Sympathetic neural reactivity to mental stress in humans: test-retest reproducibility

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Fonkoue IT, Carter JR. Sympathetic neural reactivity to mental stress in humans: test-retest reproducibility. Am J Physiol Regul Integr Comp Physiol 309: R1380–R1386, 2015. First published September 23, 2015; doi:10.1152/ajpregu.00344.2015.—Mental stress consistently increases arterial blood pressure, but this reliable pressor response is often associated with highly variable muscle sympathetic nerve activity (MSNA) responsiveness between individuals. Although MSNA has been shown to be reproducible within individuals at rest and during the cold pressor test (CPT), intraindividual reproducibility of MSNA responsiveness to mental stress has not been adequately explored. The purpose of this study was to examine MSNA reactivity to mental stress across three experimental sessions. Sixteen men and women (age 21 ± 1 yr) performed two experimental sessions within a single laboratory visit and a third experimental session 1 mo later. Each experimental session consisted of a mental stress trial via mental arithmetic and a CPT trial. Blood pressure, heart rate (HR), and MSNA were measured, and the consistencies of these variables were determined using intraclass correlation (Cronbach’s α coefficient). MSNA, mean arterial pressure (MAP), and HR were highly reproducible across the baselines preceding mental stress (Cronbach’s α ≥ 0.816, P ≤ 0.001) and CPT (Cronbach’s α ≥ 0.782, P ≤ 0.001). Across the three mental stress trials, changes in MSNA (Cronbach’s α = 0.875; P = 0.001), MAP (Cronbach’s α = 0.749; P < 0.001), and HR (Cronbach’s α = 0.919; P < 0.001) were reproducible. During CPT, changes in MSNA (Cronbach’s α = 0.805; P = 0.008), MAP (Cronbach’s α = 0.878; P < 0.001), and HR (Cronbach’s α = 0.927; P < 0.001) remained consistent across the three sessions. In conclusion, our findings demonstrate that MSNA reactivity to mental stress is consistent within a single laboratory visit and across laboratory sessions conducted on separate days.

A number of studies, including extensive work from our laboratory (3, 8, 16, 27), have examined muscle sympathetic nerve activity (MSNA) responsiveness to mental stress in humans. A recent review by Carter and Goldstein (5) highlights a total of 55 studies over the past 40 years that have examined MSNA responsiveness to mental stress in humans. It is widely acknowledged that MSNA, primarily governed by the baroreflex, is a key modulator of acute arterial blood pressure regulation. Although it seems reasonable to suggest MSNA responsiveness to mental stress contributes to CVR, there is actually little evidence to support this notion. Since the early work of Wallin et al. (29), mental stress has been shown to consistently increase arterial blood pressure, but this reliable pressor response is often associated with highly variable MSNA responsiveness. More recently, Carter and Ray (8) pooled data across several studies and reported that changes in arterial blood pressure during mental stress were not associated with changes in MSNA.

It is important to acknowledge that exposure to acute autonomic stressors can engage a variety of feedback (i.e., chemoreflex, baroreflex, etc.) and feed-forward (i.e., central command) mechanisms that strongly influence MSNA, and consequently blood pressure. Therefore, the CVR associated with acute mental stress is likely a complex response dependent upon multiple factors. Taken together with the unusually high interindividual variability of MSNA responsiveness to mental stress and the lack of rigorous prospective studies, it should not be assumed that MSNA reactivity to mental stress has similar predictive power as CVR. Interestingly, there is some limited evidence that MSNA reactivity to mental stress may be directly relevant to hypertensive risk (23). Specifically, Noll et al. (23) reported that individuals with a family history of hypertension (FHH+) demonstrated a significant increase of MSNA during mental stress, while individuals with no family history of hypertension (FHH−) demonstrated no change in MSNA reactivity to mental stress.

The preliminary findings of Noll et al. (23) suggest that studies examining MSNA reactivity to mental stress may, indeed, be clinically relevant, but advancing a “MSNA reactivity hypothesis” would require the same rigorous testing that the CVR hypothesis has undergone, beginning with the establishment of a stable MSNA responsiveness to mental stress within a subject (i.e., intraindividual reproducibility). This seems particularly timely, given the recent and accumulating evidence that MSNA reactivity to mental stress demonstrates unusually high interindividual variability and that this MSNA responsiveness is not associated with CVR (8, 29). Despite 55 published studies on MSNA and mental stress (5), there has not been a single test-retest study to determine reproducibility. Therefore, the objective of the present study was to determine whether MSNA reactivity to mental stress is reproducible.
using a standard test-retest study design. CPT trials were also performed as a control because MSNA reactivity to CPT has been shown to be reproducible (12). We hypothesized that MSNA reactivity to mental stress would be reproducible within a single laboratory visit and across laboratory visits separated by ~1 mo.

**METHODS**

**Participants.** Nine men and seven women (aged 21 ± 1 yr; height, 170 ± 2 cm; weight, 69 ± 3 kg; body mass index, 23 ± 1 kg/m²) were studied. All subjects were required to abstain from exercise, caffeine, and alcohol for 12 h prior to the experiment. Exclusion criteria included smoking, diabetes, cardiovascular disease, pregnancy, breastfeeding, and hormonal contraceptives. All female subjects reported menstrual cycles ranging between 26 and 30 days and were tested during their early follicular phase (i.e., 2–5 days after the onset of menstruation) due to the known impact of the menstrual cycle on sympathetic nerve activity (4). All subjects received an orientation session and provided written informed consent. The study was approved by the Michigan Technological University Institutional Review Board.

**Experimental design.** Figure 1 summarizes the experimental design for this study. Participants completed three experimental sessions over the course of two laboratory visits separated by ~1 mo (to ensure that all females were tested in their early follicular phase during both visits). On the first laboratory visit, the subjects underwent two experimental sessions; during the second laboratory visit, subjects underwent one experimental session. Each experimental session consisted of a mental stress trial via mental arithmetic and a CPT trial that are detailed below. The two experimental sessions during the first laboratory visit were separated by a 45-min rest interval.

For each visit, subjects arrived to the laboratory at 7:30 AM after an overnight fast. A standard, light breakfast that would not interfere with autonomic recordings was provided. Each visit began with three seated blood pressures after 5 min of quiet rest using an automated sphygmomanometer, followed by the evaluation of state anxiety using the State-Trait Anxiety Inventory questionnaire for adults. The subjects were then comfortably positioned on cushioned laboratory table in the supine position for the experimental sessions. Arterial blood pressure, MSNA, and heart rate (HR) were recorded during each trial (i.e., mental stress and CPT). Each mental stress trial consisted of 5 min of baseline, 5 min of mental arithmetic, and 5 min of recovery. Each CPT trial consisted of 5 min of baseline, 2 min of CPT, and 5 min of recovery. Mental stress trials always preceded CPT trials within each of the three experimental sessions.

**Mental stress.** Mental stress was experimentally evoked using standard mental arithmetic. Briefly, mental arithmetic involved the continuous subtraction of the number 6 or 7 from a two- or three-digit number. During the first visit, both numbers were used in a randomized order. During the second visit, one number (6 vs. 7) was randomly selected. During all mental stress trials, subjects answered verbally and were pressured by the investigators to answer as quickly as possible. A new number from which to subtract from was provided by an investigator every 5–10 s. Importantly, the same investigator administered the mental stress for all three trials with a subject. Subjects were asked to rate their perceived stress during the mental stress trial using a standard five-point scale: 0, not stressful; 1, somewhat stressful; 2, stressful; 3, very stressful; and 4, very, very stressful.

**Cold pressor test.** CPT was performed by submerging the subject’s hand in an ice-cold bucket with a temperature between 1°C and 3°C for 2 min.

**Measurements.** Three consecutive supine blood pressures were taken prior to each baseline using an automated sphygmonanometer (Omron HEM-907XL, Omron Healthcare, Kyoto, Japan). Beat-to-beat arterial blood pressure was recorded continuously during all baselines, stress tasks, and recoveries using a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands). Arterial blood pressure was expressed as systolic, diastolic, and mean arterial blood pressure (MAP). HR was recorded continuously via a three-lead electrocardiogram, and respiratory rate was measured continuously with the use of a pneumobelt.

Multifiber recordings of MSNA were obtained by inserting a tungsten microelectrode (FHC, Bowdoin, ME) into the peroneal nerve of the right leg, while a reference electrode was inserted 2–3 cm subcutaneously from the recording electrode. Both electrodes were connected to a differential preamplifier and then to an amplifier (total gain of 80,000), where the nerve signal was band-pass filtered (700-2,000 Hz) and integrated (time constant, 0.1). Quality recordings of MSNA were considered to be spontaneous, pulse-synchronous bursts that increased during end-expiratory apnea and remain unchanged during auditory stimulus or stroking of the skin.

**Data analysis.** Data were imported and analyzed using the WinCPRS software program (Absolute Aliens, Turku, Finland). R-waves were detected and marked in the time series. Bursts of MSNA were automatically detected on the basis of amplitude using a signal-to-noise ratio of 3:1, with a 0.5-s search window centered on a 1.3-s expected burst peak latency from the previous R-wave. Potential bursts were displayed and edited by one trained investigator (J. R. Carter). MSNA was expressed as burst frequency (bursts/min), burst incidence (bursts/100 heartbeats), and total MSNA (i.e., the sum of the normalized burst areas/min). During the first laboratory visit, we successfully obtained high-quality MSNA recordings throughout both experimental sessions in 13 of 16 subjects. Of those 13 subjects (9 men and 4 women), we successfully obtained high-quality MSNA recordings during experimental session 3 (i.e., one month later) in 11 (9 men and 2 women). Despite preexperiment coaching by the investigators, some subjects inadvertently moved or tensed their leg during mental stress. In some cases, this random leg movement and/or muscular contraction results in dislodgement of the tungsten electrode and/or a noisy signal. This is not uncommon during mental stress trials and has been previously documented (9, 32). Accordingly, we successfully obtained high-quality MSNA recordings throughout all stress trials [mental stress and CPT and all three experimental trials in 7 subjects (6 men and 1 woman)].

**Statistical analysis.** All data were analyzed statistically using commercial software (SPSS 22; IBM SPSS, Armonk, NY). Intraclass correlation (ICC) analyses were performed to determine the consistency (i.e., reproducibility) of the primary variables (MSNA, HR, and blood pressure) at three time points of the study: 1) baseline, 2) during each stress task (i.e., mental stress and CPT), and 3) recovery from stress task. The stress reactivity was calculated as the stress response (5-min mean value for mental stress or 2-min mean value for CPT)
minus the corresponding 5-min mean baseline. The recovery was calculated as the mean 5-min recovery minus the corresponding 5-min baseline. To probe for temporal patterns, we also performed ICC analyses on the minute-by-minute and peak MSNA reactivity during mental stress. The Cronbach’s alpha (α) value of the ICC (i.e., the coefficient of consistency) was reported with two-tailed P values.

In addition to the ICC analyses, Pearson correlations were used to examine the correlations within laboratory visit 1. This was done to increase sample size for the within-day comparisons, since we were unable to obtain all MSNA signals during experimental session 3. Results are expressed as means ± SE, Cronbach’s α coefficient, and correlation coefficient (r). Results were considered significant when P value was less than 0.05.

RESULTS

Baseline hemodynamics and MSNA. Table 1 depicts baseline hemodynamic (blood pressure and HR; n = 16) and MSNA (n = 11) during supine rest, and the respective ICC values for each variable. During the baselines preceding the three mental stress trials, arterial blood pressure, HR, and MSNA were remarkably consistent (Cronbach’s α ≥ 0.816; P ≤ 0.001). Likewise, baseline hemodynamics and MSNA before the three CPT trials were significantly consistent (Cronbach’s α ≥ 0.782; P ≤ 0.002). Importantly, consistencies in baseline MSNA (Cronbach’s α ≥ 0.825; P ≤ 0.005), arterial blood pressure (Cronbach’s α ≥ 0.644; P ≤ 0.060), and HR (Cronbach’s α ≥ 0.911; P ≤ 0.001) remained significant when we analyzed the subset of seven subjects with complete MSNA reactivity data during mental stress and CPT trials. Consistent with the ICC analyses, which take into account all three sessions, the Pearson correlation analyses of the 13 subjects with complete MSNA recordings within laboratory visit 1 (i.e., session 1 vs. 2) revealed highly significant resting MSNA correlations during the premental stress baselines (burst frequency: r = 0.961, P < 0.001; burst incidence: r = 0.957, P < 0.001) and pre-CPT baselines (burst frequency: r = 0.932, P < 0.001; burst incidence: r = 0.938, P < 0.001). Figure 2 provides representative neurogram tracings from one subject during all three mental stress trials. State anxiety levels were not different between laboratory visits.

Neural and cardiovascular reactivity. Figure 3 demonstrates that MSNA responsiveness to mental stress and CPT were consistent across the three experimental sessions. Figure 4 shows that arterial blood pressure and HR reactivity to mental stress and CPT were also significantly consistent across the three experimental sessions. Importantly, ICCs for arterial blood pressure and HR reactivity to mental stress (Cronbach’s α ≥ 0.757; P ≤ 0.018) and CPT (Cronbach’s α ≥ 0.809; P ≤ 0.007) remained highly significant when we analyzed the subset of seven subjects with complete MSNA reactivity data during mental stress and CPT. When changes in total MSNA burst during mental stress were quantified as absolute change, the values were not consistent [Δ2320 ± 919 vs. Δ2795 ± 1221 vs. Δ1969 ± 1326 arbitrary units (au); Cronbach’s α = 0.099, P = 0.411] across the three experimental sessions. However, when the changes in total MSNA during mental stress were calculated as a percent change, total MSNA reactivity to mental stress showed consistency across all experimental sessions (Δ49 ± 23 vs. Δ44 ± 18 vs. Δ36 ± 28%; Cronbach’s α = 0.740, P = 0.023). Similarly, total MSNA responsiveness to cold pressor test was not consistent when quantified as absolute change (Δ8259 ± 1389 vs. Δ12129 ± 2057 vs. Δ17223 ± 2886 au; Cronbach’s α = 0.223; P = 0.333), but were highly consistent across the three experimental sessions when quantified as a percent change (Δ272 ± 111 vs. Δ316 ± 85 vs. Δ263 ± 102%; Cronbach’s α = 0.903; P < 0.001). Consistent with ICC analyses, Pearson correlation analyses of the 13 subjects with complete MSNA recordings within laboratory visit 1 revealed significant correlation of MSNA reactivity during mental stress (burst frequency: r = 0.619, P = 0.012; burst incidence: r = 0.665, P = 0.006) and CPT (burst frequency: r = 0.789, P < 0.001; burst incidence: r = 0.649, P = 0.008).

ICC subanalyses of minute-by-minute and peak MSNA burst frequency reactivity to mental stress revealed highly significant reproducibility for peak (Cronbach’s α = 0.910; P < 0.001), minute 1 (Cronbach’s α = 0.895; P = 0.001), minute 4 (Cronbach’s α = 0.855; P = 0.002), and minute 5 (Cronbach’s α = 0.837; P = 0.004) reactivity. The MSNA burst frequency reactivity to mental stress was modestly consistent during minute 2 (Cronbach’s α = 0.621) and minute 3 (Cronbach’s α = 0.620), but the ICC analysis did not quite reach statistical significance (P = 0.072 and 0.073, respectively). Results were similar when minute-by-minute and peak

<table>
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<th>Session 3</th>
<th>Cronbach Alpha</th>
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<td>SAP, mmHg</td>
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<td>79 ± 2</td>
<td>78 ± 2</td>
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<td>HR, beats/min</td>
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<td>59 ± 3</td>
<td>59 ± 3</td>
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<td>MSNA, bursts/min</td>
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<td>113 ± 3</td>
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<td>MSNA, bursts/100 hb</td>
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<td>24 ± 4</td>
<td>30 ± 4</td>
<td>0.782</td>
<td>=0.002</td>
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Values are expressed as means ± SE; n = 16 for blood pressure and heart rate (HR); n = 11 for muscle sympathetic nerve activity (MSNA). Two-tailed P values.
MSNA reactivity to mental stress were calculated as burst incidence (not reported).

Recovery responses. Table 2 depicts the hemodynamic and MSNA recoveries from the mental stress and cold pressor trials, as well as respective ICCs, across the three experimental sessions. The recovery from mental stress was not consistent across the three experimental sessions for MSNA, arterial blood pressure, or HR. In contrast, MSNA and HR recoveries from CPT were significantly consistent across the three experimental sessions; MAP recovery from CPT was not consistent.

Changes in total MSNA during the mental stress recovery were quantified and were not consistent across the three experimental trials when expressed as either absolute change or percent change. In contrast, total MSNA recovery from CPT was significantly consistent when quantified as either absolute change or percent change. Consistent with ICC analyses, Pearson correlation analyses of the 13 subjects with complete MSNA recordings within laboratory visit 1 revealed significant correlation of MSNA recovery responses post-CPT (burst frequency: \( r = 0.574, P = 0.025 \); burst incidence: \( r = 0.560, P = 0.029 \)), but not postmental stress (burst frequency: \( r = 0.225, P = 0.229 \); burst incidence: \( r = 0.363, P = 0.111 \)).
DISCUSSION

The present study investigated the reliability of neural and cardiovascular responses to mental stress and CPT across three experimental sessions. Our results demonstrate that measures of neural and cardiovascular reactivity to mental stress and CPT are consistent across sessions, both within a single laboratory visit and across laboratory visits separated by at least 1 mo. Given the wide interindividual variability of MSNA responsiveness to mental stress, establishing intraindividual reproducibility is essential to past, present, and future work aimed at examining potential associations between the sympathoexcitatory responsiveness to mental stress and cardiovascular risk.

Consistent with previous studies (13, 14, 28, 31), we found strong reproducibility of MSNA during supine rest. Consistency of resting MSNA is a hallmark of microneurography and reinforces the value of this unique methodology within human autonomic research. However, what often remains overlooked in the field is the consistency of the MSNA responsiveness to a given laboratory stressor. To our knowledge, only two studies have adequately detailed MSNA test-retest reproducibility during an acute, sympathoexcitatory laboratory stress. Kimmerly et al. (19) and Fagius et al. (12) reported that MSNA reactivity was reproducible during head-up tilt and CPT, respectively. Two other studies performed subanalyses to examine MSNA reproducibility during CPT (26) and mental stress (18), but neither performed any correlation analyses. Instead, both defined reproducibility strictly by the lack of difference between mean values of MSNA reactivity between test sessions (18, 26). This is problematic because it is possible to obtain similar mean values despite wide intraindividual variability. Indeed, the data reported by Shoebel et al. (26) illustrates this concept. The authors reported remarkably consistent mean values of resting MSNA (21.9 ± 2.1 vs. 20.3 ± 2.5 bursts/min) between two sessions separated by 3 wk to 14 mo (26). However, upon closer inspection of the data, we noted an unusually high intraindividual variability (26), and a correlation analysis on the raw data reveals that the two resting MSNA sessions were not correlated ($r = 0.33, P = 0.21$). This is unusual because as previously noted, most studies (including data presented in this article) report strong reproducibility across sessions with resting MSNA. In summary, similar mean values across sessions are not sufficient to demonstrate reproducibility; thus, the present study is the first to examine the reproducibility of MSNA responsiveness to mental stress.

We found strong reproducibility of both MSNA and blood pressure reactivity to mental stress within a single laboratory visit, as well as across sessions separated by ~1 mo. Moreover, when we examined the mental stress MSNA reactivity for temporal patterns (i.e., minute-by-minute or peak responsiveness), we observed strong reproducibility. The consistency between two laboratory visits separated by 1 mo is particularly important because longitudinal studies have focused on interventions that might lower MSNA reactivity to mental stress (9, 24). Our mental stress findings are bolstered by the significant consistency observed during CPT in the same subjects, which is consistent with Fagius et al. (12) and was included in the present study as a control. A study looking at the reproducibility of SSNA (skin sympathetic nerve activity) reactivity to mental stress within a day and a week later came to a similar conclusion (22).

Fig. 4. Changes in mean arterial pressure (MAP) and heart rate (HR) during mental stress and CPT across the three experimental sessions ($n = 16$). The solid line represents the median, the solid circle represents the mean, and open circles represent outliers to the mean (included in ICC analysis). MAP reactivity and HR reactivity remained consistent across the mental stress and CPT trials.
The rationale for studying MSNA reactivity to mental stress has never been clearly defined. As outlined by Carter and Goldstein (5), justification has primarily focused on MSNA responsiveness as a potential mechanistic link to the CVR hypothesis and catastrophic cardiovascular events associated with stress, such as myocardial infarction, stroke, and sudden death. There is actually very little evidence to support such claims, particularly given that recent work has suggested the lack of direct association between MSNA and blood pressure reactivity to mental stress (8). However, Noll et al. (23) reported that individuals with a family history of hypertension (FHH+) demonstrated a significant increase of MSNA during mental stress, while individuals with no family history of hypertension (FHH−) demonstrated no change in MSNA during mental stress. Unfortunately, the study (23) was limited to 16 young, healthy medical students (n = 8 FHH+ vs. n = 8 FHH−; sex ratios not reported), thus limiting generalizability. Nevertheless, Noll et al. (23) suggests that MSNA reactivity may be associated with FHH, but this reported association is strongly dependent upon intraindividual consistency of MSNA reactivity to mental stress. Therefore, our findings of a reproducible MSNA reactivity to mental stress both within and across laboratory sessions is an important step in advancing the work of Noll et al. (23), as well as providing a foundation for future research that might elucidate whether MSNA reactivity might have similar, or perhaps even superior, predictive value compared with the CVR hypothesis.

In addition to the mental stress work of Noll et al. (23), there have been a number of other studies that have examined the association between FHH and MSNA reactivity during CPT. Calhoun and Mutinga (2) reported that MSNA reactivity to CPT was greater in normotensive African-Americans with FHH+ compared with African-Americans with FHH−. Greaney et al. (15) demonstrated greater MSNA reactivity to CPT, handgrip exercise, and postexercise muscle ischemia in young women with FHH+ compared with women with FHH−. In contrast, Lambert and Schlaich (20) reported that MSNA reactivity to CPT was actually heightened in FHH− compared with FHH+ subjects. So while there are several lines of evidence beginning to converge toward what we would like to propose as a “MSNA reactivity hypothesis,” such a concept is far from definitive (2, 15, 20, 23). To advance such a hypothesis will require the same rigorous scientific scrutiny and testing that the CVR hypothesis has undergone, and the MSNA reactivity reproducibility observed in this study during both mental stress and CPT is a critical first step.

Finally, it has been our observation that while the MSNA responsiveness to mental stress is widely variable, the MSNA recovery response is fairly consistent (5–7). This is largely believed to be related, in part, to the baroreflex being overridden and/or reset during mental stress, and the related sympathoexcitation as blood pressure begins to fall during recovery from mental stress. Some studies have focused on the MSNA recovery response because this stress recovery period is often associated with high sympathetic outflow during bradycardia, which has been suggested as a potential trigger for myocardial infarction (6, 11, 25). Our present findings demonstrate that MSNA recovery responses to mental stress were not reproducible across the three laboratory sessions. Thus, the usefulness of examining MSNA recovery responses to mental stress must be called into question.

### Perspectives and Significance

The present study demonstrates that MSNA and hemodynamic responses to mental stress and CPT are reproducible within individuals. Given the emerging evidence that MSNA reactivity might be associated with FHH, these findings are timely and critical to advancing research related to MSNA reactivity and cardiovascular health. Any potential predictive utility of MSNA reactivity to mental stress (or other stressors) critically depends upon future prospective studies, and might well depend upon relative MSNA reactivity. For example, is it possible that MSNA reactivity to mental stress could have predictive value in MSNA responders, but have little to no predictive utility in MSNA nonresponders? Does MSNA reactivity offer any advantage over the cardiovascular reactivity hypothesis, which is well established and easier to quantify?
Longitudinal studies are clearly needed to answer such questions and potentially advance the idea that MSNA reactivity might help predict cardiovascular risk.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: I.T.F. and J.R.C. analyzed data; I.T.F. and J.R.C. interpreted results of experiments; I.T.F. prepared figures; I.T.F. and J.R.C. edited and revised the manuscript; I.T.F. and J.R.C. approved final version of manuscript; J.R.C. conception and design of research; J.R.C. performed experiments.

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