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Association between oxygen consumption and nitric oxide production during the relaxation response

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Summary

Background:

Mind/body practices that elicit the relaxation response (RR) are currently practiced by over 30% of American adults. RR elicitation reduces volumetric oxygen consumption (VO_2) from rest and counteracts the effects of stress, although the mechanisms mediating the RR remain unknown. This study was designed to investigate whether RR elicitation is mediated by nitric oxide (NO). We developed a method to quantify depth of RR using change in VO_2 (slope) during RR elicitation. We evaluated whether depth of RR elicitation was correlated with changes in NO, as measured by percentage changes in fractional exhaled nitric oxide ($F_E NO$).

Material/Methods:

We conducted a randomized, controlled trial, in which 46 subjects were randomized to either 8-weeks of RR training using audiotapes ($n=34$) or 8-weeks of exposure to a control condition – receiving health-education by audiotapes ($n=12$). Prior to randomization, VO_2 and $F_E NO$ were measured while subjects listened to a control audiotape. Eight weeks later, VO_2 and $F_E NO$ were measured while the RR group listened to a RR-eliciting audiotape and the control group listened to a control audiotape.

Results:

Prior to receiving any training, there was no association between VO_2 slope and $F_E NO$. After training, there was an inverse correlation between VO_2 slope and $F_E NO$ in the RR group ($r=-0.41$, $P=0.037$, $n=26$), but not in the control group ($r=0.12$, $P=0.78$, $n=8$).

Conclusions:

Depth of RR elicitation was associated with increased concentrations of $F_E NO$ after RR training. The RR may be mediated by NO helping to explain its clinical effects in stress-related disorders.

key words:

relaxation response • fractional exhaled nitric oxide • volumetric oxygen consumption • stress

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BACKGROUND

Mind/body practices that elicit the relaxation response (RR) have been practiced for thousands of years to promote health and well-being [1]. A recent national survey indicates that mind/body techniques are used by 30% of the US adult population [2] affirming the continued popularity of these practices.

Numerous mind/body approaches can elicit the RR including: meditation, repetitive prayer, yoga, tai chi, autogenic training, deep breathing exercises, progressive muscle relaxation, biofeedback, guided imagery and Qi gong [1,3]. The RR can be elicited as individuals repeat a word, sound, phrase, prayer or focus on their breathing and disregard intrusive everyday thoughts [4].

The RR [5] is described as a coordinated physiological response that is characterized by decreased arousal, diminished heart rate, respiratory rate, and blood pressure, in association with a state of "well-being" [4,6]. The physiological responses of the RR occur in the opposite direction from those of the stress response, described as the "fight or flight" response and the "general adaptation response" to stress by Cannon [7] and Selye [8] respectively. Accordingly, a conceptual model of the RR as a mind/body state that can oppose or counteract the physiological changes of the stress response has been hypothesized [4].

The elicitation of the RR is characterized by measurable, predictable and reproducible physiological decreases in volumetric oxygen consumption (VO_2) [4,6,9-13]. In addition to decreased VO_2 , other consistent physiological changes include decreased carbon dioxide elimination [6,10,11,13] reduced heart and respiration rates [14], lower arterial blood lactate [6], reduced systolic and diastolic blood pressure [15-18], decreased responsivity to norepinephrine [19], decreased theta and beta waves and increased alpha frontal activity on EEG [20,21], prominent low frequency heart rate oscillations [22,23] and alterations in cortical and sub-cortical brain regions [20,24,25].

Clinically, the RR has been shown to counteract the negative effects of long-term stress. The RR is often utilized as an adjunct to medical treatment, in conditions that are caused or exacerbated by stress [26]. These conditions represent a broad range of physiological systems and include: premature ventricular contractions in stable ischemic heart disease [27]; hypertension [15,16,28,29]; myocardial ischemia [30]; chronic heart failure [31,32]; cardiac rehabilitation [33]; anxiety [34-37]; psychosomatic complaints [37-40]; insomnia [41-43]; headache [44-46]; back/neck pain [47]; chronic pain [47,48]; musculoskeletal disorders [49]; osteoarthritis [47]; rheumatoid arthritis [50,51]; fibromyalgia [52]; premenstrual syndrome [53]; and infertility [54,55]. The clinical effect of the RR has also been shown in improved outcomes after cardiac and other surgery [56,57]; wound healing [58]; pain relief and anxiety reduction in femoral arteriography and other invasive medical procedures [59,60] and symptoms related to cancer treatment [61-65]. Despite these clear physiological and clinical observations, the underlying mechanisms of the RR remain undefined.

Nitric oxide (NO), a short-lived nitrogenous free radical, has been shown to mediate diverse physiological processes including cardiovascular, immune and nervous system functions [66] as well as decreased hypothalamic-pituitary-adrenal (HPA) axis activation [67]. NO is well known as an endothelium-derived relaxing factor [68, 69] and plays a prominent role in vascular dilatation [70-72]. On the basis of the well-established vasodilatory effects of both NO and RR elicitation, the broad range of physiological systems regulated by NO and the diverse clinical applications of various RR-eliciting practices, Stefano et al. hypothesized that elicitation of the RR is associated with the synthesis and liberation of NO by constitutive isoforms of nitric oxide synthase (NOS), neuronal (NOS-1) and endothelial (NOS-3) [73]. Stefano et al. indicate that NO, released from autonomic nerve terminals throughout the cardiovascular system, produces a vasodilatation, mediated by the second messenger cyclic guanosine monophosphate (cGMP), that overcomes basal sympathetic tone, predominantly mediated by norepinephrine (NE). Furthermore, sustained exposure to NO inhibits the release of NE [73]. Additional studies have conclusively demonstrated NO release from vascular terminals of the autonomic nervous system [74,75].

As a gaseous free radical, endogenous NO is difficult to measure directly in plasma or other bodily fluids [76]. A variety of methods have been developed to provide quantitative measures of NO and we have elected to obtain quantitative NO data by measuring fractional exhaled nitric oxide ($F_E NO$). This method reliably measures NO in exhaled breath [77,78], and has been widely used according to standards developed by the American Thoracic Society (ATS) [79]. Interestingly, it has recently been demonstrated that NO levels, as measured by $F_E NO$, are altered in stress-related conditions, such as hypertension [80] and NO is clearly linked to regulation of blood pressure as 4 of the 5 classes of anti-hypertensive drugs have NO-releasing capacities [81].

Decreases in VO_2 have been consistently reported during elicitation of the RR [4,6,9-13]. In these studies, VO_2 measurements were typically reported as a mean value for the whole RR elicitation period and these individual values then averaged for an entire group of subjects. Although the findings suggest that VO_2 data may be used as an indicator of RR elicitation, they do not take into account the dynamic nature of oxygen consumption or individual differences in ability to elicit the RR [82]. There are pronounced differences in individuals' response to stress in terms of both HPA/autonomic activity and physiological changes [83,84]. Considering a model where the RR is a counter to the stress response, it is not unreasonable to suggest that there may be similar individual differences in ability to elicit the RR [82].

Revisiting the use of VO_2 to assess depth of the RR while addressing the limitations of reporting only mean VO_2 data, we decided to analyze the change in VO_2 over time (as VO_2 slope) when study subjects were either attempting to elicit the RR or were listening to a control audiotape. This novel approach captures the dynamic nature of VO_2 responses over time and provides the opportunity to assess individual differences in RR elicitation. Consequently, the VO_2 slope was used in regression analysis with real-time $F_E NO$ data to explore subtle, physiological changes in NO related to RR elicitation.

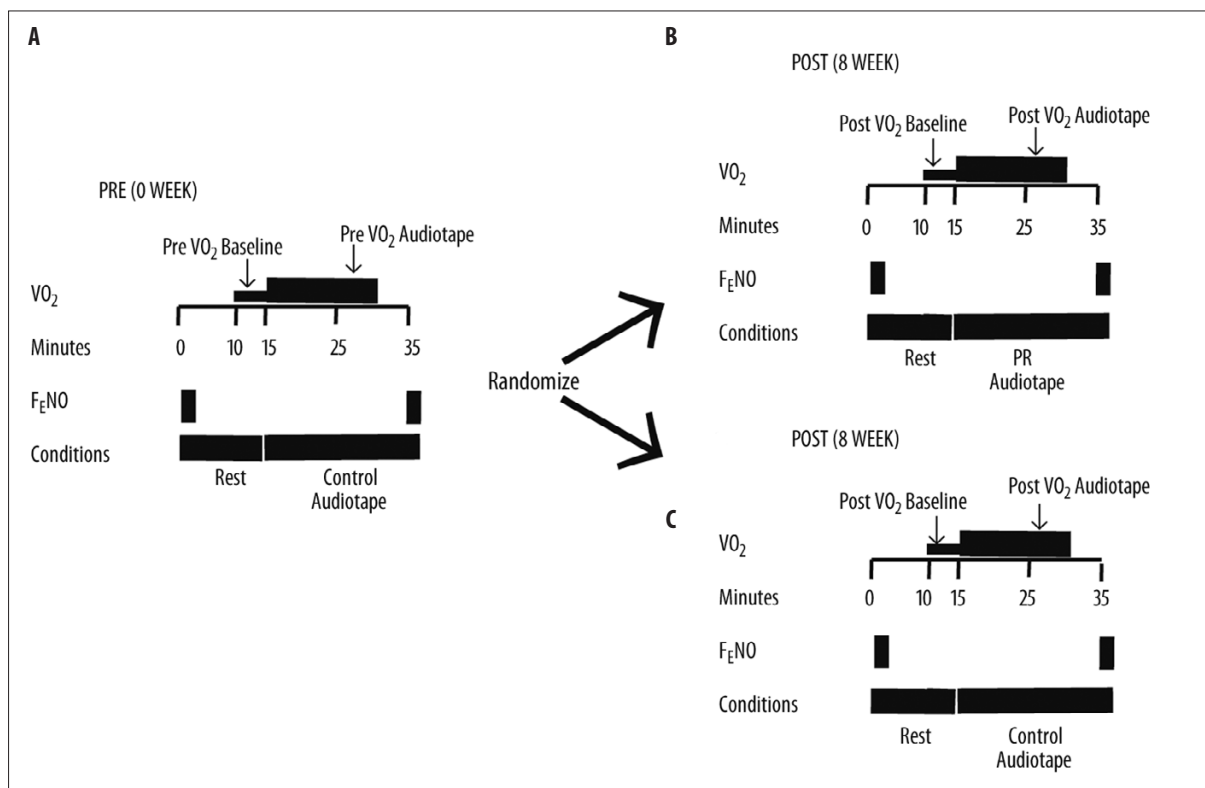


Figure 1. Study Procedures. A mask was placed on subjects at minute 10 and removed at minute 31. VO_2 was measured continuously at baseline (minutes 10 to 15), and while listening to an audiotape (minutes 15 to 31). VO_2 data were not collected from minutes 31 to 35. Fractional exhaled nitric oxide ($F_E NO$) was measured at minutes 0 and 35 (vertical bars). (A) Pre-training: All subjects listened to a health-education audiotape from minutes 15 to 35. (B) Post-training: Relaxation Response subjects listened to the RR-eliciting audiotape from minutes 15 to 35. (C) Post-training: Control subjects listened to a health-education audiotape from minutes 15 to 35.

We conducted a randomized, controlled trial by using this new approach to test the hypothesis that there is an association between depth of elicitation of the RR (as measured by VO_2 slope), and changes in NO production, as measured by percentage changes in $F_E NO$ during the time that the RR was being elicited.

MATERIAL AND METHODS

Study protocol

This blinded randomized control trial included two study groups. Interested individuals were informed that the purpose of the study was to compare two Health Management training programs. The Committee for Clinical Investigations, Beth Israel Deaconess Medical Center (BIDMC), Boston, MA, approved the study protocol and all subsequent amendments.

Blinding

Since subjects were informed that they were participating in a study comparing 2 different Health Management programs, they were blind to their treatment assignments and which program was experimental or control. Only the clinical trainers and the scheduler were aware of individual subject's treatment assignment, whereas all other research personnel, including those involved in data collection, were unaware of any individual's group assignment.

Subjects/Screening

Potential subjects responded to advertisements posted on-line and in Boston, MA newspapers. An initial phone screen excluded subjects who were current smokers, had asthma, severe seasonal allergies or other respiratory conditions which may effect exhaled NO levels (e.g., pneumonia, obstructive bronchitis), and those who had previous experience with any RR-eliciting techniques. Individuals were excluded for: history or presence of neurological, psychiatric or musculoskeletal disorders; pregnancy; prescription, non-prescription or herbal medication usage (except oral contraceptives). After providing written informed consent, individuals were screened by a physician (HB), and had fasting blood drawn. Those with a hematocrit below 32%; glucose <50 or >450 mg/dl; creatinine >1.3 mg/dl; human chorionic gonadotropin greater than 5 mIU were ineligible to participate. Those who had an acute upper respiratory illness (URI) were also excluded for the duration of their URI. A total of 46 healthy young adults met all eligibility criteria and were scheduled for a pre-training visit at the General Clinical Research Center (GCRC) of the BIDMC and were instructed to refrain from strenuous exercise, consuming caffeine and use of any over the counter medications in the 48 hours prior to the visit.

Pre-training visits

All subjects completed a pre-training (Pre) visit (week 0) at the GCRC. GCRC visits routinely began at 9 am to control

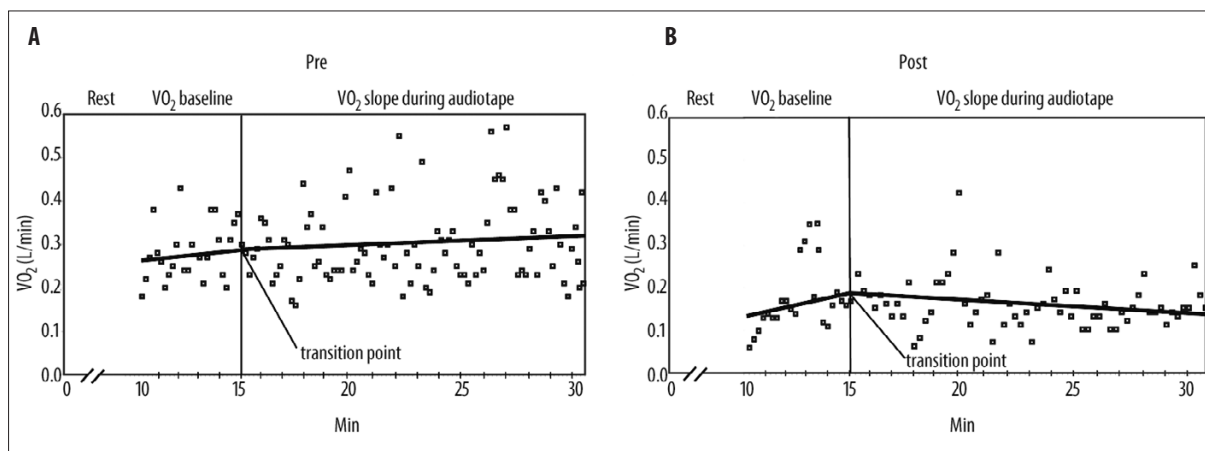


Figure 2. VO_2 data for a representative RR subject. The VO_2 average for every 3-breath interval is represented by open squares. **(A)** VO_2 data versus time (minutes) with slope lines for VO_2 baseline (minutes 10–15) and VO_2 audiotope periods (minutes 15–31, subject listened to a control audiotope). **(B)** VO_2 data versus time (minutes) with slope lines for VO_2 baseline and VO_2 audiotope periods (subject listened to a RR-eliciting audiotope).

for diurnal variation. Subjects answered questions regarding illness and usage of caffeine or medications. GCRC visits were rescheduled for subjects who reported caffeine/medication usage or illness. Those who were eligible for data collection were trained how to breathe into the NO analyzer before the Pre-training visit started.

The Pre visit included sampling subject's VO_2 levels for 21 minutes as they listened to a control health-education audiotope. F_eNO was evaluated at time 0 and immediately after listening to the audiotope (time 35) (Figure 1).

Randomization

After completing their Pre visit, subjects were randomly assigned at a 3:1 ratio to "Health Management Group 1" ($n=34$) in which they received an 8-week RR training program or to "Health Management Group 2" ($n=12$) group in which they received 8-weeks of health-education information. Such a design allows for more statistical power to test our study hypothesis in the experimental (RR) group.

Post-training visits

After completing their 8-week training program, subjects came into the GCRC for a Post-training visit. Subjects were again instructed to avoid strenuous exercise, consuming caffeine and use of any over the counter medications in the 48 hours prior to the post study visit in the GCRC. The same procedures were repeated during the Post visit, with the exception that subjects in the RR group listened to the RR-eliciting audiotope and control subjects listened to a different health-education control audiotope.

Volumetric oxygen consumption (VO_2)

VO_2 was measured and analyzed by a portable metabolic measurement system using galvanic cell oxygen measurement (V02000: MedGraphics Corporation, St. Paul, MN) [85]. Automatic 2-point calibration was conducted before each testing session. Room temperature, barometric pressure and relative humidity were also recorded. For collec-

tion of VO_2 , subjects wore a snug lightweight headgear supporting a nose clip to prevent nasal artifacts in metabolic measures, and a mouthpiece attached to a PreVent™ pneumotachometer. Subjects were instructed to remain still and breathe normally during data collection. BreezeSuite™ software recorded continuous data for every breath interval. Instantaneous metabolic measures for every 3 consecutive exhalations were automatically calculated and averaged (Figure 2).

Calculation of VO_2 slope

Using the ordinary least squares regression method [86], each subject's VO_2 data were fit into the following 2-slope regression model.

$$VO_2 \text{ data} = \beta_0 + \beta_1 * m + \beta_2 * m1$$

where "m" is the time (in minutes) when the VO_2 was collected, "m" ranges from 0 to 21 (the period of minutes 10 to 31 when VO_2 was measured), $m1=0$ if $m < 5$ minutes, $m1=m$ if $m \geq 5$ minutes. The time variable m was re-coded to be from 0 to 21 minute for the ease of calculation. The estimates of β_1 and β_2 were not affected due to the subtraction of a constant value 10 from the original time of minutes of 10 to 31.

In this 2-slope model, the slope of the regression line for the data collected during the 5-minute VO_2 baseline period is β_1 , while the slope of the regression line for the data collected during the VO_2 audiotope period is $\beta_1 + \beta_2$. The transition point is the time point when subjects switch from sitting at rest without listening to an audiotope (minutes 10–15) to listening to an audiotope (minutes 15–31). The transition point is the starting point of the regression line of the VO_2 audiotope period. Since VO_2 data were collected continuously over time, it is essential to have the ending point of the first regression line join the starting point of the second regression line. This feature of the 2-slope regression could not be achieved by using separate 1-slope regression models for the VO_2 baseline and VO_2 audiotope periods. The slope of both the VO_2 baseline and VO_2 audiotope periods indi-

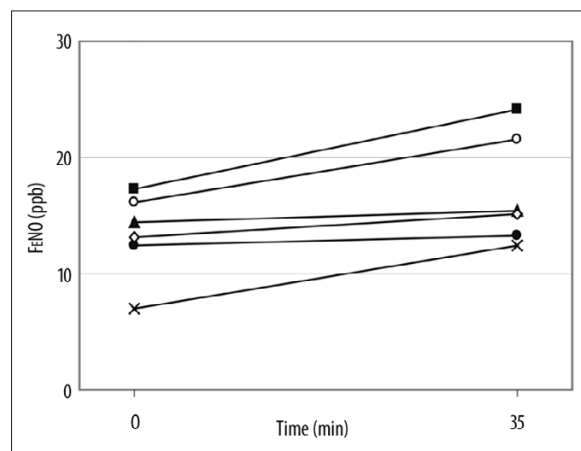


Figure 3. F_eNO raw values in parts per billion (ppb) collected during the Post visit are plotted at minutes 0 and 35 in 6 RR subjects with the most negative post VO_2 slopes. Each line represents a single subject and each point represents an average F_eNO of at least 3 breaths. The measurements at time 0 and 35 reflect F_eNO values prior to and immediately after listening to RR-eliciting audiotape respectively.

cate VO_2 change (in liters/minute) over time (Figure 2). Since RR elicitation is a gradual process that leads to deeper and deeper states over time, a greater VO_2 decreasing rate indicates a deeper RR elicitation.

The slopes for the VO_2 baseline and VO_2 audiotape periods were calculated separately for each subject. The same algorithm was applied to the VO_2 data collected during the Pre (Figure 2A) and the Post visits (Figure 2B).

Fractional Exhaled Nitric Oxide (F_eNO)

A rapid response chemoluminescent Nitric Oxide Analyzer (NOA Model 280i, Sievers instruments; Boulder, CO) was used for measuring real-time exhaled F_eNO . This is a valid method for measuring real-time F_eNO [76,87]. Before each testing session, two point calibrations were performed according to American Thoracic Society (ATS) recommendations [79] using a zero air filter and 45 parts per million nitrogen based calibration gas. Ambient NO levels were recorded before each measurement.

On-line data was collected using Sievers NOAnalysis™ Software: Restricted Exhaled Breath (version 3.21) to exclude nasal artifacts. Subjects inhaled ambient air through a filter to reduce NO concentrations to <5 parts per billion. Subjects inhaled without a nose clamp to total lung capacity and immediately exhaled, targeting a constant pressure of 16 cm H_2O with the aid of a visual feedback display. Exhalations at a flow rate of 50 mL/s for at least 6 seconds were collected, according to ATS guidelines [79] to ensure an end-expiratory plateau where flow varied $\pm 10\%$ of the target flow. Subjects repeated the procedure until at least 3 exhalations met the ATS standards and had a standard deviation of 10% or less. The average of the acceptable exhalations was recorded as single value for a given timepoint (see Figure 3).

Changes in F_eNO were expressed as percentages of values at Minute 0, to adjust for individual differences at baseline.

The percent change in F_eNO was calculated using the following formula:

$$F_eNO \text{ percent change} = (F_eNO \text{ after audiotape} - F_eNO \text{ before audiotape}) / F_eNO \text{ before audiotape} * 100$$

To test the study hypothesis that RR elicitation is associated with F_eNO , we estimated the correlation between the slope for the VO_2 audiotape period (a measure of depth of RR elicitation) and the percent change in F_eNO . A weighted regression approach [86] was used to correct for the heterogeneity in variance of the VO_2 slopes, which were estimated for each individual subject separately. Separate weighted regression models were used to fit data collected during the Pre and Post visits in both groups. Data were analyzed with SAS 8.2 (SAS Institute Inc, Cary NC).

Interventions

Subjects in both groups attended 8 weekly, 1 hour sessions with a clinical trainer. In addition to the weekly sessions, all subjects were provided with audiotapes/CD to listen to every day for 20 minutes and asked to keep a diary card recording their compliance. Clinical trainers, although not blind to an individual's treatment group, devoted the same amount of time and presented information in a standardized fashion, following a clearly specified protocol to both groups over the 8-week study period.

Relaxation response training

Those randomly assigned to Health Management Group 1, the RR intervention, completed 1-hour weekly individual sessions and 20-minute daily RR elicitation (guided by tape/CD). Sessions consisted of reviewing the previous week's diary card and discussing difficulties/problems with RR practice with suggestions on how to handle them, receiving information about the effects of stress on health and a 20-minute elicitation of the RR guided by a trainer. Both the trainer and the tape/CD employed body scan, focus word and mindfulness techniques to elicit the RR. During the body scan, subjects were instructed to slowly move their attention through the body, sequentially focusing on relaxing different regions. During the focus word segments subjects were instructed to focus on the silent repetition of a word, sound or phrase and to return to this repetition when thoughts or other distractions occurred. These approaches are consistent with those used in which decreased VO_2 has been described [4,6,9–13]. Topics covered in the informational part of the sessions included: description of stress, the stress response and ways stress can influence health/wellness; information on the RR (definition, practice guidelines and effects); conceptual model of mind/body medicine; description of coping skills that can limit the impact of stress/interrupt the stress response (stress hardness – positive cognitive orientation, social support and humor). The tape/CD used for home practice has been used in our clinical practice and research studies for over 10 years [37].

Control training

Subjects randomized to Health Management Group 2, control training, completed 1-hour weekly individual sessions, and 20-minute daily listening to a series of health educa-

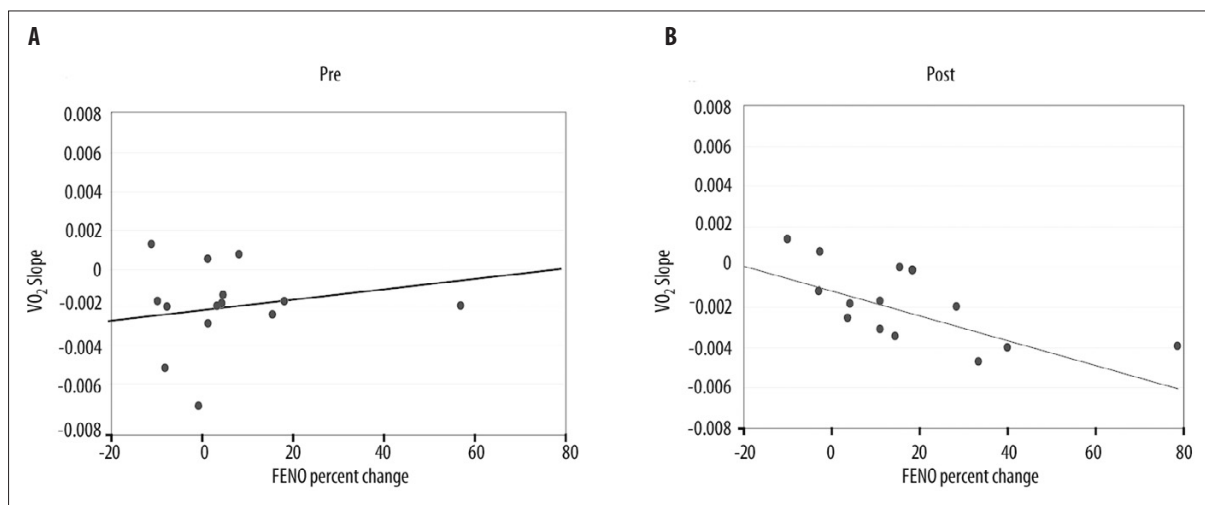


Figure 4. Interim analysis results: RR subjects. VO_2 slopes plotted versus percentage changes in $F_E NO$ in 14 RR subjects. Each data point represents a single subject. **(A)** No significant association between VO_2 slopes during the Pre-audiotape period and $F_E NO$ percentage change ($r=0.14$, $P=0.60$). **(B)** A significant inverse association between VO_2 slopes during the Post-audiotape period and $F_E NO$ percentage change ($r=-0.70$, $P=0.005$).

tion audiotapes. Sessions consisted of reviewing the previous week's diary card and receiving information about the effects of stress on health. The weekly sessions and education audiotapes did not contain any RR-eliciting instructions. Topics covered in the informational part of these sessions included (in greater detail than in the RR group): description of stress, the stress response and ways stress and negative emotions can influence health/wellness (specific conditions discussed: hypertension, insomnia, cardiovascular disease immunity); conceptual model of mind/body medicine; description of the negative stress cycle, touching on coping skills; factors that can limit the impact of stress (stress hardness, altruism, humor, the placebo effect) and guidelines for optimizing one's personal health care. The educational tapes contained information about nutrition and positive perspectives on lifestyle and emotions. To ensure compliance with the daily listening requirements, subjects were asked to write a brief summary of the material that they listened to each day on a weekly diary card.

RESULTS

Subject characteristics

Twenty six of the 34 RR subjects completed both Pre and Post visits: 2 dropped-out of the study, 4 had missing data due to equipment malfunctions, and 2 had acute upper respiratory infections during the Post visit. Eight of the 12 control group subjects completed Pre and Post visits: 3 dropped out of the study and 1 had an acute upper respiratory infection during the Post visit. Subjects with complete data were similar to those with missing data in age, gender, and race (all P 's >0.5) and Pre VO_2 and $F_E NO$ data (P 's >0.4).

The mean age of subjects in Health Management Group 1 (RR group: $n=26$) was 25.9 years (8.5 sd), which was almost identical to the age in the Health Management Group 2 (control group: $n=8$, 25.4 years (7.5 sd), $P=0.88$). The groups had similar distributions of gender and race (46% of RR group and 63% of control group participants were female

($P=0.69$) and 81% of the RR group and 88% of the control group reported their race as white/Caucasian ($P=0.99$).

Interim analysis

To test the feasibility of our approach, we decided to conduct an interim analysis after 21 subjects had been enrolled in the study. Data were available on 14 of 17 subjects randomized to the RR group and 3 of 4 subjects randomized to the control group. Interim analyses were not conducted on data from the control group due to the small sample size ($n=3$). For the RR group, separate weighted regression models were used to fit data collected during the Pre and Post visits. The Pre visit VO_2 slope of the audiotape period was not associated with changes in $F_E NO$ in the RR group ($r=0.14$, $P=0.60$, Figure 4A). During the Post visit, the VO_2 slopes of the audiotape period were inversely associated with percentage change in NO ($r=-0.70$, $P=0.005$, Figure 4B). The investigators were aware of these interim results and then elected to complete the enrollment to the original planned sample size of 46 subjects.

Final analysis

The final analysis included data from 26 RR and 8 control subjects. In separate weighted regression models, we found there was no association between VO_2 slope of the audiotape period and percentage changes in $F_E NO$ for either the RR ($r=0.13$, $P=0.53$, Figure 5A) or control ($r=-0.03$, $P=0.94$, Figure 6A) groups during the Pre visit.

In contrast, during the Post visit, VO_2 slopes of the audiotape period were inversely associated with $F_E NO$ percent change ($r=-0.41$, $P=0.037$, Figure 5B) for the RR group. However, for the control group, there was no association between VO_2 slope and $F_E NO$ percent change ($r=0.12$, $P=0.78$, Figure 6B).

To demonstrate the individual changes in $F_E NO$ in relation to RR slope, in Figure 3, we present data from the 6 RR subjects who had the most negative post VO_2 slopes indicating

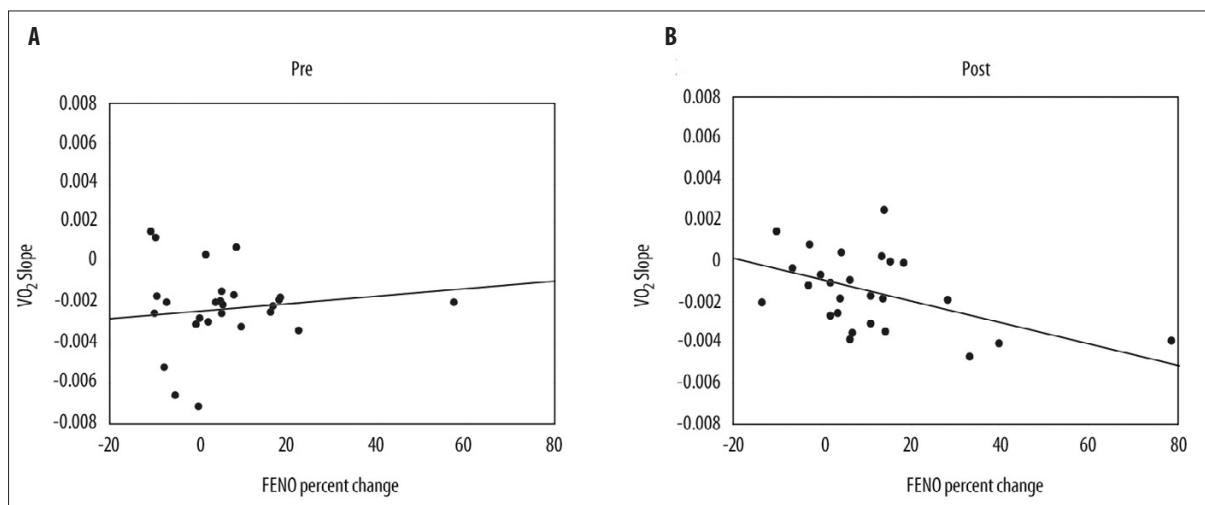


Figure 5. Final analysis results: RR subjects. VO_2 slopes plotted versus percentage changes in $F_E NO$ in 26 RR subjects. Each data point represents a single subject. (A) No association between the VO_2 slope during the Pre-audiotape period and $F_E NO$ percent change ($r=0.13$, $P=0.53$). (B) A significant inverse association between the VO_2 slope during the Post-audiotape period and $F_E NO$ percent change ($r=-0.41$, $P=0.037$).

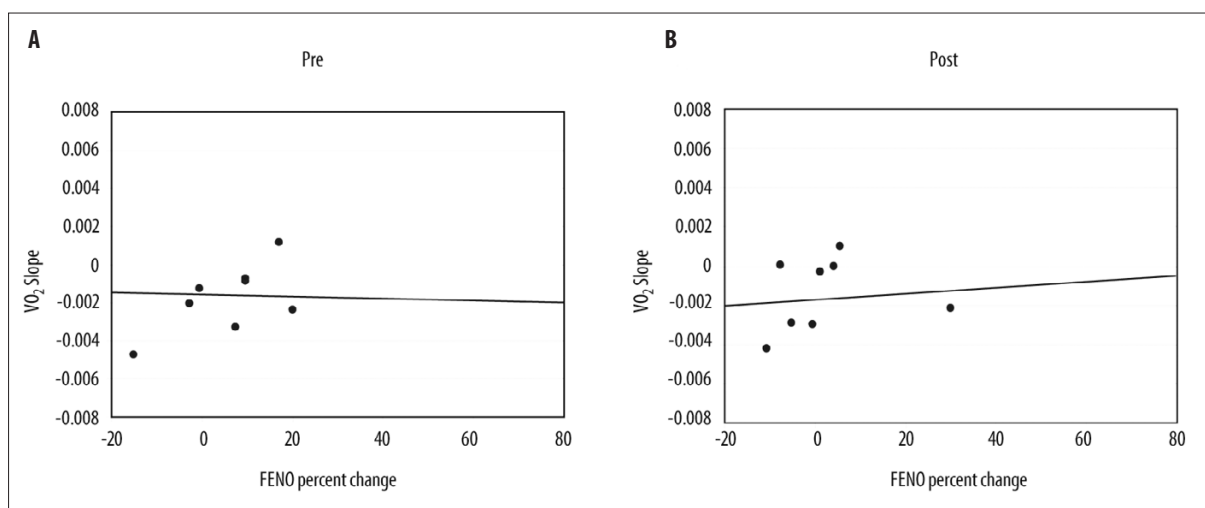


Figure 6. Final analysis results: Control subjects. VO_2 slopes plotted versus percentage changes in $F_E NO$ in 8 control subjects. Each data point represents a single subject. (A) There was no association between the VO_2 slope during the Pre-audiotape period and $F_E NO$ percent change ($r=-0.03$, $P=0.94$). (B) No association between the VO_2 slope during the Post-audiotape period and $F_E NO$ percent change ($r=0.12$, $P=0.78$).

the deepest degree of RR elicitation. $F_E NO$ data (shown in parts per billion) was collected prior to (0 min) and immediately after (35 min) listening to RR-eliciting audiotape. As shown in the figure, all these subjects had $F_E NO$ increased from 0 min to 35 min.

DISCUSSION

We developed a new method to quantify the depth of RR elicitation using the VO_2 slope and in a randomized controlled trial of 8 weeks of RR training vs. control, observed that depth of RR elicitation is correlated with percent change of $F_E NO$.

Our rationale for developing a method to quantify RR elicitation was based on well recognized individual differences in reaction to stress [83,84,88–90] and in abilities to elicit

the RR [82]. Prior studies which reported that VO_2 changes were associated with the RR had only enrolled experienced practitioners, averaged the observed changes for the whole group, disregarding individual differences, and reported the average VO_2 value for the entire RR eliciting period thus not capturing the dynamic nature of VO_2 measurement. The presently reported regression analysis captures the dynamic nature of VO_2 – calculating VO_2 slope is a refinement of prior VO_2 data collection methods and confirms previous findings. It also provides a novel understanding of individual differences in RR elicitation by offering a quantitative indication of RR depth.

There are limitations of the current study. First, an interim analysis was conducted approximately half-way through the trial, at which point the results for the first 21 subjects were made known to all members of the investigative team. We

are conscious of the possible influence of conducting the interim analysis on the subsequent data collected. Since the investigators were aware of the preliminary results of the interim analysis, it is possible that these results may have been communicated implicitly or explicitly to the remaining 25 subjects. However, since neither subjects nor study personnel responsible for data collection were aware of individual subjects' group assignments, we consider this unlikely. A related concern was the slightly more pronounced results from the interim analysis than in the final analysis. We are aware that results of the interim analyses may not be as reliable as those of the final study results due to random variability inherent in that smaller sample size.

Second, as a gaseous free radical, NO is rapidly oxidized by superoxide and other oxygen radicals [91] and has proven difficult to measure directly in the blood stream [76]. Several methods have been developed to quantify NO levels indirectly including measurement of oxidized NO metabolites such as nitrites and nitrates in plasma or other bodily fluids [92,93] and direct chemoluminescent detection of gaseous NO. We elected to evaluate NO in exhaled breath through real-time measurement of $F_E\text{NO}$. This method has been widely used and guidelines for accurate data collection have been established by the American Thoracic Society [79]. Drawbacks to this approach include the inability to determine which isoforms of NOS (constitutive: NOS-1, NOS-3 and inducible NOS-2) contribute to $F_E\text{NO}$. Also, it remains unclear to what extent $F_E\text{NO}$ captures systemic NO changes [94–96]. Exhaled NO is produced in the lungs and airways; derived from vascular endothelium, pulmonary epithelium, neurons and alveolar macrophages [97]. These tissues can express NOS-1, NOS-3 and NOS-2. Consequently, we can not determine that RR elicitation influences NO levels exclusively through the constitutive isoforms of NOS (NOS-1 and NOS-3), as proposed by Stefano et al. [73], that RR elicitation affects systemic NO levels or whether changes in NO simply accompany the RR.

A third limitation involves the possible influence of estrogen on NO levels. Although high estrogen levels associated with ovulation have been associated with increases to roughly 150 ppb in $F_E\text{NO}$ [98], all subjects with $F_E\text{NO}$ exceeding 60 ppb were excluded from this study. Therefore, it is likely that female subjects with high $F_E\text{NO}$ levels due to ovulation were excluded. A related concern is the fact that five RR subjects were taking oral contraceptives (OCP). However, since there are no studies reporting the influence of OCP on $F_E\text{NO}$, it remains unclear to what extent our results have been influenced by synthetic hormones of OCP. To minimize these concerns in future studies, however, we plan to collect data only during the early/mid-follicular phase (i.e. days 3–10) when estrogen levels are low and to exclude females taking OCPs.

NO is described as endothelium-derived relaxing factor, and is a well known regulator of vasomotor tone [68,69]. This small free radical is also an established biological mediator of the hypothalamic-pituitary-adrenal (HPA) and sympathetic-medullar-adrenal (SMA) axes [99,100], as well as the autonomic nervous system, reviewed in [75]. It is possible that RR-stimulated release of NO produces a series of cellular, biochemical and physiological changes in various organ systems that results in clinical effects of the RR in patient populations (e.g., hypertension). In future studies, we also plan to evaluate whether

biological mediators of the HPA and SMA axes are associated with VO_2 slope, the degree to which other RR-eliciting techniques such as yoga and different forms of meditation influence VO_2 slope and $F_E\text{NO}$, and identification of baseline characteristics predictive of deeper RR elicitation.

CONCLUSIONS

Our current data demonstrate that depth of RR elicitation (as defined by the VO_2 slope) is associated with increased percentage changes of $F_E\text{NO}$. This observation suggests that NO may serve as a biological mechanism underlying the RR and provides the first empirical support for the hypothesis that NO is a mediator of the RR [73]. Our study provides evidence of a possible mechanism underlying the widely-reported clinical effects of RR. Future, larger scale studies are needed to confirm our study findings.

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