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A Kinetic Study on Functional Impairment of Nitric Oxide-Exposed Rat Erythrocytes

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In acute *in vivo* exposure of rats to 25 to 250 ppm nitric oxide (NO) by use of a small exposure chamber for a single rat, the kinetic parameters of nitrosylhemoglobin (Hb-NO) and methemoglobin (MetHb) formation were estimated (with the aid of computer simulation) on the basis of experimental data. The biochemical and rheological injuries of erythrocytes were also examined.

The time course of Hb-NO and MetHb formation in blood was compared with that simulated by a simplified kinetic model. The rate of MetHb formation from Hb-NO was much faster than MetHb reduction to ferrous form and dissociation of Hb-NO; thus, MetHb content was always greater than Hb-NO content. The activity of MetHb reduction decreased on exposure to a high concentration of NO, but the activity was recovered when rats were placed in clean air.

Rheologically, the blood viscosity was scarcely altered, but a few undeformed cells were detected at high shear stress. Morphologically, echinocytic transformation was observed to some extent. Biochemically, the crosslinking of membrane proteins and the alteration of acyl chain composition of membrane phospholipids were not detected in the *in vivo* exposure, though the *in vitro* exposure of rat erythrocytes to high concentrations of NO revealed remarkable oxidative crosslinking among membrane proteins and hemoglobin.

In conclusion, both for persistent methemoglobinemia and for membrane damage, the maintenance of reductive activity in erythrocytes is the most important determinant factor for the protection of NO-induced oxidative injury.

Introduction

It is now known that in *in vivo* exposure of an animal, nitric oxide (NO) enters through the lungs into the blood and binds with hemoglobin in erythrocytes to form nitrosylhemoglobin (Hb-NO) (*1–5*). In contrast, nitrogen dioxide (NO_2) mainly causes various pathological changes on the surface of airways and alveoli (*5,6*). To study the toxicity of NO, detailed knowledge of the interaction of NO with erythrocytes is necessary.

The following impairment by NO on the oxygen transport capacity of hemoglobin may be expected theoretically, according to the *in vitro* studies: (a) NO binds with hemoglobin very strongly [i.e., 3×10^5 stronger than oxygen (*7,8*)] and competes with oxygen, e.g., on exposure to 0.4 ppm NO, about 50% of hemoglobin will be converted to Hb-NO. (b) The homotropic allosteric interaction due to partially occupied NO in tetrameric hemoglobin further reduces oxygen dissociation. According to our previous *in vitro* study on human erythrocytes (*1*), the oxygen dissociation from 50% NO-liganded hemoglobin decreases to 60 to 70% of normal hemoglobin; thus, the oxygen transport will decrease to one-third of normal erythrocytes by the duplicate effect of reactions (a) and (b) ($0.5 \times 0.6–0.7 = 0.3–0.35$). (c) Ferrous Hb-NO is easily oxidized to ferric methemoglobin (MetHb) in the presence of oxygen, and the resultant MetHb further decreases the oxygen transport of erythrocytes (*1,4*). In summary, the oxygen transport ability of hemoglobin exposed to 0.4 ppm NO will decrease to less than 25%, and this is lethal.

In contrast, such severe toxicity has not been found in *in vivo* exposure, either with experimental animals (*9–11*) or with the workers in underground highways (*12*). In these cases, little or no Hb-NO has been detected, in spite of the increase of MetHb content. These discrepancies between the calculated toxicity of hemoglobin function and the *in vivo* observations must arise from some restoring ability in blood. The conversion of Hb-NO to MetHb is fast in the presence of oxygen [the first-order rate constant in human hemoglobin was 0.075 min^{-1} at 37°C (*4*)], but the conversion rate was slightly retarded for erythrocytes because of the reduction of MetHb to ferrous hemoglobin by MetHb reductase. Therefore, the steady-state concentration of Hb-NO during NO exposure becomes quite low, but MetHb increases.

In addition to the functional impairment of hemoglobin, NO and nitrite ion act as oxidizing reagents; thus, oxidative injury of erythrocyte constituents may be ex-

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pected. If the erythrocyte membrane is oxidatively injured by various mechanisms (*13–19*) and hemoglobin is denatured (*10,20*); the rheological impairment, especially decreased erythrocyte deformability under high shear stress (*21–23*), may develop as the degree of NO intoxication proceeds. In addition to slight methemoglobinemia, transformed erythrocytes, if increased by oxidative injury, result in retarded oxygen release (*24*).

In this study, the kinetics of Hb-NO and MetHb formation in the *in vivo* experiment determined with the aid of computer simulation as well as the biochemical and rheological injury of erythrocytes in acute NO-exposed rat, are reported.

Materials and Methods

Animals

Specific pathogen-free, Sprague-Dawley rats (male, body weight of 320 ± 20 g) were used. Blood was collected from the abdominal artery under anesthesia with ethyl ether, and the blood was heparinized (5 units heparin/mL blood were used).

Exposure to NO Gas

An exposure chamber (1400 mL in volume) for a single rat was used (*25*), as shown in Figure 1. The chamber enables shortening the contact of NO with oxygen (to keep NO_2 concentration to a minimum) and enables anesthetizing the rat in NO-containing air inside of the chamber (without breathing laboratory air). The exposure chambers were placed in a draft chamber. NO (800 ppm, balanced with nitrogen) and air were mixed in appropriate proportion outside the exposure chamber by a gas mixer and flowed at a rate of 400 mL/min. After both inlet and outlet of gas was stopped, the rat was anesthetized by ether introduced into the chamber through a stopcock. The blood was collected in an airtight syringe within 100 sec after placing the rat in the room air.

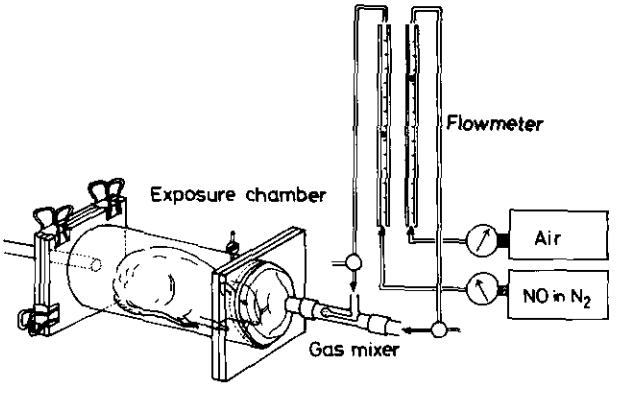

FIGURE 1. Exposure chamber for a single rat and gas flow system.

Biochemical and Rheological Analyses

Determination of Hb-NO and MetHb content in blood was carried out by electron paramagnetic resonance, using a Varian E-3 spectrometer (Palo Alto, CA) at 77°K (*4,9,26–29*). The blood was transferred into the sample tube (4.5 mm in diameter) under nitrogen gas stream, then frozen in liquid nitrogen. The doubly integrated signal of $g = 2$ for Hb-NO and the signal amplitude of $g = 6$ for high spin MetHb were compared with those of freshly prepared Hb-NO (100%) and MetHb (100 %) in phosphate buffer (pH 7.4).

Blood viscosity was measured by a cone-plate viscometer (Tokyo Keiki Co., Type E, Tokyo) at 37°C. Erythrocyte deformability was monitored with a homemade rheoscope (*21,30,31*) in isotonic phosphate-buffered saline containing 20% Dextran T-40 (pH 7.4, 20 cP) at shear stress of 4 to 150 dyn/cm^2.

The crosslinking of membrane proteins was examined by means of polyacrylamide gel electrophoresis containing 1% sodium dodecyl sulfate (without disulfide reducing agent) (*21,32*). The analysis of acyl chains of membrane phospholipids was performed with a gas chromatograph (Hitachi, model 163) after hydrolysis and esterification of lipids extracted with 10% HCl-methanol (*33,34*).

The shape change of erythrocytes was observed with a scanning electron microscope (Hitachi, model S-500A) after fixing with 1% glutaraldehyde and then with 1% OsO_4.

Computer simulation

The simulation of kinetic pattern was performed using a FACOM 180IIAD (FACOM, Tokyo) with SLCS IV program (Simulation Language for Continuous System IV). The differential equations, the rate constants, and the initial concentrations were input, and the numerical solutions were calculated by means of fourth order Runge-Kutta method and were printed (*25*).

Results

Hb-NO and MetHb in Blood of Acute NO-Exposed Rats

Animal Experiment. The representative time course of Hb-NO and MetHb formation in blood of an NO-exposed rat (100 and 200 ppm NO) is shown in Figure 2. The concentration of Hb-NO reached a steady state after 30 to 60 min, and MetHb reached a steady state afterward. In this process, the amount of MetHb was always greater than that of Hb-NO. Obviously, the oxidation process of Hb-NO to MetHb was faster than the reduction process of MetHb to ferrous hemoglobin.

As the concentration of NO (25–250 ppm) in inspired gas increased, both Hb-NO (0.08–3%) and MetHb (0.6–20%) increased after 60 min of exposure, and the molar ratio of MetHb to Hb-NO increased (7–15 times). In addition, the concentrations of these hemoglobins in ar-

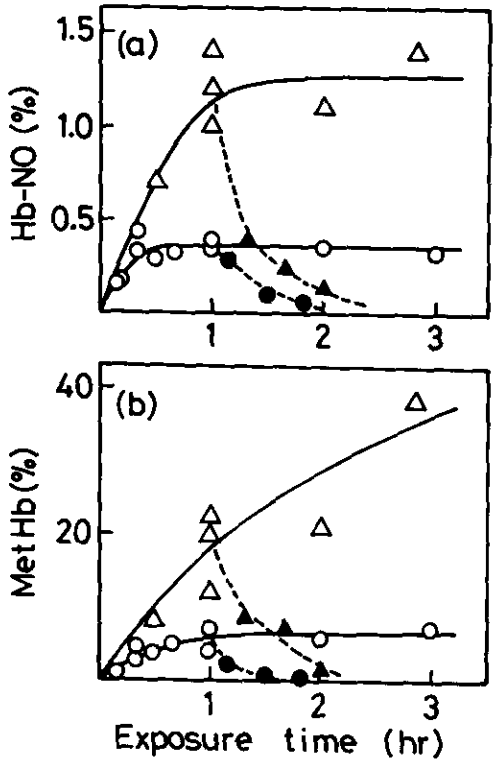

FIGURE 2. Time courses of the formation of Hb-NO (*a*) and MetHb (*b*) during exposure to NO. (○) 100 ppm NO; (△) 200 ppm NO; (- - -) time of the disappearance of Hb-NO and MetHb during successive exposure to fresh air after 1-hr exposure to 100 ppm NO (●) and 200 ppm NO (▲).

terial blood (from aorta abdominalis) were the same as those in venous blood (from vena cava inferior); thus, the uniform concentration of Hb-NO and MetHb in blood of whole body was ascertained independently of deoxyhemoglobin (Hb) and oxyhemoglobin ($Hb\text{-}O_2$) concentration.

The recovery process in clean air is also shown in Figure 2. After exposing rats to NO for 60 min, the rats were placed in clean air. Both Hb-NO and MetHb disappeared, with a half-life of about 20 min. These kinetic results were similar to those observed for NO-exposed mice (*10*).

Computer Simulation. Based on the above experimental results and previous *in vitro* studies on NO-exposed human erythrocytes (*4*), a simplified kinetic model (*25*) on the formation of Hb-NO and MetHb in blood of NO-exposed rat has been proposed. A refined model is shown in Figure 3. In this model, we dealt with the kinetic process in the erythrocytes on the basis of some assumptions, as shown in Table 1. The following five differential equations were computed to obtain the numerical solutions by the fourth order Runge-Kutta method.

$$d[NO]/dt = k_0([NO]_0 - [NO]). \quad (1)$$

$$d[Hb\text{-}O_2]/dt = k_1[Hb][O_2] - k_2[Hb\text{-}O_2], \quad (2)$$

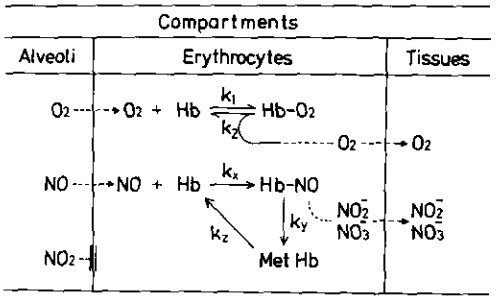

FIGURE 3. Reaction scheme for the computer simulation. The rate constants used for the computer simulation are summarized in Table 1.

$$d[Hb\text{-}NO]/dt = k_x[Hb][NO] - k_y[Hb\text{-}NO], \quad (3)$$

$$d[MetHb]/dt = k_y[Hb\text{-}NO] - k_z[MetHb], \quad (4)$$

$$d[Hb]/dt = -k_1[Hb][O_2] + k_2[Hb\text{-}O_2] - k_x[Hb][NO] + k_z[MetHb] \quad (5)$$

[Hb], [$Hb\text{-}O_2$], [Hb-NO], and [MetHb] are the concentrations of deoxy-, oxy-, NO- and methemoglobin in the erythrocytes, respectively; $[NO]_0$ is the concentration (ppm) of inspired NO gas; [O_2] and [NO] are the concentrations (ppm) of O_2 and NO gas in the hypothetical gaseous phase which is in equilibrium with the liquid phase in erythrocytes. The estimated ranges of the rate constants for the computer simulation are summarized in Table 1.

A set of simulated time courses is shown in Figure 4 with varying k_y and k_z and fixing other rate constants. Compared with the experimental results (Fig. 2), the computed time course with $k_y = 0.3\ min^{-1}$ and $k_z = 0.02\ min^{-1}$ (Fig. 4, center) is close to the experimental results of 100-ppm NO exposure. If other rate constants except k_y and k_z were changed, the simulated time course did not fit experimental results. Therefore, the rate constant of MetHb formation from Hb-NO should be about 10 times greater than that of MetHb reduction to Hb. However, in order to fit the data of 200-ppm NO exposure, k_z should be decreased to below $0.01\ min^{-1}$, i.e., the higher concentration of NO·led to the lower activity of reduction of MetHb to Hb. These situations (i.e., the MetHb reduction was the rate limiting step and was retarded with increasing NO concentration) explained qualitatively the increase of MetHb/Hb-NO ratio as a function of inspired NO concentration. However, in order to fit the data of dose-reaction relation, one must change k_y with NO-exposure time.

Computer simulation on the decay of Hb-NO and MetHb in clean air was conducted with the parameters used in Figure 3. In order to obtain similar kinetic patterns, the differential Equation 1 was replaced by $d[NO]/dt = k_0[NO]$, and the rate constant k_z was increased to $0.07–0.1\ min^{-1}$ in the present model; i.e., the accelerated reduction of MetHb was needed.

Table 1. Estimation of rate constants for computer simulation.

Rate constant	Dimension[a]	Basis for estimation
$k_0 = 0.08$	min^{-1}	$[NO] = [NO]_0 \{1 - \exp(-k_0 t)\}$, in which k_0 includes the overall diffusion process from inspired air into erythrocytes and is varied within the range of 0.05–10 min^{-1}. $k_0 = 0.08\ min^{-1}$ is chosen in order to obtain the reasonable fit to the experimental results.[b]
$k_1 = 5.67\ k_2/[O_2]$	$ppm^{-1} \cdot min^{-1}$	To maintain $[Hb - O_2]/[Hb] = 85/15$ (= 5.67) in average, $k_1[Hb] \cdot [O_2] = k_2 [Hb - O_2]$, thus $[Hb - O_2]/[Hb] = k_1[O_2]/k_2$, neglecting the homotropic allosteric interaction.
$k_2 = 0.7$	min^{-1}	Assuming O_2 consumption = 0.9 mL/g/hr [4.5 mL/min for 300 g rat, (*35*)], blood volume = 20 mL (*35*) and hematrocrit = 50%, the maximal O_2 capacity of total blood = 4 mL and then half-life of $Hb–O_2$ disappearance may be ~ 1 min ($k_2 = 0.7\ min^{-1}$).
$k_x = 4\ k_1$	$ppm^{-1} \cdot min^{-1}$	According to *in vitro* study on human hemoglobin (*8,36*), $k_x/k_1 = 3–5$, disregarding the allosteric effect of hemoglobin. In addition, the reverse reaction is slow (*8,36*), and thus neglected.
$k_y = 0.01–0.5$	min^{-1}	According to our *in vitro* study on human hemoglobin (*4*), $k_y = 0.075\ min^{-1}$ in the presence of dissolved oxygen.
$k_z = 0.005–0.05$	min^{-1}	Half-life of MetHb decay in erythrocytes (Fig. 2) = ~ 20 min.

[a]The concentration of gas (ppm) is the hypothetical concentration in gaseous phase which is in equilibrium with the liquid phase in erythrocytes.
[b]For inert gas (G) entering the circulation blood, for example, the concentration in erythrocytes, [G], may be calculated as follows: $[G] = [G]_0 \{1 - \exp(-k_a t)\} \cdot \{1 - \exp(-k_b t)\}$; where $[G]_0$ is the concentration in the inspired air; k_a is the rate constant of invasion of gas into alveolar gas phase and equal to (tidal volume, 1.8 mL) × (respiratory frequency, 80 min^{-1}) / (total lung capacity, 18 mL), (*35*) = 8 min^{-1}, neglecting the dead space volume; k_b is the rate constant of diffusion process of gas from alveolar gas space into erythrocytes. For NO gas, the chemical reactions of NO, yielding NO_2 in gaseous phase and NO_2^- and NO_3^- in blood (*3,4,37*) are ignored, and a simple form is given as shown above, since k_a is supposed to be much larger than k_b.

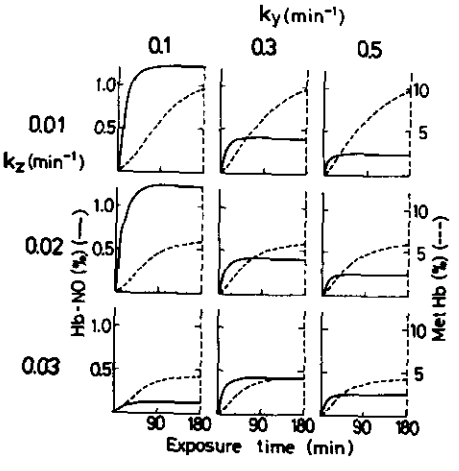

FIGURE 4. Simulated time courses of Hb-NO and MetHb formation in blood of 100 ppm NO-exposed rat. (———) Hb-NO (%); (---) MetHb (%). Note added in proof: the solid line in left bottom panel ($k_y = 0.1$, $k_z = 0.03$) is in error, the line should be drawn as high as other two left panels ($k_y = 0.1$).

Changes in Blood Rheology and in Erythrocyte Membranes

Blood Viscosity. We have shown that the suspension viscosity of NO-exposed human erythrocytes increases *in vitro* (*23*). This phenomenon has been explained by the echinocytic transformation and the oxidative crosslinking of membrane proteins. However, the change in blood viscosity of NO-exposed rats (25–250 ppm for 60 min) was scarcely detected.

Erythrocyte Morphology and Deformability. With increasing concentration of NO in aspirating gas, the incidence of echinocytes increased in rat blood (*23*). Using high shear rheoscopy, cellular deformability was measured. A low percentage of undeformed cells appeared in the blood of NO-exposed rats (200 ppm for 60 min).

Biochemical Changes of Erythrocyte Membrane. As shown in Figure 5, the electrophoretic pattern of ghost-membrane proteins of rat erythrocytes exposed to pure NO, then to nitrogen (to remove NO gas), and finally to atmospheric oxygen (*in vitro*) revealed a remarkable decrease of monomeric spectrin (both bands 1 and 2); the appearance of new bands with slightly higher molecular weight than spectrin; diminution and broadening of band 3; and increase of globin band. These changes increased with prolonged exposure to air (*23*). Furthermore, the changes were perfectly reversed upon addition of mercaptoethanol to ghost-membrane preparation prior to electrophoresis. The results suggest that the oxidative crosslinking at cysteine residues in membrane proteins takes place in NO-exposed rat

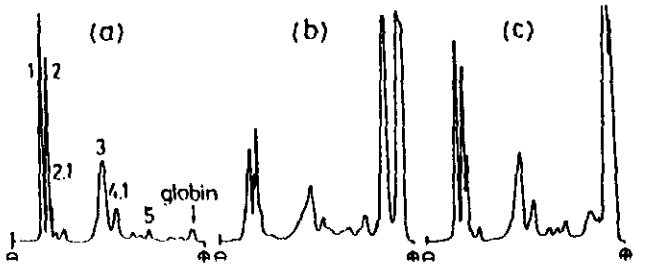

FIGURE 5. Densitograms of polyacrylamide gel electrophoretic pattern of membrane proteins of rat erythrocytes. (*a*) Control (not exposed to NO); (*b*) exposed to air for 60 min at 37°C, after exposing to pure NO; and (*c*) treated further with mercaptoethanol at 25°C for 5 min. Numbers in (*a*) show the band number of membrane proteins (*32*).

erythrocytes in contact with oxygen *in vitro*. However, such crosslinking could not be detected in the *in vivo* experiment.

In addition, no changes were observed in acyl chain composition of membrane phospholipids extracted from both *in vitro* and *in vivo* NO-exposed rat erythrocytes (*23*).

Discussion

Functional Injury of Hemoglobin

The functional injury of hemoglobin in erythrocytes of NO-exposed rats is the formation of MetHb, and to a lesser extent, persistence of Hb-NO. As expected from the *in vitro* experiment, the oxygen transport capacity of erythrocytes in the presence of 0.4 ppm NO decreases to less than one-fourth of the normal capacity by the formation of Hb-NO and MetHb and their homotropic allosteric interaction (*1,4*). However, the *in vivo* studies (*9–12*) have revealed the Hb-NO concentration in the steady state is far lower than expected and the influence of NO is not lethal. This is due to the following factors: inspired NO is converted to NO_2 in the airway by the reaction with oxygen; the conversion of Hb-NO to MetHb takes place quickly in the presence of oxygen (*4,25*); MetHb can be reduced to ferrous hemoglobin by MetHb reductase in erythrocytes; and nitrite and/or nitrate ions, which further oxidize ferrous hemoglobin (*38*), are excreted in urine (*3,37*). The regenerative process of hemoglobin (via MetHb) is much faster than NO dissociation from Hb-NO. Therefore, the oxidative conversion from Hb-NO to MetHb in the presence of oxygen (*4,5*), and the following reduction of MetHb by MetHb reductase have a protective role for NO intoxication. In short, the conversion of Hb-NO to MetHb prevents the hazardous effect of NO, as far as MetHb reductase activity is maintained.

The present kinetic study, both animal experiment and computer simulation, demonstrated that the rate of Hb-NO conversion to MetHb is about 10 times faster than the rate of MetHb reduction (which is thus the rate limiting process). It also demonstrated that the rate of MetHb reduction decreases with increasing NO concentration (though MetHb is reduced by at least 1 hr exposure to clean air). These kinetic considerations explain why the increasing MetHb/Hb-NO ratio paralleled NO concentrations. For example, at 25 ppm NO, Hb-NO was less than 0.1% and MetHb was 0.6%; thus, practically no change in oxygen transport was expected. On the other hand, at 250 ppm NO, MetHb elevated to 25% and Hb-NO was 3%; thus, more than 30% decrease in oxygen transport should occur.

The quick regain of the decreased rate of MetHb reduction as observed in the recovery experiments is noteworthy. This suggests that the main cause of the reduced enzyme activity in acute NO-exposure would probably be the decreased generation of NADH and/or the inactivation of cytochrome b_5, not the injury of MetHb reductase. The NADH-MetHb reductase (characterized as NADH-cytochrome b_5 reductase) in human erythrocytes uses NADH generated in the glyceraldehyde-phosphate dehydrogenase reaction to reduce the iron of MetHb from the trivalent to the divalent form, where cytochrome b_5 is an efficient electron carrier protein (*39–42*). The maintenance of reductive activity is important in preventing the decrease of oxygen transport of hemoglobin.

The extension of the computer simulation for a lower concentration range (using the same rate constants as for higher NO concentration) revealed that, at 1 ppm NO exposure for 3 hr, the concentration of Hb-NO and MetHb would be 0.005 and 0.02%, respectively. This estimate for a rat may not fit long-term NO exposure, e.g., Oda et al. (*10*) demonstrated that in the life-long NO-exposed (2.4 ppm) mice, the persistent Hb-NO was only 0.01% and MetHb (0–0.3%) did not increase, but Heinz bodies in erythrocytes were detected. Ayres et al. (*12*) found a statistically significant increase of MetHb content (0.43 g/dL) in highway tunnel workers (exposed to 1.38 ppm total nitrogen oxides containing 0.07 ppm NO_2 in an average 30-day period), compared with that of control group (0.34 g/dL). In these cases, the activity of the reductive enzyme system might be decreased by such long-term NO exposure.

Rheological Injury of Erythrocytes

Erythrocyte membrane proteins have an important role in maintaining the ability of passive deformation of erythrocytes (*21,22,43*), since the crosslinking of membrane proteins sensitively affects the deformability (*19,21*) and increases the suspension viscosity of erythrocytes (*21*). Morphologically, echinocytic transformation increases the blood viscosity (*24,44,45*). Some echinocytes were detected in blood of NO-exposed rats and some undeformed cells were observed under high shear stress by rheoscopy, though no influence in blood viscosity was observed. As demonstrated in an *in vitro* study (*23*), the main cause of these injuries may be the oxidative crosslinking among membrane proteins and hemoglobin. These oxidative changes explain the appearance of Heinz bodies in long-term NO-exposed animals (*10*).

The molecular mechanism of such oxidative crosslinking of membrane proteins may occur by many ways: (a) by the direct action of NO as an oxidant; (b) by the oxidative action of nitrite and nitrate ions which are products of NO in erythrocytes; (c) by reduced protection against oxidative attack due to the decrease of NADH or glutathione and/or the inactivation of cytochrome b_5; (d) by oxidative action of Hb-H_2O_2 (*14,18,19*), suggested as an intermediate in the reaction between Hb-O_2 and nitrite ion (*46–48*); (e) by the Schiff base formation by malondialdehyde, which is a peroxidative product of lipid (*13,16,17*) (though this was not considered because of reversibility of crosslinking by mercaptoethanol and lack of change of acyl chain composition of membrane phospholipids). At the moment, we

are not aware of the exact mechanism of oxidative crosslinking.

On the other hand, the crosslinking of membrane proteins was not detectable in the NO-exposed rat blood in this study. This may be because the sensitivity of polyacrylamide gel electrophoresis is quite low; the repairing mechanism of crosslinking is strong enough to overcome the effects of crosslinking; or the undeformed erythrocytes may be always trapped and destroyed in spleen. Conclusively, the main rheological injury of NO-exposed rat blood is due to the occurrence of oxidative crosslinking of erythrocyte membrane proteins. Such injury, if any, can be rapidly repaired by anti-oxidative mechanism of erythrocytes.

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