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ABSTRACT

The Effect of Aerobic Exercise Versus Inactivity on Nitric Oxide Concentration and Synthesis in an Elderly Population

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CONTEXT: Nitric Oxide (NO) is an endothelial-derived vasoactive molecule that causes an increase in blood flow and oxygen delivery to tissue. A reduction in NO bioavailability has been found to occur in adults over the age of 60 and can be reversed pharmacologically by improving NO synthase (NOS) activity. Reversing these age-related changes with alternative interventions, such as aerobic exercise, has shown some promising results.

OBJECTIVE: To quantify blood NO-bioavailability (as measured by blood nitrite levels) in a population of aerobically trained elderly men and compare these data to a group of age-matched, inactive individuals. In addition, we measured the cutaneous vasodilator response to local skin heating as a bioassay for NO-mediated cutaneous dilation.

SETTING: BYU Human Performance Research Center (HPRC).

PARTICIPANTS: 16 healthy elderly men (age = 66 ± 7.07 years) were divided into two groups based on physical fitness levels and estimated $\dot{V}O_2max$ in ml $O_2 \bullet kg^{-1} \bullet min^{-1}$ (Trained = 39.1 ± 1.21, Untrained = 29.0 ± 2.70).

INTERVENTIONS: A blood sample was collected and analyzed for NO. A microdialysis study was performed and dialysate was collected at 32°C and at 42°C. During the heating process, skin blood flow (skin vasomotor activity) was monitored and reported as cutaneous vascular conductance (CVC).

MAIN OUTCOME MEASURES: Whole blood nitrite concentrations, pre- and post-heat nitrite concentrations, and CVC_{max} were compared between trained and untrained groups.

RESULTS: Whole blood nitrite concentration was similar in trained subjects and untrained subjects averaging 25.77 ± 6.75 and 21.43 ± 7.20 µM, respectively ($F_{1,13} = 0.19$; $P = 0.6671$]. Local skin heating had no impact on the concentration of nitrite in dialysate samples ($[NOx]_{dialysate}$ $F_{1,26} = 0.01$; $P = 0.7567$). In addition, the plateau in % CVC_{max} following 30 minutes of local heating was similar for trained and untrained subjects averaging 67.7 ± 5.8 and 68.0 ± 6.2 % CVC_{max}, respectively ($F_{1,13} = 0.00$; $P = 0.9673$).

CONCLUSIONS: The results of this study indicate that age-mediated reductions in whole blood NO-bioavailability and decrements in NO-mediated cutaneous vasodilation during local heating were similar in aerobically fit and sedentary adults 60 years old or older. We conclude that a commitment to aerobic fitness was unable to overcome the age-related dysfunction of the NOS system.

Keywords: microdialysis, skin vasomotor activity, cutaneous vascular conductance

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Introduction

Nitric oxide (NO) is an endothelial-derived vasoactive molecule that causes an increase in blood flow and oxygen delivery to tissue. The benefits of increasing the bioavailability of NO in circulation of the blood via various treatments (8, 15, 21, 26, 33, 38) can improve vascular function (21) and blood pressure regulation (44, 45) which can lead to a range of health benefits, especially in the elderly. Alternatively, a reduction in NO bioavailability can reduce oxygen delivery contributing to the development of hypertension (6), coronary artery disease (24), or congestive heart failure (30). Reduced NO bioavailability can be caused by a reduction in the activity of vascular endothelial cell nitric oxide synthase (NOS) (39). This latter problem is typically seen in the cutaneous circulation of adults over the age of 60 years (2, 18, 34, 39) and can be reversed by pharmacologically improving nitric oxide synthase activity (1, 3, 16, 39, 45).

Reversing age-related declines in NO bioavailability and/or NOS activity using nonpharmacological interventions, such as aerobic exercise, has shown some promising results; for example, bioavailable NO was increased following several weeks of aerobic exercise training in older adults (> 60 years old) (3, 8, 13). The authors speculated that aerobic exercise elevated NO production from endothelial cells due to increased vascular shear forces during exercise. Zago et al. (45) showed an acute increase in whole blood NO bioavailability measured as a change in total blood nitrite concentration. NO is highly reactive and is quickly oxidized to nitrite (4) thus blood nitrite levels are measured as an assessment of NO bioavailability in the blood. This change was measured at rest and during exercise after a few months of aerobic training in a group of formerly sedentary elderly adults. An alternative method to evaluate changes in NO bioavailability is to examine the cutaneous vasodilator response to local heating. The plateau in the amount of skin vasomotor activity during 30–40 minutes of local heating is

primarily mediated (> 90%) by NO (13). An attenuated plateau in skin vasomotor activity during local skin heating is noted in older adults and reflects a reduction in endothelium-dependent vasodilation (2, 18, 19).

While we know that the NO-dependent vasodilator response to local skin heating is decreased in the elderly (2, 18, 19), it is unclear if this decrement is reversed in elderly subjects with an active lifestyle. The purpose of our study was to quantify blood NO bioavailability (as measured by blood nitrite levels) in a population of aerobically trained elderly men as compared to a group of age-matched, sedentary elderly individuals. In addition, we measured the cutaneous vasodilator response to local skin heating as a bioassay for NO-mediated cutaneous dilation. We tested the hypothesis that active elderly individuals have a greater amount of bioavailable NO in whole blood and have a larger NO-mediated cutaneous vasodilation during local heat stress compared to their inactive elderly counterparts.

Methods

Subjects

We recruited men over the age of 60 years. All subjects were required to be generally healthy, normotensive or pre-hypertensive, nonsmokers, not diabetic, and not taking any medications that could alter blood flow. During the prescreen interview, we asked detailed questions about their physical activity habits—type of exercise, frequency, and how many years they have consistently participated. If the subject reported that they were sedentary, we asked how long they had been inactive and if there was an event such as an injury or illness that caused them to cease exercise.

A physician confirmed the health status of all individuals prior to participation. Institutional Review Board approval was obtained, and each subject gave written informed

consent before participation. Subjects who volunteered to participate in this study were asked to fill out a Rapid Assessment of Physical Activity questionnaire (42). This questionnaire was used to determine a basic, self-reported physical activity level. This assessment focused primarily on current activity level. If their responses placed them in the sedentary category (score of 1 or 2) or the active category (score 6 or 7) they met the first criterion for the study and were referred to the physician for further consideration. Individuals who scored a 3, 4, or 5 on the questionnaire were excluded from the study.

The physician reviewed the health history and medication usage of each person. If they had a significant health condition that may affect the results of this study (heart condition, disease, diabetes, or major surgical history) or were using medication that alters blood flow, they were excluded from the study. Blood pressure, heart rate, height, and weight were measured as population descriptors. Those subjects who met the all inclusion criteria and were medically cleared by the physician to walk on the treadmill were allowed to proceed with testing. Twelve subjects were rejected from this portion of the screening test.

Procedures

Subjects reported to the Human Performance Research Center to participate in a sub-maximal treadmill exercise test. The purpose of this test was to estimate $\dot{V}O_2max$ and to verify that the subjects' predicted physical activity score was matched to an appropriate level of aerobic fitness level. Heart rate was measured during treadmill exercise using an electronic heart rate monitor (Polar Heart Rate Monitor, Polar Electro Inc., Lake Success, NY) and maximal heart rate was estimated using the equation (35): $HR_{max} = 205.8 - 0.685*(age)$.

The submaximal exercise test began by walking for 4 minutes at a 0% grade at a self-selected comfortable speed (usually between 2 and 4.5 mph). Next, the grade of the treadmill

was increased to 5% while the speed was maintained. This pace and grade was maintained for 4–5 minutes with the heart rate of the patient measured at the end of minutes 3, 4, and 5. The average HR between minutes 3 and 5 was used to estimate $\dot{V}O_2max$ using the following equation (43): $\dot{V}O_2max$ (ml $O_2 \bullet kg^{-1} \bullet min^{-1}$) = 58.687 + (7.520 × Gender; 0 = woman and 1 = man) + (4.334 × mph) − (0.211 × kg) − (0.148 × HR) − (0.107 × Age).

Qualified subjects reported to the laboratory for the second visit having fasted for 8 hours and had a blood sample drawn for measurement of blood glucose and NO concentration. Those with fasting glucose levels of 126 mmol/L or higher were excluded from the study (12). At this point the subjects were divided into two groups: aerobically trained (n = 7, age = 66 ± 5 years, BMI = 25.7 ± 1.7 $kg \bullet m^{-2}$, estimated $\dot{V}O_2max$ of 39.1 ± 1.2 ml • min^{-1} • kg^{-1}) and sedentary controls (n = 8, age = 66 ± 9 years, BMI = 30.1 ± 4.6 7 $kg \bullet m^{-2}$, estimated $\dot{V}O_2max$ of 29.0 ± 2.7 ml • min^{-1} • kg^{-1}). Data from one untrained subject were removed from the results and statistics after it was discovered that he was taking medication that altered blood flow, leaving eight trained subjects and seven untrained subjects. A power analysis was completed prior to beginning the study to confirm that we would be able to detect a difference at .80 power at .05 level of significance.

Following the fasting blood glucose test, the subject assumed a semi-recumbent position in a comfortable room ($T_{room} \approx 21$ °C). After resting for at least 30 minutes, a 3 ml blood sample of blood was collected into a Li-heparin vacutainer. The blood sample was immediately combined with a nitrite preservation solution of 0.75 ml. The preservation solution consisted of 0.8 M ferricyanide, 0.1 M N-ethylmaleimide, and 500 uL IGEPAL®CA-630. The solution was gently inverted and then stored at -80°C until analysis.

Next, the subject was prepped for measuring the NO-mediated cutaneous vasodilator response to local heating. Two linear intradermal microdialysis probes (2.5 cm hollow fiber with molecular weight cut-off of 18 kilo-Dalton kDa) were placed in the dermis of the skin of the dorsal aspect of the nondominant forearm at <1 cm apart. The microdialysis probes were assembled in our laboratory, following guidelines established in previous research (11) and gas sterilized before use. Placement of the microdialysis fibers were performed by first inserting a 27 g 3.50 inch needle just underneath the skin (≈1 mm) with entry and exit sites approximately 3 cm apart. The microdialysis probes were threaded through the needle. Once the probes were threaded, the needle was carefully removed, leaving the probe in place. A 3 x 3 cm peltier controlled thermomodule was placed directly over the two microdialysis probes with a laser Doppler flow probe (model: VP7a, Moore Instruments, Wilmington, DE) placed in a central access point in the peltier. The peltier was secured to the forearm using a double stick disc. The laser Doppler output and thermomodule temperature were monitored using a Powerlab system (ADInstruments Inc., Colorado Springs, CO).

The microdialysis probes were flushed with sterile saline (5.0 µl/ minute) for ≈ 90 minutes with a computer controlled infusion pump to reach a plateau, which differed for each subject, (model VPF; Harvard Apparatus, Holliston, MA) while local skin temperature under the thermomodule was held at 32°C. An automated noninvasive brachial blood pressure monitor (model: Tango+, SunTech Medical Instruments, NC, USA) was placed on the dominant arm, opposite of the microdialysis site. The arm was placed in a relaxed, pronated position at heart level. Blood pressure was recorded every 5 minutes throughout the local skin heating protocol.

After 90 minutes of flushing with saline and maintaining a skin temperature of 32°C, we collected a preheating microdialysis sample. The microdialyis probes were perfused with sterile

saline at a rate of 1 µl/minute. The dialysate from the two microdialysis probes were collected into 1.5 ml amber Eppendorf tubes for 30 minutes. After the 30 minutes of collection at 32°C, the thermomodule temperature was increased to 42°C at a rate of 0.1°C per second and was held at 42°C for an additional 30 minutes. A second dialysate sample was collected throughout this 30-minute heating period while we monitored skin blood flow continuously using laser Doppler flowmetry. The dialysate samples were collected into Eppendorf tubes which contained the same nitrite preservation solution used for the whole blood sample in a 4:1 ratio (v/v). After collection, the dialysate samples were gently inverted and frozen at -80°C until analysis.

Following the collection of the pre- and post-heat dialysate collection, the microdialysis probes were perfused with 28 mM sodium nitroprusside (SNP) at 5µl/minute until a second plateau in skin vasomotor activity was reached, while maintaining the skin temperature at 42°C. Twenty-eight mM of SNP at a skin temperature of 42°C is sufficient to elicit a maximum cutaneous vasodilation (28). After a plateau was reached, perfusion of SNP stopped and a blood pressure cuff was inflated on the nondominant arm to occlude blood flow in the forearm. This provided a measurement of 0 skin vasomotor activity and was verified by the graph in the Powerlab system. Having both a minimum and maximal skin vasomotor activity value during each trial allowed for appropriate normalization of the skin vasomotor activity responses to local heating. Vasomotor activity in the skin was reported as cutaneous vascular conductance (CVC) by dividing skin vasomotor activity by the average mean arterial blood pressure during the local heating protocol. These data were normalized to maximal CVC and then expressed as percent of CVC_{max} (%CVC_{max}).

Blood and Dialysate Analysis

Each sample was rapidly thawed in a 37°C water bath, deproteinated with 100% methanol (1:1 vol:vol) and then centrifuged at 15000 rpm for 3 minutes. The supernatant was removed and placed in a clean Eppendorf tube. The Eppendorf tube was then centrifuged again and the supernatant transferred to a second tube for analysis.

Sample nitrite levels were detected using an ozone based chemiluminescence nitric oxide analyzer (Sievers Instruments, Boulder, CO, USA). Before analysis, the NO analyzer was calibrated according to the manufacturer protocols using 5 levels of sodium nitrite. The glass purge vessel contained a tri-iodide solution that consisted of 50 mg potassium iodide, 2000 µl of ultrapure water, and 5 ml of Glacial acetic acid. We performed each analysis in triplicate using 100 µl of the supernatant of the deproteinized blood sample and 10 µl of the supernatant of the deproteinized microdialysate sample.

We compared the results to the sample nitrite peaks on our calibration curve to determine the nitrite concentrations in each sample.

Statistical Analysis

Whole blood nitrite concentrations were analyzed using a one-way ANOVA. The pre- and post-heat dialysate samples were compared using a two-way ANOVA, concentrating on the interaction to see if there was a difference from pre- to post-heat nitrite concentrations for the trained versus the untrained population. A one-way ANOVA was performed on the $\%CVC_{Max}$ to compare the difference between trained and untrained individuals. The raw data for both the blood and the dialysate nitrite concentrations were not normally distributed. Therefore, a log transformation of the data was performed before performing the ANOVA. The level of significance was set at $P < 0.05$.

Results

Subject characteristics are presented in Table 1. The average age, BMI, DBP, and fasting blood glucose was similar in both trained and sedentary groups. However, the trained subjects had a higher estimated $\dot{V}O_2max$ and a lower resting SBP than the sedentary group ($p < 0.05$). The average fasting blood glucose levels were below 100 mg • 100 ml^{-1} plasma indicating reasonable glucose control in all subjects.

Whole blood nitrite concentration was similar in trained subjects and untrained subjects averaging 25.77 ± 6.75 and 21.43 ± 7.20 μM, respectively ($F_{1,13} = 0.19$; $P = 0.6671$). Local skin heating had no impact on the concentration of nitrite in dialysate samples ($[NOx]_{dialysate}$. Table 2, $F_{1,26} = 0.01$; $P = 0.7567$). In addition, the plateau in $\%CVC_{max}$ following 30 minutes of local heating was similar (Table 2) for trained and untrained subjects averaging 67.7 ± 5.8 and 68.0 ± 6.2 $\%CVC_{max}$, respectively ($F_{1,13} = 0.00$; $P = 0.9673$).

Discussion

The purpose of our study was to evaluate the relationship between physical activity levels and bioavailability of NO in a population of elderly men. Understanding how aerobic exercise affects NO levels in men over the age of 60 could provide insight into the role of exercise on age-related vascular changes. Our data did not support the idea that aerobic training in the elderly influences resting $[NOx]_{blood}$ or intradermal $[NOx]_{dialysate.}$ In addition, we saw no improvement in the NO-dependent vasodilator response to local heating in aerobically trained elderly subjects.

Previous studies (18-20, 34, 36, 38) have documented an improved NO-dependent vasodilator response in elderly subjects following several weeks of aerobic training. Those studies typically used a mix of men and women and defined elderly as ages 50 and older. In contrast, we only used men and all subjects were $\geq$ 60 years old. In older women, menopausal

status (premenopausal versus postmenopausal) and use of hormone replacement therapy can impact NO bioavailability. Menopausal status is the main factor explaining the gender differences of serum nitric oxide concentrations in middle-aged population (40). Tehrani et al. noted that the variability in $[NOx]_{blood}$ in a middle-aged women was associated with menopausal status. Postmenopausal women had a higher $[NOx]_{blood}$ than premenopausal women or older men.

The age range of subjects may have also impacted our findings. In normotensive or hypertensive elderly adults, a reduction in NO bioavailability is associated with a reduction in activity of the NOS pathway in vascular endothelial cells (39). These data indicate that NO bioavailability is compromised by the age of 60 years old (39). As such, we selected only subjects at 60 years and older. Previous studies, however, have used different criteria for defining the age of their subjects and this factor may account for how each study defines age-related change in NO bioavailability with aging. Kenney et al. compared the cutaneous vasodilator effect of heating on skin blood flow in older and younger men (18), defining younger men as ages 50–53. This group had the highest increase in skin blood flow (3.5 $l \cdot min^{-1}$) while the older men (ages 61–73) had the lowest increase in skin blood flow (1.6 $l \cdot min^{-1}$).

Our study used an ozone-based chemiluminescence detector to measure the $[NOx]_{blood}$ levels. The majority of previous studies (26, 44, 45) have used chemical analysis (Griess reaction) to measure $[NOx]_{blood}$. Studies using the Griess reaction assay have yielded varying results. For example, Maeda et al. recruited 7 sedentary women, ages 59–69, and placed them on a 3-month aerobic exercise program (26). The $[NOx]_{blood}$, analyzed using the Griess assay, increased significantly. Zago et al. recruited 16 male and female subjects, ages 59 ± 6 years, placing them on a 6-month aerobic exercise program (45). They found no change in $[NOx]_{blood}$

using the Griess assay.

Local heat stress to the skin was used to assess vascular endothelial-mediated NO production in skin blood vessels and thus can serve as proxy to NO bioavailability. The normal response to local heating includes increased vasodilation through relaxation of the blood vessels and increased blood flow. The plateau in skin blood flow over a time period of 25–30 minutes (expressed as $\%CVC_{max}$) is mediated primarily by NO and can be blocked by administration of a NOS inhibitor. In our study, the average CVC_{max} was similar for trained and untrained subjects (67.7% ± 5.9% and 68.0% ± 6.3%, respectively). Nitrite concentration in dialysate of pre- and post-heating also failed to show a significant difference. Clough et al. (9, 10) reported basal concentrations of $[NOx]_{dialysate}$ of 0.63 ± 0.09 μM and 0.49 ± 0.06 μM. Kellogg et al. (16) reported basal values of diffusible NO in cutaneous interstitial tissue of 0.54 ± 0.11 μM. In both of these studies, the amount of NO present in the tissue increased in response to heat or pharmacological agents known to activate the NOS pathway. In contrast, basal concentrations of $[NOx]_{dialysate}$ in the current study ranged from 2.45 to 2.72 μM. Crandall and MacLean (14) reported basal values of $[NOx]_{dialysate}$ of 7.6 ± 0.5 μM. The significance of the higher basal levels of $[NOx]_{dialysate}$ is that in these studies the NO levels were unchanged by whole body or local heating. The inability to increase $[NOx]_{dialysate}$ during local heating may reflect the high basal levels of $[NOx]_{dialysate}$.

Minson et al. (28) tested the cutaneous vasodilator response to local heating in groups of young and old individuals. In the older group (5 men and 5 women, ranging in age from 69–84 years) the plateau CVC during 40 minutes of local heating averaged 61.1 ± 4.5% CVC_{max}. The local heat stress produced an average plateau of CVC levels at around 68% CVC_{max}, similar to

the data by Minson et al. (21) for a mixed group of older men and women. Our data indicate that the skin vasomotor activity response to local heating may not be improved by aerobic training.

Conclusion

The results of this study indicate that age-mediated reductions in whole blood NO bioavailability and decrements in NO-mediated cutaneous vasodilation during local heating were similar in aerobically fit and sedentary older adults ($\geq$ 60 yrs). We conclude that a commitment to aerobic fitness was unable to overcome the age-related dysfunction of the NOS system.

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Table 1. Subject characteristics

Group	Age years	BMI $kg \bullet m^{-2}$	SBP mmHg	DPB mmHg	Glucose $mmol \bullet L^{-1}$	Estimated $\dot{V}O_2max$ $ml\ O_2 \bullet kg^{-1} \bullet min^{-1}$
Trained (n = 8)	66 ± 4.94	25.7 ± 1.71	113 ± 6.80*	69 ± 4.88	98.3 ± 8.81	39.1 ± 1.21*
Untrained (n = 7)	66 ± 9.20	30.1 ± 4.63	124 ± 12.59	72 ± 8.32	115.9 ± 8.93	29.0 ± 2.70

Values represent mean ± 1 SD for each group.
BMI = body mass index; SBP = systolic blood pressure; DBP = Diastolic blood pressure; $\dot{V}O_2max$ = oxygen consumption.
* $p < 0.05$ different from Untrained

Table 2. Normalized skin blood flow and nitric oxide response to local heating

Group	$[Nitrite]_{Blood}$ µM	$[Nitrite]_{Dialysate}$ Pre Heat µM	$[Nitrite]_{Dialysate}$ Post Heat µM	Plateau CVC % Max
Trained (n = 8)	2.72 ± 0.42	5.12 ± 0.37	4.94 ± 0.37	67.8 ± 5.9
Untrained (n = 7)	2.45 ± 0.45	4.94 ± 0.39	5.00 ± 0.39	68.0 ± 6.3

Values represent mean ± 1 SD for 8 subjects in the trained group and 7 subjects in the untrained group.
CVC (cutaneous vascular conductance) % Max= SkBF/average mean arterial blood pressure
*$p < 0.05$ different from Untrained