Respiratory alkalosis does not alter NOx concentrations in human plasma and erythrocytes

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Ishibashi, Takaharu, Kaname Kubota, Mariko Himeno, Taku Matsubara, Tomoyuki Hori, Kazuyuki Ozaki, Masaru Yamozoe, Yoshifusa Aizawa, Junko Yoshida, and Matomo Nishio. Respiratory alkalosis does not alter NOx concentrations in human plasma and erythrocytes. Am J Physiol Heart Circ Physiol 281: H2757–H2761, 2001.—To test the hypothesis that NOx (NO2 and NO3, metabolites of NO) accumulates in red blood cells (RBC) in response to changes in PCO2 and bicarbonate (HCO3) concentration in blood, we examined the effect of changes in PCO2 and HCO3 induced by hyperventilation in healthy adults on partitioning of NOx in whole blood. NOx in hemolysate was measured by a high-performance liquid chromatography-Griess system equipped with a C18 reverse phase column to trap hemoglobin, which enables determination of whole blood NOx concentration and calculation of NOx concentration in RBC with high accuracy and reproducibility. NOx in hemolysate was measured by a high-performance liquid chromatography-Griess system equipped with a C18 reverse phase column to trap hemoglobin, which enables determination of whole blood NOx concentration and calculation of NOx concentration in RBC with high accuracy and reproducibility. NOx concentration in RBC was lower than that in plasma, and equilibrium between plasma and RBC was achieved rapidly after addition of NO3. Changes in PCO2 and HCO3 by hyperventilation failed to influence NOx concentrations in both plasma and RBC. Plasma NOx concentrations correlated with whole blood NOx and RBC NOx concentrations. Our results indicate that changes in PCO2 or HCO3 induced by hyperventilation do not influence NOx compartmentalization in plasma and RBC.

nitrate; hemolysate; nitric oxide metabolite measurement; hyperventilation

NITRIC OXIDE (NO) metabolites in plasma [NO2 and NO3 (NOx)] have been used as an index of endothelial NO production in physiological and pathological conditions, especially in local circulation, based on the rapid oxidization of NO to NOx in the blood (2). In support of this notion, higher NOx concentrations have been reported in coronary sinus blood than in arterial blood in the conscious dog model (11, 16). In the human heart, however, no increase in NOx across the normal coronary circulation has been noted (7), and this finding has been supported by a recent study in the conscious dog model (14). Furthermore, a number of studies have shown a negative NOx balance in the coronary circulation in certain pathological conditions such as pacing-induced heart failure in conscious dogs (11), angina with significant organic stenosis, or with vasospasm in human subjects (5, 7). These findings seem unreasonable. Even when endothelial cell function is attenuated in some pathological conditions, NO would be released at levels that are detectable in the coronary circulation as an attenuated increment in NOx production. It has been also noted that addition of exogenous NO3 to whole blood (ex vivo) results in a smaller increase in plasma NO3 concentration than the estimated value based on volume of plasma as a sole compartment for distribution (7, 8, 11). From these observations, it is conceivable to consider that NOx may localize into other compartments apart from plasma under certain circumstances. As pointed out by Recchia et al. (11), red blood cells (RBC) may act as a second compartment for NOx in the blood. They recently showed that the NOx concentration in RBC is several times higher than that in plasma and indicated that the PCO2 and bicarbonate concentration of plasma may regulate NOx levels in both compartments (12). However, in their measurement procedure, they used an ultrafiltration unit that was known to be contaminated by NOx (6), leading to incorrect (higher) estimation of NOx concentration in RBC. Therefore, we have examined

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distribution of NOx in plasma and in RBC with a new technique, avoiding NOx contamination in the procedure. In addition, we examined whether dynamic fluctuations in potential regulatory factors (PCO2 and bicarbonate concentration of plasma) induced by hyperventilation alter NOx concentration in the plasma and RBC of normal human subjects, whereas conclusions by Recchia et al. (12) were based on static blood samples collected from different vascular beds in conscious dogs.

MATERIALS AND METHODS

Subjects. To evaluate our system in determining the NOx concentration in blood (see below), venous blood was sampled from healthy volunteers (aged 25–50 yr of both sexes) from the laboratory staff of the Department of Pharmacology, Kanazawa Medical University. For the hyperventilation study, nine healthy male volunteers (age, 27–36 yr; height, 172 ± 1 cm; weight, 67 ± 3 kg) were recruited from the laboratory staff of the First Department of Internal Medicine, Niigata University School of Medicine. Each volunteer was informed about the purpose and the procedure of the study before giving written consent to participate. Both studies were approved by the Human Ethics Committee of the respective university. All subjects, including smokers, were normotensive, took no medication, and had no evidence of metabolic or cardiovascular disease. In addition, fresh venous blood was taken from mongrel dogs (weighing from 5 to 28 kg of both sexes, n = 7) in the animal laboratory of Kanazawa Medical University. Animals were handled in a humane way according to the “Guiding Principles for the Care and Use of Laboratory Animals” approved by The Japanese Pharmacological Society.

Hyperventilation study. After the human volunteers were allowed a period of bed rest, control samples of venous and arterial blood were withdrawn from the cephalic vein and brachial or radial artery, respectively. The volunteers were then asked to take deep breaths at 30/min over a period of 5 min using a metronome (3). Immediately after this period of hyperventilation, blood sampling was performed again. Blood gases and hematocrit were analyzed immediately by a blood gas analyzer (ABL625, Radiometer; Copenhagen, Denmark), and a portion of the blood was transferred to 9 vol of hypotonic solution (Tris 10 mM, pH 7.4) to prepare the hemolysate. After vortexing was performed, the hemolysate was centrifuged at 10,000 g for 10 min, and the supernatant was collected and stored at 2°C for 10 min at 4°C to remove proteins. The supernatant was centrifuged at 10,000 g for 10 min, and the supernatant was collected and kept at −80°C. Plasma was obtained after centrifugation of the blood at 1,600 g for 5 min at 4°C and was mixed with methanol (1:1) by 10.22 ± 0.33.5 on June 28, 2017 http://ajpheart.physiology.org/ Downloaded from
RESULTS

Verification of the method. Recovery experiment (ex vivo) was performed using fresh venous blood samples obtained from five volunteers. NOx concentration in the hemolysate was 25.4 ± 6.3 μM in the control state. Addition of 3 μl of NaNO3 solution (10 mM) to 1 ml of whole blood (expecting a resultant increase of 30 μM of NO3 in whole blood) followed by gentle agitation at room temperature resulted in an increase of NOx concentration to 54.3 ± 6.0 μM in the hemolysate. The increase was 28.7 ± 1.8 μM, and the recovery ratio was 96.5 ± 4.6%. The increases in NOx in both plasma and RBC were parallel and stable for 60 min (Fig. 2). Standard (mixed) plasma and hemolysate were prepared from blood samples of three volunteers and served to verify the quantification system. Areas under NO3 and NO2 curves of the standard hemolysate relative to those of standard solutions (10 μM each) in the absence of ODS column (1.28 ± 0.12% and 75.43 ± 0.29%, respectively, n = 6) were significantly (P < 0.01) smaller than those in the presence of ODS column (2.02 ± 0.08% and 77.11 ± 0.25%, respectively, n = 6). The means ± SE and intra-assay coefficient of variance (in parentheses) of NOx in the standard (n = 6) were 36.30 ± 0.04 μM (0.26%) for plasma, 30.32 ± 0.11 μM (0.80%) for hemolysate, and 22.99 ± 0.23 μM (2.25%) for calculated RBC NOx concentration. Values for interassay (determined 6 days later) were 35.97 ± 0.15 μM (0.95%) for plasma, 30.78 ± 0.28 μM (2.07%) for hemolysate, and 24.41 ± 0.55 μM (5.03%) for calculated RBC NOx concentration. Venous blood freshly drawn from seven mongrel dogs was also subjected to the evaluation. Mean NOx concentration of RBC (10.03 ± 2.06 μM) was significantly (P < 0.05) lower than that of plasma (21.38 ± 5.18 μM), and the resultant NOx ratio (RBC NOx concentration/plasma NOx concentration) was 0.50 ± 0.04 (0.41–0.63 in range).

Hyperventilation study. As shown in Table 1, arterial Pco2 and HCO3 significantly decreased, whereas Po2 and pH increased after 5 min of hyperventilation, indicative of respiratory alkalosis. A similar result was also obtained in venous blood. However, no significant change by hyperventilation was observed in arterial and venous plasma NOx concentration, or in the RBC NOx concentration (Table 1). Furthermore, the NOx ratio remained stable at around 0.5 (Table 1). Linear regression analysis showed a significant correlation between plasma NOx concentration and whole blood NOx concentration or RBC NOx concentration (Fig. 3, A and B). However, there was no significant correlation between Pco2 and the natural logarithm of NOx ratio [ln (NOx ratio)] and between plasma HCO3 concentration and between plasma HCO3 and venous plasma NOx concentration, or in the RBC NOx concentration (Table 1). Furthermore, the NOx concentration in RBC was lower than in plasma. Our results are inconsistent with those reported in the study of Recchia et al. (12), who measured NOx concentration in RBC of conscious dogs. The first difference is the NOx ratio, which was markedly higher (4.38–14.6) than that in our present study with canine venous blood (0.41–0.63) and with human arte-
The NOx concentration in red blood cells (RBC NOx) are shown. *Significantly (P < 0.01) different from arterial blood. † and ‡Significantly (P < 0.05 and <0.01, respectively) different from prehyperventilation.

Table 1. Hyperventilation-induced changes in blood gases and NOx concentration

<table>
<thead>
<tr>
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<th>Arterial Blood</th>
<th>Venous Blood</th>
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<tbody>
<tr>
<td>pH</td>
<td>Pre: 7.41 ± 0.01</td>
<td>7.35 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Post: 7.59 ± 0.01</td>
<td>7.45 ± 0.01</td>
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<tr>
<td>PCO2, mmHg</td>
<td>Pre: 41.4 ± 0.6</td>
<td>52.4 ± 1.6</td>
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<tr>
<td></td>
<td>Post: 23.5 ± 1.0</td>
<td>39.1 ± 1.5</td>
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<tr>
<td>PO2, mmHg</td>
<td>Pre: 100.3 ± 4.0</td>
<td>35.7 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>Post: 121.2 ± 3.6</td>
<td>43.5 ± 3.0</td>
</tr>
<tr>
<td>HCO3, mmol/l</td>
<td>Pre: 25.8 ± 0.4</td>
<td>28.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Post: 22.7 ± 0.5</td>
<td>26.8 ± 0.6</td>
</tr>
<tr>
<td>Plasma NOx, μM</td>
<td>Pre: 50.4 ± 10.9</td>
<td>49.7 ± 10.6</td>
</tr>
<tr>
<td></td>
<td>Post: 49.6 ± 10.5</td>
<td>49.2 ± 10.4</td>
</tr>
<tr>
<td>RBC NOx, μM</td>
<td>Pre: 29.4 ± 9.0</td>
<td>25.2 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>Post: 25.6 ± 7.7</td>
<td>30.1 ± 8.3</td>
</tr>
<tr>
<td>NOx ratio, RBC/PL</td>
<td>Pre: 0.48 ± 0.05</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Post: 0.50 ± 0.08</td>
<td>0.49 ± 0.04</td>
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</table>

Values are means ± SE. Blood samples were taken before (Pre) and after (Post) hyperventilation (for 5 min, n = 9), and blood gas profiles as well as sum of NO2 and NO3 in plasma (Plasma NOx) and in red blood cells (RBC NOx) are shown. *Significantly (P < 0.01) different from arterial blood. † and ‡Significantly (P < 0.05 and <0.01, respectively) different from prehyperventilation.

The reported volume of distribution in dogs is 21.5% of body weight (16). Roughly estimated volumes of plasma and RBC are 4.4 and 3.6% of body weight, respectively, assuming an Hct of 45%. Because the Vd has been determined by NOx concentration in venous blood (16), the mean NOx ratio calculated in peripheral venous blood based on the reported values (6.76, n = 6) (12) should be applied. A simple calculation that 4.4% (apparent Vd in plasma) + 6.76 × 3.6% (apparent Vd in RBC) = 28.7% (apparent Vd within only circulating blood), in excess of Vd (21.5%), indicates that the NOx concentration in RBC reported in their study may be too high, whereas our value obtained by canine venous blood (0.5) is fairly reasonable by the same estimation. Similarly, the calculated NOx ratio in our human study indicates that the Vd within circulating blood (8% of body weight) is 6.7% of body weight, being acceptable when the Vd of 28–33% of body weight in humans (1, 9, 13, 15) is taken into consideration.

A possible cause of the high NOx concentration in whole blood in the study of Recchia et al. (12) would be the use of an ultrafiltration unit to remove hemoglobin, because we could not recognize species difference between dog and human in this study. As we have reported previously (6), ultrafiltration units (especially filters) are heavily and variably contaminated with NO3 and to a lesser degree with NO2. Indeed, significant amounts of NO2 (13 ± 1 pmol; range, 10–15 pmol, n = 8) and NO3 (400 ± 26 pmol; range, 332–557 pmol, n = 8) in the filtrate (around 150 μl) through a 50-kDa cutoff filter (Millipore) for removal of hemoglobin were noticed in our preliminary studies (phosphate buffer was used as a substitute for hemolysate). The above contamination would be sufficient to result in an erroneously high concentration of NOx in the filtrated hemolysate, because the smaller volume of the hemolysate filtrate (about one-third compared with phosphate buffer in our preliminary studies) would result in higher concentration of NOx in the filtrate and the above calculation (multiplication by dilution factor) would magnify the contamination and error.

Because the RBC NOx values reported by Recchia et al. (12) are difficult to accept, it would not be surprising that close relationships between the natural logarithm of the NOx ratio (their index of the ratio of NOx distribution between plasma and RBC) and PCO2 or plasma NO3 concentration (12) are not recognized in our study. Instead, a close relationship between plasma NOx and hemolysate NOx and a rather rough but significant relationship between plasma NOx and RBC NOx were recognized. However, our results do not necessarily exclude the possible redistribution of NOx between plasma and RBC. As shown in Fig. 3B, there are some data points that are far away from the regression line between plasma NOx and RBC NOx.
centrations. In addition, our preliminary study showed that the NOx ratio sometimes varied in the same subject under certain conditions (data not shown). Therefore, certain factor(s), other than PCO2 and HCO32, may be operative in determining the NOx ratio between plasma and RBC. Further studies are required to determine these factors.

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