NO modulates norepinephrine release in human skeletal muscle: implications for neural preconditioning

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Received 10 July 2000; accepted in final form 10 January 2001

Costa, Fernando, Niels J. Christensen, Ginnie Farley, and Italo Biaggioni. NO modulates norepinephrine release in human skeletal muscle: implications for neural preconditioning. Am J Physiol Regulatory Integrative Comp Physiol 280: R1494–R1498, 2001.—The purpose of this study was to estimate muscle interstitial norepinephrine (NE) levels during exercise and to determine whether nitric oxide (NO) modulates NE release in the skeletal muscle in humans. We measured interstitial dialysate concentrations of NE with two microdialysis probes inserted into the forearm. Probes were perfused with saline and the NO synthesis inhibitor N⁵-monomethyl-L-arginine (L-NMMA), respectively. Dialysate samples were collected during two sequential 20-min intense dynamic handgrip periods, preceded by 40-min baseline periods. On a different day, forearm ischemia was performed instead of the first exercise period. Exercise increased dialysate NE from 172 ± 42 to 270 ± 45 pg/ml (83% increase, P < 0.02, n = 6). Probes perfused with L-NMMA had a 136 ± 39% greater dialysate NE compared with probes perfused with saline (225 ± 25 vs. 125 ± 25 pg/ml, P < 0.001, n = 9). The exercise-induced increase in NE (125 ± 52%) was attenuated if preceded by exercise (34 ± 34%) or ischemia (40 ± 36%; P = 0.06, n = 6), suggesting a neural preconditioning effect. This attenuation was not observed in probes perfused with L-NMMA. We propose that NO modulates NE release in skeletal muscle, that ischemic exercise increases muscle interstitial NE, and that this increase can be attenuated by a preconditioning effect mediated in part by NO.

Norepinephrine (NE) is released mainly from the sympathetic nerves, acts as a main neurotransmitter of the sympathetic nervous system, and is used as a marker of overall sympathetic nervous system activity. Sympathetic activity, and therefore NE release, is tightly controlled by redundant reflex mechanisms. In addition, NE release is also regulated locally, mostly through presynaptic receptors that increase (e.g., β2-adrenoreceptors) or inhibit (e.g., α2-adrenoreceptors) vesicular release. One potential local modulator of NE release is nitric oxide (NO). Animal studies suggest that release of NE is regulated, at least in part, by NO-dependent mechanisms, as shown in the heart (15) and exercising skeletal muscle (18). Local modulation of sympathetic activity is particularly important during ischemia. NE is released during ischemia in metabolically active tissues by vesicular and nonvesicular release and may have potential deleterious effects. NE, e.g., may play a role in the genesis of arrhythmias during myocardial ischemia. It would be important to determine, therefore, if NO restrains NE release during ischemia.

Another process that modulates NE release during ischemia is preconditioning. Preconditioning is defined as the process by which brief periods of ischemia limit infarct size from subsequent sustained ischemia (10). Reduced NE release is observed in preconditioned tissues, and this may contribute to a reduction in energy consumption and cell death (9, 17). Furthermore, there is evidence suggesting that NO plays a protective role during ischemia and preconditioning (11, 13).

The goals of this study were to 1) estimate the increase in interstitial NE levels in skeletal muscle in humans induced locally by intense (ischemic) exercise, 2) to determine if ischemic exercise will induce a “preconditioning” effect, as evidenced by a blunted increase in interstitial NE during a subsequent exercise period, and 3) to determine if NE release during these conditions is modulated by NO. For this purpose, we estimated interstitial NE at rest and during intense dynamic exercise using microdialysis probes introduced into the exercising muscle. Probes were perfused with saline or N⁵-monomethyl-L-arginine (L-NMMA) to determine the effect of inhibition of NO synthesis in the tissues surrounding the probe.

MATERIALS AND METHODS

Subjects
We studied nine healthy volunteers, 19–41 yr of age. Volunteers gave written informed consent. The protocol was approved by the Institutional Review Board.

Study Protocol
Subjects were studied fasted and in the supine position. Two microdialysis probes (CMA/20, 10 × 0.5 mm in size with the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
a 20,000 molecular weight cutoff; CMA, Acton, MA) were introduced into the flexor digitorium superficialis muscle of the nondominant forearm. The characteristics of the probe (2) and the insertion technique (4) have been detailed previously. One of the probes was perfused with saline and the other with L-NMMA (10 mg/ml) at 2 μl/min (“perfusate”). The effluent (“dialysate”) was collected from both probes simultaneously to obtain 40-μl samples over 20-min periods.

In six subjects, after instrumentation and 1-h equilibration period, two consecutive 20-min dialysate samples were collected from each probe to measure baseline NE levels. Subjects were then asked to perform intermittent dynamic handgrips using the same arm where the probes were introduced. A dynamometer (Lafayette Instrument, Lafayette, IN) was used to produce 5-s contractions every 10 s at 50% of maximal voluntary contraction for 20 min. One 20-min dialysate sample was collected from each probe during exercise. The dialysate collection period was shifted by 1 min in relation to exercise to account for the lag time produced by the length of the collecting tubing. Two 20-min recovery samples were collected immediately after exercise. The exercise and recovery periods were then repeated, and dialysate samples for NE determinations were collected as described above.

In six subjects, the protocol described above was performed in a different study day, at least 7 days apart, but instead of the first exercise period, forearm ischemia was induced by inflating a proximal pneumatic cuff to 50 mmHg above the systolic blood pressure for 20 min. The rest of the protocol remained the same. Three of the subjects participated in both protocols.

Methods

In vitro calibration to estimate NE recovery. An in vitro calibration was performed at the end of each study to estimate the fraction of NE recovered across the microdialysis membrane. The probes removed from the muscle were placed in a solution containing 1,000 pg/ml NE, EGTA, reduced glutathione, and 6% albumin. Probes were perfused at 2 μl/min, and dialysates were collected in two 20-min fractions for each probe. Samples were also collected directly from the NE solution and processed and analyzed as the dialysates. The relative recovery of the probe was calculated as the fraction of NE recovered across the microdialysis membrane divided by the concentration of NE measured in the calibration solution.

Analytic methods and drugs. Samples for NE were analyzed by a radioenzymatic assay as outlined previously (7), with some modifications. Dialysate samples were collected in ice-cooled tubes containing EGTA and reduced glutathione. In addition, 20 μl of 6% albumin were added, and the samples were stored at −80°C until analyzed. The samples were freeze-dried before analysis, thereafter 100 μl of 6% albumin were added. Samples were precipitated with equal volume of 6 N perchloric acid, and 100 μl of the supernatant were used. Appropriate blanks and standards were included. The 3H-labeled product of NE, normetanephrine, was isolated by high-pressure liquid chromatography, oxidized to vanillin, and counted by liquid scintillation spectrometry. The sensitivity of the assay was 0.5 pg/assay calculated as three times the value of the standard deviation of the analytic blank.

L-NMMA was purchased from Calbiochem-Novabiochem and prepared for human use in Vanderbilt's Investigational Drug Pharmacy at a concentration of 10 mg/ml in saline. ±NE HCl was purchased from RBI (Natick, MA) and dissolved in 6% albumin at a concentration of 1,000 pg/ml. EGTA and glutathione were purchased from Sigma Chemical.

Statistics

Results are expressed as means ± SE. Statistical analysis was performed using t-test for single comparisons and ANOVA for multiple comparisons.

RESULTS

Effects of Exercise and L-NMMA on NE Concentrations

Intense dynamic handgrip increased muscle dialysate NE concentrations from 172.5 ± 42.5 to 270 ± 45 pg/ml (Fig. 1A, control vs. exercise 1, P < 0.02 by paired t-test, n = 6). After correction from recovery from the microdialysis probe, these dialysate levels correspond to estimated interstitial NE concentrations of 543 ± 31 and 853 ± 145 pm/ml, respectively. Probes perfused with L-NMMA had a 136 ± 39% greater dialysate NE concentration at rest, compared with parallel probes perfused with saline in the same subjects (225 ± 25 vs. 125 ± 25 pg/ml, P < 0.001 by paired t-test, n = 9). Dialysate NE levels were higher in probes perfused

![Graph](http://ajpregu.physiology.org/)
with l-NMMA during all study periods (Fig. 1A, \(P < 0.01\) for control vs. l-NMMA curves by ANOVA, \(n = 6\)).

The relative change in dialysate NE produced by exercise from the preceding resting period is shown in Fig. 1B. In probes perfused with saline (control), the first exercise period increased dialysate NE from 172 ± 42 to 270 ± 46 pg/ml (83 ± 30% increase). This increase was significantly blunted during the second exercise period (from 200 ± 130 to 213 ± 36 pg/ml, 4 ± 12% increase, \(P = 0.02\) by paired \(t\)-test compared with the increase observed during the first exercise period). In parallel probes perfused with l-NMMA, the first exercise period increased dialysate NE from 228 ± 46 to 282 ± 38 pg/ml (34 ± 15% increase), and a similar increase was observed in the second exercise period (from 242 ± 46 to 297 ± 53 pg/ml, 26 ± 7% increase, \(P = \) not significant by paired \(t\)-test compared with the increase observed during the 1st exercise period).

**NE Concentrations During Exercise Preceded by Exercise or Ischemia**

In a different set of experiments, dynamic exercise produced a 125 ± 52% increase in dialysate NE concentrations. This increase was substantially lower if exercise was preceded by dynamic handgrip (a 34 ± 34% increase) or forearm ischemia (a 40 ± 36% increase) (Fig. 2, \(P = 0.06\) by ANOVA, \(n = 6\)).

**Effect of Ischemia on NE Concentrations**

Forearm ischemia did not produce significant changes in dialysate NE concentrations either in probes perfused with saline (from 65 ± 9 to 77 ± 19 pg/ml, an 8 ± 14% increase) or l-NMMA (from 212 ± 39 to 136 ± 22 pg/ml, a −23 ± 17% change).

**In Vitro NE Recovery**

Average recovery of NE for the probes perfused with saline and l-NMMA were 31.7 ± 3.9 and 30.9 ± 3.5%, respectively.

**DISCUSSION**

**Effects of Exercise on NE Concentrations**

A new finding of this study is that intense dynamic exercise increased interstitial NE concentrations. The source of this interstitial NE is likely to be vesicular release from sympathetic nerve terminals. However, because of the intensity of the stimulus (most subjects reached muscle fatigue toward the end of the exercise period), it is possible that nonvesicular release also occurs, as seen in metabolically active tissues during ischemia (14). Previous studies have shown increase in venous plasma NE during intense dynamic exercise (5, 6), and our results confirm, for the first time, an increase in muscle interstitial NE during dynamic exercise in humans.

**Modulatory Effect of NO on NE Release**

Inhibition of NO synthesis with l-NMMA resulted in increased interstitial NE levels at rest, implying that endogenous NO tonically inhibits NE release in vivo in skeletal muscle. Perfusion of l-NMMA could induce local vasoconstriction in tissues surrounding the microdialysis probe. It could be argued, therefore, that the increase in interstitial NE was not due to increased NE release but to a decrease in clearance due to reduced blood flow. We do not believe this to be the case, because complete circulatory arrest of the forearm had no effect on NE levels. It is possible, however, that the large vessel occlusion induced by our paradigm may not reproduce the effects of microcirculatory vasoconstriction induced by l-NMMA. Higher levels of NE during l-NMMA perfusion could also be due to a decrease in neuronal uptake of NE. The observation that NO synthase inhibitors still enhance NE release in the presence of the NE uptake inhibitors (15) argues against this possibility.

**Role of NE and NO in Preconditioning**

There was an attenuated increase in dialysate NE in response to dynamic exercise when preceded by either exercise or ischemia, a phenomenon resembling a neural “preconditioning” effect. NE has been suggested to contribute to preconditioning (1) and, conversely, less NE is released during ischemia in preconditioned myocardium (16). If present, neural preconditioning can contribute to protection against ischemia, as attenuated NE release would decrease myocardial workload and arrhythmogenesis. It could be argued that the attenuation in dialysate NE we found during the second exercise period was simply due to depletion of NE stores at the sampling site induced by the preceding exercise. This, however, cannot be the case when exercise is preceded by ischemia, inasmuch as the latter does not induce an increase in dialysate NE.
The increase in resting dialysate NE produced by L-NMMA confounds the interpretation of the role of NO in mediating preconditioning. It is noteworthy, however, that in probes perfused with L-NMMA, the increase in dialysate NE was similar during both exercise periods, suggesting that a preconditioning effect did not occur. This was true whether results were expressed as absolute values or as relative changes from preceding resting values (see Fig. 1). We speculate, therefore, that NO contributes to this neural preconditioning effect, as has already been suggested by others (8, 12).

Adenosine is considered an important mediator of preconditioning and could certainly contribute to the reduced dialysate NE during the second exercise period. Inhibition of NO synthesis with L-NMMA, if anything, may increase adenosine release (19). This would result in a greater preconditioning effect during L-NMMA, rather than the lack of effect observed in our studies. Adenosine mechanisms, therefore, cannot explain the loss of preconditioning during L-NMMA, supporting the involvement of NO in this process.

Finally, the role of NO in the modulation of NE release during ischemic exercise is less certain. If NO provided a negative feedback mechanism for NE release, then inhibition of NO synthesis should result in potentiation of NE release during exercise. This clearly was not the case in our study, as NE reached similar levels during exercise in muscle perfused with saline or L-NMMA. Nevertheless, it could be argued that relative changes in NE from the preceding baseline were lower during L-NMMA perfusion and that further NE increases were not possible because of a “ceiling” effect. Also, it is possible that the nonvesicular component of NE release is more prominent during ischemia and is not subject to NO modulation. Therefore, we are reluctant to completely exclude a role of NO in inhibiting NE release during ischemic exercise.

Limitations

It is important to emphasize that dialysate values represent estimates rather than absolute interstitial NE concentrations. We are confident, however, on the directional changes produced by our interventions and the differences detected between parallel probes perfused with saline or L-NMMA. Also, we did not measure the actual rate of NE release, and, as discussed previously, interstitial NE is also influenced by changes in NE clearance. Finally, our purpose was to study local mechanisms that modulate NE release. However, intense dynamic exercise produces systemic reflex sympathetic activation (3) and we cannot account for the potential influence this may have on the exercising muscle.

Perspectives

The main conclusions from this study are that intense exercise increases interstitial NE concentrations and that NO modulates the release of NE in the skeletal muscle in humans. Our results also suggest that ischemia and intense (ischemic) exercise have a neural preconditioning effect on subsequent exercise in humans, expressed as an attenuated increase in muscle interstitial NE. This phenomenon was not observed when NO synthesis was blocked. NO, therefore, may contribute to protective mechanisms engaged during ischemia. These findings may be of particular relevance in conditions associated with impaired endothelial and NO functions, such as atherosclerosis, hypercholesterolemia, hypertension, and diabetes. These patients may lose the restraining effect of NO mechanisms and the protection rendered by neural preconditioning. This may lead to greater NE release at rest and during ischemia, with the deleterious consequences this entails.

REFERENCES