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Effects of Nitric Oxide Donor, Isosorbide Dinitrate, on Energy Metabolism of Rat Reticulocytes

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Summary

Since nitric oxide (NO) in many cells is involved in energy metabolism, the aim of this study was to evaluate the role of isosorbide dinitrate (ISDN), a NO donor, in energy metabolism of rat reticulocytes, particularly due to their high content of hemoglobin – an effective scavenger of NO. Rat reticulocyte-rich red blood cell suspensions were aerobically incubated in the absence (control) or in the presence of different concentrations of ISDN. ISDN decreased total and coupled oxygen consumption ($p<0.05$) while increased uncoupled oxygen consumption ($p<0.05$) in a dose- and time-dependent manner. This was followed by enhancement of glycolysis, as measured by increased glucose consumption and lactate accumulation ($p<0.05$). Levels of all glycolytic intermediates in the presence of ISDN indicate only stimulation of pyruvate kinase activity. ISDN did not alter the concentration of ATP, while increased ADP and AMP levels ($p>0.05$). In rat reticulocytes under steady-state conditions, 95.4 % of overall energy was produced by oxidative phosphorylation but only 4.6 % by glycolysis. Due to a reduced coupled oxygen consumption in the presence of ISDN, ATP production *via* oxidative phosphorylation was significantly diminished. A simultaneous increase of glycolytic ATP production is not enough to ensure constant ATP production. The calculated mean ATP turnover time was prolonged by 199 % in the presence of 1.5 mmol/l ISDN. In conclusion, ISDN a) inhibited total and coupled respiration but enhanced uncoupled respiration, b) stimulated glycolysis, c) decreased ATP production and d) prolonged ATP turnover time in rat reticulocytes. These effects were mediated by NO as the effector molecule.

Key words

Rat reticulocytes • Energy metabolism • Oxidative phosphorylation • Glycolysis • Nitric oxide • Isosorbide dinitrate

Introduction

In recent years, organic nitrates have experienced a remarkable revival with the finding that they have to be regarded as prodrugs acting *via* the release of nitric oxide (NO), a discovery which coincided with the demonstration that the endothelium-derived relaxing factor (EDRF) is chemically identical to NO (Furchgott and Zawadzki 1980). Several enzyme systems have been shown to be capable of biotransformation of organic nitrates to an active compound – NO and the velocity of degradation to NO increased with the number of nitrate groups in the molecule (Feelisch and Noack

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1987, Feelisch 1996). The 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, Crow and Beckman 1995).

The reticulocyte is a stage of maturation of the erythroid cells which is well defined by morphological and biochemical criteria. In addition, these cells are characterized by elimination of the nucleus in mammals, but they still posses functional mitochondria and process of glycolysis (Rapoport 1986), which make these cells an excellent experimental model for determination of the processes involved in energy metabolism. Glycolysis is the only energy producing process in mammalian erythrocytes, while energy in reticulocytes is provided by glycolysis (10 %), as well as by oxidative phosphorylation (90 %) (Rapoport 1986). Recent studies have shown that in many cells NO inactivates aconitase and mitochondrial respiratory chain at the complex I and II (proteins with iron-sulfur centers) (Drapier and Hibbs 1986, 1988) and cytochrome oxidase (hem protein) (Brown 1995, 1997, Reid 1998, Stewart *et al.* 1998, Torres *et al.* 1998). NO at a low concentrations can substantially lower energy production by the isolated liver, brain (Schweizer and Richter 1994) and myocardial mitochondria (Kelm *et al.* 1997) at oxygen concentrations that prevail in cells and tissues. In addition, NO causes ADP-ribosylation and inhibition of glyceraldehyde 3-phosphate dehydrogenase (GA3PDH), a glycolytic enzyme (Dimmeler *et al.* 1992, Brüne *et al.* 1994, Mohr *et al.* 1996). Contrary to these results, Mallozzi *et al.* (1996,1997) showed NO-induced phosphorylation of band 3 with consequent activation of GA3PDH in erythrocytes.

However, no data are available concerning the effects of NO on energy metabolism of red blood cells, especially regarding their high content of hemoglobin –an effective scavenger of NO (Wennmalm *et al.* 1992). Therefore, the aim of this study was to investigate the role of isosorbide dinitrate (ISDN), a NO donor (Feelisch 1996, Ignarro 1996), on rat reticulocytes energy metabolism.

Methods

Rat reticulocyte-rich red blood cell suspensions (Wistar albino rats of 250-350 g body mass) were used in this study. Reticulocytosis was induced by phenylhydrazine hydrochloride treatment (35 mg/kg body weight for three days) (Kostić *et al.* 1990). After 7-8 days, when blood was obtained by exsanguination, reticulocytes amounted to 70-100 %. Three times washed red blood cells were resuspended in an incubation buffer containing: 50 mmol/l HEPES, 100 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l NaH_2PO_4, 5 mmol/l glucose and 2 mmol/l $CaCl_2$, pH 7.4 at 37 ^{0}C (Kostić *et al.* 1990). Cell suspensions (final hematocrit value about 0.20) were incubated aerobically for 2 h in the absence (control) or in the presence of different concentrations of ISDN (Schwarz Pharma, Monheim, Germany): 0.1, 0.25, 0.5, 1.0 and 1.5 mmol/l. Chemicals for solutions were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany), while all enzymes were Boehringer products (Mannheim, Germany).

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 the difference between total and oligomycin (5 μmol/l) resistant oxygen consumption (Siems *et al.* 1984).

The aliquots of red blood cell suspensions for extraction of glucose, lactate, 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, pyruvate = PYR) and adenine nucleotides (ATP, ADP and AMP) taken from the start and after two hours of aerobic incubation. Extraction was done with 1 volume of ice-cold 0.6 mol/l perchloric acid and extracts were neutralized with 0.25 volume of 1 mol/l TRA - 2.3 mol/l K_2CO_3.

Glucose and lactate were determined in neutralized perchloric acid extracts, enzymatically by means of the spectrophotometric technique (Bergmeyer *et al.* 1974, Gutmann and Wahlefeld 1974). Glucose consumption and lactate accumulation (in certain time) were then calculated. Enzymatic determination of G6P, F6P (Lang and Michal 1974), FDP and TP (Michal and Beutler 1974) were performed using the spectrofluorometric technique (Lowry and Passonneau 1972). 2,3-DPG (Michal 1974), 3PG, 2PG, PEP (Czok and Lamprecht 1974) and pyruvate (Jaworek *et al.* 1974) were determined enzymatically in neutralized perchloric

acid extracts by spectrophotometry. Concentrations of ATP (Lamprecht and Trautschold 1974), ADP and AMP (Jaworek *et al.* 1974) were also determined enzymatically.

Energy production through oxidative phosphorylation (OxP) was calculated on the basis of coupled oxygen consumption and a P/O ratio of 2.5 (1 mol/l of consumed atom of oxygen = 2.5 mol/l of produced ATP) (Siems *et al.* 1982). The glycolytic energy production was calculated on the basis of a lactate/ATP ratio equal to 1 (1 mol/l produced lactate = 1 mol/l produced ATP) (Siems *et al.* 1982). Total energy production in reticulocytes was estimated by adding glycolytic and mitochondrial ATP production. The ATP turnover time was calculated from the ratio between total ATP production and ATP concentration (Siems *et al.* 1982).

All values are expressed as means ± S.E.M. Statistical evaluation was performed by Student's t-test for paired observations. For all comparisons $p<0.05$ was regarded as significant. The concentration of ISDN that produced a 50 % response of the maximal effect (EC_{50}) was evaluated by linear regression in a dose-response manner by the method of Bowman and Rand (1980).

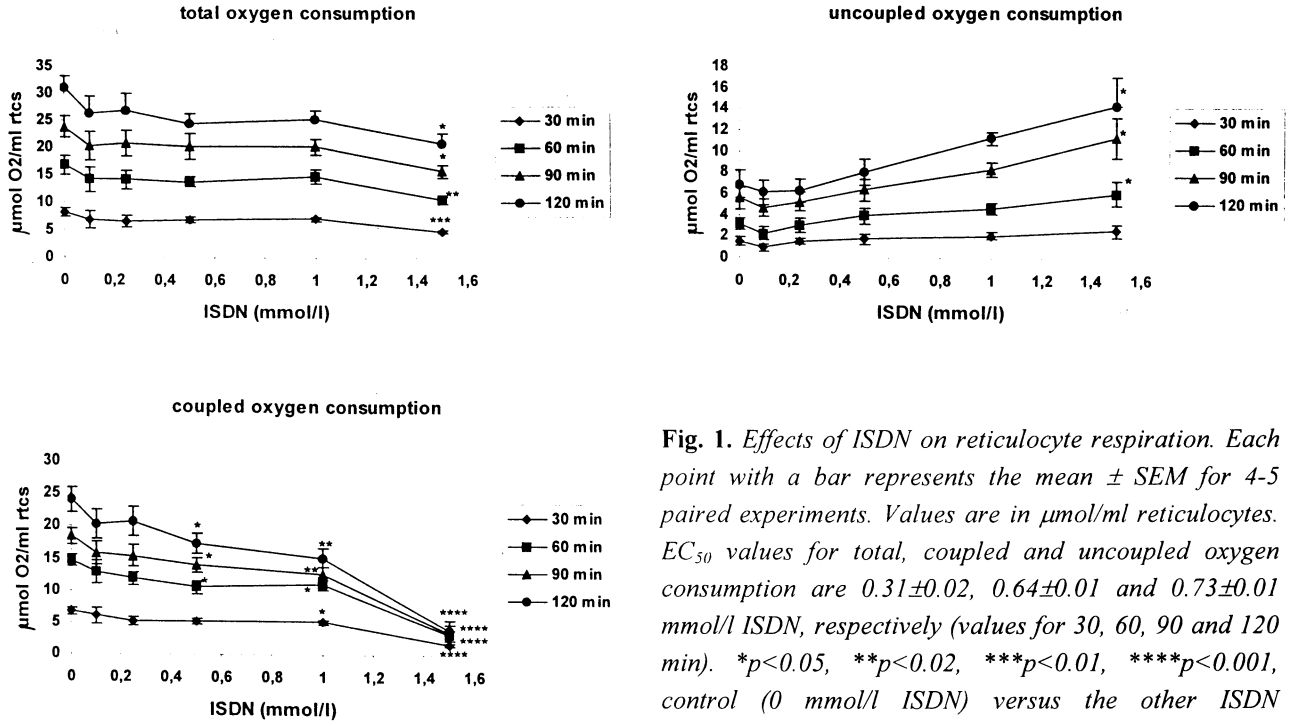

Fig. 1. *Effects of ISDN on reticulocyte respiration. Each point with a bar represents the mean ± SEM for 4-5 paired experiments. Values are in µmol/ml reticulocytes. EC_{50} values for total, coupled and uncoupled oxygen consumption are 0.31±0.02, 0.64±0.01 and 0.73±0.01 mmol/l ISDN, respectively (values for 30, 60, 90 and 120 min). *p<0.05, **p<0.02, ***p<0.01, ****p<0.001, control (0 mmol/l ISDN) versus the other ISDN concentrations.*

Results

Our results indicated that total, coupled and uncoupled oxygen consumption in reticulocyte-rich red blood cell suspensions amount to 30.94±2.16, 24.10±1.94 and 6.84±1.31 µmol/ml reticulocytes/2 h, respectively (Fig. 1). The effects of ISDN appear to depend on the time of incubation and the dose of ISDN. Significant reduction of total and coupled oxygen consumption ($p<0.05$) occurs after 30 min of aerobic incubation in the presence 1.5 and 0.5, 1.0, 1.5 mmol/l ISDN, respectively (Fig. 1). Elevation of uncoupled oxygen consumption ($p<0.05$) occurs after 60 min of aerobic incubation only in the presence of a high concentration of ISDN (1.5 mmol/l) (Fig. 1). The concentrations of ISDN that produce a 50 % response of the maximal effect (EC_{50}) on reticulocyte respiration in 30, 60, 90 and 120 min were for total oxygen consumption: 0.34, 0.35, 0.33 and 0.23; for coupled oxygen consumption: 0.67, 0.66, 0.65 and 0.60 and for uncoupled oxygen consumption: 0.73, 0.72, 0.77 and 0.69 mmol/l ISDN, respectively. These values indicate that total (EC_{50} = 0.31±0.02 mmol/l ISDN; for all times; Fig. 1) and coupled oxygen consumption (EC_{50} = 0.64±0.01 mmol/l ISDN) were more sensitive to ISDN influence than uncoupled oxygen consumption (EC_{50} = 0.73±0.01 mmol/l ISDN).

Table 1. Effects of ISDN on glucose consumption and lactate accumulation in rat reticulocytes after 2 h of aerobic incubation.

ISDN (mmol/l)	Glucose consumption (μmol/ml cells/2 h)	Lactate accumulation (μmol/ml cells/2 h)
0	7.01±1.63	7.11±0.40
0.1	8.41±0.70	7.39±0.77
0.25	8.34±0.53	7.96±0.89
0.5	9.46±0.64	8.18±0.36
1.0	8.27±1.37	8.91±0.40*
1.5	16.40±1.91***	16.71±2.60***

*Values represent mean ± S.E.M. for 4-5 paired experiments. EC_{50} values for glucose consumption and lactate accumulation are 0.95 and 1.0 mmol/l ISDN, respectively. *p<0.05, ***p<0.01, control (0 mmol/l ISDN) versus the other ISDN concentrations.*

Table 2. Effects of ISDN on glycolytic intermediate levels in rat reticulocytes.

	ISDN (mmol/l) 0	0.1	0.25	0.5	1.0	1.5
G6P	192.9±9.6	220.7±9.1	221.2±31.1	198.4±7.9	165.9±22.2	146.7±14.6
F6P	30.9±2.8	33.2±2.0	42.8±4.3	30.6±3.7	29.1±4.8	27.8±2.5
FDP	86.2±24.4	80.7±8.1	77.4±11.7	75.3±7.5	90.9±9.1	77.6±22.0
TP	97.7±8.5	90.1±9.0	60.8±6.1	65.1±6.5	93.0±9.3	87.9±7.6
2,3-DPG	7.1±0.7	7.2±0.5	6.7±0.2	7.0±0.4	6.8±0.3	6.5±0.5
3PG	66.0±1.8	91.7±12.6	101.0±8.9*	104.9±14.5	137.2±22.7*	147.8±21.6*
2PG	25.5±9.7	27.8±7.2	36.0±2.8	25.8±3.5	28.3±1.4	18.4±0.5
PEP	38.1±6.3	53.7±1.1	45.0±3.5	70.9±6.0	77.3±4.2***	75.1±8.1***
PYR	124±33	141±47	260±27	247±26	609±62**	2026±170***
LAC	9.44±0.36	9.81±1.54	10.10±1.19	10.38±0.49	11.23±0.45*	22.65±2.57***

*Values represent mean ± S.E.M. 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. *p<0.05, **p<0.02, ***p<0.01, control (0 mmol/l ISDN) versus the other ISDN concentrations.*

Reduction of coupled oxygen consumption indicates that the energy production (ATP) in the oxidative phosphorylation (OxP) is reduced. The rate of glycolysis is increased when the process of OxP is inhibited, which represents an example of the Pasteur effect (Rapoport 1986). Namely, glucose consumption and lactate accumulation during 2 h of aerobic incubation (7.01±1.63 and 7.11±0.40 μmol/ml cells/2 h, respectively) were significantly increased in the presence of 1.0 and 1.5 mmol/l ISDN ($p<0.05$) (Table 1). In addition, EC_{50} of ISDN for coupled oxygen consumption and glycolysis (0.64±0.01 and 1.00 mmol/l, respectively) indicate that mitochondrial respiration was more sensitive to ISDN than glycolysis. The levels of all glycolytic intermediates are shown in Table 2. The increased level

of pyruvate ($p<0.01$) indicates that pyruvate kinase (PK) activity is enhanced.

ISDN did not influence adenine nucleotide values, except for a slight increase of ADP and AMP levels ($p>0.05$) (Table 3).

According to our results obtained in rat reticulocytes (rtcs) under steady-state conditions, 95.4 % of overall energy was produced by OxP (73.25±4.65 μmol ATP/ml rtcs/h), but only 4.6 % by glycolysis (3.55±0.20 μmol ATP/ml rtcs/h) (Table 4). The ATP level was 1.52±0.10 μmol/ml reticulocytes and a mean turnover time of 1.19 min was calculated (Table 4). Due to the decrease of coupled oxygen consumption in the presence of ISDN, ATP production *via* OxP was significantly diminished ($p<0.05$; EC_{50} = 0.66 mmol/l). The simultaneous increase of glycolytic ATP production ($p<0.05$; EC_{50} = 1.00 mmol/l) is not enough to maintain a constant ATP production. Calculated mean ATP turnover time was prolonged by 199 % in the presence of 1.5 mmol/l ISDN (Table 4), which indicates that ATP consumption in ISDN-treated reticulocytes is inhibited.

Table 3. Effects of ISDN on adenine nucleotide (ATP, ADP, AMP) levels in rat reticulocytes.

ISDN (mmol/l)	ATP (μmol/ml cells)	ADP (μmol/ml cells)	AMP (μmol/ml cells)	TAN (μmol/ml cells)
0	1.52±0.10	0.115±0.014	0.023±0.004	1.43±0.12
0.1	1.31±0.16	0.110±0.028	0.025±0.017	1.35±0.18
0.25	1.38±0.23	0.114±0.016	0.027±0.021	1.48±0.25
0.5	1.49±0.20	0.115±0.009	0.026±0.013	1.41±0.21
1.0	1.41±0.17	0.154±0.019	0.034±0.023	1.61±0.15
1.5	1.41±0.03	0.179±0.036	0.043±0.023	1.66±0.21

TAN - total adenine nucleotides; Values represent mean ± S.E.M. for 4-5 paired experiments. Values are in μmol/ml cells.

Table 4. Effects of ISDN on energy production, ATP concentration and ATP turnover time in rat reticulocytes.

ISDN (mmol/l)	OxP ATP prod. (μmol/ml rtcs/h)	Glyc ATP prod. (μmol/ml rtcs/h)	Total ATP prod. (μmol/ml rtcs/h)	ATP conc. (μmol/ml rtcs)	ATP turnover (times/h)	(min)
0	73.25±4.65	3.55±0.20	76.80±2.42	1.52±0.10	50.53	1.19
0.1	64.45±8.95	3.69±0.38	68.14±4.66	1.31±0.16	52.01	1.15
0.25	59.35±5.10	3.98±0.44	63.33±2.77	1.38±0.28	45.89	1.31
0.5	52.75±5.05	4.09±0.18	56.84±2.61***	1.49±0.20	38.15	1.57
1.0	55.65±5.25*	4.45±0.20*	60.10±2.72****	1.41±0.17	42.62	1.41
1.5	15.40±3.85****	8.35±0.89***	23.75±2.37****	1.41±0.03	16.84	3.56

*Values represent means ± S.E.M. for 4-5 paired experiments. ATP production in reticulocytes is the sum of that provided by oxidative phosphorylation (OxP ATP production, calculated on the basis of P/O = 2.5) and glycolysis (Glyc ATP production, calculated on the basis of lactate/ATP = 1) (Siems et al. 1982). ATP turnover time is calculated on the basis of the ratio between ATP production and ATP concentration (Siems et al. 1982). EC_{50} values for OxP ATP production, Glyc ATP production and Total ATP production are 0.66, 1.00 and 0.63 mmol/l ISDN, respectively. *$p<0.05$, ***$p<0.01$, ****$p<0.001$, control (0 mmol/l ISDN) versus the other ISDN concentrations.*

Discussion

In this work we analyzed the effects of isosorbide dinitrate, a NO donor, on energy metabolism of rat reticulocytes. Our interest in oxidative phosphorylation (OxP) and glycolysis, processes involved in ATP production of reticulocytes, is based on the results of other authors, who showed NO-induced alteration of OxP and glycolysis (Drapier and Hibbs 1986, 1988, Dimmeler *et al.* 1992, Brüne *et al.* 1994, Brown 1995, 1997, Mallozzi *et al.* 1996, 1997). On the other hand, the reticulocyte is a stage of maturation of erythroid cells well defined by morphological and biochemical criteria. In addition, these cells are characterized by the absence of the nucleus in mammals, and also by the presence of functional mitochondria and glycolysis (Rapoport 1986), which make these cells an excellent experimental model for the determination of processes involved in energy metabolism. However, the high content of hemoglobin in these cells may serve as an effective scavenger of NO (Wennmalm *et al.* 1992) liberated by ISDN. What is happening in reticulocytes in the presence of experimental doses of ISDN?

Our results showed that reticulocyte respiration is significantly altered by ISDN in a dose- and time-dependent manner. There was a decrease of total and coupled oxygen consumption (EC_{50} = 0.31±0.02 and 0.64±0.01 mmol/l) and an increase of uncoupled oxygen consumption (EC_{50} = 0.73±0.01 mmol/l). The inhibition of total and coupled oxygen consumption by ISDN was probably a consequence of NO-induced inhibition of proteins with iron-sulfur centres (complex I and II) (Drapier and Hibbs 1986, 1988) and hem protein – cytochrome oxidase (Brown 1995, 1997, Torres *et al.* 1998) in the mitochondrial respiratory chain. However, free oxygen radicals are generated due to mitochondrial activity (Sies 1991, Blasig *et al.* 1996, Keller *et al.* 1998), as well as the superoxide anion radical ($O_2^{\cdot-}$) which is formed in mitochondria under the influence of NO (Poderoso *et al.* 1996, Giulivi *et al.* 1998). Hence, NO liberated from ISDN reacts with $O_2^{\cdot-}$ to produce peroxynitrite ($ONOO^-$) (Crow and Beckman 1995), which irreversibly inactivates mitochondrial electron transport (at complex I-III) and influences ATP-synthase (Deckers-Hebestreit *et al.* 1987, Inoue *et al.* 1996, Moro *et al.* 1996). Besides NO, peroxynitrite also contributes to the inhibition of reticulocyte respiration with consequent inhibition of OxP and mitochondrial ATP production. Furthermore, hydrogen peroxide (H_2O_2), a product of superoxide anion inactivation by superoxide dismutase, inhibits, but only in high concentrations (2 and 5 mmol/l) reticulocyte respiration and increases the rate of glycolysis (Maletić *et al.* 1997). The results in this study were in accordance with earlier findings of Richter and coworkers (Richter *et al.* 1994, Schweizer and Richter 1994), which indicated that NO at low concentrations can potently deenergize isolated liver and brain mitochondria at oxygen concentrations that prevail in cells and tissues.

Uncoupled oxygen consumption in reticulocytes was a consequence of lipoxygenase-dependent oxygenation of unsaturated fatty acids, hemoglobin and glutathione autooxidation and of NADH-cytochrome b_5 reductase, NADPH-oxidase and catalase activities (Rapoport 1986). The augmentation of these processes in the presence of NO, a free oxygen radical compound, probably induced an increase of uncoupled oxygen consumption in ISDN-treated rat reticulocytes.

The reduction of coupled oxygen production was followed by enhanced glycolysis, as measured by lactate accumulation and glucose consumption. However, even a 2.35-fold stimulation of reticulocyte glycolysis (in the presence of 1.5 mmol/l ISDN), providing 35 % of total energy production, was not sufficient to compensate the decreased energy production due to inhibition of oxidative phosphorylation. What is the reason for the stimulation of glycolysis in ISDN-treated reticulocytes? The levels of all glycolytic intermediates and application of the "cross-over" theorem (Rapoport 1986) indicate that PK activity is stimulated. Inhibition of OxP, stimulation of glycolysis and simultaneous rise of ADP and AMP levels, all together represent an example of the Pasteur effect (Rapoport 1986) occurring in ISDN-treated reticulocytes. However, ISDN dose-dependently increased the glycolytic rate in mature erythrocytes (Maletić and Kostić, unpublished data), which is in accord with the results of Mallozzi *et al.* (1996, 1997) that indicate NO-induced phosphorylation of band 3 and consequently stimulation of GA3PDH activity. These data indicate that ISDN itself stimulates glycolysis, but not only through the Pasteur effect which could not be induced in mature erythrocytes. In addition, our data showed that NO released by ISDN did not influence GA3PDH, while Dimmeler *et al.* (1992), Brüne *et al.* (1994) and Mohr *et al.* (1996) showed NO-induced ADP-ribosylation and inhibition of GA3PDH.

According to the present results obtained in rat reticulocytes under steady-state conditions, 95.4 % of overall energy was produced by OxP, but only 4.6 % by glycolysis. Due to the decreased coupled oxygen consumption in the presence of ISDN, ATP production *via* OxP was significantly diminished. The concomitant increase of glycolytic ATP production is not enough to ensure constant ATP synthesis. The calculated mean ATP turnover time was prolonged by 199 % in the presence of 1.5 mmol/l ISDN, which indicates an inhibition of ATP consuming processes, namely globin synthesis, Na^+,K^+-ATPase activity and proteolysis (Rapoport 1986, Kostić and Živković 1994, Stojadinović *et al.* 1996) in ISDN-treated reticulocytes. Recent studies have shown that NO-donors inhibit Na^+,K^+-ATPase activity (Sato *et al.* 1995) and H^+-ATPase (Tojo *et al.* 1994) in brain cells. Furthermore, NO inhibited the hemoglobin expression in the K562 erythroleukemic cell line (Rafferty *et al.* 1996).

The results presented in this study have shown ISDN (NO)-induced alterations in energy metabolism of rat reticulocytes. However, reticulocytes have a high content of hemoglobin (Rapoport 1986), an effective scavenger of NO (Feelisch and Noack 1987, Wennmalm *et al.* 1992). Wennmalm *et al.* (1992) reported in healthy human donors that the incubation of plasma or whole blood (both venous or artetrialized) with NO resulted in the formation of methemoglobin, nitrosyl hemoglobin, nitrite and nitrate, i.e. inactivated forms of NO. However, NO reacts with SH groups of hemoglobin to form S-nitrosohemoglobin under physiological conditions (Crow and Beckman 1995, Jia *et al.* 1996, Gow and Stamler 1998, Pawloski *et al.* 1998). There was a possibility that NO liberated from ISDN reacted with hemoglobin and produced S-nitrosohemoglobin, which again liberated NO and preserved the NO-induced effects on energy metabolism of rat reticulocytes.

On the other hand, the metabolic effects of ISDN were not mimicked by 8-Br-cGMP (exogenous analogue of cGMP), a second messenger of NO (Feelisch and Noack 1987, Murad 1994, Hampl *et al.* 1995, Kostić *et al.* 1996), except for the slight, dose-independent inhibition of OxP which was not accompanied by any changes of 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 $NaNO_3$ (nitrites and nitrates), which are secondary products of NO in aerobic aqueous solutions (Feelisch 1991, Lewis and Deen 1994, Fukuto 1995), did not alter OxP and glycolysis in rat reticulocytes (Maletić and Kostić 1999). In addition, reticulocytes synthesized cytochrome P450 (Patil *et al.* 1992) and glutathione-S-transferase (Schroder *et al.* 1996, Vodela and Dalvi 1997). These enzymes are involved in the biotransformation of organic nitrates to active compound of nitric oxide (Tsuchida *et al.* 1990, Schröder 1992). Kosaka *et al.* (1996) showed that the major metabolic activity of ISDN in the blood is present in red blood cells.

On the basis of the implications emerging from our study, we have concluded that ISDN a) inhibited total and coupled respiration and stimulated uncoupled respiration, b) enhanced glycolysis, c) decreased ATP production and d) prolonged ATP turnover time in rat reticulocytes. These effects were mediated by NO as an effector molecule.

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