Mechanisms of Hemolytic Anemia during Experimental Methemoglobinemias

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A complex study of the peripheral erythron component was performed during methemoglobinemias induced by single administration of sodium nitrate and phenylhydrazine in LD_{50}. Administration of methemoglobin-forming agents to rats induced the development of hemolytic anemia. The pathogenesis of this disorder included significant long-term modifications of the erythrocyte membrane. The severity and duration of anemia syndrome depended on chemical structure of xenobiotics, blood methemoglobin level, and the duration of the acute period of methemoglobinemia.

Key Words: *methemoglobinemia; hemolytic anemia; erythrocyte; membrane*

Methemoglobin-forming agents are widely distributed in the human environment, which explains high incidence and poor outcome of intoxication with these compounds. Polyorgan complications develop during the postintoxication period and include persistent disturbances in the nervous, cardiovascular, respiratory, and endocrine system, liver, kidneys, and red blood [3].

The toxic effect of methemoglobin-forming agents results in hemolytic anemia [5,11]. The bone marrow and peripheral erythron compartment are involved in the pathological process. The hemoglobin component of the erythrocyte is first affected during intoxication. Qualitative changes in hemoglobin modulate structural and functional characteristics of the erythrocyte membrane (EM) [6]. The pathogenesis for dysfunction of the peripheral erythron compartment during acute exposure to methemoglobin-forming agents remains unknown.

Here we evaluated the role of structural and functional changes in EM in the pathogenesis of experimental hemolytic anemia induced by methemoglobin-forming agents.

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MATERIALS AND METHODS

Experiments were performed on 240 male Wistar rats weighing 190-250 g. Methemoglobinemia was induced by single intraperitoneal injection of 0.6% $NaNO_2$ (LD_{50}=90 mg/kg, 95 rats) and 2% phenylhydrazine hydrochloride (PH, LD_{50}=150 mg/kg, 109 rats). The control group included 36 intact animals. The blood was taken from the caudal vein of ether-anesthetized rats 1.5 h and 1, 3, 5, 7, 13, and 21 days after administration of $NaNO_2$ and PH. The blood was stabilized with 50 U/ml heparin.

Blood methemoglobin level was measured. The degree of intravascular hemolysis was estimated by the index of erythrocyte destruction. The content of membrane-bound hemoglobin in erythrocytes was measured as described elsewhere [8].

EM were isolated by hyposmotic hemolysis of the erythrocyte suspension. Protein concentration in the membrane suspension was estimated by the microbiuret method. Neutral lipids and phospholipids of EM in lipid extracts were separated by thin-layer chromatography [1] in heptane/diethyl ether/ethyl acetate (80:20:1.5) and chloroform/methanol/water (32:12.5:2) systems, respectively, on Sorbfil plates and identified using Sigma standards.

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Na^+/K^+-ATPase activity in EM was measured as described previously [4].

Spectral characteristics of the interaction between EM and fluorophore pyrene were recorded on a MPF-4 spectrofluorometer (Hitachi). Microviscosity of the EM lipid phase was estimated by pyrene excimerization at an excitation wavelength (λ_e) of 340 nm [2]. The pyrene excimerization coefficient was calculated as the ratio between maximum fluorescence of excimer (λ=470 nm) and monomer probes (λ=370 nm).

The results were analyzed by Student's *t* test and Mann—Whitney *U* test.

RESULTS

Blood methemoglobin level in rats significantly increased 1.5 h after single injection of $NaNO_2$ (51.95±1.59 *vs*. 1.19±0.24% in the control, $p<0.001$), but at later terms this parameter did not differ from the control. Blood methemoglobin level in rats sharply increased 30 min after single injection of PH (22.31± 1.10%, $p<0.001$), remained high by the 24th hour after xenobiotic treatment (7.50±0.19%, $p<0.001$), progressively decreased in the follow-up period, and did not differ from the control on days 3-21.

Our previous studies showed that single administration of $NaNO_2$ is followed by the development of anemia in the acute period of methemoglobinemia [7]. Signs of anemia syndrome were transient. The number of circulating erythrocytes, hemoglobin concentration, and hematocrit returned to normal on day 3 after treatment. Prolonged anemia in animals with PH-induced methemoglobinemia was revealed over 7 days of study.

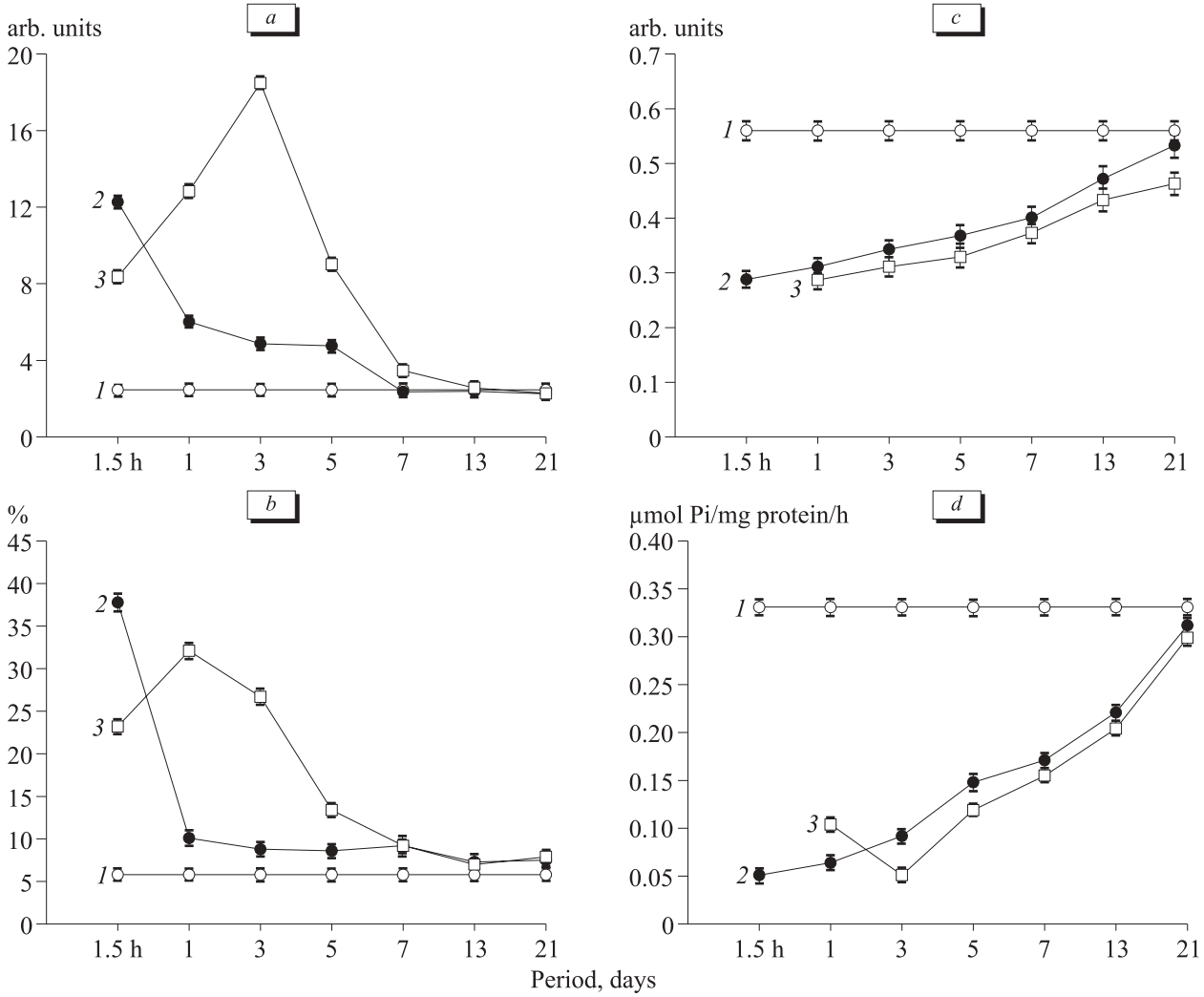

Fig. 1. Comparative characteristics of the peripheral erythron component in rats after single intraperitoneal injection of $NaNO_2$ and PH. Erythrocyte destruction index (*a*); membrane-bound hemoglobin concentration in erythrocytes (*b*); pyrene excimerization coefficient (I_{470}/I_{370}, λ=340 nm) in the erythrocyte membrane (*c*); Na^+/K^+-ATPase activity in the erythrocyte membrane (*d*). Control (*1*) and administration of $NaNO_2$ (*2*) and PH (*3*).

We concluded that administration of $NaNO_2$ and PH results in the development of hemolytic anemia. For example, extraerythrocytic hemoglobin concentration and erythrocyte destruction index remained high over a long period after treatment (up to 7 days). The degree of hemolysis was higher after PH administration (Fig. 1, *a*). The differences in the dynamics of $NaNO_2$- and PH-induced anemias are probably related to not only dysfunction of the glycolytic pathway for methemoglobin reduction under the influence of PH, but also to high antioxidant activity of this compound [10]. PH is slowly eliminated from the organism (1 day), while $NaNO_2$ disappears from the circulation over 1 h. Destruction of circulating erythrocytes is promoted by hemoglobin aggregation under oxidative degradation of globin (Heinz bodies) and decrease in deformability and abnormal shape of erythrocytes during oxidative modification of protein and lipid molecules in EM [6,9].

The content of membrane-bound hemoglobin significantly increased over 7 days after administration of xenobiotics, particularly of PH (Fig. 1, *b*). The observed changes are probably associated with the interaction between structural components of EM and hemoglobin, which depends on numerous factors (structural peculiarities of the EM lipid compartment, microviscosity of the lipid bilayer, nature of lipid polar groups, surface charge density, and structural and functional characteristics of the hemoglobin molecule) [8]. Methemoglobin accumulation induced by $NaNO_2$ probably results in the formation of a membrane-bound complex due to the reversible ionic interaction. However, the effect of PH is related to the formation of strong covalent bonds.

TABLE 1. Fractional Composition of EM Total Lipids in Rats after Single Injection of $NaNO_2$ and PH

Group, period	Total phospholipids	Cholesterol	Cholesterol esters
Control	44.8	38.5	16.7
$NaNO_2$, 90 mg/kg			
1.5 h	33.3*	40.3	26.4*
1 day	31.8*	41.8	26.4
3 days	30.9*	44.8*	24.3*
5 days	32.5*	43.4*	24.1*
7 days	35.2*	41.1	23.7*
13 days	39.6*	40.2	20.2*
21 days	42.1	39.5	18.4
PH, 150 mg/kg			
1.5 h	—	—	—
1 day	25.3*	42.3*	32.4*
3 days	27.4*	60.1*	12.5*
5 days	30.5*	55.7*	13.8*
7 days	34.5*	50.7*	14.8*
13 days	38.2*	45.9*	15.9
21 days	41.3*	41.9*	16.8

Note. Here and in Table 2: *$p \leq 0.05$ compared to the control.

The fractional composition of EM lipids in animals underwent significant changes after administration of methemoglobin-forming agents: the relative content of cholesterol increased against the background of decreased total phospholipid content and increased percent of cholesterol esters (Table 1). Signs for disorganization of EM phospholipids consisted in increased content of lysophosphatidylcholine, sphingomyelin, and phosphatidylserine and decreased percentage phosphatidylcholine and phosphatidylethanolamine (Table 2). Changes in EM lipids induced by PH were more significant and persisted for a longer period compared to those caused by $NaNO_2$.

Changes in EM structure in rats receiving $NaNO_2$ and PH were detected by the fluorescent method using hydrophobic probe pyrene. These structural changes persisted throughout the study. The pyrene excimerization coefficient in treated rats was lower than in control animals. These changes reflect an increase in the arrangement of the EM lipid phase (Fig. 1, *c*).

A prolonged increase in activity of membrane-bound ion transport enzyme Na^+/K^+-ATPase in EM was revealed in both animal groups (up to 21 days). Na^+/K^+-ATPase activity in EM was minimum 1.5 h (15% of the mean value) and 3 days after $NaNO_2$ administration (6.7-fold lower compared to the control, Fig. 1, *d*).

Our results illustrate complex pathogenesis of anemia syndrome during toxic methemoglobinemias (Fig. 2). Acute treatment with methemoglobin-forming agents is followed by severe disorganization of EM, which results in hemolytic anemia. Signs for structural modification of EM occur over a long period after administration of $NaNO_2$ and PH in LD_{50}. They are observed after the disappearance of methemoglobinemia and quantitative recovery of the peripheral erythron component. Methemoglobinemias induced by $NaNO_2$ and PH in LD_{50} are accompanied by similar structural changes in EM. However, the severity and duration of anemia syndrome depend on the chemical structure of xenobiotics, blood methemoglobin level, and duration of the acute period of methemoglobinemia.

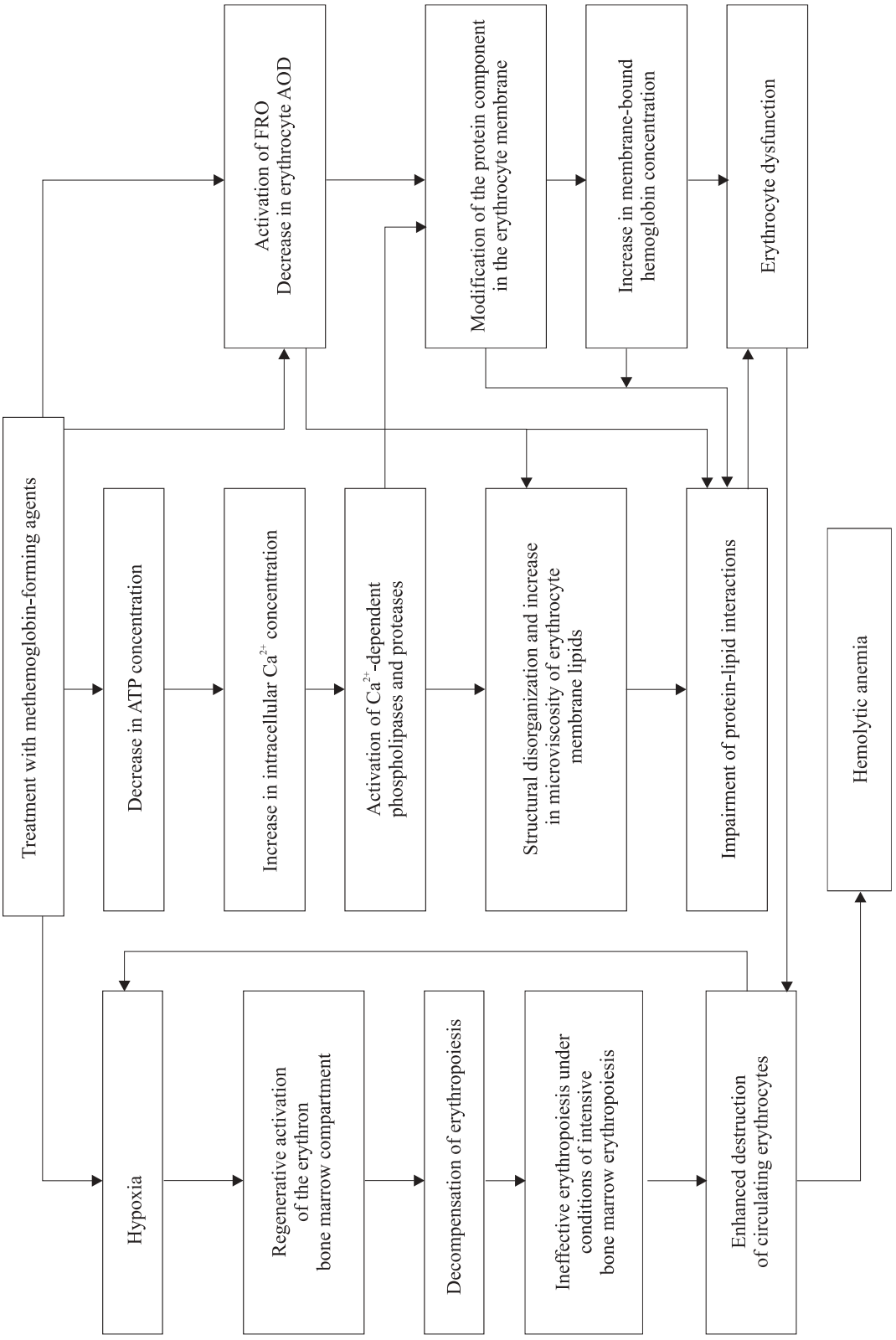

Fig. 2. Pathogenesis of hemolytic anemia during experimental methemoglobinemias. FRO, free radical oxidation; AOD, antioxidant defense.

TABLE 2. Fractional Composition of EM Phospholipids in Rats after Single Injection of $NaNO_2$ and PH

Group, period	Lysophosphatidylcholine	Phosphatidylinositol	Sphingomyelin	Phosphatidylcholine	Phosphatidylserine	Phosphatidylethanolamine
Control	4.2	7.9	11.5	39.2	17.4	19.8
$NaNO_2$, 90 mg/kg						
1.5 h	12.8*	7.1	16.3*	32.7*	19.2*	11.9*
1 day	10.9*	7.1	16.2	34.6*	18.7	12.5
3 days	8.9*	6.9	15.8*	35.5*	18.0	14.9*
5 days	6.9*	7.3	14.9*	37.1*	19.8*	14.0*
7 days	6.3*	7.2	15.9*	35.5*	20.1*	15.0*
13 days	4.7	7.6	14.0*	37.6	19.2*	16.9*
21 days	4.2	7.4	13.3	38.0	18.3	18.8
PH, 150 mg/kg						
1.5 h	—	—	—	—	—	—
1 day	12.4*	6.5	17.4*	31.1*	22.2*	10.4*
3 days	11.9*	7.4	16.9*	32.5*	19.8*	11.5
5 days	9.4*	7.5	15.3*	34.6*	19.4	13.8*
7 days	7.2*	7.4	14.5*	35.8*	19.6*	15.5*
13 days	6.5*	7.7	12.7	37.1*	18.7	17.3*
21 days	5.2	7.6	11.3	38.9	18.1	18.9

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