Physiol. Res. 53: 439-447, 2004

Effects of Exogenous Donor of Nitric Oxide - Sodium Nitroprusside on Energy Production of Rat Reticulocytes

S. D. MALETIĆ, L. M. DRAGIĆEVIĆ-DJOKOVIĆ[1], B. I. OGNJANOVIĆ, R. V. ŽIKIĆ, A. Š. ŠTAJN, M. B. SPASIĆ[2]

Institute of Biology, Faculty of Sciences, [1]Institute of Physiology, Faculty of Medicine, University of Kragujevac, Kragujevac and [2]Institute for Biological Research "Siniša Stanković", Department of Physiology, Belgrade, Serbia and Montenegro

Received April 22, 2003
AcceptedAugust 24, 2003

Summary

The effects of the sodium nitroprusside (SNP), a nitric oxide (NO) donor clinically used in the treatment of hypertensive emergencies on the energy production of rat reticulocytes were investigated. Rat reticulocyte-rich red blood cell suspensions were aerobically incubated without (control) or in the presence of different concentrations of SNP (0.1, 0.25, 0.5, 1.0 mM). SNP decreased total and coupled, but increased uncoupled oxygen consumption. This was accompanied by the stimulation of glycolysis, as measured by increased glucose consumption and lactate accumulation. Levels of all glycolytic intermediates indicate stimulation of hexokinase-phosphofructo kinase (HK-PFK), glyceraldehyde 3-phosphate dehydrogenase (GAPD) and pyruvate kinase (PK) activities in the presence of SNP. Due to the decrease of coupled oxygen consumption in the presence of SNP, ATP production *via* oxidative phosphorylation was significantly diminished. Simultaneous increase of glycolytic ATP production was not enough to provide constant ATP production. In addition, SNP significantly decreased ATP level, which was accompanied with increased ADP and AMP levels. However, the level of total adenine nucleotides was significantly lower, which was the consequence of increased catabolism of adenine nucleotides (increased hypoxanthine level). ATP/ADP ratio and adenylate energy charge level were significantly decreased. In conclusion, SNP induced inhibition of oxidative phosphorylation, stimulation of glycolysis, but depletion of total energy production in rat reticulocytes. These alterations were accompanied with instability of energy status.

Key words

Rat reticulocytes • Energy production • Energy status • Sodium nitroprusside • Nitric oxide

Introduction

Corresponding to their intermediate position in the differentiation program, reticulocytes (rtcs) do not possess a full range of metabolic pathways of proliferating cells, since some pathways are lost with the disappearance of the nucleus, endoplasmatic reticulum and Golgi apparatus. On the other hand, reticulocytes are still equipped with a set of metabolic pathways (corresponding to the presence of mitochondria and ribosomes), most of which are lost during their transition to the mature erythrocytes (Rapoport 1986). The

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

mitochondria are primary consumers of oxygen and primary ATP generators in cells (Duchen 1999). Hence, energy production in mammalian reticulocytes is provided mainly by oxidative phosphorylation (OxP) in mitochondria (90 %), whereas the contribution of glycolysis is less than 10 % (Rapoport 1986). In consideration of their primary role in ATP production, mitochondrial dysfunction is an irreversible step on the pathway to cell death (Duchen 1999).

The nitric oxide (NO) is a small hydrophobic molecule with chemical properties that make it uniquely suitable as both an intra- and intercellular messenger (Moncada and Higgs 1993). Primary reactions of NO are almost exclusively limited to other species possessing unpaired electrons, such as the iron in hem proteins, as well as non-hem iron (proteins with iron-sulfur centers), thiols, molecular oxygen and superoxide (Kostić 1993, Moncada and Higgs 1993). Recent studies showed that the influence of NO on the processes in mitochondria, particularly on the respiratory chain, has many consequences on cell function, even cell death. Namely, nanomolar concentrations of NO immediately, specifically and reversibly inhibit cytochrome oxidase in competition with oxygen, while higher concentrations of NO and its derivatives (peroxynitrite, nitrogen dioxide or S-nitrosothiols – R-SNO) can cause irreversible inhibition of the respiratory chain (Brown and Borutaite 2002). On the other hand, NO induced stimulation of glycolysis (Maletić *et al*. 2000) and direct influence of NO on glycolytic enzymes was documented only for glyceraldehyde 3-phosphate dehydrogenase (GAPD) (Mallozzi *et al*. 1997, Galli *et al*. 1998, 2002).

Diverse and important physiological roles of NO implicate that exogenous NO donors may be useful in the treatment of some diseases (Farghali *et al.* 1997, Gerová and Kristek 2001). Transition metal NO complex, sodium nitroprusside (SNP) spontaneously releases NO, mainly as the nitrosonium ion – NO^+ (Hou *et al*. 1999), which generates R-SNO in reaction of nitrosation with low molecular weight thiols and which may store or release NO (Hogg 2000). SNP also gives rise to important quantities of cyanide ions, which react with hem-containing proteins (Bates *et al*. 1991).

In this study we investigated the effects of SNP as donor of NO^+ included in S-nitrosation reactions on energy production and energy status of reticulocytes. We used reticulocytes for two reasons. The first, reticulocytes are an excellent experimental model for the determination of energy metabolic pathways. The second, physiological functioning of reticulocytes is an important factor in the normal course of erythropoiesis, as well as in normal functioning of cardiovascular system. Hence, this study and investigation of organic nitrate effects (Maletić and Kostić 1999, Maletić *et al*. 1999a,b) make our knowledge of the mechanism of NO donors' effects on energy metabolism of red blood cells more complete.

Methods

In this study reticulocyte-rich red blood cell suspensions of rats (Wistar albino rats of 250-350 g body mass) were used. Reticulocytosis was induced by phenylhydrazine hydrochloride treatment (35 mg/kg body mass during three days) (Kostić *et al*. 1990). After 7-8 days, rats were anesthetized by ether and blood was obtained taken by exanguination. Reticulocytes amounted to 65-80 %. Three times washed red blood cells were resuspended in the 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 (Kostić *et al*. 1990). Cell suspensions (final hematocrit value about 0.20) were incubated for 2 h, aerobically without (control) or in the presence of different concentrations of SNP: 0.1, 0.25, 0.5 and 1.0 mM. The SNP was added at the start of incubation (0 min).

Oxygen consumption was measured by the Warburg technique (Umbreit *et al*. 1964). Coupled oxygen consumption (the part of total oxygen consumption used for ATP production in oxidative phosphorylation) was calculated as a difference between total and oligomycin (5 μM) resistant (uncoupled) oxygen consumption (Siems *et al*. 1982).

The aliquots of red blood cell suspensions for extraction of glucose, lactate (LAC), glycolytic intermediates (glucose 6-phosphate = G6P, fructose 6-phosphate = F6P, fructose 1,6-diphosphate = FDP, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate as triosephosphates = TP, glycerate 2,3-diphosphate = 2,3-DPG, glycerate 3-phosphate = 3PG, glycerate 2-phosphate = 2PG, phosphoenolpyruvate = PEP and pyruvate = PYR), adenine nucleotides (ATP, ADP and AMP) and hypoxanthine (HX) were taken at the beginning and after two hours of aerobic incubation. Extraction was carried out with 1 volume of ice-cold 0.6 M perchloric acid and extracts were neutralized with 0.25 volume of 1 M triethanolamine / 2.3 M K_2CO_3.

Glucose and lactate were determined in neutralized perchloric acid extracts, enzymatically by

means of spectrophotometric technique (Bergmeyer *et al*. 1974, Gutmann and Wahlefeld 1974) and thereafter glucose consumption and lactate accumulation (in desired time) were calculated. Glucose 6-phosphate, F6P (Lang and Michal 1974), FDP and TP (Michal and Beutler 1974) were determined enzymatically by using spectrofluorometric technique (Lowry and Passonneau 1972). In neutralized perchloric acid extracts 2,3-DPG (Michal 1974), 3PG, 2PG, PEP (Czok and Lamprecht 1974) and PYR (Jaworek *et al*. 1974) were determined enzymatically by means of spectrophotometric technique. Concentrations of ATP (Lamprecht and Trautschold 1974), ADP, AMP (Jaworek *et al*. 1974) and HX (Jorgensen 1974) were also determined enzymatically by using spectrophotometric technique.

On the basis of given parameters of glycolysis – the LAC/PYR ratio (a representative NADH/NAD ratio), as well as energy status – the total adenine nucleotide (TAN) level, level of HX added to TAN (TAN+HX), ATP/ADP ratio and AEC (ATP+1/2ADP)/TAN) level were calculated.

Energy production through oxidative phosphorylation was calculated on the basis of the estimated coupled oxygen consumption and the estimated P/O ratio of 2.5 (1 M atoms of oxygen consumed equivalent to 2.5 M produced ATP) (Siems *et al*. 1982). Glycolytic energy production was calculated on the basis of LAC/ATP ratio of 1 (1 M produced lactate equivalent to 1 M produced ATP) (Siems *et al.* 1982). Total energy production in reticulocytes was calculated by adding of glycolytic to mitochondrial ATP production. On the basis of the given parameters of oxygen consumption the coupled/total oxygen consumption ratio was calculated.

Chemicals for solutions were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany), while all enzymes were products from Boehringer (Manheim, Germany). SNP purchased from Gensia Pharmaceuticals (Irvine, USA).

All values are expressed as mean ± S.E.M. Statistical evaluation was calculated by Student's t-test for paired observations. For all comparisons $p<0.05$ was considered as significant.

Results

Results of our investigations show that total, coupled and uncoupled oxygen consumption in reticulocyte-rich red blood cell suspensions amount to 35.27±1.80, 27.46±1.60 and 8.18±1.54 μmol/ml rtcs/2 h, respectively (Figs 1a-c). Significant reduction of total and coupled oxygen consumption ($p<0.01$ and $p<0.05$, respectively) occurred after 30 min of aerobic incubation in the presence of 0.1 mM SNP and this effect persisted during prolonged incubation and at increasing dose of SNP (Figs 1a,b). Basal participation of coupled in total oxygen consumption (C/T ratio) amounted to 78.5 % during 60 min (Fig. 1). This is in accordance with literary data suggesting that energy production, due to mitochondrial respiration, consumed about 80 % of the oxygen consumption of reticulocytes (Rapoport 1986). The SNP induced a dose-dependent decrease of coupled/total oxygen consumption ratio (C/T ratio for 0, 0.1, 0.25, 0.5 and 1.0 mM SNP in 60 min amounted to 0.785, 0.869, 0.650, 0.476 and 0.336, respectively), indicating the strong inhibition of oxidative phosphorylation.

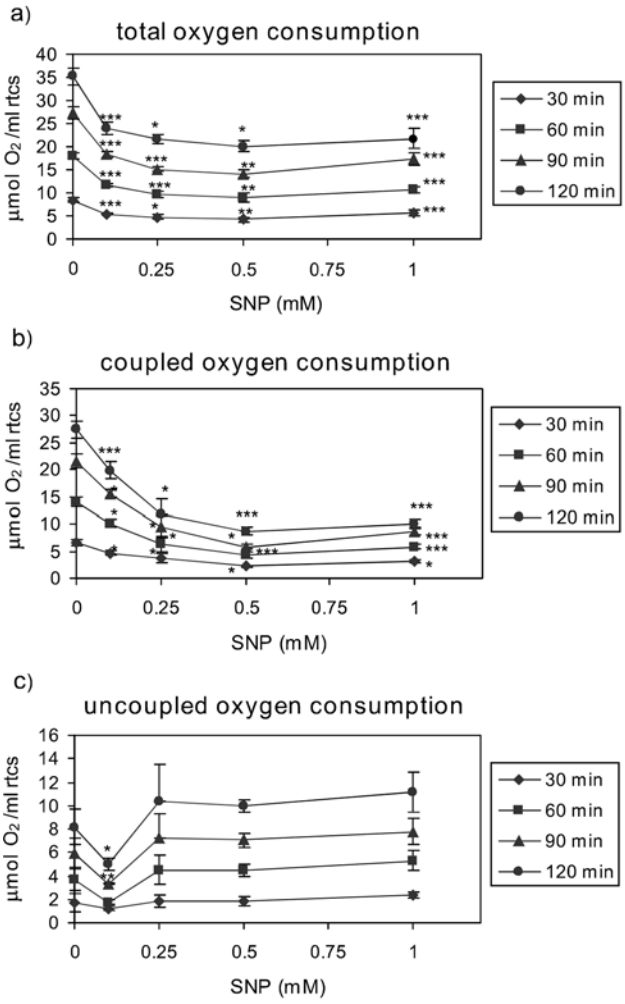

Fig. 1a, b, c. Effects of SNP on reticulocyte respiration. Each point with a bar represents the means ± S.E.M. for 4 paired experiments. Significantly different from controls (0 mM SNP): *p<0.05, **p<0.02, ***p<0.01.

The minimum applied dose of SNP (0.1 mM) significantly decreased ($p<0.05$), while other doses slightly but non-significantly ($p>0.05$) elevated uncoupled oxygen consumption (Fig. 1c).

Reduction of coupled oxygen consumption indicates a decreased energy production (ATP) by the OxP. It is accompanied by stimulation of glycolysis, which represents an example of the Pasteur effect (Rapoport 1986). Namely, glucose consumption and lactate accumulation during 2 h of aerobic incubation (4.90±0.43 and 7.87±0.44 μmol/ml cells/2 h, respectively) (Fig. 2) were significantly increased even in the presence of a low concentration (0.1 mM) of SNP ($p<0.01$). Levels of all glycolytic intermediates are shown in Table 1. Decreased levels of G6P ($p < 0.05$), F6P, FDP and TP ($p>0.05$), as well as increased levels of PYR ($p<0.01$) in the presence of SNP and application of "cross-over" theorem (Fig. 3) indicate stimulation of HK-PFK, GAPD and PK activities. SNP significantly increased 2,3-DPG level ($p<0.02$). The LAC/PYR ratio was decreased even 12.6-fold in the presence of 0.5 mM SNP (Table 1).

According to the results of this work, 94.7 % of overall energy was produced by OxP (70.70±4.10 μmol ATP/ml rtcs/h), but only 5.3 % by glycolysis (3.93±0.22 μmol ATP/ml rtcs/h) in rat reticulocytes under steady-state conditions (Table 2). Due to a decrease of coupled oxygen consumption in the presence of SNP, ATP production *via* OxP was significantly diminished ($p<0.05$). The simultaneous increase of glycolytic ATP production ($p<0.01$) was not sufficient to provide constant ATP production. Hence, the total energy production significantly decreased in a dose-dependent manner ($p<0.05$) in the presence of SNP (Table 2).

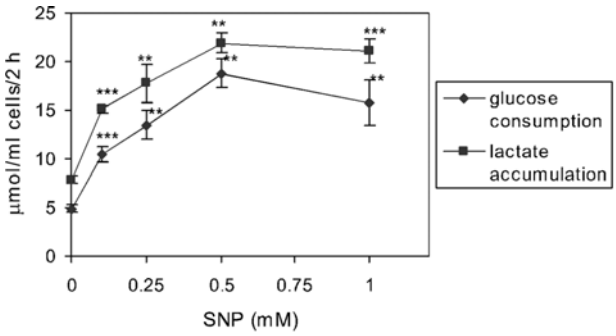

Fig. 2. Effects of SNP on glucose consumption and lactate accumulation in rat reticulocytes after 2 h of aerobic incubation. Each point with a bar represents the means ± S.E.M. for 4 paired experiments. Significantly different from controls (0 mM SNP): **p<0.02, ***p<0.01.

Table 1. Effects of SNP on glycolytic intermediates levels in rat reticulocytes after 2 h of aerobic incubation.

SNP (mM)	0	0.1	0.25	0.5	1.0
G6P	219.0±4.1	152.0±18.3*	165.1±9.4**	127.7±12.8***	68.5±6.8***
F6P	55.9±8.5	42.5±9.8	58.1±1.6	41.9±4.2	39.1±3.9
FDP	110.3±5.1	137.3±9.4	124.7±7.6	93.0±9.3	88.2±4.1
TP	145.8±20.6	103.5±18.9	100±13.7	102.1±14.4	108.6±21.0
2,3-DPG	7.2±0.4	8.6±0.2	9.7±0.3**	11.9±0.4***	13.0±0.6***
3-PG	94.2±11.7	112.1±19.4	107.4±16.3	160.1±11.6*	121.5±10.2
2-PG	34.1±8.8	41.3±5.3	47.7±10.6	56.6±9.7	44.0±16.3
PEP	35.6±1.4	33.8±4.9	51.6±3.8*	55.7±12.1	89.8±1.3***
PYR	59.7±9.4	74.64±11.4	167.1±14.3**	1587.0±358.8**	1343±9.64***
LAC	11.28±1.36	17.02±2.32***	20.22±2.45***	23.90±0.88***	26.01±1.34***
LAC/PYR	189	284	121	15	19

Values represent mean ± SEM for 3-4 paired experiments. Values for all glycolytic intermediates are in nmol/ml cells, except for 2,3-DPG and LAC in μmol/ml cells. Significantly different from controls (0 mM SNP): *p < 0.05, **p < 0.02, ***p < 0.01.

Apart from the reduction of cell respiration, stimulation of glycolysis, and consequently inhibition of total energy production, SNP induced marked alterations of energy status in rat reticulocytes. A dose-dependent decrease of ATP level ($p<0.05$) was accompanied by elevation of ADP and AMP levels ($p<0.05$; Fig. 4). However, the level of total adenine nucleotides was significantly lower in the presence of SNP ($p<0.05$; Table 3). Due to the fact that SNP-induced a decrease of TAN levels, which can be the consequence of increased

catabolism of adenine nucleotides, we determined the concentration of hypoxanthine, a final product of adenine nucleotide catabolism in red blood cells (Rapoport *et al.* 1990). HX concentration increased in a dose-dependent manner in the presence of SNP ($p<0.01$, Fig. 4). This elevation was sufficient to compensate the decrease of TAN level (Table 3). In addition, ATP/ADP ratio and adenylate energy charge (AEC) were decreased in the presence of SNP (Table 3), indicating an instability of energy status in rat reticulocytes.

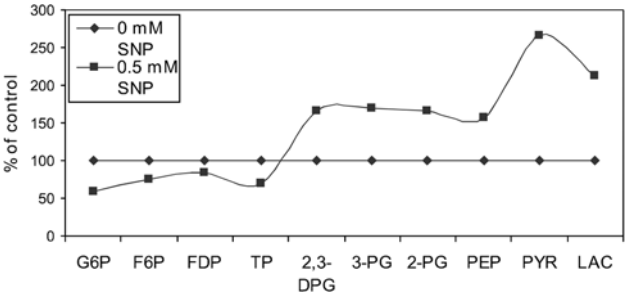

Fig. 3. Crossover plot of the glycolytic intermediate levels in the presence of 0.5 mM SNP in rat reticulocytes. Control values (0 mM SNP) are expressed as 100 percent. Value for PYR is x 10^{-1}.

Table 2. Effects of SNP on energy production in rat reticulocytes.

SNP (mM)	0	0.1	0.25	0.5	1.0
OxP ATP prod.	70.70±4.10	50.90±3.35*	31.45±7.65*	21.25±2.65***	29.40±1.90***
Glyc ATP prod.	3.93±0.22	7.54±0.22***	8.87±0.99**	10.97±0.54**	10.56±0.62***
Total ATP prod.	74.63±4.32	58.44±3.57**	40.32±8.64**	32.22±3.19***	39.96±2.52***

Values represent mean ± S.E.M. for 4 paired experiments. Values in μmol/ml cells/h. Significantly different from controls (0 mM SNP): *p < 0.05, **p < 0.02, ***p < 0.01.

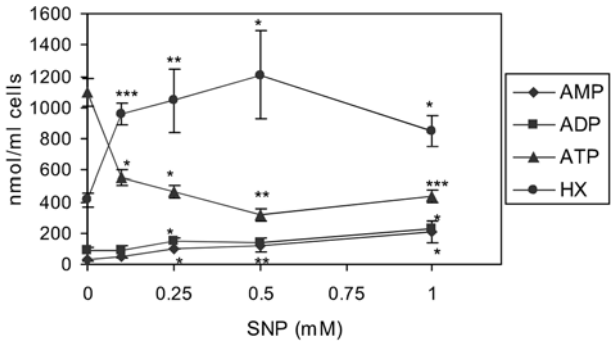

Fig. 4. Effects of SNP on adenine nucleotide (ATP, ADP, AMP) and HX levels in rat reticulocytes after 2 h of aerobic incubation. Values represent means ± S.E.M. for 4 paired experiments. Significantly different from controls (0 mM SNP): *p<0.05, **p<0.02, ***p<0.01.

All the investigated parameters were influenced by SNP. Sodium nitroprusside in low concentrations (0.1-0.5 mM) had dose-dependent effects, while at higher doses (0.5-1.0 mM) it showed saturable characteristics with maximum effects at the concentration of 0.5 mM.

Discussion

Considering that mitochondria are primary consumers of oxygen and, consequently, primary ATP generators in cells, the central role of mitochondrial dysfunction in cell death was not surprising (Duchen 1999). Hence, the inhibitory effects of exogenous NO-donors on rat reticulocyte respiration may have far-reaching implications. The results of this study indicated that SNP induced the inhibition of total and coupled oxygen consumption and the reduction of participation of coupled oxygen consumption in total oxygen consumption. According to Brown and Borutaite (2002), physiological nanomolar NO concentrations immediately, specifically and reversibly inhibit cytochrome oxidase in competition with oxygen, while higher concentrations of NO and its derivatives (peroxynitrite, nitrogen dioxide or R-SNO) can cause irreversible inhibition of the respiratory chain. On the basis of these data, SNP-induced inhibition of the respiratory chain in rat reticulocytes seems to be irreversible and is mediated by NO derivatives (probably by R-SNO), since high (mM) doses of SNP were applied in this experiment. On the other hand, the cyanide ion liberated from SNP (Bates *et al.* 1991) also contributed to the inhibition of mitochondrial respiration.

The extramitochondrial oxygen consumption in reticulocytes was a consequence of lipoxygenase-dependent oxygenation of unsaturated fatty acids, hemoglobin and glutathione autooxidation, as well as NADH-cytochrome b5 reductase, NADPH-oxidase and catalase activities (Rapoport 1986). The augmentation of these processes in the presence of NO and its reactive

derivatives probably induced a non-significant elevation of uncoupled oxygen consumption in SNP-treated rat reticulocytes. These data are in accordance with previous results of a dose-dependent, significant increase of uncoupled respiration in rat reticulocytes in the presence of organic NO-donors (nitroglycerine - NTG, isosorbide dinitrate – ISDN and molsidomine – MO) (Maletić and Kostić 1999, Maletić *et al*. 1999a, b).

Table 3. Effects of SNP on energy status in rat reticulocytes after 2 h of aerobic incubation.

SNP (mM)	0	0.1	0.25	0.5	1.0
TAN	1208±110	694±90*	712±50**	573±60**	875±170
TAN+HX	1618±70	1650±160	1755±160	1781±200	1853±550
ATP/ADP	12.73	6.26	3.04	2.22	1.87
AEC	0.94	0.86	0.75	0.67	0.63

Values represent mean ± S.E.M. for 4 paired experiments. Values for TAN and TAN + HX are in nmol/ml cells. Significantly different from controls (0 mM SNP): *p < 0.05, **p < 0.02.

Reduction of coupled oxygen production was accompanied by stimulation of glycolysis, as measured by lactate accumulation and glucose consumption. However, even a 2.8-fold stimulation of reticulocyte glycolysis (in the presence of 0.5 mM SNP), providing 34 % of whole energy production, was not sufficient to compensate for the decreased energy production due to inhibition of OxP. What was the reason for the stimulation of glycolysis in SNP-treated reticulocytes? Levels of all glycolytic intermediates and application of the "cross-over" theorem (Rapoport 1986) indicate the stimulation of HK-PFK, GAPD and PK activities. Stimulation of glycolysis accompanied with inhibition of the OxP, activation of HK-PFK, decrease of ATP level and simultaneous rise of ADP and AMP levels, with the reduction of ATP/ADP ratio, altogether represent an example of the Pasteur effect (Rapoport 1986) occurring in SNP-treated reticulocytes.

We demonstrated that SNP dose-dependently increased glycolytic rate in mature erythrocytes (Maletić *et al*. 2000). Low *et al*. (1993) showed that reversible binding of glycolytic enzymes to the cytoplasmic domain of membrane protein band 3 may play a role in regulation of glycolysis. In addition, SNP- and R-SNO-induced S-nitrosylation of GAPD (Galli *et al*. 1998, 2002), as well as peroxynitrite-induced tyrosine nitration of band 3 (Mallozzi *et al*. 1997), resulted in a decrease of the enzyme affinity to erythrocyte membrane and GAPD translocation/activity and the glycolytic flux. These data indicate that SNP itself stimulates glycolysis not only through the Pasteur effect because it could not appear in mature erythrocytes.

Interesting implications of this study are SNP-induced significant elevations of 2,3-DPG level, which is in contrast with previous investigations (Maletić and Kostić 1999, Maletić *et al*. 1999a,b), showing no alteration or slight (non-significant) depletion of 2,3-DPG level in organic nitrate-treated reticulocytes. Stimulation of 2,3-diphosphoglycerate bypass and ATP depletion on the other hand, may be the consequence of the competition of 2,3-DPG and ATP for the available phosphate (Rapoport 1986). The increased level of 2,3-DPG indicates stimulation of GAPD activity and consequently higher production of NADH. However, even a 12.6-fold decrease of the LAC/PYR ratio, a representative NADH/NAD ratio, occurred in the presence of 0.5 mM SNP. These data indicate a dysbalance in prooxidative-antioxidative metabolism (Maletić *et al*., unpublished data).

In rat reticulocytes, 94.7 % of overall energy was produced by OxP under steady-state conditions, while only 5.3 % was produced by glycolysis, indicating a dominant mitochondrial energy production. Due to a decrease of coupled oxygen consumption in the presence of SNP, ATP production *via* OxP was significantly diminished, which is in accordance to previous results (Maletić and Kostić 1999, Maletić *et al*. 1999a,b). The simultaneous increase of glycolytic ATP production, being a consequence of the Pasteur effect (Rapoport 1986), was not sufficient to compensate for decreased ATP production due to inhibition of OxP. Hence, total energy production was significantly decreased, indicating that the mitochondrial respiratory chain is the primary site of SNP effects.

Apart from lower ATP production, a dose-dependent decrease of ATP level in the presence of SNP was also found. However, increase of ADP and particularly of AMP content, was not sufficient to prevent the loss of ATP. Our results showed that decreased levels of total adenine nucleotides were the consequence of their increased catabolism. In addition, dose-dependent lowering of ATP/ADP ratio also indicated a decrease of energy production, as well as instability of energy status in SNP-treated reticulocytes. This was accompanied with low activity of energy-consuming processes, corresponding with the lowering of AEC level (Larner 1971). It was documented in earlier studies that protein synthesis in reticulocytes amounted to 30 %, Na, K-ATPase to 23 % and ATP-dependent proteolysis to 18 % of ATP consumed, i.e. to about 70 % of total energy production (Rapoport 1986, Kostić and Živković 1994, Ognjanović *et al.* 2003). This is in accordance with reports that NO-donors induce inhibition of Na, K-ATPase (Boldyrev *et al*. 1997), and that NO directly inhibits hemoglobin expression (protein synthesis) in the K562 erythroleukemic cell line (Rafferty *et al*. 1996), or indirectly through regulation of iron-regulatory protein activity (Pantopoulos and Hentze 1995).

On the basis of the implication of this study, the question is why the dose of 0.5 mM of SNP achieved the maximum effects on investigated parameters. The clue lies in dose-dependent liberation of NO from SNP, documented by Feelisch and Noack (1987). Namely, the relationship of thiol-independent NO released together with the concentration of SNP showed a saturation characteristic (maximum level is at 0.5 mM SNP). These implications were exactly mimicked by the dose-dependent effects of SNP on the investigated parameters of energy production and energy status in this study.

The metabolic effects of SNP were not mimicked by 8-Br-cGMP, except for the slight, dose-independent inhibition of OxP with no changes of the glycolytic rate (Maletić and Kostić 1999). However, Xie *et al*. (1996) showed that NO and 8-Br-cGMP influenced OxP by two different mechanisms. Exogenous $NaNO_2$ and $NaNO_3$ did not alter OxP and glycolysis in rat reticulocytes (Maletić and Kostić 1999).

The results presented in this study showed SNP (NO)-induced alterations in energy metabolism of rat reticulocytes. However, reticulocytes have a high content of hemoglobin, an effective scavenger of NO (Feelisch and Noack 1987, Moncada and Higgs 1993, Gross and Lane 1999). The question is why NO liberated from SNP was not inactivated by hemoglobin in rat reticulocytes under our experimental conditions? There are three possible answers to this question. (i) The first is that high experimental doses of SNP (far above scavenging capacity of hemoglobin) were applied. (ii) The second is that the NO reaction with RBCs was nearly 500-1000 fold slower in comparison to the reaction with cell-free hemoglobin (Huang *et al*. 2001). (iii) The third concerns the significant physiological role of nitrosated derivate of hemoglobin, S-nitrosohemoglobin, which can be formed *via* a transnitrosation reaction between oxyhemoglobin and R-SNO. Moreover, S-nitrosohemoglobin preserve capacity of spontaneous liberation of NO and mediation the NO-induced effects (Jia *et al*. 1996, Gow and Stamler 1998, Patel *et al*. 1999, Wolzt *et al*. 1999). Further investigations should explain this matter.

On the basis of the implications presented in this study we concluded that SNP induced a) inhibition of total and coupled, as well as stimulation of uncoupled respiration, b) stimulation of glycolysis, c) decrease of total energy production, d) instability of energy status, e) increased catabolism of adenine nucleotides and f) depletion of AEC level (indicating inhibition of energy-consuming processes) in rat reticulocytes. These effects were mediated by NO^+ (RSNO) and cyanide (mostly as an oxidative phosphorylation inhibition) as effector molecules.

Acknowledgements

This work was supported by the Serbian Ministry of Science, Technologies and Development, Grants No 1669. The authors are thankful to Prof. Dr Dragic Banković for statistical evaluation of the data, Prof. Radmila Štajn for proofreading and Mr. Predrag Ravić for excellent technical assistance. This work is dedicated in memory of the late Prof. Dr M. M. Kostić.

References

BATES JN, BAKER MT, GUERA RJR, HARRISON DG: Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss is required. *Biochem Pharmacol* **42** (Suppl): S157-S165, 1991.

BERGMEYER HU, BERNT E, SCHMIDT F, STORK H: Determination of glucose with hexokinase and glucose-6-phosphate dehydrogenase. In: *Methods of Enzymatic Analysis*. HU BERGMEYER (ed), Academic Press, New York, 1974, pp 1196-1201.

BOLDYREV AA, BULYGINA ER, KRAMARENKO GG, VANIN AF: Effect of nitroso compounds on Na/K-ATPase. *Biochim Biophys Acta* **1321**: 243-251, 1997.

BROWN GC, BORUTAITE V: Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radic Biol Med* **33**: 1440-1450, 2002.

CZOK R, LAMPRECHT W: Pyruvate, phosphoenolpyruvate and d-glycerate-2-phosphate. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York, 1974, pp 1446-1451.

DUCHEN MR: Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signaling and cell death. *J Physiol Lond* **516**: 1-17, 1999.

FARGHALI H, ZÍDEK Z, HYNIE S: Effects of nitroprusside as a nitric oxide donor on anoxia/reoxygenation and D-galactosamine hepatic injuries: a study in perfused hepatocytes. *Physiol Res* **46**: 336-369, 1997.

FEELISCH M, NOACK E: Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* **139**: 19-30, 1987.

GALLI F, ROVIDATI S, GHIBELLI L, CANESTRARI F: S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase decreases the enzyme affinity to the erythrocyte membrane. *Nitric Oxide* **2**: 17-27, 1998.

GALLI F, ROSSI R, SIMPLICIO P, FLORIDI A, CANESTRARI F: Protein thiols and glutathione influence the nitric oxide-dependent regulation of the red blood cell metabolism. *Nitric Oxide* **6**: 186-199, 2002.

GEROVÁ M, KRISTEK F: Efficiency of NO donors in substituting impaired endogenous NO production: a functional and morphological study. *Physiol Res* **50**: 165-173, 2001.

GOW AJ, STAMLER JS: Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* **391**: 169-173, 1998.

GROSS SS, LANE P: Physiological reactions of nitric oxide and hemoglobin: a radical rethink. *Proc Natl Acad Sci USA* **96**: 9967-9969, 1999.

GUTMANN I, WAHLEFELD AN: L-(+)-lactate. Determination with lactate dehydrogenase and NAD. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York, 1974, pp 1464-1468.

HOGG N: Biological chemistry and clinical potential of S-nitrosothiols. *Free Radic Biol Med* **28**: 1478-1486, 2000.

HOU YC, JANCZUK A, WANG PG: Current trends in the development of nitric oxide donors. *Curr Pharm Des* **15**: 417-441, 1999.

HUANG KT, HAN TH, HYDUKE DR, VAUGHN MW, HERLE HV, HEIN TW, ZHANG C, KUO L, LIAO JC: Modulation of nitric oxide bioavailability by erythrocytes. *Proc Natl Acad Sci USA* **98**: 11771-11776, 2001.

JAWOREK D, GRUBER W, BERGMEYER HU: Adenosine-5'-monophosphate. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York, 1974, pp 2127-2131.

JIA L, BONAVENTURA C, BONAVENTURA J, STAMLER JS: S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* **380**: 221-226, 1996.

JORGENSEN S: Hypoxanthine. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York, 1974, pp 1941-1945.

KOSTIĆ MM: A new bioregulatory system: nitric oxide from L-arginine. *Iugoslav Physiol Pharmacol Acta* **29**: 3-34, 1993.

KOSTIĆ MM, ZIVKOVIĆ RV: Energy metabolism of reticulocytes: two different sources of energy for Na^+K^+-ATPase activity. *Cell Biochem Funct* **12**: 107-112, 1994.

KOSTIĆ MM, DRAGIĆEVIĆ LJ, ŽIVKOVIĆ R, MÜLLER M, RAPOPORT SM: Stimulation of rat red blood cell glycolysis by phenylhydrazine hydrochloride. *Biomed Biochim Acta* **49**: 17-25, 1990.

LAMPRECHT W, TRAUTSCHOLD I: Adenosine-5'-triphosphate. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York, 1974, pp 2101-2110.

LANG G, MICHAL G: D-glucose-6-phosphate and D-fructose-6-phosphate. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York., 1974, pp 1238-1242.

LARNER J: Integration and control of energy metabolism. In: *Intermediary Metabolism and Its Regulation*. J LARNER (ed), Prentice-Hall, Englewood Cliffts, New Jersey, 1971, pp 248-267.

LOW PS, RATHINAVELU P, HARRISON ML: Regulation of glycolysis via reversible enzyme binding to the membrane protein, band 3. *J Biol Chem* **268**: 14627-14631, 1993.

LOWRY OH, PASSONNEAU JV: *A Flexible System of Enzymatic Analysis*. Academic Press, New York, 1972.

MALETIĆ SD, KOSTIĆ MM: Effects of nitroglycerin on energy metabolism of rat reticulocytes. *J Physiol Pharmacol* **50**: 75-87, 1999.

MALETIĆ SD, DRAGIĆEVIĆ LJM, ŽIKIĆ RV, ŠTAJN AŠ, KOSTIĆ MM: Effects of nitric oxide donor, isosorbide dinitrate, on energy metabolism of rat reticulocytes. *Physiol Res* **48**: 417-427, 1999a.

MALETIĆ SD, DRAGIĆEVIĆ-DJOKOVIĆ LJM, JAKOVLJEVIĆ VLJ, MILOVANOVIĆ DR, ROSIĆ MA, KOSTIĆ MM: Energy metabolism alterations in rat reticulocytes under the influence of molsidomine. *Exp Clin Cardiol* **4**: 152-158, 1999b.

MALETIĆ SD, DRAGICEVIĆ-DJOKOVIĆ LJM, ZIKIĆ RV, ŠTAJN AŠ, MILENKOVIĆ P, KOSTIĆ MM: Effects of nitric oxide donors on energy metabolism of rat erythrocytes. *J Environ Pathol Toxicol Oncol* **19**: 383-390, 2000.

MALLOZZI C, DI STASI AM, MINETTI M: Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. *FASEB J* **11**: 1281-1290, 1997.

MICHAL G: D-glycerate-2,3-diphosphate. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York, 1974, pp 1433-1438.

MICHAL G, BEUTLER HO: D-fructose-1,6-diphosphate, dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. In: *Methods of Enzymatic Analysis.* HU BERGMEYER (ed), Academic Press, New York, 1974, pp 1314-1319.

MONCADA S, HIGGS A: The L-arginine-nitric oxide pathway. *N Engl J Med* **329**: 2002-2012, 1993.

OGNJANOVIĆ BI, PAVLOVIĆ SZ, MALETIĆ SD, ŽIKIĆ RV, ŠTAJN AŠ, RADOJIČIĆ ZS, SAIČIĆ ZS, PETROVIĆ VM: Protective influence of vitamin E on antioxidant defense system in the blood of rats treated with cadmium. *Physiol Res* **52**: 563-570, 2003.

PANTOPOULOS K, HENTZE MW: Nitric oxide signaling to iron-regulatory protein: direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. *Proc Natl Acad Sci USA* **92**: 1267-1271, 1995.

PATEL RP, HOGG N, SPENCER NY, KALYANARAMAN B, MATALON S, DARLEY-USMAR VM: Biochemical characterization of human S-nitrosohemoglobin. Effects on oxygen binding and transnitrosation. *J Biol Chem* **274**: 15487-15492, 1999.

RAFFERTY SP, DOMACHOWSKE JB, MALECH HL: Inhibition of hemoglobin expression by heterologous production of nitric oxide synthase in the K562 erythroleukemic cell line. *Blood* **88**: 1070-1078, 1996.

RAPOPORT SM: *The Reticulocyte.* CRC Press, Boca Raton, Florida, 1986.

RAPOPORT I, DRUNG I, RAPOPORT SM: Catabolism of adenine nucleotides in rabbit red blood cells. *Biomed Biochim Acta* **49**: 11-16, 1990.

SIEMS W, MÜLLER M, DUMDEY R, HOLZHÜTTER HG, RATHMANN J, RAPOPORT SM: Quantification of pathways of glucose utilization and balance of energy metabolism of rabbit reticulocytes. *Eur J Biochem* **124**: 567-576, 1982.

UMBREIT WW, BURRIS RH, STAUFFER F: *Manometric Techniques*. Burgess Publishing Co. Minneapolis, 1964, pp 1-17.

WOLZT M, MACALLISTER RJ, DAVIS D, FEELISCH M, MONCADA S, VALLANCE P, HOBBS AJ: Biochemical characterization of S-nitrosohemoglobin. Mechanisms underlying synthesis, NO release, and biological activity. *J Biol Chem* **274**: 28983-28990, 1999.

XIE YW, SHEN W, ZHAO G, XU X, WOLIN MS, HINTZE TH: Role of endothelium-derived nitric oxide in the modulation of canine myocardial mitochondrial respiration in vitro. *Circ Res* **79**: 381-387, 1996.

Reprint requests

Assist. Snežana D. Maletić, MSc., Institute of Biology, Faculty of Sciences, Radoje Domanović 12, 34000 Kragujevac, P.O.Box 60, Serbia and Montenegro. Fax: +381 34 335 040. E-mail: maletic@knez.uis.kg.ac.yu