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METABOLISM OF SIN-1, PEROXYNITRITE DONOR IN RAT RED BLOOD CELLS

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The aim of this study is elucidation of the molecular mechanism of the 3-morpholino-sydnonimine-hydrochloride (SIN-1) action, particularly its metabolism in rat erythrocytes and reticulocytes. SIN-1 is active metabolite of molsidomine, an established vasodilator drug, clinically used in the treatment of coronary artery disease. Rats erythrocyte and reticulocyte-rich suspensions were aerobicaly incubated without (control) or in the presence of SIN-1 (0.1, 0.25, 0.5, 1.0 and 1.5 mM). The concentrations of reactive nitrogen species (RNS),

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reactive oxygen species (ROS) and parameters that indicate oxidative damage of red blood cells were determined after incubation. In rat erythrocytes SIN-1 in high doses increased concentrations of nitrite (NO^+ ion indicator), hydroxylamine (NO^- ion indicator) and 3-nitrotyrosine (peroxynitrite indicator). Concentration of superoxide anion radical ($O_2^{\cdot}$) increased in the presence of SIN-1 on dose-dependent way (except in the presence of higher applied dose), while level of hydrogen peroxide (H_2O_2) did not alter. In rat reticulocytes SIN-1 significantly increased 3-nitrotyrosine concentrations only. In addition, low doses of SIN-1 induced decrease of $O_2^{\cdot}$ level, while concentration of H_2O_2 increased dose-dependently. These data indicate that SIN-1-induced nitrosative and oxidative stress in rat red blood cells. The strong oxidative damage of erythrocytes and reticulocytes (increased level of methemoglobin, Heinz bodies and lipid peroxides concentrations) are appeared as consequence of SIN-1-induced oxidative stress.

Key words: erythrocytes, oxidative damage, oxidative stress, reticulocytes, SIN-1

INTRODUCTION

Corresponding to their intermediate position in the differentiation program the reticulocytes do not possess full range of metabolic pathways of proliferating cells, but they are still equipped with a set of metabolic pathways (corresponding to presence of mitochondria and ribosomes), most of which are lost during their transition to the mature erythrocytes (RAPOPORT, 1986). Reactive oxygen species (ROS) and their derivatives are present in living tissues at low but measurable concentrations, which are determined by the balance between the rates of radical production and their corresponding rates of clearance (DROGE, 2002). In conditions of disturbed prooxidant-antioxidant balance in favour of the prooxidants leading to potential damage of cells the oxidative stress occurs (SIES, 1991).

Nitric oxide (NO) is a small hydrophobic molecule with chemical properties which make it uniquely suitable for both intra- and intercellular messenger. In the reactions with $O_2^{\cdot}/O_2$, NO generated reactive nitrogen species (RNS) which affected almost every molecule in cells (WINK and MITCHELL, 1998). In addition, NO reacts with oxy- and deoxyhemoglobin in erythrocytes, generating both methemoglobin (MetHb) and nitrozylhemoglobin (NOHb), respectively (PAWLOSKI and STAMLER, 2002). Due to these reactions NO is inactivated. The third kind of reactions between NO and Hb included SH-groups of globin chains forming S-nitrosohemoglobin (SNOHb) the form that retained NO-regulator function (PAWLOSKI and STAMLER, 2002).

Diverse and important physiological roles of NO implicate that exogenous donation of NO may be useful in the treatment of some disease states. 3-morpholino-sydnonimine-hydrochloride (SIN-1) is active metabolite of molsidomine a drug clinically used in the treatment of coronary artery disease

(FEELISCH *et al.*, 1989; REDEN, 1990). Investigation of molsidomine/SIN-1 action indicates that molecular oxygen initiates NO formation through a one-electron abstraction from the intermediate. SIN-1 has also been shown to liberate superoxide anion radicals ($O_2^{\cdot-}$) concomitantly with NO, which rapidly react to form peroxynitrite (FEELISCH *et al.*, 1989; REDEN, 1990).

The aim of this study is further elucidation of the molecular mechanism of the SIN-1 action, particularly its metabolism in rat erythrocytes and reticulocytes.

MATERIAL AND METHODS

In this study erythrocyte and reticulocyte-rich red blood cell suspensions of male 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 anaesthetized by ether and blood was taken by exanguination. Reticulocytes amounted to 86.57 ± 1.28 %. Three times washed red blood cells were resuspended in 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°C (KOSTIĆ *et al.*, 1990). Cell suspensions (final hematocrit value about 0.20) were aerobically incubated for 2 hours without (control), or in the presence of different concentrations of SIN-1: 0.1, 0.25, 0.5, 1.0 and 1.5 mM. The SIN-1 was added at the beginning of incubation (0 min). Samples extractions were carried out after incubation.

Concentrations of RNS and ROS were determined in L-Arg extracts: ½ vol 3 M perchloacetic acid and 2 vol 20 mM EDTA were added to 1 vol cell suspension. After extraction on ice (15 min) and centrifugation 4 min / 15000 rpm, extracts were neutralized by 2 M K_2CO_3. Spectrophotometric determination of nitrites (NO^+ indicator), (GREEN *et al.*, 1982), hydroxylamine (NO^-indicator), (ARNELLE and STAMLER, 1996), 3-nitrotyrosine (peroxynitrite indicator), (RIORDAN and VALLEE, 1972), superoxide anion radical ($O_2^{\cdot-}$),(AUCLAIR and VOISIN, 1985) and hydrogen peroxide (H_2O_2) concentrations (PICK and KEISARI, 1980) were performed in L-Arg extracts.

The followed parameters indicated oxidative damage of red blood cells were determined. Concentrations of methemoglobin (MetHb), (HEILMEYER, 1943) and Heinz bodies (HB), (BATES and WINTERBOURN, 1984) in cells suspensions by spectrophotometric techniques. Lipid peroxide levels were measured on the basis of lipid peroxidation products (malondialdehydes) reaction with thiobarbituric acid (thiobarbituric acid reactive substances- TBARS), (OHKAWA *et al.*, 1979).

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

RESULTS AND DISCUSSION

Results presented in Fig. 1 showed that SIN-1 in high doses increased concentrations of nitrite (NO^+ ion indicator), hydroxylamine (NO^- ion indicator) and 3-nitrotyrosine (peroxynitrite indicator) in rat erythrocytes. These data indicate that SIN-1 spontaneously metabolized in NO-species, which is in accordance with literature data (FEELISCH *et al.*, 1989; REDEN, 1990). In addition, there is clear that peroxynitrite is not dominant product of SIN-1 metabolism in rat erythrocytes. Experimental doses of SIN-1 (0.1 - 1.5 mM) generated high flux of NO which is in reactions with O_2^-/O_2 generated RNS according to WINK and MITCHELL (1998).

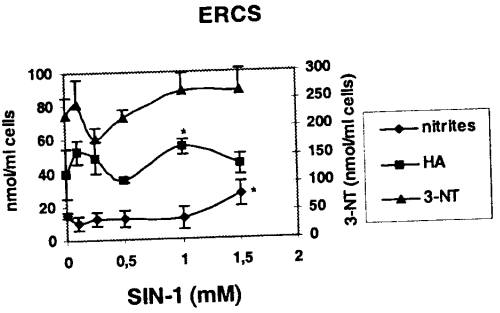

Fig. 1. - Alterations of nitrite, hydroxylamine, S-nitrosothiols and 3-nitrotyrosine concentrations in the presence of SIN-1 in rat erythrocytes. Values represent mean ± SEM for 4 experiments. *p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

Concentration of O_2^- increased in the presence of SIN-1 on dose-dependent way (except in the presence of higher applied dose) as a consequence of O_2^- liberation from SIN-1 (FEELISCH *et al.*, 1989; REDEN, 1990) or inhibition of superoxide dismutase (SOD) an antioxidant enzyme which dismutate O_2^- to H_2O_2. Level of H_2O_2 did not alter in the presence of SIN-1 which is in accordance with the last hypothesis (Fig. 2).

The presented results indicate that SIN-1-induced nitrosative and oxidative stress in rat erythrocytes. These conditions were followed by strong oxidative damage of erythrocytes.

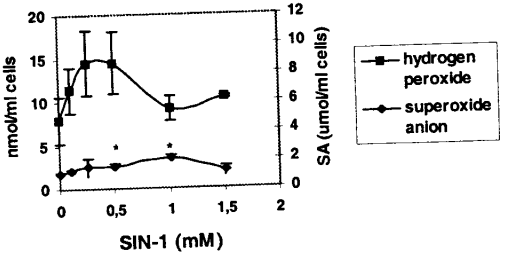

Fig. 2. - Alterations of superoxide anion radical and hydrogen peroxide concentrations in the presence of SIN-1 in rat erythrocytes. Values represent mean ± SEM for 4 experiments. Values for superoxide anion are in μmol/ml cells. *p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

Data presented in Table 1. showed a dose-dependent increase of methemoglobin, Heinz bodies and lipid peroxides concentrations. The

methemoglobin generation is consequence of primary reaction of NO (liberated from SIN-1) with hemoglobin (PAWLOSKI and STAMLER, 2002), as well as hemoglobin oxidation by $O_2^{\cdot-}$ (STERN, 1989) or peroxynitrite (ALAYASH *et al.*, 1998), both of them also liberated from SIN-1. Increased lipid peroxidation in erythrocytes is probably the consequence of toxic effects of all RNS and ROS liberated from SIN-1 (DROGE, 2002).

Table 1. - Alterations of methemoglobin, Heinz bodies and lipid peroxides concentrations in the presence of SIN-1 in rat erythrocytes. Values represent mean ± SEM for 4 experiments

	SIN-1 (mM)					
	0	0.1	0.25	0.5	1.0	1.5
TBARS nmol/ml cells	6.3 ± 0.6	7.9 ± 0.9	8.7 ± 0.6	10.6 ± 1.9*	11.9 ± 4.0*	15.2 ± 4.0*
MetHb %	9.3 ± 2.2	10.4 ± 2.3*	12.1 ± 2.0*	12.9 ± 1.5*	16.3 ± 0.9*	22.4 ± 1.9*
HB	43.3 ± 8.9	47 ± 8.5*	49 ± 8.7*	46 ± 9.4*	49 ± 8.3*	55.3 ±7.7*

*p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

In rat reticulocytes SIN-1 significantly increased only 3-nitrotyrosine concentrations (Fig. 3).

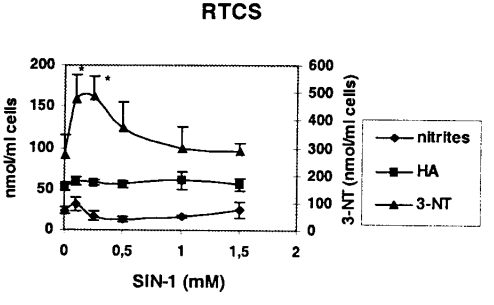

Fig. 3. - Alterations of nitrite, hydroxylamine and 3-nitrotyrosine concentrations in the presence of SIN-1 in rat reticulocytes. Values represent mean ± SEM for 4 experiments. *p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

In addition, low doses of SIN-1 induced decrease of $O_2^{\cdot-}$ level (Fig. 4). This results together with unaltered nitrite level indicate peroxynitrite generation (FEELISCH *et al.*, 1989; REDEN, 1990). These data indicate that SIN-1 dominatly metabolised in peroxynitrite in rat reticulocytes. On the other hand, decrease of $O_2^{\cdot-}$ level followed by dose-dependent increase of H_2O_2 concentration, may be also a consequence of SOD eficacy.

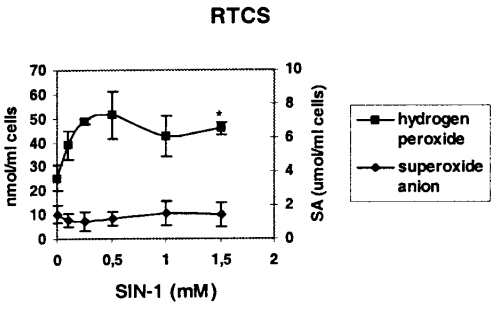

Fig. 4. - Alterations of superoxide anion radical and hydrogen peroxide concentrations in the presence of SIN-1 in rat reticulocytes. Values represent mean ± SEM for 4 experiments. Values for superoxide anion are in ?mol/ml cells. *p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

The nitrosative and oxidative stress induced with SIN-1 were followed by strong oxidative damage of reticulocytes (Table 2). The increase of methemoglobin, Heinz bodies and lipid peroxides concentrations have the same explanation as in erythrocytes.

Table 2. - Alterations of methemoglobin, Heinz bodies and lipid peroxides concentrations in the presence of SIN-1 in rat reticulocytes. Values represent mean ± SEM for 4 experiments

	SIN-1 (mM)					
	0	0.1	0.25	0.5	1.0	1.5
TBARS nmol/ml cells	8.5 ± 0.9	11.2 ± 1.7*	10.9 ± 1.5	11 ± 1.7*	13.2 ±3.1*	15.2 ± 4.9*
MetHb %	57.4 ± 1.9	58.4 ± 3.6	58.8 ± 2.2	59.9 ± 2.6	64.4 ±3.3*	70.9 ± 3.2*
HT	539 ± 45	516 ± 44	541 ± 41	529 ± 44	543 ± 49	565 ± 44*

*p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

Our previous study has been showed that molsidomine-induced inhibition of the process of oxidative phosphorylation in rat reticulocyte mitochondria and stimulation of the process of glycolysis in rat reticulocytes (MALETIĆ *et al.*, 1999) and erythrocytes (MALETIĆ *et al.*, 2000). Data presented in this study indicate that molsidomine-induced alteration of energy production in rat red blood cells are mediated by RNS and ROS liberated from SIN-1 as reactive metabolite of molsidomine.

On the basis of obtained data presented in this study, we can conclude that SIN-1 spontaneously liberated RNS in rat erythrocytes and reticulocytes. In addition, applied experimental doses of SNP induced strong nitrosative and oxidative stress in these cells.

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METABOLIZAM SIN-1 (DONOR PEROKSINITRITA) U CRVENIM KRVNIM ĆELIJAMA PACOVA

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Izvod

Cilj ovog rada je da se objasne molekularni mehanizmi delovanja 3-morfolino-sidnonimin-hidrohlorida (SIN-1) sa posebnim akcentom na metabolizam ovog leka u eritrocitima i retikulocitima pacova. SIN-1 je aktivni metabolit molsidomina, leka koji se klinički koristi u terapiji bolesti koronarnih arterija. Suspenzije eritrocita i crvenih krvnih ćelija bogate retikulocitima su aerobno inkubirane bez (kontrola) ili u prisustvu različitih koncentracija SIN-1 (0.1, 0.25, 0.5, 1.0 i 1.5 mM). Koncentracije reaktivnih vrsta azota (RNS) i reaktivnih vrsta kiseonika (ROS), kao i koncentracije parametara koji ukazuju na oksidaciona oštećenja crvenih krvnih ćelija, određivane su nakon inkubacije. U eritrocitima pacova, SIN-1 samo u visokim dozama povećava koncentracije nitrita (indikator NO^+ jona), hidroksilamina (indicator NO^- jona) i 3-nitrotirozina (indikator peroksinitrita). Koncentracija superoksid anjon radikala ($O_2^{\cdot-}$) je povećana na dozno-zavisan način (osim u prisustvu maksimalne primenjene doze SIN-1), dok se nivo vodonik peroksida (H_2O_2) ne menja. U retikulocitima pacova SIN-1 značajno povećava samo koncentraciju 3-nitrotirozina. Niske doze SIN-1 indukuju smanjenje $O_2^{\cdot-}$ nivoa, dok koncentracije H_2O_2 rastu na dozno-zavisan način. Na osnovu iznetih podataka, možemo zaključiti da SIN-1 indukuje nitrozacioni i oksidacioni stres u crvenim krvnim ćelijama pacova. Ovakvo stanje je posledično praćeno snažnim oksidacionim oštećenjem eritrocita i retikulocita (povećanjem koncentracija methemoglobina, Heinz-ovih telašaca i lipidnih peroksida).

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