

# Impact of 2-nitrooxy-ethyl-2-amino-3-methylbutanoate on blood pressure and plasma nitrate/nitrite following acute exercise in normotensive men

**Running Title:** Nitric oxide and exercise

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## **ABSTRACT**

**Objective:** We recently reported a small increase in plasma nitrate/nitrite (NO<sub>x</sub>) following oral intake of the novel nitric oxide donor, 2-nitrooxy-ethyl-2-amino-3-methylbutanoate (2-NO). However, in that study subjects remained sedentary during the 60 minute data collection time. It is now believed that the 2-NO molecule may be activated via increased blood pressure, secondary to physical stress. We compared the effects of 2-NO on blood pressure, heart rate (HR), and NO<sub>x</sub> in normotensive men.

**Methods:** 15 healthy men (28.5±0.8 yrs) reported to the lab on two different days separated by two weeks. They were randomly assigned in a double-blind, cross-over manner to receive either a 2-NO tablet or a placebo tablet before performing two, two-minute sets of sit-up exercise (exercise sets performed 20 minutes apart). Systolic (SBP) and diastolic (DBP) blood pressure and HR were measured before and for 20 minutes following each set of exercise. Blood was collected via an indwelling venous catheter at the same times and analyzed for NO<sub>x</sub>.

**Results:** For both conditions, subjects performed a near identical number of repetitions (2-NO: 101; placebo: 100). HR was not different between conditions ( $p>0.05$ ). However, SBP was lower with 2-NO compared to placebo ( $p=0.03$ ), in particular during 20 minute recovery period following set 2 ( $p=0.04$ ; ~6 mm Hg lower). 2-NO also resulted in a lower DBP during the same time period following set 2 ( $p=0.02$ ; ~5 mm Hg lower). NO<sub>x</sub> was increased approximately 10% above baseline with 2-NO ( $p=0.09$ ) but remained stable or decreased slightly with placebo.

**Conclusions:** These data indicate that 2-NO can lower blood pressure in response to acute exercise in a sample of healthy, normotensive men. The change in blood pressure may be associated with an increase in circulating NO<sub>x</sub>.

**KEY WORDS:** nitric oxide, dietary supplements, blood pressure, nitrate, exercise

## 1. INTRODUCTION

It is well described that nitric oxide (NO) functions as a signaling molecule and can promote vasodilation by acting on vascular smooth muscle [1]. The downstream effects of NO-mediated vasodilation include greater control of blood pressure [2] and a possible enhancement in blood flow at rest [3] and during exercise [4]. The reduction in blood pressure may be of great interest to those with hypertension, as well as to clinicians who treat patients with this disorder. The improvement in blood flow may be of interest to those with clinical conditions in which blood flow is compromised (e.g. peripheral vascular disease, sickle cell disease), as well as those who seek enhanced blood flow for purposes of athletic performance and exercise recovery [5,6].

The molecule 2-nitrooxy-ethyl-2-amino-3-methylbutanoate (2-NO) was developed with the objective of providing NO to the circulation via transmucosal delivery. Initial work using this molecule demonstrated a ~6.7% increase in circulating nitrate/nitrite (NOx) during the initial 15 minute post-intake period [7]. No differences were noted in blood pressure between the 2-NO and placebo. However, in that study subjects remained sedentary during the 60 minute data collection period. Further work using the 2-NO molecule in animals has noted that shearing forces on the vasculature are responsible for fully activating the molecule (unpublished findings). Therefore, use of the 2-NO within hypertensive patients may yield more robust findings for blood pressure reduction. Moreover, use of 2-NO in conjunction with strenuous physical work that elicits an acute hypertensive response may also allow for the molecule to yield a blood pressure lowering effect. Considering the above, the present study was designed to compare the effects of 2-NO on blood pressure, heart rate (HR), and NOx in normotensive men before and following acute, strenuous exercise. We hypothesized that the 2-NO would allow for a reduction in

blood pressure during the post-exercise period, possibly due to an increase in circulating NOx.

## 2. MATERIALS AND METHODS

### 2.1. Subjects

Fifteen healthy men between the ages of 25 and 34 years were enrolled in this study. Subjects were normotensive (resting blood pressure <140/90 mm Hg) and without excessive body fat. Subjects were not current smokers and did not have a history of substance abuse. Subjects did not have diagnosed cardiovascular, metabolic, or neurological disorders including but not limited to heart disease, diabetes, or seizures. Subjects were not using any medication that might have impacted testing (e.g. nitrates). Subjects could not have musculoskeletal problems, including back pain, which could have impacted their ability to perform the exercise protocol (described below). Health history, medication and dietary supplement usage, and physical activity questionnaires were completed by all subjects to determine eligibility. Prior to participation, each subject was informed of all procedures, potential risks, and benefits associated with the study through verbal and/or written form in accordance with the procedures approved by the University Institutional Review Board for Human Subjects Research (protocol # 3557). Subjects provided written informed consent prior to being admitted to participate.

### 2.2 Initial Laboratory Visit: screening visit

During the initial visit to the laboratory, subjects completed the informed consent form, health history, and physical activity questionnaires. Subjects' heart rate and blood pressure, height, weight, waist, and hip circumference were measured using standard procedures. Subjects completed a familiarization trial for the exercise protocol and were instructed how to record dietary intake. Upon completion of the screening, subjects were scheduled for their initial testing visit.

### 2.3. Independent Variable

Subjects were randomly assigned in double-blind manner using a cross-over design to one of two conditions. Subjects reported to the lab on two different days (separated by approximately two weeks) and received one of the following conditions on each day: one 2-nitrooxy-ethyl-2-amino-3-methylbutanoate (2-NO) tablet or one placebo tablet. The 2-NO tablets contained 30 mg of 2-nitrooxy-ethyl-2-amino-3-methylbutanoate; similar to the dosage we have used in one other study involving sublingual intake of this agent [7]. The placebo tablet contained an inert substance. All tablets were near identical in appearance and only differed slightly in color. Subjects were instructed to place the tablet under their tongue and allow it to completely dissolve (approximately 10-15 minutes). Subjects were then provided with 12 ounces of water to consume.

### 2.4. Test Visit Procedures

Subjects reported to the lab during the morning hours (6:00-9:00am) and the time was similar for both visits. Subjects reported in a fasted state, without having consumed alcohol or caffeine within the past 24 hours. Subjects were instructed to obtain at least 7 hours of sleep during the night prior to testing. Subjects should not have performed strenuous physical exercise within the past 24 hours.

Upon arrival at each visit, subjects rested quietly for 10 minutes and then had baseline data collected (as described below for testing battery). Following this, they received a standardized liquid meal of approximately 225 calories (8 ounces of orange juice and 25 grams of protein powder) and consumed this within 1-3 minutes. Subjects then rested quietly for 20 minutes. They were then provided with one of the two assigned conditions and followed the protocol as indicated below (starting at number 7).

#### Timeline for data collection

1. Arrive at lab and place indwelling venous catheter (see below for blood collection)

2. Rest for 10 minutes
3. **Collect baseline data (Pre Meal)**
4. Provide standardized meal
5. Rest for 20 minutes
6. Provide assignment condition (2-NO or placebo)
7. Rest for 15 minutes
8. **Collect data (Pre Exercise)**
9. Perform exercise (2 min. of abdominal sit-up to failure—as many reps as possible in 2 min)
10. **Collect data (immediately post exercise)**
11. **Collect data (5 minutes post exercise)**
12. **Collect data (10 minutes post exercise)**
13. **Collect data (20 minutes post exercise)**
14. Perform exercise (2 min of abdominal sit-up to failure—as many reps as possible in 2 min)
15. **Collect data (immediately post exercise)**
16. **Collect data (5 minutes post exercise)**
17. **Collect data (10 minutes post exercise)**
18. **Collect data (20 minutes post exercise)**

Subjects were allowed to consume as much water as desired throughout the lab period, but they were not allowed to ingest any food or calorie containing beverages other than what was provided by investigators. The water intake was matched for both lab visits.

### 2.5. Testing Battery (outcomes)

The following were collected from subjects at all times indicated above: heart rate, blood pressure, and blood sample (for measurement of NO<sub>x</sub>). The total number of repetitions performed was recorded for each exercise trial. Heart rate and blood pressure were measured using an automated digital unit (Omron HEM 907XL). Duplicate readings were taken from subjects at each collection time and the mean of the two

readings was used in data analysis. At the immediate post exercise times only, the initial reading was also recorded in an attempt to capture the “peak” blood pressure increase resulting from exercise. Rate pressure product was calculated as an indicator of myocardial work using the equation: (mean) systolic blood pressure x (mean) heart rate.

### **2.6. Blood Collection and Analysis**

On both lab visits for testing, blood was collected from subjects using a 20 or 22 gauge venous catheter placed into a forearm vein. The catheter remained patent by flushing regularly with Lactated Ringer’s injection. Blood samples were collected at the times indicated above, for a total of 10 samples at each of the two lab visits. Following the removal of approximately 2 mL of “waste” from the catheter, approximately 5 mL of blood was collected at each time period via syringe and placed immediately into a glass collection tube containing EDTA. The blood was then mixed and centrifuged at 4°C to obtain plasma. Aliquots were stored at -70°C until analyzed for NO<sub>x</sub> using a colorimetric assay kit (Catalog#: 780001; Caymen Chemical, Ann Arbor, MI). After being thawed, samples were centrifuged at 10,000g for 5 minutes in a refrigerated centrifuge (4°C). Nitrate reductase and an enzyme cofactor were added to each diluted sample and the mixture was incubated. Greiss reagent was then added and the absorbance was detected at 540 nm using a PowerWave microplate spectrophotometer (BioTek Instruments, Winooski, VT). Quantification was performed with a calibration curve using Gen5 software. The detection limit, as per the manufacturer, is  $\geq 2.5 \mu\text{M}$ . The coefficient of variation for the assay was 5.4%. All assays were performed in duplicate on first thaw.

### **2.7. Dietary Intake and Records**

All subjects were instructed to consume their usual diet throughout the study period and to record all food and drink consumed during the 24 hours prior to each test day. Diet records were analyzed for

nutrient intake using computer software (Food Processor, ESHA Research, Salem, OR).

### **2.8. Statistical Analysis**

Data were analyzed using a 2 (condition) x 10 (time) repeated measures analysis of variance (ANOVA). Post hoc testing using the method of Tukey was performed as necessary, with contrasts analyzed during the time between baseline and pre exercise, as well as during post exercise periods. A one-way ANOVA was used to analyze dietary intake and repetitions performed during the exercise bouts. The data are presented as mean  $\pm$  SEM. All analyses were performed using JMP statistical software (version 4.0.3, SAS Institute, Cary, NC). Statistical significance was set at  $p \leq 0.05$ .

## **3. RESULTS**

All 15 subjects successfully completed the study and subject characteristics are presented in Table 1. A few data points were missing, as follows: the number of repetitions performed was not recorded for two subjects when assigned to 2-NO (for set 1 and 2 for one subject; for set 2 only for one subject) and for two subjects when assigned to placebo (for set 1 and 2 for one subject; for set 2 only for one subject). Heart rate and blood pressure data were not available at the 10 minute post exercise time point (set 2) for one subject when assigned to 2-NO. Nitrate/nitrite data were not available at the 10 minute post exercise time point (set 2) for two subjects when assigned to 2-NO, as well as at the 0 minute post exercise time point (set 1) for one subject when assigned to placebo. Data were missing due to difficulty in obtaining blood samples at the above times. In addition, NO<sub>x</sub> values were not included for one subject due to values being considerably higher than all other subject values and far exceeding the standard curve for the assay.

Dietary intake was not different during the day before each test day ( $p > 0.05$ , Table 2). Subjects consumed  $21 \pm 3$  ounces of water

during the study periods. The number of repetitions performed during the sit-up test was nearly identical for 2-NO (51 and 50 reps for sets 1 and 2, respectively) and placebo (50 and 49 reps for sets 1 and 2, respectively) ( $p > 0.05$ ).

Heart rate and rate pressure product data are presented in Figure 1. For heart rate, no condition ( $p = 0.30$ ) or interaction ( $p = 0.99$ ) effect was noted. A time effect was noted ( $p < 0.0001$ ), with values at all times following sets 1 and 2 higher than baseline (Pre Meal) and Pre Exercise. For rate pressure product, no condition ( $p = 0.65$ ) or interaction ( $p = 0.98$ ) effect was noted. A time effect was noted ( $p < 0.0001$ ), with values at all times following sets 1 and 2 higher than baseline (Pre Meal) and Pre Exercise.

Blood pressure data are presented in Figure 2. For systolic blood pressure, a condition effect was noted ( $p = 0.03$ ), with values for 2-NO lower than those for placebo. A time effect was noted ( $p < 0.0001$ ), with values at 0 and 5 min following set 1 higher than baseline (Pre Meal) and Pre Exercise, and higher at 0 min following set 2 than baseline (Pre Meal) and Pre Exercise. No interaction effect was noted ( $p = 0.89$ ). Systolic blood pressure was lower for 2-NO compared to placebo at the Pre exercise time ( $p = 0.059$ ) and also during the 5-20 minute post exercise period following set 2 ( $p = 0.04$ ). The peak systolic blood pressure following exercise was not statistically different between conditions ( $p = 0.31$ ) but was lower for 2-NO compared to placebo following set 1 ( $151 \pm 4$  mm Hg vs.  $154 \pm 4$  mm Hg) and set 2 ( $148 \pm 5$  mm Hg vs.  $154 \pm 4$  mm Hg). For diastolic blood pressure, no condition ( $p = 0.12$ ), time ( $p = 0.18$ ) or interaction ( $p = 0.64$ ) effects were noted. However, diastolic blood pressure was lower for 2-NO compared to placebo during the 5-20 minute post exercise period following set 2 ( $p = 0.02$ ).

Plasma NO<sub>x</sub> data are presented in Figure 3. For NO<sub>x</sub>, no condition ( $p = 0.11$ ), time ( $p = 0.99$ ) or interaction ( $p = 0.99$ ) effects were noted. When comparing NO<sub>x</sub> values

during the 20 minutes following set 1 ( $p = 0.25$ ) and set 2 ( $p = 0.22$ ) of exercise, values were higher for 2-NO compared to placebo but were not of statistical significance. When comparing all post exercise time points between conditions, the higher NO<sub>x</sub> values for 2-NO approached statistical significance ( $p = 0.09$ ), which corresponded to the trend for a condition effect ( $p = 0.11$ ).

#### 4. DISCUSSION

Data from the present investigation indicate that the molecule known as 2-nitrooxy-ethyl-2-amino-3-methylbutanoate 1) can reduce both SBP and DBP in response to acute physical stress, 2) can increase circulating NO<sub>x</sub> by approximately 10% in response to acute physical stress, and 3) has little to no impact on HR. These data are specific to a sample of healthy, young, and normotensive men.

Our prior work using the 2-NO molecule noted a small but similar elevation in NO<sub>x</sub> in men (~6.7%) [7]. Subjects in that study were required to remain sedentary throughout the 60 minute data collection period, which may have impaired the "activation" of the 2-NO. That is, recent evidence in animals suggests that increased shearing forces on the vasculature are necessary to fully activate this molecule (unpublished findings). In these past studies, 2-NO appeared to increase NO<sub>x</sub> in response to the minor rise in blood pressure; quantities of NO<sub>x</sub> sufficient to counteract the stressor without inducing hypotension. Based on these initial observations, coupled with our current findings, 2-NO may be a novel nitrate-like molecule that provides a controlled and relatively safe release of NO in response to elevated blood pressure.

In the present study, our use of acute sit-up exercise resulted in an increase in SBP of approximately 25 mm Hg, which may have been the needed stimulus for the 2-NO molecule to exert its effects. This appears somewhat supported when viewing the

findings for SBP, which was similar between 2-NO and placebo during the time following set 1 but was different between conditions during the time following set 2. It is possible that the acute blood pressure increase during set 1 was needed to activate the 2-NO in such a way as to result in a blood pressure lowering effect during the recovery period following set 2 (Figure 2A). That said, both DBP and NO<sub>x</sub> values appeared different between conditions following both sets 1 and 2. The NO<sub>x</sub> values presented in Figure 3 demonstrate an approximate 10% increase from baseline, which is slightly greater than what we noted in our prior work with 2-NO. Considering that the overall increase in blood pressure in response to the sit-up exercise was rather minor (~25 mm Hg), it is possible that a greater increase in blood pressure may have yielded a more robust increase in NO<sub>x</sub>. Future studies may seek to use more strenuous and/or longer duration exercise in an attempt to further increase blood pressure.

It is presently unknown what the precise implications are for the noted increase in NO<sub>x</sub>. Indeed, it is well known that NO<sup>•</sup> functions to promote vasodilation by acting on vascular smooth muscle. In turn, peripheral resistance may be lowered with increase NO<sup>•</sup>, possibly leading to enhanced blood flow at rest [8] and during exercise [4]. It has yet to be determined whether or not such changes will definitely result in improved athletic performance and exercise recovery [6].

Related to the noted decrease in blood pressure with 2-NO, the 2-NO appeared quick to respond to the given stressor (food or exercise), as can be seen in Figure 2. This rapid time to onset may have implications in various clinical applications. Indeed, these findings may have implications for those with hypertension, as even small decreases in blood pressure (e.g. 5 mm Hg) correlate to a significant decrease in disease risk [8-10]. If future studies with 2-NO replicate the current findings for a decrease in blood pressure in response to feeding and exercise, this agent

may prove to be an adjunct therapy for treating elevated blood pressure. Moreover, the 2-NO might prove beneficial in treating clinical conditions in which blood flow is compromised, such as peripheral vascular disease. Future studies using 2-NO may focus on clinical populations who present with hypertension and associated co-morbidities. The use of 2-NO within hypertensive patients may yield more robust outcomes as related to blood pressure reduction. That said, it is known that nitrates have a dose dependent effect upon blood pressure, whether in hypertensive, normotensive or hypotensive individuals and hence even natural nitrate supplementation must be used cautiously [11].

Interesting, the 2-NO appeared to have the most pronounced effect on negating the rise in SBP in response to acute feeding. As indicated in the Methods section of this paper, the meal consisted simply of orange juice and protein powder (and essentially devoid of dietary fat), yet resulted in an increase in SBP of approximately 9 mm Hg for placebo. The use of 2-NO abolished this increase completely, with identical values noted Pre Meal and Pre Exercise (Figure 2A). Further study of the impact of 2-NO on feeding-induced blood pressure elevation may be considered.

Nitroglycerin is the most commonly used nitrate, having been in use for over 130 years for a myriad of medical applications. The extremely small dosages (e.g. 0.3-0.6 mg) promotes a vasodilatory effect in most patients, which may reduce symptoms of angina but may also lower blood pressure rapidly and to quite low levels. Considering the moderate blood pressure lowering effect of 2-NO, an effect that appears controlled based on the degree of acute blood pressure elevation, this novel agent may be considered for medical application upon further study. This may apply within the context of 2-NO use to aid hypertension, pain crises which promote hypertension [12], sexual function which promotes acute elevation in blood

pressure [13], as well as other conditions in which nitrates may prove beneficial.

## **5. CONCLUSION**

The molecule known as 2-nitrooxy-ethyl-2-amino-3-methylbutanoate can lower blood pressure in response to acute exercise in young, healthy, and normotensive men. The noted change in blood pressure may be partly explained by an increase in circulating NO<sub>x</sub>. Future studies are needed to replicate these findings and to extend this work with the use of clinical populations who may benefit from use of a blood pressure lowering agent.

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## **7. CONFLICT OF INTEREST**

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**Table 1.** Characteristics of healthy men ingesting 2-nitrooxy-ethyl-2-amino-3-methylbutanoate or placebo

Variable	Value (n=15)
Age (years)	28.5±0.8
Height (cm)	181.8±2.2
Body Weight (kg)	85.1±3.5
Body Mass Index (kg·m <sup>-2</sup> )	25.7±0.9
Waist Circumference (cm)	85.8±1.8
Hip Circumference (cm)	101.8±2.0
Waist:hip	0.84±0.01
Resting Heart Rate (bpm)	70.4±2.0
Resting Systolic Blood Pressure (mmHg)	123.3±1.6
Resting Diastolic Blood Pressure (mmHg)	80.1±1.7
Weekly Aerobic Training (hrs)	2.4±1.0
Aerobic Training History (yrs)	7.2±2.1
Weekly Anaerobic Training (hrs)	2.6±0.6
Anaerobic Training History (yrs)	5.5±1.7

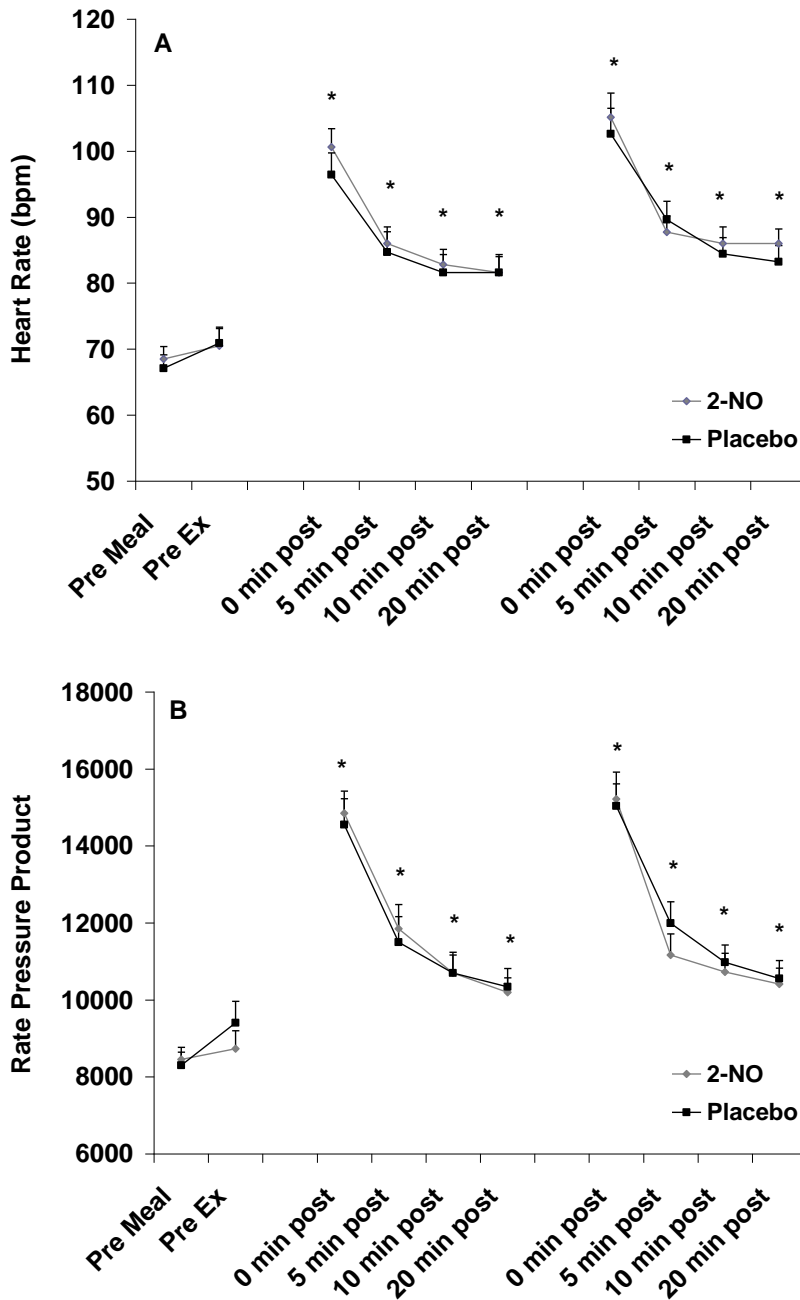
Values are mean±SEM.

**Table 2.** Dietary data of healthy men during the 24 hours prior to ingesting 2-nitrooxy-ethyl-2-amino-3-methylbutanoate or placebo

Variable	2-NO	Placebo	P-value
Kilocalories	2164±223	2503±283	0.28
Protein (g)	110±12	129±16	0.31
Carbohydrate (g)	254±34	282±36	0.56
Fat (g)	77±12	98±16	0.20
Vitamin C (mg)	83±20	87±21	0.78
Vitamin E (mg)	7±2	10±3	0.48
Vitamin A (RE)	472±81	845±190	0.07

Values are mean±SEM.

**Figure 1.** Heart rate (A) and rate pressure product (B) data of healthy men before and following ingestion of 2-nitrooxy-ethyl-2-amino-3-methylbutanoate or placebo, before and after two sets of sit-up exercise



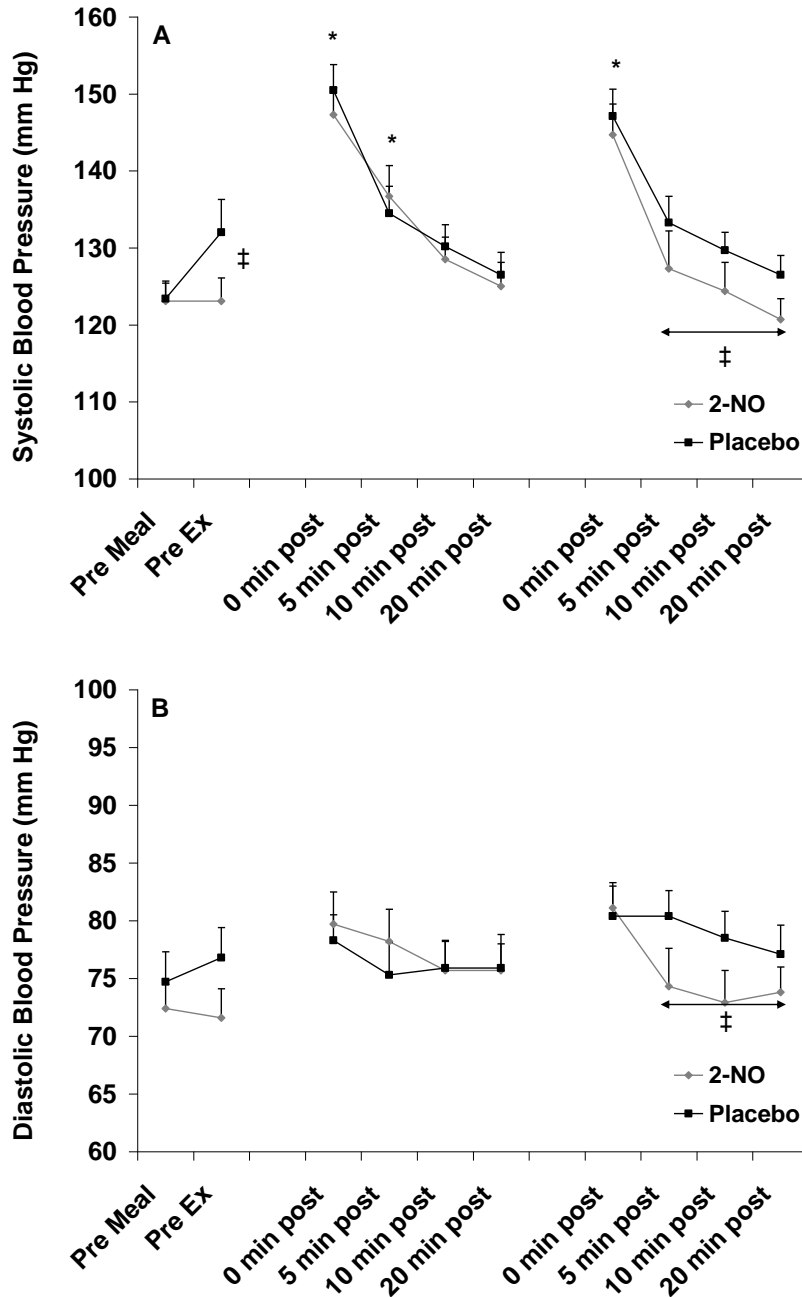
Values are mean±SEM.

N=15

*Heart rate:* \* time effect (p<0.0001); values at all times following sets 1 and 2 higher than baseline (Pre Meal) and Pre Ex.

*Rate pressure product:* \* time effect (p<0.0001); values at all times following sets 1 and 2 higher than baseline (Pre Meal) and Pre Ex.

**Figure 2.** Systolic (A) and diastolic (B) blood pressure data of healthy men before and following ingestion of 2-nitrooxy-ethyl-2-amino-3-methylbutanoate or placebo, before and after two sets of sit-up exercise



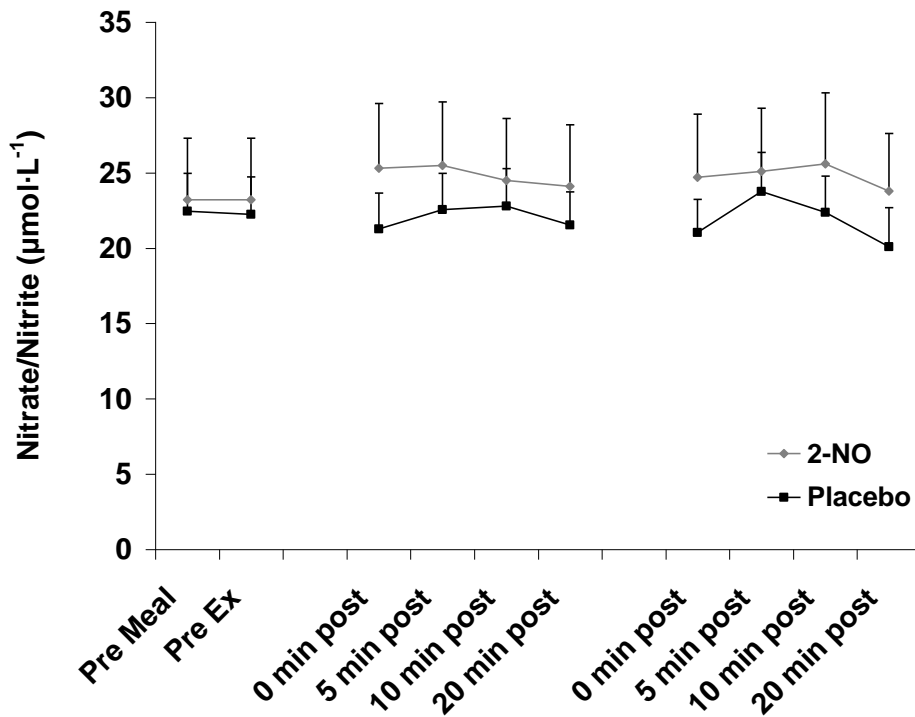
Values are mean±SEM.

N=15

*Systolic blood pressure:* condition effect (p=0.03); \* time effect (p<0.0001), values at 0 and 5 min following set 1 higher than baseline (Pre Meal) and Pre Ex, and higher at 0 min following set 2 than baseline (Pre Meal) and Pre Ex. ‡ lower for 2-NO compared to placebo at the Pre Ex (p=0.059) and during the 5-20 minute post exercise period following set 2 (p=0.04).

*Diastolic blood pressure:* ‡ lower for 2-NO compared to placebo during the 5-20 minute post exercise period following set 2 (p=0.02).

**Figure 3.** Plasma nitrate/nitrite data of healthy men before and following ingestion of 2-nitrooxy-ethyl-2-amino-3-methylbutanoate or placebo, before and after two sets of sit-up exercise



Values are mean $\pm$ SEM.

N=14