Published in final edited form as:

Nitric Oxide. 2009 June ; 20(4): 231–237. doi:10.1016/j.niox.2009.01.002.

Plasma Nitrite Response and Arterial Reactivity Differentiate Vascular Health and Performance

Jason D Allen^{a,*}, Elizabeth M Miller^a, Earl Schwark^a, Jennifer. L. Robbins^a, Brian D Duscha^a, and Brian H Annex^b

^a Department of Medicine, Duke University Medical Center, Durham, NC, USA

^b Department of Medicine, University of Virginia, Charlottesville, VA, USA

Abstract

NO is crucial for endothelial function and vascular health. Plasma nitrite (NO₂⁻) is the main oxidation product of NO and has been shown to reflect changes in eNOS activity. We hypothesized that plasma NO₂⁻ response to physical exercise stress along with physiological endothelial function would be reduced with increasing severity of vascular disease. Subject groups were; a) risk factors but no vascular disease (RF); b) Type 2 diabetes with no vascular disease (DM); c) diagnosed peripheral arterial disease (PAD); and d) DM+PAD. Venous blood was drawn at rest and 10min following maximal exercise. Plasma samples were analyzed by reductive chemiluminescence. Brachial diameters were imaged prior to, during and following 5min of forearm occlusion (BAFMD). There were no differences in resting plasma NO₂⁻ or BA diameters between groups. The PAD groups had lower age adjusted BAFMD responses ($p \leq 0.05$). Within group analysis revealed an increase in NO₂⁻ in the RF group (+39.3%), no change in the DM (-15.51%), and a decrease in the PAD (-44.20%) and PAD+DM (-39.95%). This was maintained after adjusting for age and VO_{2peak} ($p \leq 0.05$). Δ NO₂⁻ and BAFMD were the strongest independent predictors of VO_{2peak} in multivariate linear regression. These findings suggest Δ NO₂⁻ discriminates severity of cardiovascular disease risk, is related to endothelial function and predicts exercise capacity.

Keywords

Endothelium; Nitric Oxide; Plasma Nitrite; Cardiovascular Disease; Exercise; VO_{2peak}

An estimated 17.5 million people died from cardiovascular disease (CVD) in 2005, representing 30 % of all global deaths[1]. Peripheral arterial disease (PAD) is a form of CVD caused by atherosclerotic occlusions that impair the arterial blood flow to the legs. PAD affects approximately 27million people in Europe and North America, with approximately 2/3 of those suffering typical claudication, defined as pain in one or both legs on walking, which is relieved by rest[2]. The incidence of PAD development in subjects with diabetes mellitus (DM) is so greatly increased that the American Diabetes Association recommends screening should be performed even in asymptomatic subjects[3,4]. The presence of DM also alters the natural

*Address Correspondence to: Dr. Jason David Allen, Duke University Medical Center, Wallace Clinic, Center for Living Campus, DUMC 3022, Durham, NC 27710, Tel: (919) 660 6764, Fax: (919) 668 6301, e-mail: E-mail: j.d.allen@duke.edu.

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history of PAD and greatly increases the odds of progressing from intermittent claudication to critical limb ischemia[5–7].

Dysfunction of endothelial cells is an early event in the process of atherosclerotic lesion formation[8], and is associated with risk factors for CVD [9–11] and diabetes mellitus[12]. In fact, these relationships have led to the use of endothelium-mediated vascular responsiveness as a surrogate marker of cardiovascular risk. Recently, brachial artery flow-mediated dilation (BAFMD) was shown to be an independent predictor of long-term cardiovascular events in subjects with PAD[13], and to add to the predictive value of the ankle brachial-plexus index (ABI), the most powerful prognostic indicator in these patients[14]. This suggests markers of endothelial function may be useful to differentiate between subjects with increasing risk for vascular disease and those with clinically diagnosed PAD[15].

The endothelium can respond to changes in hemodynamic forces, or blood-borne signals, by membrane receptor mechanisms and to physical and chemical stimuli by synthesis or release of a variety of vasoactive substances including the endothelium-derived relaxing factor, nitric oxide (NO)[16]. Unfortunately, the rapid metabolism and short half-life of NO in human circulation makes it impractical to measure directly. Immediately upon formation NO may ; i) undergo biological activity and activate soluble guanylate cyclase; ii) nitrosylate thiol[17] or amine groups; iii) in the presence of oxygen undergo autoxidation to nitrite (NO_2^-) [18]; iv) be inactivated to nitrate (NO_3^-) via either oxyhaemoglobin-catalyzed oxidation in red blood cells[19] or interaction with superoxide anions to produce per-oxynitrite (ONOO^-) which decomposes to NO_3^- . Given that nitroso-adducts are also difficult to measure has lead to widespread use of plasma NO_2^- and NO_3^- or both (NOx) as surrogate markers of NO bioavailability.

The use of plasma NO_3^- as a marker of NO production comes mainly from its chemical stability and relatively long half life. However, NO_3^- is produced by several sources other than the vasculature, including, dietary intake, saliva formation, inhalation and production in the bowel. This results in high (μM) background levels which make it difficult to determine changes in vascular NO production at rest or following single limb perturbations [20]. Alternatively, plasma NO_2^- has a negligible background concentration due to its potential for oxidation to NO_3^- , especially in the presence of hemes and therefore requires relatively quick sample storage and/or analysis. NO_2^- has been shown to reflect acute changes in regional vascular NO bioavailability following both chemical (1-arginine and NG-monomethyl-L-arginine (L-NMMA) [21], and physiological (hyperemia) [21,22] stimuli.

To date no studies have attempted to investigate the biochemical changes reflecting NO availability in PAD compared to sub-clinical disease and link these changes to physiological responsiveness and functional capacity. Given the underlying reduction of NO bioavailability in the pathophysiology of vascular disease we hypothesized that endothelial function (BAFMD, an NO dependant process) and plasma nitrite responses (a 1st order NO metabolite) to physical stress would be reduced with increasing severity of vascular disease. In order to address this hypothesis we examined the link between plasma nitrite pre and post acute exercise stress and physiological endothelial function in subjects with; a) risk factors but no clinical vascular disease (RF); b) Type 2 diabetes mellitus with no clinical vascular disease (DM); c) diagnosed peripheral arterial disease (PAD); and d) DM+PAD.

Materials and Methods

Patient Characteristics

All subjects were recruited from the Duke University Medical Center Cardiology-Vascular clinics and surrounding area as part of ongoing enrollment for the Angiogenesis and

Mechanisms of Exercise Training in PAD (AMNESTI) clinical trial. Prior to participation all subjects signed an informed consent document approved by Duke University Medical Center Internal Review Board. All subjects were aged 40 to 75 years. Subjects were placed in the groups.

RF = greater than 2 traditional risk factors, no clinically diagnosed CVD

DM = Type 2 diabetes no clinically diagnosed CVD

PAD = intermittent claudication ≥ 3 months and ABI <0.9 at rest.

DM+PAD

The RF and DM subjects were selected on the basis of no complicating illnesses, freedom from symptomatic coronary artery disease, post-menopausal (females only), an ABI of > 1.0 , not actively enrolled in an exercise program, and resting systolic blood pressure < 170 and resting diastolic < 100 on less than 2 medications.

PAD subjects had a history of stable intermittent claudication for 3 or more months and an ABI <0.9 at rest. There were all receiving anti-platelet and lipid lowering therapy. Exclusions were based on a past medical history of gangrene, impending limb loss or osteomyelitis, lower extremity vascular surgery, angioplasty or lumbar sympathectomy within 3 months of enrollment, severe peripheral neuropathy, any condition other than PAD that limits walking, unstable angina, history of significant left main disease or three vessel coronary artery disease ($>70\%$ stenosis, unprotected by grafts) or recent myocardial infarction (6 weeks), chest pain during treadmill exercise which appears before the onset of claudication, or >3 mm ST depression during exercise.

The DM subjects had a clinical diagnosis of type 2 diabetes mellitus (by fasting blood glucose, glycosylated hemoglobin, abnormal oral glucose tolerance testing, or previous clinical diagnosis) and be on stable medications for at least eight weeks prior to entry. Subjects with an uncontrolled glucose at screening who were not referred from the endocrinology clinic were consulted by a co-investigator specializing in endocrine medicine.

A representative subset of each of the subjects groups were selected to participate in the blood draws for NO metabolite analysis (see table 1).

Arterial Vasoreactivity Measures

Prior to imaging, subjects were instructed to hold medications, fast and refrain from exercise for 12hr and alcohol for 48hr. All vascular imaging was performed between 8am and 11am, on the left arm, with the subject in a supine position with the forearm extended and slightly supinated. Brachial artery assessments were obtained using high resolution ultrasound and a 7.5MHz linear array transducer (Accuson, Sequoia 512), at baseline (following 10min of supine rest), during five minutes of forearm occlusion, and continuously on r-wave trigger for 2 minutes following cuff release (hyperemia) (for further details and reproducibility data see [23,24]). The percent change in brachial artery diameter was calculated by

$$((\text{peak post} - \text{hyperemia diastolic diameter} - \text{baseline diastolic diameter}) / \text{baseline diastolic diameter}) * 100$$

Following an additional 15 minutes rest, endothelium independent 0.4mg nitroglycerine-mediated) dilation was measured.

VO_{2peak} Measures

Subjects performed a symptom-limited maximal graded exercise test (GXT) with gas exchange analysis, consisting of treadmill walking/running with gradual increases in speed and/or incline until the subject requested to stop, could not continue or demonstrated clinical symptoms which met test termination criteria. Each test was tailored to allow subjects adequate warm-up and to achieve maximal exercise within approximately 15 min of initiation. Prior to, during and following the treadmill testing, heart rate, and 12 lead ECG were recorded using standard lead placement. Blood pressure measurements and ratings of perceived exertion were also obtained at the end of each minute throughout the test.

Nitric Oxide Metabolite Measures

Prior to initiation of the GXT, a 20gauge I.V. catheter was placed in the cephalic vein. Approximately, five ml of blood was taken prior to the GXT (Pre), and within 10 minutes of exercise termination (Post). Samples were separated into 1ml eppendorf tubes containing 5uL heparin (1 to 1000U/ml) and centrifuged at 5000g for 1 minute. Plasma samples were then removed into separate tubes, snap-frozen in liquid nitrogen and stored at -70°C until analysis.

All NO metabolite concentrations were measured (within 30mins of defrosting) by chemiluminescence using Ionics/Sievers nitric oxide analyzer (NOA 280), as per manufacturer's instructions (Sievers Instruments, Boulder, CO). The reductant used for nitrite analysis was potassium iodide in acetic acid, which has the reduction potential to convert nitrite to NO but is insufficient to reduce any higher oxides of nitrogen such as nitrate and thus is relatively specific for nitrite. To obtain concentrations of total plasma nitrogen oxides (NO_x) we used the same apparatus with a stronger reductant, vanadium chloride in HCl at 94°C . This stronger reductant reduces the sum of all nitrogen oxides with an oxidation state of +2 or higher which is predominantly nitrate [μM] but also includes both nitrite [nM] and nitrosothiols [nM].

Statistics

All statistical analyses were performed using SPSS for windows (version 15.0). Between group differences were detected by one-way analysis of variance (ANOVA) with tukey post-hoc analysis. An analysis of covariance (ANCOVA) adjusted for age was used for brachial artery endothelial function and VO_{2peak} measures. An ANCOVA adjusted for age and VO_{2peak} was used for NO metabolite measures. Fisher Exact test was used to calculate differences between groups for categorical variables.

Within group analyses were paired t-tests for changes in brachial artery endothelial function and NO metabolites pre and post stimulus. The Pearson product moment correlation and linear regression analysis was used to examine relationships between BAFMD, VO_{2peak}, NO metabolites and age. An Alpha level of $p \leq 0.05$ was required for statistical significance.

Results

Patient Characteristics

There were 41 subjects in the risk factor (RF) group, 10 in the diabetes only group (DM), 29 in the PAD only group, and 9 in the DM+PAD group. The subject characteristics are summarized in table 1. The RF group was younger, had higher fitness level, and lower resting systolic blood pressures than the other groups. As expected both the RF and DM had higher ABI measures than the two diagnosed PAD groups. There were no differences in resting diastolic blood pressures or brachial artery diameters between groups.

For NO metabolite analysis there were 21RF, 10DM, 10PAD, and 9PAD+DM subjects. Prior to testing subjects were not placed on a nitrate controlled diet. Consequently, there were differences in resting plasma nitrate but not plasma nitrite concentrations (see table 1).

Arterial Vasoreactivity

There were no differences in brachial artery baseline diameters for any of the subject populations (see table 1). All groups significantly increased brachial diameters (dilation) in response to both flow stimulus and sublingual nitroglycerine, as shown by within group paired t-test analysis.

The peak percent change in BAFMD was significantly higher for the RF group ($5.88 \pm 0.58\%$) than the PAD ($1.78 \pm 0.48\%$) and DM+PAD ($2.43 \pm 1.01\%$) groups. After statistically adjusting this data for differences in subject age these differences remained (see figure 1). There were no significant differences in blood flow velocities or calculated volumes between the groups at any stage in the protocol indicating a similar vasodilatory stimulus for all groups. There were also no differences between groups in their response to sublingual NTG (0.4mg) following adjustment for age and VO_{2peak} (NTG response at 5mins = $24.88 \pm 1.67\%$, $20.67 \pm 2.88\%$, $21.83 \pm 1.77\%$ and $22.62 \pm 4.11\%$ respectively), suggesting similar smooth muscle function in all subjects groups.

VO_{2Peak}

The RF (24.57 ± 1.07 ml/kg/min) group had a greater VO_{2peak} than the other 3 groups, as determined by GXT. Additionally, the DM (20.67 ± 1.77 ml/kg/min) subjects had higher values than the PAD+DM (14.28 ± 0.64 ml/kg/min) and the PAD group (16.67 ± 0.83 ml/kg/min, $p=0.07$ trend). After adjusting the data for age between groups the stepwise performance pattern remained but the difference between DM and PAD was no longer significant ($p=0.067$) (figure 2).

Nitric Oxide Metabolites

There were no differences in resting plasma nitrite values between groups (see table 1). However, following the GXT the RF group had significantly greater plasma nitrite concentrations than the other groups (after adjustment for both age and VO_{2peak}) (see figure 3). Furthermore, within group (pre-post) t-tests revealed a significant increase in plasma nitrite in the RF group (+39.3%), no change in the DM (−15.51%), and a significant decrease in the PAD (−44.20%) and PAD+DM (−39.95%) (see figure 3). There were no significant changes in plasma nitrate from baseline for any of the groups following treadmill exercise (see figure 4).

Relationship between Endothelial Function, Plasma Nitrite and Exercise Capacity

There were several univariate relationships between each of the primary dependant variables. Localized endothelial function measured by BAFMD (% change) was significantly correlated with both whole body exercise capacity (VO_{2peak}) ($r^2=0.12$, $p=0.005$), and absolute change in plasma nitrite during exercise (ΔnM) ($r^2=0.11$, $p=0.037$). There were also a significant correlation between the increase in plasma nitrite during exercise (ΔnM) and exercise capacity (VO_{2peak}) ($r^2=0.23$, $p=0.001$). Age and systolic blood pressure showed negative correlations with VO_{2peak} , change in plasma nitrite, and BAFMD (see table 2a). In a linear regression model, using the backward variable elimination method, both $\Delta nitrite(nM)$ and BAFMD(% ch) were the only significant predictors of exercise performance (VO_{2peak}) (see table 2b).

Discussion

The major finding of this study is that subjects with clinically diagnosed vascular disease (PAD) have significantly blunted responses in; a) localized NO-dependant endothelial function (BAFMD), b) the ability to increase plasma nitrite (ΔNO_2^-), and c) exercise tolerance ($\text{VO}_{2\text{peak}}$) in comparison to those with sub-clinical disease. Additionally, these physiological (BAFMD), biochemical (ΔNO_2^-), and physical ($\text{VO}_{2\text{peak}}$) indicators of vascular health/function are interrelated. In fact, within a relatively homogeneous patient population with all groups having a “poor” or lower cardiorespiratory fitness classification[25], ΔNO_2^- , and BAFMD were independent predictors of $\text{VO}_{2\text{peak}}$. These findings are important as we move towards development of new ways of detecting and treating endothelial dysfunction and vascular disease.

The reduced exercise performance ($\text{VO}_{2\text{peak}}$) and endothelial dysfunction in PAD subjects confirms the earlier findings of others. However, the ΔNO_2^- data is of particular interest because it may reflect vascular NO bioavailability. In this study ΔNO_2^- provides a clear distinction between groups with increasing severity of vascular risk and disease. This includes differentiating between the two sub-clinical groups (RF v DM) as well as those with clinical PAD. The RF group showed an increase in NO_2^- following GXT whereas the DM subjects, who traditionally have a greater risk for PAD development, demonstrated no change in plasma NO_2^- concentrations. Furthermore, the two groups with clinically diagnosed PAD had a significant decrease in plasma NO_2^- concentration following exercise stress. These differences remain significant even after statistically correcting for the possible confounding influences of age and fitness between groups.

Arterial Vasoreactivity

The role of the endothelium in the maintenance of vascular health and arterial tone has become evident during the past few decades. Due to its non-invasive nature and relative ease of use, brachial artery flow-mediated dilation has emerged as a candidate biomarker for the measurement of arterial endothelial function/health. Several studies have established its value as a predictor of cardiovascular events in “at risk” populations[26–29]. In peripheral arterial disease subjects BAFMD has been demonstrated to be an independent predictor of long-term cardiovascular events and adds to the prognostic value of the ankle-brachial pressure index (currently the most powerful prognostic indicator in PAD)[13,14].

In accordance with these studies we have demonstrated a graded BAFMD response, able to distinguish among RF, DM and PAD populations. Given that there were no differences in baseline arterial diameters, blood flow velocities or volumes, suggests the stimulus signal for dilation was similar for all groups. Additionally, smooth muscle function in response to sublingual nitroglycerine was also not different between groups, suggesting that this process is dependant upon increased endothelial NO bioavailability.

When our findings are considered in conjunction with previous work showing morbidity and mortality outcome is associated with BAFMD in patients with clinically diagnosed atherosclerotic disease it suggests that responsiveness of the vascular endothelium is inversely proportional to the degree of disease progression.

Nitric Oxide Metabolites

NO possesses a variety of anti-atherogenic properties and is essential for vascular health. A disturbance in the bioavailability of NO, either through reduced synthesis or increased consumption, has been linked with measures of endothelial dysfunction and progression of

atherosclerosis. As a result of its reactivity NO is difficult to measure directly in vivo[30]. Relatively stable reaction products of NO within human plasma are nitrite and nitrate.

Plasma nitrate is produced by a variety of sources, in addition to vascular NO consumption, resulting in high (μM) background concentrations which may limit its utility as a maker of NO bioavailability. In previous reports we have published changes in plasma nitrate in young ($\sim 29\text{yr}$) healthy subjects using a similar maximal stress treadmill test. Unfortunately this was prior to our ability to demonstrate adequate nitrite stability during processing and storage[31] and therefore nitrite was not assessed in this earlier work. However, there is congruency with the current findings. In the current study we report resting plasma nitrate levels in the 15 to $40\mu\text{M}$ range with the DM group being significantly higher than the RF group. This difference is most likely due to differences in diet prior to testing. Interestingly, there were no changes in plasma nitrate levels following acute exercise stimulus (see figure 4). Previously we have also shown no changes plasma nitrate in similar subjects groups with either diagnosed cardiovascular disease or >2 risk factors for cardiovascular disease[22,32]. We did however previously show an increase in plasma nitrate in young ($\sim 29\text{yr}$) healthy subjects to acute treadmill exercise but only at exercise capacities greater than twice those of the current study groups ($\sim 57\text{ml/kg/min}$), although others have shown no change [33]. We did not test a similar healthy young group in the current study. In contrast, nitrite is a redox active molecule and can be converted to a variety of other nitrogen oxides. It is important therefore, when examining differences between CVD groups to consider possible pathways of both nitrite production and consumption. Evidence suggests the majority of circulating plasma nitrite ($\approx 70\text{--}90\%$) is derived from vascular eNOS production of NO[34]. Our ΔNO_2^- (and BAFMD) findings support reduced endothelial health and NO production response to stress in the DM and PAD groups. Other sources of plasma nitrite include possibly enterosalivary circulation whereby nitrate is broken down by commensal bacteria in the mouth and may reenter circulation when swallowed[35]. It is unlikely, however that this contributes greatly during our short exercise bout condition.

More recent evidence suggests nitrite may be able to, under suitable conditions, be converted to NO and therefore impart bioactivity. Possible pathways for nitrite reduction to NO include; a) acidic conditions[36]; b) the presence of xanthine oxidase[37]; and c) by reaction with deoxygenated hemoglobin to form nitrosylated hemoglobin and S-nitrosohemoglobin which also allows for protected transport[38]. Interestingly conditions favorable for these pathways include low pH and hypoxia which occur during tissue ischemia, such as disease or exercise stress (or both). Plasma nitrite may therefore serve as a useful reserve for generating NO at sites where vasodilation and subsequent delivery is most needed. This maybe the case in the subjects with established clinical disease (PAD and PAD+DM). Recent studies suggest nitrite protects against ischemia-reperfusion injury[39,40] and may regulate gene expression[41]. In fact, maintenance of circulating nitrite levels may be associated with cardioprotection[42].

Influence of Vascular Health

Previously, plasma nitrite concentration has been shown to be inversely related to the number of cardiovascular risk factors and be related to BAFMD[43]. Additionally, the ability to increase plasma nitrite following a physiological and/or chemical stress has been shown in healthy subjects[22], but not in those with established endothelial dysfunction [44]. The current study extends these findings by differentiating physical function, vascular function and the ability of the vasculature to up-regulate NO and therefore plasma nitrite between a relatively homogeneous population of sub-clinical versus clinical atherosclerosis. Furthermore, we demonstrate that each of these dependant variables is positively correlated with each other.

The subjects with sub-clinical atherosclerotic disease are able to maintain (DM) or even increase (RF) plasma nitrite and therefore NO bioavailability under conditions which introduce

a hypoxic and/or ischemic challenge. From our current understanding of vascular physiology it is reasonable to suggest the RF subjects are better able to increase NO production via eNOS with less consumption via reactive oxygen species. Previous work has demonstrated that reduced NO bioavailability, via L-NMMA inhibition, in turn is a determinant of exercise-induced vasodilation in humans[45,46]. In contrast PAD is characterized by ischemic pain during exertion due to impaired arterial blood flow. Under PAD conditions it is logical to propose that plasma nitrite maybe reduced to NO in an attempt to normalize blood flow and oxygen delivery. This coupled with increased ROS and decreased ability to up-regulate eNOS production may explain the decrease in plasma nitrite observed here.

Relationship between Endothelial Function, Plasma Nitrite and Exercise Capacity

In 2007, Rassaf et al., presented evidence to suggest that the capacity of the vasculature to produce NO, and thus nitrite, together with age, independently predicts maximal power and duration of exercise in 55 healthy subjects (40 ± 2 yr)[42]. They also showed this increase in plasma nitrite was related to BAFMD. Our findings are in agreement with their work. In fact, the baseline and bicycle exercise-stimulated values in their study are very similar to our current findings for the RF group. Interestingly, a study of health young well-trained males showed lower oxygen consumption (VO_2) levels at matched sub-maximal cycle workloads following a doubling of plasma nitrite (124 to 226nM) via dietary supplementation of nitrate. This suggests a possible increase in efficiency of energy production. There were no differences at maximal effort [33].

We extend these findings to show the change in plasma nitrite and BAFMD response may independently predict maximal exercise capacity in our sub-clinical and clinical vascular disease population. If we consider by Poiseuilles Law that a 4-fold increase in radius can result in a 256-fold increase in flow the bioavailability of a potent vasodilator, such as NO, under physical stress may become significant. Further studies are required to determine if eNOS production of NO and nitrite or ischemic reduction of nitrite to NO are involved in increasing blood flow and performance during exercise.

Summary/Conclusions

The results of this study present a unique view into the biochemistry and physiological responsiveness of the vasculature in sub-clinical and clinical disease patients. Measures of endothelial function and physical performance were all able to differentiate between subjects at risk for CVD and those with clinical disease. The ability to up regulate plasma NO_2^- to exercise stress was also able to differentiate between the two sub-clinical “at risk” groups. Additionally, we present evidence that each of these “domains” are functionally related in that an inability to up regulate vascular NO bioavailability accompanies endothelial dysfunction and reduced exercise capacity in PAD.

Future studies are underway to determine if exercise training is accompanied by changes in endothelial function, plasma nitrite metabolites and physical performance in similar populations.

Acknowledgements

Assistance for treadmill testing and exercise training in this study was provided by staff at the Duke University Medical Center, Center for Living Campus, Durham, NC 27710

These findings were partially supported in by the NIH grant 5R01 HL-075752-05

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Figure 1. Brachial artery flow mediated dilation peak percent changes in diameter from baseline
All values are statically adjusted for differences in age between groups. * = significantly different at the $p \leq 0.05$ level.

Figure 2. VO_{2peak} (ml/kg/min) values during treadmill maximal graded exercise testing

All values are statically adjusted for differences in age between groups

* = significantly different at the $p \leq 0.05$ level. ** = significantly different at the $p < 0.01$ level

Figure 3. Changes in circulating plasma NO₂⁻ prior to and following a maximal GXT
 Samples were collected prior to (pre), and 10 min following (post) GXT. Data is represented as actual NO₂⁻ yield (nM) for each group. Values are statistically adjusted for differences between groups in age and VO_{2peak}. * = significantly different within groups at the p≤0.05 level. ** = significantly different between groups at the p≤0.01 level.

Figure 4. Changes in circulating plasma NO_3^- prior to and following a maximal GXT
 Samples were collected prior to (pre), and 10 min following (post) GXT. Data is represented as actual NO_3^- yield (μM) for each group. Values are statistically adjusted for differences between groups in age and $\text{VO}_{2\text{peak}}$. * = significantly different between groups at the $p \leq 0.05$ level.

Table 1

Subject baseline characteristics.

	RF (n=41, NO=21)	DM (n=11, NO=10)	PAD (n=29, NO=10)	DM+PAD (n=9, NO=9)
Age (yr)	53.5±1.1 *	61.2±1.7	67.2±2.1	62.9±3.8
Ht (cm)	168.9±2.0	175.8±2.5	172.1±2.5	175.4±3.9
Wt (kg)	80.2±3.2	97.0±3.2	77.9±3.4	83.5±4.9
BMI	28.58	32.17	27.87	28.84
SBP (mmHg)	129.8±3.3 *	151.9±6.7	155.5±3.7	162.6±6.1
DBP (mmHg)	80.9±1.5	81.3±3.1	79.2±1.9	80.4±2.3
ABI	1.05±0.02 †	1.08±0.11 †	0.63±0.21	0.72±0.13
CAD (%)	0 †	0 †	41	33
CVA (%)	0	0	22 *	0
CHF (%)	0	0	13 *	0
Smoking %				
Never	74 †	55 †	22	22
Former	19	45	47	44
Current	7	0 †	31	34
Medications %				
Ace Inhibitor	2 *	64	41	67
Beta Blocker	0 *	36	41	44
Statin	12 †	36 †	72	56
Aspirin	26 †	55	69	100 *
Insulin	0	0	0	33 *
Metformin	0 §	90	0 §	44
Glipizide	0 §	9	0 §	11
Glucapcharge	0	0	0	11
Actos	0	18 *	0	0
Avandia	0	18 *	0	0
Glucotrol	0	18 *	0	0
Novolin	0	9	0	0
Glimepiride	0	9	0	0
Brach Diam (mm)	3.16±0.11	3.42±0.22	3.27±0.11	3.63±0.28
Plasma NO ₂ ⁻ (nM)	93.8±12.5	79.0±8.8	112±19.8	77.6±13.1
Plasma NO ₃ ⁻ (μM)	14.2±1.6 #	40.6±5.4	25.7±4.3	26.8.6±6.0

* = significantly different than other groups ($p \leq 0.05$)† = significantly different than PAD, DM+PAD group ($p \leq 0.05$)# = significantly different than DM group ($p \leq 0.05$)§ = significantly different from DM, DM+PAD group ($p \leq 0.05$)

CVA = Cerebrovascular disease, CHF = Congestive Heart Failure

Table 2**Table 2a: Univariate Correlations with VO_{2peak} (ml/kg/min)**

	r	p
Age (yr)	-0.488	<0.001
SBP	-0.415	<0.001
DBP	+0.87	0.450
Brach Base Diam (mm)	-0.007	0.950
BAFMD (%ch)	+0.345	0.005
Rest Nitrite (nM)	-0.182	0.242
ΔNitrite (nM)	+0.477	0.001
Rest Nitrate (uM)	-0.219	0.101
ΔNitrate (uM)	-0.051	0.706
%chNTG	+0.339	<0.006

Table 2b: Multivariate Linear Regression Analysis for VO_{2peak} Prediction

	Beta	p
ΔNitrite (nM)	+0.347	0.053
BAFMD (%ch)	+0.761	0.046
SBP	-0.230	0.231
%chNTG	+0.026	0.231
Age (yr)	-0.128	0.927
Adjusted R ²	+0.287	
Significance (ANOVA)		0.005