuals and individuals with PAD show a lower blood flow response to passive movement suggesting impaired NO function [5, 6]. Similarly, reactive hyperaemia, which is also highly dependent on NO [7, 8], is lower in the aged and in PAD compared to young healthy individuals [9]. This raises questions about the control of blood flow in PAD and presents the possibility that in addition to arterial stenosis, altered NO availability, and thereby limited vasodilation, might contribute to the impairment in leg blood flow of PAD patients during exercise.

Nitric oxide is a critical agent in the control of blood flow to skeletal muscle [7, 10]. It is well established that NO availability declines with age [11, 12] and can be further compromised by the presence of atherosclerotic disease, as observed in PAD [13]. However, the cause for low NO availability in PAD is unknown. Generally, NO availability is dependent on the amount of endothelial nitric oxide synthase (eNOS) protein, the state of activation of the enzyme, and the presence of reactive oxygen species (ROS) [11, 14]. ROS reduce NO bioavailability by readily reacting with NO to form peroxynitrite, but ROS can also uncouple eNOS whereby the enzyme forms superoxide ions instead of NO [15]. One of the main contributors to ROS in the vasculature is NADPH oxidase and several inflammatory conditions and disease states have been associated with increased NADPH oxidase levels [16, 17], but its expression in skeletal muscle of PAD patients is not known.

Although NO is known to be important for vascular function, other vasoactive systems, both vasodilating and constricting, are known to contribute to vascular conductance in skeletal muscle [10, 18]. During exercise, blockade studies in healthy individuals demonstrate that NO and prostaglandins are interdependent vasodilating systems that can compensate for each other to maintain blood flow when one system is compromised [19, 20]. This interaction may be acute, but

there are also indications of more chronic redundancy between the systems; for example in diabetes and in hypertension, NO availability is low and vascular function is maintained by elevated levels of prostacyclin [21, 22]. The impact of PAD on the prostaglandin system is unknown.

Thus, the aims of this study were: 1) to establish femoral arterial blood flow responses in PAD patients compared to healthy controls during reactive hyperaemia, passive leg movement, and active knee extensor exercise; 2) to compare the amounts of vasoactive enzymes in the vastus lateralis muscle in PAD patients and healthy controls, and 3) to explore the relationships between levels of vasoactive enzymes and the blood flow responses.

Methods

Participants

Ten patients with a confirmed diagnosis of PAD (Fontaine stage II) and nine healthy control participants of similar age and weight were recruited to participate in the study (see Table 1 for participant characteristics). All participants were screened for inclusion. PAD patients were included if they had: 1) diagnosis of PAD with a medical record of stenosis and occlusion locations, 2) clinically stable (> 6 months) intermittent claudication [23], and 3) an ankle-to-brachial systolic blood pressure index (ABI) < 0.90 in the study leg [24]. Patients were excluded if they had: 1) ABI > 0.9, 2) unstable or poorly controlled conditions, such as unstable angina or poorly-managed diabetes, and 3) communicable diseases. Four patients had previously (> 12 months) undergone revascularisation procedures for PAD, but stenosis had reoccurred and inclusion criteria were clinically confirmed with haemodynamic measures and vascular imaging. Medication use was not an exclusion criteria in this study. Participants in the healthy group were included if they had: 1) an ABI > 1.0 in both legs, 2) no history of PAD, 3) no unstable or poorly managed conditions, and 4) no communicable diseases. All participants provided a full medical history including smoking and medication use (Table 1), and completed a 7-day physical activity recall and the walking impairment questionnaire [25]. All participants provided written, informed consent prior to the study, and all procedures were approved by the Royal Brisbane and Women's Hospital and University of the Sunshine Coast human research ethics committees.

Healthy controls reported no limitation in their walking ability, while the PAD group reported walking impairment (49 ± 9 out of 100, where 0 = unable and 100 = fully able). There were no significant differences in reported habitual activity level between groups (PAD: 6.1 ± 2.1 ; Healthy: 4.4 ± 2.2 hours·week⁻¹, $P = 0.58$). All PAD participants were past smokers and two were current smokers

at the time the experiment was conducted. Eight of the nine controls had also smoked previously. PAD patients tended to report a larger pack-year smoking history (PAD: 42 ± 12 ; Healthy: 14 ± 5 pack-years, $P = 0.057$).

One individual was excluded from the study due to a history of hepatitis C. Four PAD patients were excluded from the reactive hyperaemia test due to the presence of stent grafts in the common or superficial femoral arteries.

ACCEPTED MANUSCRIPT

	Healthy	PAD
n	9	10
Age (years)	62 ± 12	69 ± 7
Gender (M/F)	7/2	8/2
Height (cm)	173.9 ± 8.8	176.9 ± 4.7
Weight (kg)	83.8 ± 13.9	80.0 ± 16.0
Body mass index (kg·m⁻²)	25.4 ± 3.9	27.4 ± 2.7
Current smokers (%)	0	20
Past smokers (%)	89	100
Study leg ABI	1.22 ± 0.15	0.79 ± 0.15 *
Contralateral leg ABI	1.16 ± 0.11	0.83 ± 0.18 *
Peak power (W)	38 ± 14	23 ± 7 *
Previous lower limb revascularisation surgery (%)	0	40
Previous myocardial infarction (%)	11	30
Obese (%)	0	10
Diabetic (%)	11	10
Hypertensive (%)	33	70
Hyperlipidemia (%)	44	40
Diuretic use (%)	0	30
β-blocker use (%)	0	50
Ca²⁺ channel blocker use (%)	22	10
ACE/ARB use (%)	22	60
Statin use (%)	30	80
Antiplatelet use (%)	33	80
Aspirin use (%)	33	60

Table 1. Participant characteristics. Values are mean ± SD or percent distribution. PAD = peripheral arterial disease, ABI = ankle brachial index, Peak power (W) was determined during an incremental single leg kick test, ACE = angiotensin converting enzyme, ARB = angiotensin receptor blocker.

*denotes significant difference between the PAD group and the healthy control group, P < 0.05.

Study Overview

Following screening and test familiarisation, participants visited the laboratory on two occasions separated by at least 7 days (PAD: 25 ± 7 ; Healthy: 28 ± 12 days). All participants were instructed to avoid exercise, caffeine, and alcohol for 24 hours before their appointments. During the first visit, each participant underwent a resting biopsy of the vastus lateralis muscle of the study leg for the determination of vasoactive mediators. The second visit involved measurement of resting leg blood flow and reactive hyperaemia at the femoral artery. This was followed by the assessment of femoral artery blood flow during 5-minute bouts of passive and active isolated single-leg extension exercise.

Experimental Procedures

Ankle-Brachial Index (ABI)

Ankle and brachial systolic blood pressures were measured in triplicate using a continuous wave ultrasound probe (MD6, Hokanson, Bellevue, USA). ABI was calculated in each limb as the higher ankle artery pressure (dorsalis pedis or posterior tibial) divided by the higher brachial artery pressure (left or right).

Leg movement and exercise protocol

Seated resting measures, passive leg movement and active leg kick exercise were completed on a custom-built leg-kick ergometer. Participants sat in a semi-reclined position with their foot secured in a boot that was attached to the cycle crank of the ergometer (Velotron, RacerMate Inc, WA, USA). Passive leg movement was carried out by a member of the research team turning the cranks to move the participant's leg. Active leg kicking was performed when the participant kicked against a set load. All leg kicking was completed at a rate of $60 \text{ cycles}\cdot\text{min}^{-1}$ timed with an audible metronome. During the familiarisation visit, participants completed an incremental leg kick test to determine peak power output where the workload started at 5W and increased by 5W each 3 min, with 1 min rest between each stage. Participants continued until they were unable to maintain a cadence of $60 \text{ cycles}\cdot\text{min}^{-1}$.

Participants rested in the seat for at least five minutes before baseline measurements. They then completed two five-minute bouts of passive knee extension, each followed by five minutes of rest, and then two five-minute bouts of active knee extension against a 10W load, each followed with 10 minutes of rest. Quadriceps and hamstring EMG were observed for all participants during passive movement. If there was any activity, the participant was instructed to relax the thigh muscles (EMG data not shown).

Ultrasound blood flow

Duplex ultrasound (Mindray M5, Mindray Medical International Ltd, Shenzhen, China) was used to record vessel image and blood flow velocity spectra in consecutive cine loops initiated each 30 s throughout rest, reactive hyperaemia, and passive and active knee extension. Blood flow was measured at the common femoral artery at least three cm above the bifurcation of the superficial and profundus branches. The Doppler sample volume was set as wide as possible within the limits of the vessel lumen, and an insonation angle of $< 60^\circ$ was maintained for all data capture. Each cine loop was stored and examined off line after all data were collected. One researcher examined all files to minimise measurement variability. Blood flow velocity over the first four artefact-free cardiac cycles of each cine loop were used to calculate flow volume ($\text{L}\cdot\text{min}^{-1}$). Vessel diameter was measured in triplicate at end-diastole of each cycle using the leading-edge to leading-edge distance [26] with electronic callipers. When intimal layers of the vessel could not be clearly defined, Doppler colour filling was used to assist in the determination of vessel lumen diameter. Leg blood flow was calculated by the Mindray M5 software using the following equation:

$$\text{Volume flow} = \frac{|\text{TAMAX}| \times (\pi \times D^2)}{4} \times 60$$

Where: TAMAX is the time averaged maximum Doppler velocity ($\text{cm}\cdot\text{s}^{-1}$) and D is the diameter of the vessel (cm).

Resting supine leg blood flow and reactive hyperaemia

Participants rested supine for 10 minutes before resting blood flow was measured, which was followed (< 5 min) by the reactive hyperaemia assessment. Blood flow to the leg was occluded using a contoured thigh cuff (Hokanson, Bellevue, USA) inflated to 220 mmHg. After five minutes, the cuff pressure was rapidly released and images for reactive hyperaemia measurement were collected immediately (0s) and 30s later using duplex ultrasound (see Ultrasound blood flow). Vascular conductance was calculated as femoral flow / mean arterial pressure.

Passive movement and active exercise blood flow

Blood flow responses during the leg extension protocols were assessed as the average blood flow over the five-minute bouts. Initial blood flow responses were assessed as the area under the curve (passive) or the linear rate (slope) of rise in blood flow over the first 90 seconds. Steady state blood flow during active exercise was assessed as the average over the final two minutes of the test.

Muscle biopsy and vasoactive enzymes

Participants rested supine for 30 minutes and then the skin over the vastus lateralis of the experimental leg was anaesthetised with 1% Xylocaine. A resting muscle biopsy was taken from the vastus lateralis using a Bergstrom percutaneous biopsy needle with suction. Muscle tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Muscle tissue samples were analysed using Western Blot procedures that have been described previously [27]. Approximately 5mg dry weight of biopsied muscle tissue (free from connective tissue and fat) was homogenised, rotated for 1 hr at 4°C , centrifuged at 17000g at 4°C , and then the lysate was collected for analysis. The concentration of proteins in the lysate was determined by BSA protein assay (Pierce Biotechnology Inc, Rockford, IL). Lysate proteins were separated using 10% Tris/Tricine gels then transferred to membranes (Immobilion Transfer Membrane, Millipore, USA) and incubated with primary and secondary antibodies. Antibodies used were: Anti-eNOS-pSER¹¹⁷⁷ (482737, Merck Millipore, USA), p67phox (610912, BD Transduction Laboratories, USA), Anti-COX1 (ab53766), Anti-COX2 (ab52237), and Anti-TBX synthase (ab39362, from Abcam, Cambridge, USA), and Anti-PGI₂ synthase (sc-20933, Santa Cruz Biotechnology, USA). Following detection (Kodak Image Station, 2000 MM) and quantification (Kodak Molecular Imaging software), the protein levels were expressed in arbitrary units. GAPDH was used as the loading control.

Statistics

All data are expressed as mean \pm standard error of the mean, unless otherwise stated. All analyses were performed using SPSS (IBM SPSS Statistics, Ver22, USA). For each individual, the two passive trials were averaged and the two active trials were averaged. This had a data smoothing effect without altering the significance of the findings. ANOVA for repeated measures was used to assess group and time effects on blood flow during each exercise phase. Where ANOVA revealed a significant F test, pair-wise comparisons were examined to identify the specific location of differences. Strength of the relationships between blood flow variables and enzyme levels were assessed using Pearson correlation coefficient. Z-tests were used for comparisons of frequencies and proportions between groups. Statistical significance was set at $P < 0.05$.

Results

Rest, reactive hyperaemia, and vascular conductance

Resting leg blood flow was not significantly different between groups either in the supine or the seated positions (Table 2); however, reactive hyperaemia in PAD was blunted compared to healthy controls. There was no difference between groups for vascular conductance during seated rest, but PAD patients had significantly lower conductance during reactive hyperaemia (Table 2).

	Supine rest	Seated rest	Reactive hyperaemia		
	Blood flow (ml·min⁻¹)	Blood flow (ml·min⁻¹)	Vascular conductance (ml·min⁻¹·mmHg⁻¹)	Blood flow (ml·min⁻¹)	Vascular conductance (ml·min⁻¹·mmHg⁻¹)
PAD	205 ± 28	132 ± 19	2.27 ± 0.34	423 ± 100	4.99 ± 1.72
Healthy	246 ± 21	188 ± 26	2.60 ± 0.20	1255 ± 175	14.01 ± 1.57
P	0.27	0.07	0.44	0.002	0.004

Table 2. Leg blood flow and vascular conductance comparing peripheral arterial disease (PAD) patients with healthy controls. Values are mean ± SEM. P = level of significance.

Blood flow responses to passive movement and active leg extension exercise

There were no significant differences between groups for average leg blood flow during the 5-minute bouts of passive leg movement (P = 0.19, Figure 1A), but the initial blood flow response during the first 90s was 45% lower in PAD (P = 0.03, Figure 1B). During active leg-extensions there was no significant difference between groups in either the rate of rise in blood flow (PAD: 620 ± 98; Healthy: 993 ± 225 ml·min⁻¹, P = 0.16) nor the steady state blood flow (PAD: 1490 ± 250; Healthy: 1887 ± 349 ml·min⁻¹, P = 0.37).

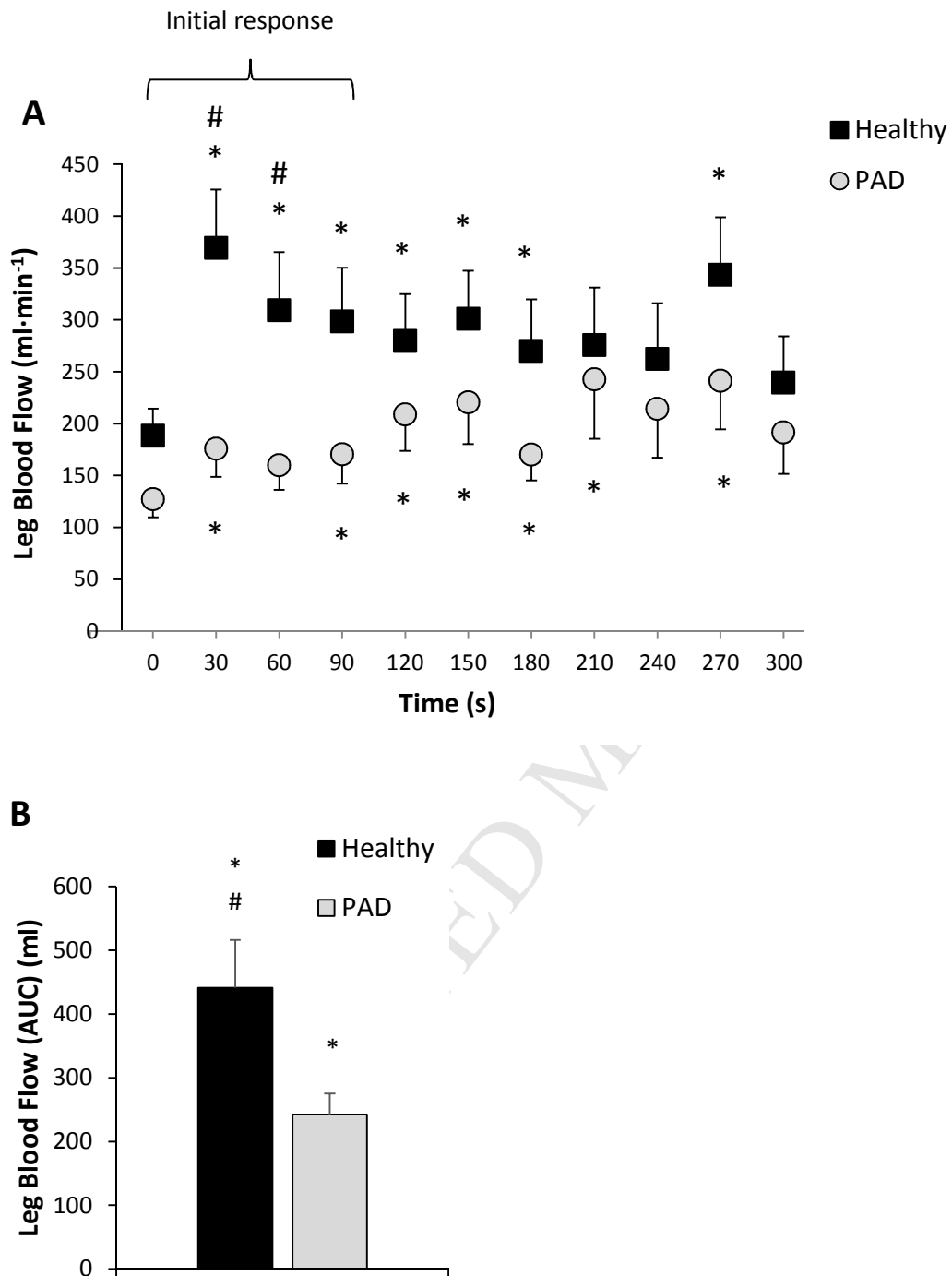


Figure 1. Femoral artery blood flow measured by Doppler ultrasound comparing peripheral arterial disease (PAD) patients ($n = 10$) with healthy controls ($n = 9$). **A:** leg blood flow during a 5 min bout of passive leg movement, **B:** Initial blood flow response, area under the curve (AUC) during the first 90 seconds of passive leg movement. Values are mean \pm SEM. # denotes significant difference between the PAD group and the healthy control group, $P < 0.05$; * denotes significant difference from baseline, $P < 0.05$.

Protein levels in skeletal muscle tissue

The level of NADPH oxidase was 2-fold higher in PAD compared to healthy controls (PAD: 1.04 ± 0.19 ; Healthy: 0.50 ± 0.06 AU, $P = 0.02$) (Figure 2A). There were no other significant differences in vasoactive enzyme levels or enzyme ratios between groups (Figures 2A and 2B).

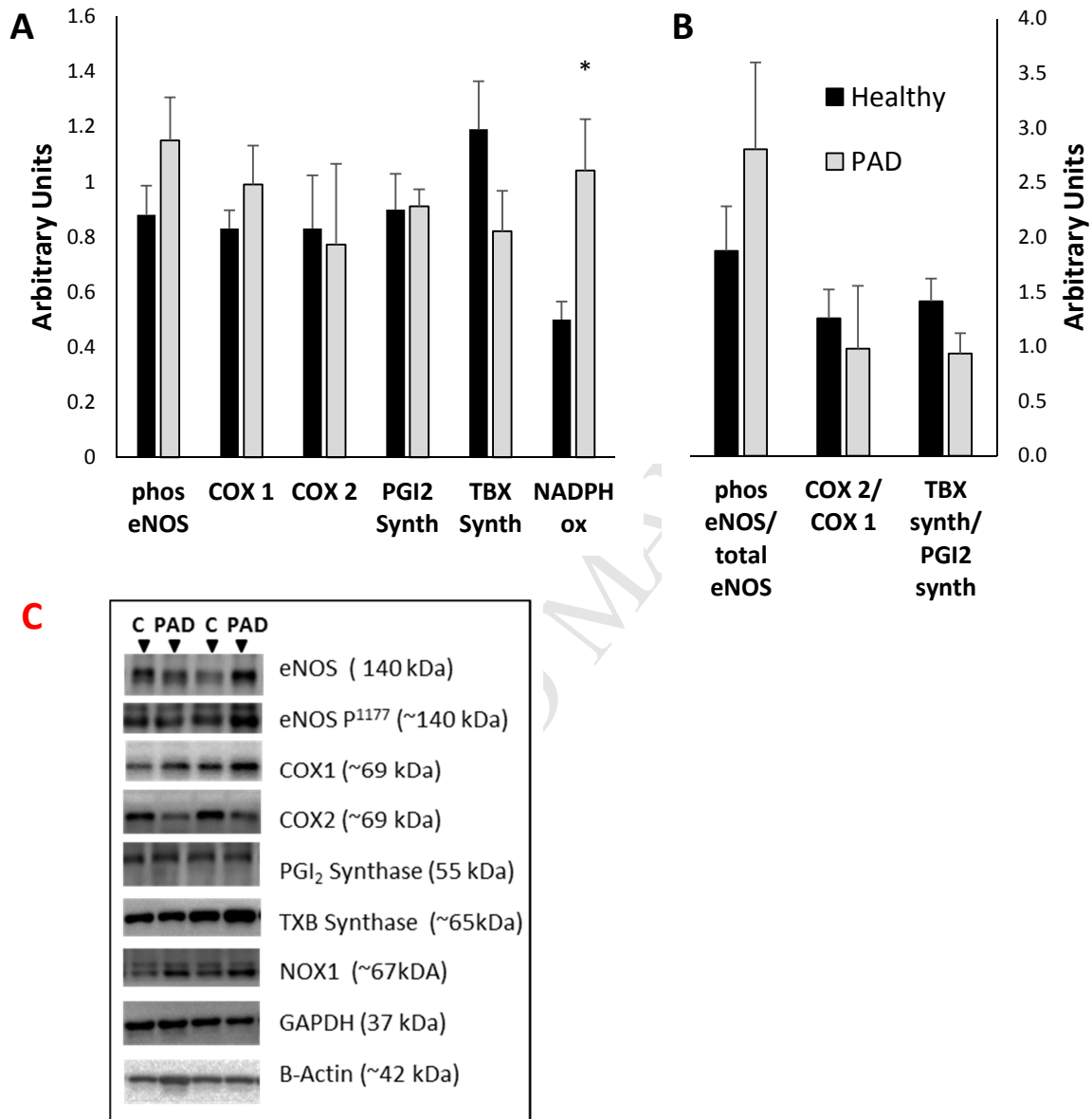


Figure 2. Vasoactive enzyme protein levels in the vastus lateralis muscle in peripheral arterial disease (PAD) patients ($n = 10$) and healthy controls ($n = 9$). **A:** enzyme levels, **B:** enzyme ratios, and **C:** Representative Western Blot images. Protein contents are expressed in arbitrary units, GAPDH was used as the loading control. PAD = peripheral arterial disease, phos eNOS = phosphorylated endothelial nitric oxide synthase, COX 1/2 = cyclooxygenase 1 or 2, PGI₂ synth = prostacyclin synthase, TBX synth = thromboxane synthase, NADPH ox = nicotinamide adenine dinucleotide phosphate oxidase. Values are mean \pm SEM. * denotes significant difference between the PAD group and the healthy control group, $P < 0.05$.

Relationships between variables

Data from the PAD patients and the healthy controls were combined for correlations. Reactive hyperaemia was significantly correlated with the initial hyperaemia during passive movement (Figure 3), the rate of rise in blood flow during active exercise and ABI (Table 3), but not with any of the vasoactive enzymes (Table 3). Prostacyclin synthase was strongly related to blood flow parameters during active exercise (Table 3).

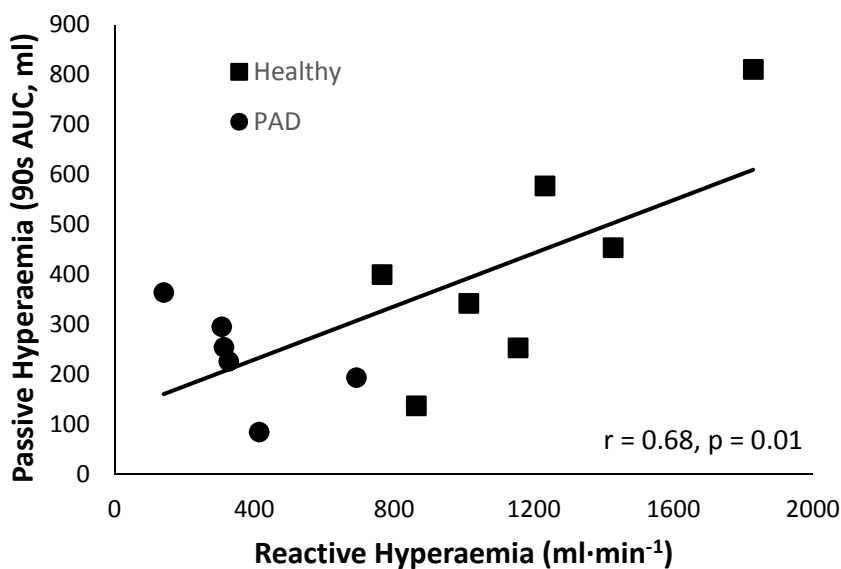


Figure 3. Relationship between passive movement hyperaemia (90s AUC) and reactive hyperaemia including both peripheral arterial disease (PAD) patients (n = 6, ●) and healthy controls (n = 7, ■).

Relationship	r	P
Phos eNOS (AU) / reactive hyperaemia (ml·min ⁻¹)	-0.04	0.89
Phos eNOS (AU) / passive hyperaemia (90s AUC, ml)	0.03	0.90
NADPH ox (AU) / reactive hyperaemia (ml·min ⁻¹)	-0.34	0.26
NADPH ox (AU) / passive hyperaemia (90s AUC, ml)	-0.30	0.21
Reactive hyperaemia (ml·min ⁻¹) / rate of rise active flow (ml·min ⁻¹)	0.57	0.04
Reactive hyperaemia (ml·min ⁻¹) / ABI	0.78	0.002
PGI ₂ synth (AU) / rate of rise active flow (ml·min ⁻¹)	0.69	0.002
PGI ₂ synth (AU) / steady state active flow (ml·min ⁻¹)	0.71	0.001
NADPH ox (AU) / COX 1 (AU)	0.80	<0.001

Table 3. Summary of linear regression analysis for leg blood flow and muscle enzyme levels. All participant data were included (n = 19, except for reactive hyperaemia, where n = 13). r = Pearson correlation, P = two-tailed significance, phos eNOS = phosphorylated endothelial nitric oxide synthase, AU = arbitrary units, AUC = area under the curve, NADPH ox = nicotinamide adenine dinucleotide phosphate oxidase, ABI = ankle brachial index, PGI₂ synth = prostacyclin synthase, COX = cyclooxygenase.

Discussion

The aims of this study were to evaluate vascular function in PAD patients who experience intermittent claudication (Fontaine II classification) compared to healthy controls of similar age by examining the hyperaemic responses to passive movement, active exercise and reactive hyperaemia. Moreover, the relationship between the functional blood flow responses and expression of vasoactive proteins and NADPH oxidase in the muscle tissue was assessed. The key findings were that: 1) in PAD patients the leg blood flow response to passive movement and the peak reactive hyperaemia were lower than in the healthy control group, whereas exercise hyperaemia was similar between groups; 2) the protein levels of several vasodilating and vasoconstricting enzymes were similar in the two groups, whereas the amount of NADPH oxidase was markedly higher in the PAD group; and 3) the level of prostacyclin synthase was correlated with leg blood flow during exercise.

This study demonstrated a positive correlation between reactive hyperaemia and passive movement hyperaemia across all participants, aligning with previous evidence that blood flow during both tests is highly dependent on the same factor, NO availability [5, 28]. PAD patients are known to have poor endothelial function [29] and reduced NO availability [30, 31], and it was therefore expected that they would exhibit an impaired flow response to these tests. Indeed, this study demonstrated significantly lower leg blood flow for PAD compared to healthy controls during passive leg movement (90s AUC) and reactive hyperaemia. These findings agree with our previous observations of a positive correlation between leg blood flow during acetylcholine infusion and passive leg movement hyperaemia, as well as a decline in passive leg movement hyperaemia with age [5]. The impairment in passive leg movement hyperaemia was most evident during the initial 90s of movement, suggesting that other vasodilator systems may contribute as movement proceeds [10]. Our findings also support the idea that, in addition to blood flow limitations imposed by stenotic plaque in PAD, poor vasodilation due to low NO availability may contribute to low reactive hyperaemia and passive movement hyperaemia for this population.

Exercise hyperaemia is the most functionally relevant test of vascular function. During active exercise, several vasoactive systems interact and contribute to the increase in blood flow, including NO and prostacyclin [19]. We selected a low absolute workload (10W) that could be achieved by both groups of participants. There was no significant difference between PAD and healthy controls in the rate of rise or steady state blood flow during the 5-minute bout of leg extension exercise, suggesting a similar vasodilator response during active exercise. This finding is consistent with vasoactive system redundancy where multiple mechanisms have been identified [10]. When data from all participants were pooled, muscle prostacyclin synthase content was correlated with both the rate of rise in blood flow at the onset of exercise and steady state flow during exercise. This aligns with previous research indicating that adults with atherosclerosis demonstrate an increased reliance on prostacyclin [21, 32], which acts as a supplementary vasodilator and inhibitor of platelet aggregation [33]. Our data indicates that under conditions of low NO availability in older adults and PAD patients, leg blood flow during exercise is closely related to prostacyclin production. Collectively, this might imply that prostacyclin production has an important role in the regulation of leg blood flow for PAD patients during exercise.

In PAD, there is evidence that NO availability is closely associated with exercise tolerance [13]. We assessed the relationship between leg blood flow and amounts of the enzymes that produce NO and ROS, as ROS production directly impacts NO bioavailability [17]. Despite blunted reactive hyperaemia and low flow during passive leg movement, there was no difference in the amount of

phosphorylated eNOS or the ratio between phosphorylated eNOS/total eNOS when PAD and healthy groups were compared. This finding concurs with our previous work showing that eNOS mRNA expression and protein content is similar between PAD patients and healthy older adults [34], suggesting that the capacity for NO production is similar and that removal of NO by ROS is likely responsible for group differences in NO availability. Indeed, PAD patients displayed significantly elevated amounts of muscle NADPH oxidase compared with healthy controls. NADPH oxidase has the sole purpose of producing ROS [17], which neutralise NO [35] and promote the uncoupling of eNOS [36]. Once uncoupled, activated eNOS produces superoxide, rather than NO, which is toxic to the cell and perpetuates the pathological sequence [15, 37]. Our finding of elevated NADPH oxidase is consistent with research demonstrating elevated oxidative stress in PAD [38] which could contribute to low NO availability. However, the lack of correlation between NADPH oxidase and blood flow in the current study would suggest that removal of NO by ROS from NADPH oxidase is not the only cause of a reduced blood flow in this population.

PAD is an inflammatory condition [39] which is known to promote COX 2 activity [40] and the abundance of atherosclerotic lesions in the vasculature has been directly linked to elevated concentrations of the potent vasoconstrictor, thromboxane [41]. We did not find a difference between groups in enzyme levels of thromboxane synthase, COX 1 or COX 2. Interestingly, however, the amount of COX 1 was correlated with the amount of NADPH oxidase. This association could support an interplay between ROS and prostanoid production, as previously described by Feletou et al [42].

Study limitations

Our focus was to compare leg blood flow and the associated enzymes that play a known role in vessel dilation and constriction at rest and/or during exercise. It was beyond the scope of this experiment to assess factors related to vascular remodelling, thus we cannot rule out the influence of pathways related to structural alterations, such as the interleukin-33/ST2 receptor pathway [43], metalloproteinases and vascular endothelial growth factor [44].

A primary aim of treatment for PAD is cardiovascular risk control and the patients in the present study had a typical medication profile. While this study was not powered to detect and determine the influence of differences in medication use, the potential impact of any differences in medication use (Table 1) should be considered. Beta blockers have a modest antihypertensive effect in some users [45], which is mediated mainly by alterations in heart rate and contractility with little impact on arterial stiffness, wave reflection, and the microcirculation [46]. In the context of the present

study, beta-blocker use is unlikely to influence vasoactive mediators at the leg or changes in leg blood flow associated with isolated leg movements. Statin use may improve flow mediated dilatation [47] and endothelial function [48], although any effect in the present study is likely to have been minimal given the significantly lower reactive hyperaemia and passive leg movement hyperaemia in the PAD patients. Chronic low dose aspirin use might reduce COX enzyme activity, but the enzyme levels would probably not be altered [49]. We found no difference between groups for COX levels, and there was also no difference between participants who did and did not report taking aspirin.

A potential limitation of our study is the prevalent smoking history among our healthy older adult group. In fact, 89% of the healthy controls reported being past smokers and cigarette smoking is known to contribute to the progression of PAD [50, 51]. Measurement of ABI confirmed that perfusion pressure at the ankle was consistent with the absence of PAD for controls, however smoking history could reduce the differences between groups.

It is possible that the assessment of blood flow and vasoactive mediators at the level of the thigh may have underestimated any differences that exist between PAD and healthy control participants. PAD blood flow limitation is known to be most pronounced at the periphery [1, 52] and claudication symptoms are typically reported in the primary mover muscles during activities like walking and cycling [53]. Our measurement site for biopsies was in the vastus lateralis muscle of the thigh, in relatively close proximity to the measurement site for leg blood flow (common femoral artery). These sites were selected because, during knee extensor exercise, the muscles of the thigh are the primary movers. Seven of the 10 participants in the PAD group had stenosis or occlusions proximal to, while all had stenosis or occlusions distal to the measurement sites. There was no relationship detected between plaque location and enzyme or blood flow results.

While our participant numbers enabled us to detect differences between groups in leg blood flow and in vasoactive enzyme levels, our ability to assess the strength of relationships between blood flow and enzyme levels was limited. The relationships we have identified provide insight into possible interactions, but further research with more participants, and possibly pharmacological blockade of prostaglandin synthesis, should be undertaken to clarify causal roles and multivariate relationships between enzyme levels and leg blood flow. Furthermore, this study specifically included PAD patients with intermittent claudication. The impact of more severe PAD, including critical limb ischemia, is not known and therefore our findings cannot be generalised to all PAD patients.

In conclusion, this study demonstrates that passive leg-movement hyperaemia and reactive hyperaemia are both lower for individuals with established PAD and intermittent claudication (Fontaine II classification) compared to healthy controls, which is consistent with impaired NO-dependent endothelial function. During exercise, leg blood flow was correlated with the amount of prostacyclin synthase in the leg muscle, supporting an important role for prostaglandins when NO availability is low. Blood flow impairment was associated with elevated concentrations of muscle NADPH oxidase for PAD patients, indicating that increased oxidative stress may be a primary cause of low nitric oxide availability and endothelial dysfunction in this population.

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Disclosures

The authors declare no conflict of interest.

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Title: Vasoactive enzymes and blood flow responses to passive and active exercise in peripheral arterial disease

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Highlights:

- Passive movement hyperaemia was low in PAD suggesting low nitric oxide availability
- Leg blood flow during exercise correlated with leg muscle prostacyclin synthase
- NADPH oxidase is elevated and leg blood flow is reduced in PAD compared to control