Both physical fitness and acute exercise regulate nitric oxide formation in healthy humans

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J üngersten, Lennart, Anneli Ambring, Björn Wall, and Ake Wennmalm. Both physical fitness and acute exercise regulate nitric oxide formation in healthy humans. J. Appl. Physiol. 82(3): 760–764, 1997.—We analyzed nitrate, a major stable end product of nitric oxide (NO) metabolism in vivo in plasma and urine from groups of healthy subjects with different working capacities. Resting plasma nitrate was higher in athletic subjects than in nonathletic controls [45 ± 2 vs. 34 ± 2 (SE) µM; P < 0.01]. In other subjects, the resting plasma nitrate level (r = 0.53; P < 0.01) and the urinary excretion of nitrate at rest (r = 0.46; P < 0.01) correlated to the subjects’ peak work rates, as determined by bicycle ergometry. Two hours of physical exercise elevated plasma nitrate by 18 ± 4 (P < 0.01) and 16 ± 6% (P < 0.01), respectively, in athletes and nonathletes, compared with resting nitrate before exercise. We conclude that physical fitness and formation of NO at rest are positively linked to each other. Furthermore, a single session of exercise elicits an acute elevation of NO formation. The observed positive relation between physical exercise and NO formation may help to explain the beneficial effects of physical exercise on cardiovascular health.

nitric oxide synthase; nitrate; vascular system; shear stress

ENDOTHELIAL NITRIC OXIDE (NO) is thought to play important roles in the local regulation of platelet-vessel wall interaction and in vascular resistance and growth (12, 13, 15, 16). On the basis of these effects, NO has been proposed to have antithrombotic, antiatherosclerotic, and antihypertensive properties.

Vascular formation of NO is directly facilitated by increased shear (4, 11). During a session of physical exercise, cardiac output is augmented and blood is redistributed to the exercising muscles. The exercise-induced augmentation of blood flow elicits an increase in shear (22), thereby providing a possible coupling between exercise and endogenous NO formation. Such a coupling would above all be limited to the exercise period and the immediate postexercise recovery.

Furthermore, regular physical exercise has been linked to an upregulated expression of the endothelial NO synthase gene in isolated specimens of vascular tissue (18, 23). Whether this mechanism, if occurring also in vivo, is the one responsible for the direct increase in NO formation developing during a single exercise session or represents another, more lasting, response of the endothelium to repeated sessions of physical activity is not known.

An effect of repeated exercise leading to an increase in the formation of NO that is maintained also between the exercise sessions might be important in explaining the beneficial effects of physical fitness on cardiovascular health (1, 3, 5, 17). Hence, a study seemed warranted to examine the possibility that subjects who exercise regularly have a higher resting level of NO formation. For this purpose, we determined the relation between peak work rate and resting levels of nitrate, a major stable metabolite of NO, in plasma and urine from subjects with different levels of physical fitness. In addition, we measured how a single session of physical exercise directly affected the levels of nitrate in plasma and urine in some of these subjects.

METHODS

Study Population

Three groups of healthy, nonsmoking volunteers were studied. Group 1 included athletic subjects (A) and their matched nonathletic controls (C). Group 2 included subjects selected with respect to physical capacity (NS). Group 3 included nonathletic subjects (NA). None of the participants was taking medication on a regular basis at the time of the study. All participants were instructed to maintain a nitrite/nitrate-restricted diet and to refrain from heavy physical exercise during 48 h before sampling for nitrate in plasma and urine. Two subjects in the NS group were excluded; their plasma nitrate levels (84 and 93 µM) clearly indicated that they had not followed the dietary instructions. All protocols were approved by the local human investigations committee, and all subjects gave informed consent.

Protocols

Group 1. Twelve A subjects, male long-distance runners (age 27–39 yr, weight 57–84 kg, height 163–189 cm) were recruited from a sports club. Matched C subjects were 12 males laboratory staff members and students (age 21–43 yr, weight 62–80 kg, height 176–188 cm). None of the C subjects exercised regularly. Venous blood samples from a cubital vein were taken from C at rest and from A at rest and immediately after a training session (135 min of jogging a distance of ~25 km).

Group 2. Twenty-four NS subjects (age 20–34 yr, weight 52–88 kg, height 161–196 cm; 16 men, 8 women), nonselected with respect to physical capacity, were recruited from hospital staff members and students. To determine the individual’s peak work rate, the subjects performed a graded exercise test on a bicycle ergometer. The initial work intensity was 70 W and was increased by 20 W/min until exhaustion. Blood pressure (standard cuff technique) and heart rate (from electrocardiogram recordings) were monitored continuously. A venous blood sample from a cubital vein was taken from C at rest and from A at rest and immediately after the exercise test (10 min after exercise).

Group 3. Fifteen NA subjects (age 20–31 yr, weight 55–88 kg, height 164–192 cm; 11 men, 4 women) were recruited among students. None of the participants exercised regularly. Venous blood samples from a cubital vein were taken at the end of each of three consecutive 120-min periods: after a period of rest, after bicycling a distance of 35 km (exercise), and after postexercise rest, respectively. Urine samples were also collected in separate portions during these periods.
Analysis of Nitrate in Plasma and Urine

Blood samples (5 ml) were collected in heparin tubes. Plasma was immediately separated by centrifugation for 7 min at 1,500 g. Plasma and urine samples were stored at −20°C until they were analyzed.

Nitrate in plasma and urine samples was analyzed with a gas chromatography-mass spectrometry method (21) that has been further developed in our laboratory. A known amount of plasma or urine sample was added with a known amount of K\(^{15}\)NO\(_3\) (Sigma Chemical, St. Louis, MO) as internal standard. Endogenous and \(^{15}\)N nitrate in the plasma and urine samples was converted to nitrobenzene by shaking a 50- or 20-µl portion, respectively, for 30 min with 750 or 1,000 µl of benzene and 110 or 40 µl trifluoromethanesulfonic acid (TFMS; Sigma Chemical). Before addition of the plasma or urine samples, the benzene- and TFMS-containing tubes were kept at −20°C. The organic phase was separated and washed with 0.5 M NaCO\(_3\). A 1-µl portion was injected into a Varian 3400 gas chromatograph equipped with an XTi-5 capillary column operated with a temperature program (60–260°C). It was connected to a Varian Saturn II mass spectrometer operated in the chemical ionization mode. Methane was used as the reactant gas, and mass equivalent (meq) 124 for endogenous nitrate and meq 125 for the \(^{15}\)N-labeled internal standard was selectively monitored. The detection limit for endogenous nitrate was 0.1 µM, and the variation coefficient was <5%.

Data Analysis and Statistics

The results are expressed as means ± SE. Statistical analyses were performed with Student’s t-test for paired and unpaired data, as appropriate. Correlation analyses were performed by using simple regression. A P value <0.05 was considered significant.

RESULTS

Group 1

The plasma nitrate level at rest in C was 34 ± 2 µM. The plasma level at rest before exercise in A was 45 ± 2 µM, which is significantly higher (P < 0.01). Immediately after exercise, plasma nitrate in A had increased to 56 ± 3 µM (P < 0.01 compared with the level at rest before exercise; Fig. 1).

Group 2

In NS, the mean peak work rate was 286 ± 9 W (range 180–350 W). Plasma nitrate was 32 ± 1 µM, and the urinary excretion rate of nitrate was 1,258 ± 124 µmol/24 h. In NS, both the basal plasma nitrate level (r = 0.53, P < 0.01) and the urinary excretion rate of nitrate (r = 0.46, P < 0.01) correlated significantly with the peak work rate (Fig. 2).

Group 3

Plasma nitrate in NA at rest was 32 ± 3 µM. It increased (P < 0.05) to reach a level of 37 ± 4 µM immediately after exercise and to 42 ± 6 µM (P < 0.01) 120 min after exercise (Fig. 1). The urine excretion rate of nitrate at rest before exercise was 124 ± 15 µmol/120 min. During exercise, nitrate excretion rate was 105 ± 12 µmol/120 min (not significant compared with before exercise), and at rest after exercise it was 146 ± 16 µmol/120 min (not significant compared with before exercise).

DISCUSSION

In the present study, the resting level of plasma nitrate was higher in A subjects than in matched NA controls. In other NS subjects, both the resting plasma nitrate level and the urinary excretion of nitrate at rest correlated with the subjects’ working capacity. Furthermore, a period of physical exercise increased the plasma nitrate level both in athletic and nonathletic subjects.

The fate of endogenous NO reaching the blood has previously been studied in animals as well as in humans. In rats exposed to inhalation of \(^{15}\)NO, a metabolic sequence comprising reaction with hemoglobin (Hb) to NOHb and subsequent conversion to nitrite and nitrate was assumed to take place (26). About <55% of the inhaled \(^{15}\)N was excreted in the urine, 75% as \(^{15}\)NO\(_3\) and 24% as urea (25). Similar figures were obtained in our laboratory after exposure of humans to inhalation of \(^{15}\)NO; \(^{15}\)N-labeled nitrate appeared as a major plasma metabolite, and \(^{15}\)NO\(_3\) in urine covered >70% of the amount of isotope inhaled (14).

However, NO liberated into (or formed in) the blood may also react with specific protein thiols to form S-nitrosothiols, of which a major part is made up by S-nitrosoproteins (19). Although appearing in human plasma only at a moderate concentration (~7 µM), such S-nitrosothiols have been proposed to serve as a reservoir for free NO, thereby having an important physiological role in maintenance of vascular tone (19). More recently, a cycle involving S-nitrosylation of Hb in the lungs has been proposed as a characteristic step in NO-mediated control of both blood pressure and oxygen.
The total plasma concentration of S-nitrosylated and Fe(II)-nitrosylated Hb in that study was 1 µM. Despite the low levels, significant effects were demonstrated with these NO-Hb adducts. Hence, it appears that NO or its metabolites materialize in plasma in several biologically active or inactive forms. Apparently, their respective plasma concentrations do not reflect their biological activity. It may be assumed that these NO adducts or metabolites exist in the blood in some equilibrium with each other.

On the basis of the observation that nitrate appears as a major and stable metabolite of NO in plasma and urine (14), we and others (2, 7, 10, 20, 24) have adopted nitrate as an index of the overall formation of NO in vivo. Such an adoption has some limitations that must be taken into account. First, the nitrate present in the blood may derive from exogenous sources such as nitrate- or nitrite-containing food (e.g., vegetables, roots, ham). Dietary restriction is therefore an absolute requirement if plasma or urine nitrate is to be used as an index of NO. Based on the calculated half-life ($t_{1/2}$) for nitrate in plasma ($t_{1/2} = 451$ min; 8), diet restriction must be maintained for at least 48 h, (i.e., $>6 \times t_{1/2}$) to eliminate the confounding influence of exogenous nitrate/nitrite. Consequently, a nitrate/nitrite-restricted diet was maintained for 48 h before sampling of blood and urine in the present study.

Second, nitrate is present in the body in an amount of $\sim 600$ µmol [plasma level, 30 µM; distribution volume, 20 liters (8)], whereas NO in S-nitrosylated proteins appears in an amount of $\sim 20$ µmol [plasma level, 7 µM; distribution volume, 3 liters (19)] and NO in S-nitrosylated Hb in a total of $<6$ µmol [plasma level $<1$ µM; distribution volume, 5–6 liters (6)]. The different NO metabolites and adducts consequently appear in pools of different sizes, and it may be assumed that these pools also differ in turnover rate. The use of plasma nitrate as an index of the endogenous NO formation has the advantage that it probably reflects a large fraction of all NO metabolites or adducts in the body. The limitation may be that plasma nitrate represents biologically inactive NO and that this pool possibly has a slower turnover than the S-nitrosylated protein and Hb pools.

With these considerations in mind, the presently observed higher resting plasma nitrate levels in A compared with C seem to permit the interpretation that the resting formation of NO was higher in A than in C. However, our data do not allow any conclusions as to whether such an elevated NO formation at rest would be localized to the vascular endothelium or to any other NO-forming cells or tissues. Furthermore, the data do not indicate whether regular exercise would enhance NO formation or, alternatively, whether an enhanced NO formation would yield a higher physical capacity.

The concept of an association between physical fitness and resting NO formation was further supported by the direct observation in the NS subjects, i.e., by the observed correlation between the peak work rate on the one hand and the resting plasma nitrate or the urine excretion of nitrate at rest on the other. To our knowledge, such a correlation has not been reported previously. The association strengthens our finding of an increased resting plasma nitrate in A compared with sedentary C. It does, however, not explain the etiological basis for the link between increased endogenous NO formation and high physical capacity.

In addition to assessing the resting plasma nitrate levels in different groups of subjects, we also studied the acute effect of a single exercise session on plasma nitrate in some of them. The plasma nitrate level in A as well as in NA was elevated immediately after compared with before exercise. Such a transient elevation of the nitrate level after exercise has been reported by others as well (2, 10). When evaluating short-lasting changes in plasma levels of various compounds, their renal excretion must be taken into account; a temporary increase or decrease in excretion may lower or
elevate, respectively, the plasma level independently of any change in formation rate. To assess the possibility that the present exercise-induced increase in plasma nitrate was based on lowered excretion, we determined nitrate excretion before, during, and after the exercise session. The excretion of nitrate at rest before exercise was \( \sim 62 \) µmol/h (renal excretion, 124 µmol/120 min). The average [i.e., \((105 + 146)\div2 = 126 \) µmol/120 min] excretion of nitrate in NA during the pooled periods during (105 µmol/120 min) and after (146 µmol/120 min) exercise did not differ from that during the period before exercise. Hence, the observed increased plasma nitrate after exercise was evidently not based on a decreased excretion of nitrate but rather seemed to be explained by an enhanced formation rate of NO.

The difference between the plasma nitrate levels before and after exercise in NA, i.e., \( 10 \) µM, may be assumed to reflect the additional formation of NO that occurred during the 4 h elapsed between these blood samplings. The distribution volume for nitrate is \( \sim 30\% \) of the body weight (8), in the present subjects corresponding to \( \sim 20 \) liters. Assuming complete distribution of NO converted to nitrate in these 20 liters of distribution volume, the additional amount of nitrate thus retained may be estimated as \( 10 \) µM \( \times 20 \) liters = 200 µmol. Hence, an additional 50 µmol/h were formed during the 4 h after onset of exercise in NA. Because the basal formation of NO was estimated to be \( \sim 62 \) µmol/h (see above), the data imply that the endogenous nitrate formation was increased by \( \sim 50\% \) above the basal level during and shortly after exercise.

On the basis of previous observations in animals (18, 23), the increased basal plasma nitrate in A compared with C, as well as the increase in nitrate after exercise both in A and in NA, may have been of vascular origin. If so, and if the increase was limited to the vasculature of the hyperemic tissues, the 80% elevation of the overall nitrate formation estimated above markedly underestimated the local increase in vascular NO formation in the exercising tissues, which may have been several times higher. However, the nitrate level in plasma and the excretion rate of nitrate reflects not only the vascular production of NO. Other endogenous sources for the increased nitrate formation during exercise must be considered as well. Recently, it was proposed that NO is involved in the relaxation process in skeletal muscle (9). The extent to which NO formation is increased in skeletal muscle during exercise is not known. Hence, further studies are required to identify the source of the increased plasma and urinary nitrate presently observed after physical exercise.

In conclusion, the present study indicates that physical fitness and basal NO formation are positively linked to each other. If the higher NO formation at rest presently observed in A and well-trained subjects is due to the regular physical activity performed by these subjects, the observation may help to explain the beneficial effects of exercise on cardiovascular health (1, 3, 5, 17). Furthermore, our data demonstrate that a single session of exercise elicits a transient elevation of plasma nitrate, possibly due to a temporary increase in endogenous NO formation. This observation confirms previous results from studies in animals and humans (2, 10, 18, 23).

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