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The Effects of Nitroglycerine on the Redox Status of Rat Erythrocytes and Reticulocytes

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Summary

The effects of nitroglycerine (NTG) are mediated by liberated nitric oxide (NO) after NTG enzymatic bio-transformation in cells. The aim of this study was to evaluate some products of NTG bio-transformation and their consequences on the redox status of rat erythrocytes and reticulocytes, considering the absence and presence of functional mitochondria in these cells, respectively. Rat erythrocyte and reticulocyte-rich red blood cell (RBC) suspensions were aerobically incubated (2 h, 37 ^{0}C) without (control) or in the presence of different concentrations of NTG (0.1, 0.25, 0.5, 1.0 and 1.5 mM). In rat erythrocytes, NTG did not elevate the concentrations of any reactive nitrogen species (RNS). However, NTG robustly increased concentration of methemoglobin (MetHb), suggesting that NTG bio-transformation was primarily connected with hemoglobin (Hb). NTG-induced MetHb formation was followed by the induction of lipid peroxidation. In rat reticulocytes, NTG caused an increase in the levels of nitrite, peroxinitrite, hydrogen peroxide, MetHb and lipid peroxide levels, but it decreased the level of the superoxide anion radical. Millimolar concentrations of NTG caused oxidative damage of both erythrocytes and reticulocytes. These data indicate that two pathways of NTG bio-transformation exist in reticulocytes: one generating RNS and the other connected with Hb (as in erythrocytes). In conclusion, NTG bio-transformation is different in erythrocytes and reticulocytes due to the presence of mitochondria in the latter.

Key words

Erythrocytes • Nitric oxide • Nitroglycerine • Oxidative stress • Reticulocytes

Introduction

Nitroglycerine (NTG) has been used in the treatment of headache and angina pectoris long before its biochemical significance was understood. Today it is known that the action of NTG is mediated by the liberation of nitric oxide ($NO^{\cdot}$), after NTG enzymatic bio-transformation in cultured vascular smooth muscle cells as well as endothelial cells from different species (Feelisch and Kelm 1991). Previously, glutathione S-transferase (Lau *et al.* 1992), the cytochrome P-450 system (McDonald and Bennett 1993) and xanthine

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oxido-reductase (O'Byrne *et al.* 2000) were candidates for the NTG bio-transformation enzyme. Recently, Chen *et al*. (2002) and Sydow *et al*. (2004) identified mitochondrial aldehyde dehydrogenase (mtALDH) as a NTG reductase that specifically catalyzed the formation of 1,2-glyceryl dinitrate and nitrite. In acidic conditions, nitrites can generate $NO^{\cdot}$ non-enzymatically (McKnight *et al.* 1997). In addition, according to Feelisch and Kelm (1991), direct interactions of NTG with low-molecular-weight thiols may produce vasodilator S-nitrosothiols. Furthermore, bio-transformation of NTG by erythrocytes is, at least in part, due to the interaction with hemoglobin (Hb) (Bennett *et al.* 1985, Chong and Fung 1989).

Physiologically $NO^{\cdot}$ is a key component of the respiratory cycle and is the third gas transported by erythrocytes (Pawloski and Stamler 2002). In addition, red blood cells (RBCs), including reticulocytes and mature erythrocytes are the main site of $NO^{\cdot}$ metabolism, either endogenously synthesized or exogenously supplied by $NO^{\cdot}$ donors. $NO^{\cdot}$ reacts with oxyhemoglobin and deoxyhemoglobin in RBCs, generating methemoglobin (MetHb) and nitrosylhemoglobin, respectively (Pawloski and Stamler 2002). $NO^{\cdot}$ is thereby inactivated due to these reactions. The third kind of reaction between $NO^{\cdot}$ and Hb involves the SH-groups of the globin chains and the subsequent generation of S-nitrosohemoglobin, the form that retains $NO^{\cdot}$-regulator function (Pawloski and Stamler 2002).

Reticulocytes do not possess a full range of metabolic pathways compared to proliferating cells. This is because some pathways are missing due to the loss of the nucleus, endoplasmic reticulum and the Golgi apparatus. However, reticulocytes are still equipped with a set of metabolic pathways, due to the presence of mitochondria and ribosomes (Rapoport 1986). In experimental conditions, reticulocytosis is induced by bleeding or by phenylhydrazine-hydrochloride (PhCl) treatment (Rapoport 1986). The amount of bleeding-induced reticulocyte generation (primary in rabbits) is 30-40 % (Grune *et al.* 1990, Živković *et al.* 1990) whereas PhCl-induced reticulocytosis in rats is over 80 % (Kostić *et al.* 1990a, Maletić and Kostić 1999, Maletić *et al.* 1999ab, 2004). Studies *in vitro* showed that PhCl induced significant changes in energy metabolism, stimulation of the oxidative pentose pathway (Kostić *et al.* 1990b, Živković *et al.* 1990) as well as the induction of lipid peroxidation and oxidative damage of both erythrocytes and reticulocytes (Stern 1989, Grune *et al.* 1990).

Reactive oxygen species (ROS) and their derivatives exist in living tissues at low but measurable concentrations. Their levels are determined by the balance between their rates of production and clearance (Dröge 2002). ROS play an important role as regulatory mediators in signalling processes (Dröge 2002). However, when there is a disturbance in the antioxidant defence system/oxidizing environment balance shifting to the latter, oxidative stress of cells occurs (Sies 1991). Metabolic pathways of endogenous synthesis and degradation of NO are closely connected with metabolic pathways of redox and antioxidative metabolism (Dröge 2002). High concentrations of $NO^{\cdot}$ may react with $O_2^{\cdot-}/O_2$ to generate reactive nitrogen species (RNS), which affect almost all molecules in the cells (Wink and Mitchell 1998). The primary consumers of oxygen and primary ATP generators are the mitochondria which are a permanent source of ROS and RNS in cells (Brown and Borutaite 2002, Dröge 2002, Škárka and Ošťádal 2002, Nohl *et al.* 2003).

In this study we identified some products of NTG bio-transformation and their effects on the redox status of rat RBCs. Erythrocytes and reticulocytes were used as experimental models to assess the role of functional mitochondria in the latter on the bio-transformation of NTG, and subsequent effects on the redox status of RBCs.

Methods

Animals

Erythrocyte and reticulocyte-rich red blood cell suspensions from *Wistar* rats (250-350 g body weight) were used in this study. The animals were kept at 21±1 ^{0}C and exposed to a 12 h light/12 h dark cycle. All rats were housed in individual cages and given standard diet and water *ad libitum*. Reticulocytosis was induced by PhCl treatment (35 mg/kg body mass during three days) (Kostić *et al.* 1990a). Rats were divided into two experimental groups: untreated (for erythrocyte-rich RBC suspension collection) and PhCl-treated rats (for reticulocyte-rich RBC suspension collection). During experiments on animals our in-house ethical committee-approved protocol was followed.

Experimental protocol

Untreated and PhCl-treated (after 7-8 days of the first PhCl injection) rats were anesthetized using ether and blood was withdrawn by exsanguination. Blood was pooled from two or three animals in each experimental

group. Reticulocytes amounted to 86.57±1.28 % of total RBCs in blood obtained from PhCl-treated rats. Three times washed erythrocytes (obtained from untreated rats) as well as reticulocyte-rich RBC suspensions (obtained from PhCl-treated rats) were resuspended in an incubation buffer containing: 50 mM HEPES, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM glucose and 2 mM $CaCl_2$, pH 7.4 at 37 ^{0}C. Cell suspensions (final hematocrit value about 0.20) obtained in this way were aerobically incubated for 2 hours, without (control), or in the presence of different concentrations of NTG (0.1, 0.25, 0.5, 1.0 and 1.5 mM). To elucidate NTG biotransformation in rat RBCs, we used high experimental doses of this NO^- donor. NTG was added at the beginning of the incubation (at 0 min). Extractions were performed after incubations.

Analytical methods

The concentrations of RNS and ROS were determined after extraction using the following protocol: 0.5 vol 3 M perchloracetic acid and 2 vol 20 mM EDTA were added to 1 vol cell suspension. After extraction on ice (15 min) and centrifugation 4 min/15,000 rpm, extracts were neutralized using 2 M K_2CO_3.

The spectrophotometric determination of nitrites (the product of a rapid reaction of nitrosonium ion - NO^+ with water) was performed using the Griess method (Green *et al.* 1982). The level of hydroxylamine as an indicator of the nitroxyl ion (NO^-) was determined by indooxine formation from 8-hydroxyquinoline as described by Arnelle and Stamler (1996). The concentration of 3-nitrotyrosine (3-NT) as an indicator of the peroxynitrite ion was performed using Riordan and Vallee's method (1972). The spectrophotometric determination of the superoxide anion ($O_2^{\cdot-}$) was based on the reduction of nitro blue tetrazolium in the presence of superoxide anion (Auclair and Voisin 1985). The determination of the hydrogen peroxide (H_2O_2) concentration was based on the oxidation of phenol red in the presence of horseradish peroxidase as a catalyst (Pick and Keisari 1980).

The concentrations of MetHb (Heilmeyer 1943) and Heinz bodies (Bates and Winterbourn 1984) were determined in RBC suspensions by spectrophotometric techniques. The level of lipid peroxide products was determined on the basis of the reaction of lipid peroxidation products (malondialdehydes) with thiobarbituric acid (TBA) (thiobarbituric acid reactive substances – TBARS) (Ohkawa *et al.* 1979).

Statistical analysis

All values are expressed as mean ± S.E.M. Statistical evaluation was calculated by one way ANOVA. For all comparisons $p<0.05$ was considered as significant.

Reagents

NTG (a Nirmin ampule) was obtained from Zorka (Šabac, Serbia). PhCl, HEPES, nitro blue tetrazolium, phenol red, horseradish peroxidase and thiobarbituric acid were purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest purity commercially available.

Results

The effect of NTG on RNS and ROS concentrations in rat erythrocytes and reticulocytes

Aerobic *in vitro* incubation of erythrocytes and reticulocytes in the presence of NTG caused changes in the redox status parameters that indicated pathways of NTG metabolism in these cells. According to the data presented in Table 1, NTG did not alter the concentrations of RNS in rat erythrocytes. However, NTG caused a significant increase ($p<0.05$) in the level of both nitrite and 3-NT (peroxinitrite) (Table 2) in rat reticulocytes. These results indicate enzymatic bio-transformation of NTG to RNS in reticulocytes.

After a 2-hour incubation of rat RBCs with NTG, the concentrations of ROS ($O_2^{\cdot-}$ and H_2O_2) were also determined. In rat erythrocytes, NTG caused a dose-dependent decrease in $O_2^{\cdot-}$ ($p<0.05$) and an increase in H_2O_2 ($p>0.05$) (Table 1).

On the basis of the data presented in Tables 1 and 2, the basal concentration of H_2O_2 was 2.33-fold higher in reticulocytes, compared with erythrocytes [as a consequence of PhCl metabolism and induced oxidative stress (Stern 1989)]. In rat reticulocytes, NTG caused a significant decrease ($p<0.05$) in the concentration of $O_2^{\cdot-}$ and an increase in the concentration of H_2O_2 (Table 2), indicating the presence of oxidative stress in these cells.

NTG-induced oxidative damage in rat erythrocytes and reticulocytes

NTG-induced oxidative stress preceded oxidative damage of rat RBCs. The basal levels of MetHb and Heinz bodies amounted to 4.59±1.71 % and 70.00±3.77 in rat erythrocytes, respectively. NTG caused a significant ($p<0.05$) and dose-dependent increase in

Table 1. The effect of NTG on the concentrations of nitrite (NO_2^-), hydroxylamine (HA), 3-nitrotyrosine (3-NT), superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) in rat erythrocytes.

	NTG (mM)					
	0	**0.1**	**0.25**	**0.5**	**1.0**	**1.5**
NO_2^- (nmol/ml cells)	7.13 ± 2.02	10.26 ± 3.50	7.88 ± 3.55	4.65 ± 1.87	4.86 ± 3.06	6.01 ± 1.84
HA (nmol/ml cells)	35.48 ± 3.56	44.33 ± 3.95	39.70 ± 4.10	48.8 ± 4.19*	41.24 ± 3.25	35.17 ± 3.77
3-NT (nmol/ml cells)	145.7 ± 17.8	139.1 ± 32.2	121.9 ± 11.3	163.4 ± 37.6	133.5 ± 12.5	141.1 ± 20.6
$O_2^{\cdot -}$ (μmol/ml cells)	1.81 ± 0.34	1.57 ± 0.35	1.19 ± 0.34	0.77 ± 0.18*	0.93 ± 0.31*	0.85 ± 0.49*
H_2O_2 (nmol/ml cells)	6.27 ± 0.74	6.73 ± 0.80	6.73 ± 0.80	8.83 ± 2.03	12.19 ± 2.08	10.20 ± 1.05

Values represent mean ± SEM from 4 experiments. *p<0.05, control (0 mM NTG) versus NTG (other concentrations)

Table 2. The effect of NTG on the concentrations of nitrite (NO_2^-), hydroxylamine (HA), 3-nitrotyrosine (3-NT), superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) in rat reticulocytes.

	NTG (mM)					
	0	**0.1**	**0.25**	**0.5**	**1.0**	**1.5**
NO_2^- (nmol/ml cells)	3.89 ± 2.34	8.08 ± 2.54	8.11 ± 2.83	9.64 ± 2.06*	8.42 ±2.87	8.80 ± 2.85*
HA (nmol/ml cells)	52.53 ± 7.25	48.32 ± 9.46	59.64 ± 2.71	57.13 ± 4.04	40.21 ± 5.76	43.36 ± 5.97
3-NT (nmol/ml cells)	192.1 ± 29.9	248.3 ± 75.4	299.6 ± 48.1	377.1 ± 44.7*	282.8 ± 32.7	338.0 ± 38.4*
$O_2^{\cdot -}$ (μmol/ml cells)	1.11 ± 0.28	0.64 ± 0.06	0.61 ± 0.04*	0.66 ± 0.15	0.66 ± 0.05*	0.48 ± 0.09*
H_2O_2 (nmol/ml cells)	14.61 ± 2.02	22.24 ± 5.44	25.37 ± 4.24*	26.86 ± 4.57*	32.76 ± 2.98*	31.70 ± 3.27*

Values represent mean ± SEM from 4 experiments. *p<0.05, control (0 mM NTG) versus NTG (other concentrations)

both MetHb and Heinz bodies (Fig. 1).

A higher basal level of both MetHb and Heinz bodies in reticulocytes (5.90- and 3.18-fold, respectively) (Fig. 2), compared with erythrocytes (Fig. 1) was the consequence of PhCl-induced methemoglobinemia in the former cells (Stern 1989).

In rat reticulocytes, there was also evidence of hemoglobin damage (by oxidation reactions) and a dose-dependent elevation of the level of MetHb (Fig. 2). Consequently, Heinz bodies accumulated (Fig. 2). In addition, the level of MetHb increased 7.19-fold in erythrocytes and 2.37-fold in reticulocytes when the maximum (1.5 mM) dose of NTG was used. These data indicated that NTG bio-transformation in erythrocytes was primarily linked to Hb.

The lipid bilayer of RBC membranes is another location for the damaging effects of NTG and/or its metabolic products. The concentration of TBARS was significantly elevated in the presence of NTG in both erythrocytes and reticulocytes (p<0.05; Figs. 3ab).

Discussion

Diverse and important physiological roles of $NO^{\cdot}$ implicate that exogenous $NO^{\cdot}$ donors may be useful in the treatment of some diseases (Gerová and Kristek 2001).

NTG has been used in the treatment of cardiovascular diseases for the last 150 years. The information about its molecular mechanism in cells and organisms has contributed to the adequate clinical application of this drug. Recently, Chen *et al*. (2002) and Sydow *et al*. (2004) identified mitochondrial aldehyde dehydrogenase as a NTG reductase. In addition, direct interactions of NTG with low molecular weight thiols (Feelisch and Kelm 1991), as well as with reduced Hb (Bennett *et al.* 1985, Chong and Fung 1989) may also mediate NTG bio-transformation. Considering the above findings, in this study two experimental models were used: erythrocytes and reticulocytes (without and with functional mitochondria, respectively). We found that NTG failed to elevate any type of RNS, but it robustly increased MetHb in erythrocytes. These data suggest that NTG biotransformation in erythrocytes was primarily linked to Hb, in accordance with data in the literature (Bennett *et al.* 1985, Chong and Fung 1989). A significant increase in the level of MetHb is probably the result of NTG-induced oxidation of Hb, according to the

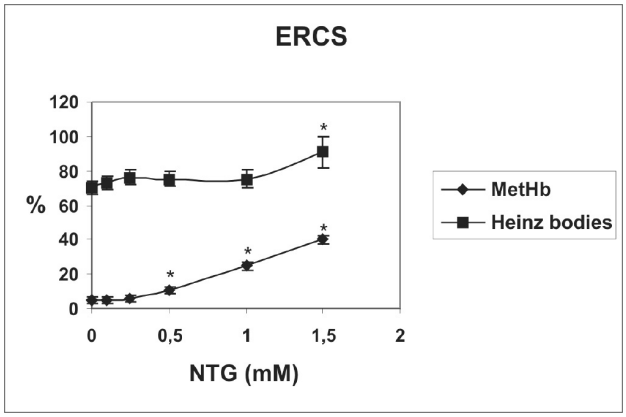


Fig. 1. The effect of NTG on the levels of MetHb and Heinz bodies in rat erythrocytes (ERCS). Values represent mean ± SEM from 4 experiments. *p<0.05, control (0 mM NTG) versus NTG (other concentrations).

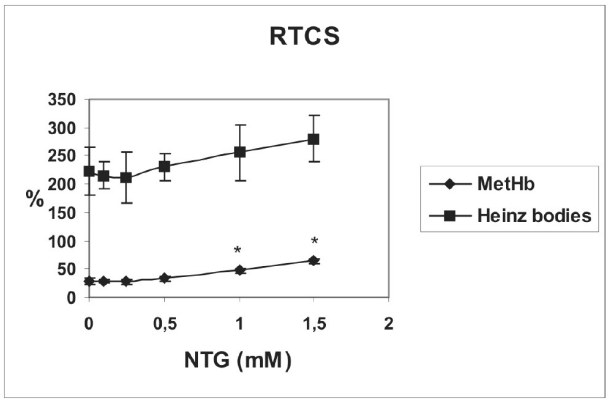
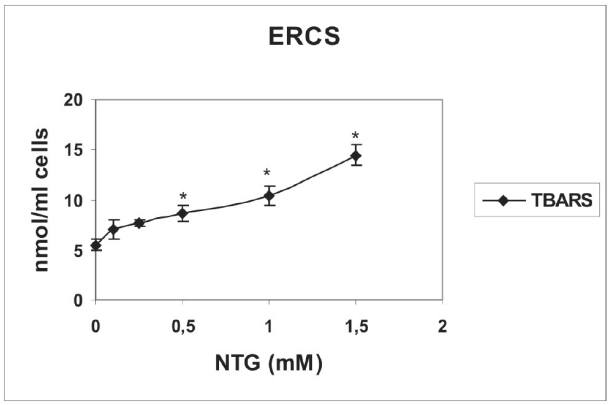


Fig. 2. The effect of NTG on the levels of MetHb and Heinz bodies in rat reticulocytes (RTCS). Values represent mean ± SEM from 4 experiments. *p<0.05, control (0 mM NTG) versus NTG (other concentrations).



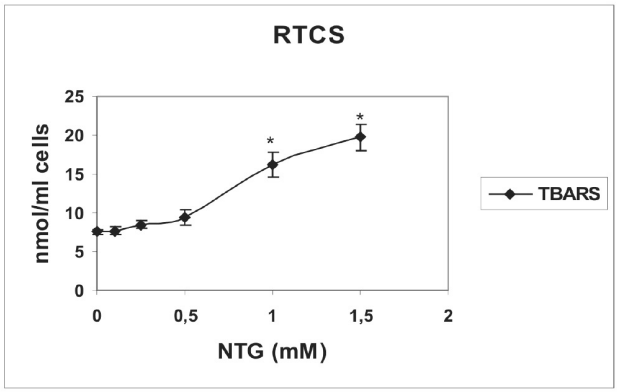


Fig. 3. The effect of NTG on the concentration of TBARS in rat erythrocytes (a) and reticulocytes (b). Values represent mean ± SEM from 4 experiments. *p<0.05, control (0 mM NTG) versus NTG (other concentrations).

proposed Equation 1.

However, NTG caused a significant decrease in the $O_2^{\cdot-}$ concentration, probably due to the reaction of superoxide dismutase (Fridovich 1995), which generates H_2O_2 in its dismutation reaction. The other important result of this study was the NTG-induced elevation of TBARS. Considering that NTG did not cause an elevation of RNS or ROS (the increase in H_2O_2 was not significant), the elevation of lipid peroxidation was the result of an oxidative process caused by $O_2^{\cdot-}$ generation (Rifkind *et al.* 2003). This is shown in Equation 1.

$$\mathbf{Hb(Fe^{2+}) - O_2 \longrightarrow MetHb(Fe^{3+}) - O_2^{\bullet-}} \qquad \textbf{Eq. 1}$$

We found elevations in the basal level of H_2O_2, MetHb and Heinz bodies in reticulocytes, when compared with erythrocytes. These changes are the consequences of PhCl metabolism and induced methemoglobinemia in the former cells (Stern 1989). However, the results of our study have shown similar changes in the oxidative stress parameters in both erythrocytes and reticulocytes in the presence of NTG. Hence, the PhCl-induced reticulocytes represent an experimental system with up-regulated oxidative stress. When compared with untreated erythrocytes, reticulocytes are a system adapted to oxidative stress.

Due to the fact that reticulocytes contain functional mitochondria (Rapoport 1986), we determined the possible role of mitochondria in NTG bio-transformation. The results presented in this study indicated that NTG caused an increase in the concentration of nitrite and peroxynitrite in rat reticulocytes. In addition, NTG caused a decrease in the level of $O_2^{\cdot-}$, which may have been the consequence of the reaction between $NO^{\cdot}$ and $O_2^{\cdot-}$ to form peroxynitrite (Wink and Mitchell 1998), as indicated by our results. Importantly, the experimental doses of NTG that were used (0.1-1.5 mM) caused a high flux of $NO^{\cdot}$, which reacted with $O_2^{\cdot-}/O_2$ to form RNS, according to Equations

2 and 3 (Wink and Mitchell 1998, Jay-Gerin and Ferradini 2000).

$$2^{\bullet}NO + O_2 \longrightarrow 2^{\bullet}NO_2 \qquad \text{Eq. 2}$$

$$^{\bullet}NO + O_2^{\bullet -} \longrightarrow ONOO^- \qquad \text{Eq. 3}$$

Together, all our data justify the role of mitochondria in NTG bio-transformation in reticulocytes and (at least in part) support the role of mitochondrial ALDH as NTG-reductase (Chen *et al.* 2002, Sydow *et al.* 2004). NTG also caused the formation of MetHb in a dose-dependent manner in reticulocytes. Taking this into consideration, together with the increased level of nitrite, it may be concluded that the generated $NO^{\bullet}$ directly reacts with oxyHb to form MetHb and nitrate (Pawloski and Stamler 2002), according to Equation 4 (Wink and Mitchell 1998, Jay-Gerin and Ferradini 2000).

$$^{\bullet}NO + Hb(Fe^{2+}) - O_2 \longrightarrow MetHb(Fe^{3+}) + NO_3 \qquad \text{Eq. 4}$$

In addition to erythrocytes, NTG caused lipid peroxidation in reticulocytes. On the basis of the data presented in this work, the increased level of TBARS was probably due to the toxic effects of peroxynitrite (Gadelha *et al.* 1997) and H_2O_2 (Maletić *et al.* 1998), as well as to oxidative stress caused by $O_2^{\bullet -}$ due to MetHb generation in reticulocytes.

We previously demonstrated that the basal ATP production during oxidative phosphorylation (OxP) in mitochondria in PhCl-induced reticulocytes amounted to 54.45±5.30 μmol/ml cells/h (Maletić and Kostić 1999). NTG caused significant inhibition of OxP energy generation, as well as stimulation of uncoupled oxygen consumption in rat reticulocytes (Maletić and Kostić 1999). Our current results clearly show an elevation of RNS and ROS (H_2O_2) in rat reticulocytes under the influence of NTG. Taking everything into account, the depletion of energy production during OxP in reticulocytes was the consequence of irreversible inhibition of enzymatic complexes (I-V) of the respiratory chain by RNS (Brown and Borutaite 2002) as well as by H_2O_2 (Maletić *et al.* 1997). In feedback, high production of ROS was a consequence of NTG-induced inhibition of OxP (Brown and Borutaite 2002).

In conclusion, NTG bio-transformation is primarily connected with Hb in erythrocytes. NTG-induced oxidation of Hb resulted in MetHb formation and $O_2^{\bullet -}$ generation, which caused lipid peroxidation. On the other hand, two pathways of NTG bio-transformation exist in reticulocytes: one causing RNS production and the other connected with Hb (as in erythrocytes). The results of this study indicate the significant role of mitochondria in the enzymatic bio-transformation of NTG. NTG caused oxidative stress in rat erythrocytes and reticulocytes leading to significant oxidative damage in these cells. This is one of the principal reasons for suggesting only acute clinical treatment with NTG.

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