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# EFFECT OF SIN-1 ON ANTIOXIDATIVE DEFENCE SYSTEM IN RED BLOOD CELLS OF RATS

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The main objective of this research is to establish the effect of 3-morpholino-sydnonimine-hydrochloride (SIN-1) on antioxidative defense system (AOS) in rat red blood cells. 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 non-enzymatic components and activities of enzymatic component of AOS were determined after incubation. In rat erythrocytes SIN-1 did not alter vitamin C (Vit C), while induced increase of vitamin E (Vit E) concentrations. Unaltered concentration of reduced glutathione (GSH) following by decrease of oxidized glutathione (GSSG) resulted in decrease of reduction potential of this redox couple (decrease of GSSG/2GSH ratio). These data indicate that GSH redox couple is

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included in defence of rat erythrocytes from oxidative damage. Activities of all AOS enzyme did not altered in the presence of SIN-1 in rat erythrocytes. In reticulocytes a high doses of SIN-1 induced a decrease of Vit C and increase of Vit E concentrations, indicating defence role of antioxidative vitamins. Level of GSH redox couple is not changed. SIN-1 inhibited activity of manganese containing superoxide dismutase (Mn SOD) which was followed by stimulation of copper zinc containing superoxide dismutase (CuZn SOD) activity. SIN-1 did not alter activities of other AOS enzyme in rat reticulocytes.

*Key words:* antioxidative defense system, erythrocytes, reticulocytes, SIN-1

## INTRODUCTION

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. Relatively high intracellular concentrations of glutathione (GSH) and the other antioxidative compounds provide a strong basal scavenging capacity (DROGE, 2002). However, 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 $O_2^{\cdot-}$ / $O_2$ nitric oxide generated reactive nitrogen species (RNS) which affected almost every molecule in cells (WINK and MITCHELL, 1998). According to literature data GSH is included in NO metabolism (HOGG, 2002). Generally, GSH-dependent enzymes of antioxidative defense system (AOS) have SH-groups in active centers. NO-species in the primary reaction with SH-groups inactivated activities of this enzyme (BECKER *et al.*, 1998). Results of NIKETIĆ *et al.* (1999) showed that NO did not affect copper zinc containing superoxide dismutase (CuZn SOD), while it inhibited manganese containing superoxide dismutase (Mn SOD) activity with liberation of $NO^+$ and $NO^-$ ions.

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 and vasodilator 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 radical ($O_2^{\cdot-}$) concomitantly with NO, which rapidly react to form peroxynitrite (FEELISCH *et al.*, 1989; REDEN, 1990).

The aim of this research is to establish the effects of SIN-1 on AOS in rat erythrocytes and reticulocytes.

## MATERIAL AND METHODS

Erythrocyte and reticulocyte-rich red blood cell suspensions of male *Wistar* albino rats (weighing 250-350g) 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 animals 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.

Non-enzymatic components of AOS were determined by using spectrophotometric techniques. After saponification and esterification of samples vitamin E (Vit E) level was measured according to DESAI (1984). Vitamin C (Vit C) was assayed according to DAY *et al.* (1979). Levels of reduced glutathione (GSH), (BEUTLER, 1975a) were determined on the basis of GSH oxidation with 5,5-dithio-bis-6,2-nitrobenzoic acid. Concentrations of oxidized glutathione (GSSG) were measured enzymatically by glutathione reductase (BEUTLER, 1975b), after inhibition of GSH oxidation by N-ethylmaleimide.

Activities of AOS enzyme were determined in reticulocyte-rich red blood cells suspension lysates. Activities of: copper zinc containing superoxide dismutase (CuZn SOD) and manganese containing superoxide dismutase (Mn SOD) (MISRA and FRIDOVICH, 1972), catalase (CAT), (BEUTLER, 1982), glutathione peroxidase (GSH-Px), (MARAL *et al.*, 1977), glutathione reductase (GR), (GLATZLE *et al.*, 1974) and glutathione S-transferase (GST), (HABIG *et al.*, 1974) were carried out by standard spectrophotometric techniques.

All values are expressed as mean ± SEM. Statistical evaluation was performed by paired Student's t-test. The value of $p < 0.05$ was taken as the least degree of significance.

## RESULTS AND DISCUSSION

Results presented in Table 1. showed that SIN-1 did not alter Vit C, while induced increase of Vit E concentrations in rat erythrocytes, indicating protective role of Vit E as the main liposoluble antioxidant (PACKER *et al.*, 2001). Unaltered concentration of GSH, following by decrease of GSSG level in the presence of SIN-1 resulted in decrease of reduction potential of GSH redox couple (decrease of GSSG / 2GSH ratio). These data indicate that GSH redox couple (SCHAFER and BUETTNER, 2001) is included in antioxidative defence of rat erythrocytes from SIN-1-induced oxidative damage.

*Table 1. - Effects of SIN-1 on Vit C, Vit E, GSH and GSSG concentrations in rat erythrocytes*

| | SIN-1 (mM) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.1 | 0.25 | 0.5 | 1.0 | 1.5 |
| Vit C Mmol/ml cells | 110 ± 7.3 | 145.6 ±14.7 | 126.1 ±30.3 | 106.3 ± 5.9 | 111.3±12.1 | 132.6±24.2 |
| Vit E µg/ml cells | 6.5 ± 1.2 | 7.5 ± 1.4 | 7.4 ± 0.8 | 7.6 ± 0.9 | 7.4 ± 1.1 | 8.1 ± 1.5 |
| GSH Mmol/ml cells | 2.1 ± 0.6 | 2.9 ± 0.3 | 2.7 ± 0.8 | 1.8 ± 0.3 | 2.7 ± 0.9 | 2.4 ± 0.5 |
| GSSG nmol/ml cells | 0.8 ± 0.1 | 0.6 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.04 | 0.5 ± 0.1 | 0.5 ± 0.05* |
| GSSG/GSH x $10^{-3}$ | 0.14 | 0.11 | 0.10 | 0.16 | 0.10 | 0.10 |

Values represent mean ± SEM for 4 experiments
*p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

Activities of all AOS enzyme did not altered significantly in the presence of SIN-1 in rat erythrocytes (Table 2). However, there is slightly stimulation of CAT, GSH-Px and GR activities.

*Table 2. Effects of SIN-1 on the activites of AOS enzyme in rat erythrocytes*

| | SIN-1 (mM) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.1 | 0.25 | 0.5 | 1.0 | 1.5 |
| SOD x $10^4$ U/ml cells | 6.5 ± 2.3 | 7.6 ± 1.6 | 6.8 ± 2.4 | 6.8 ± 0.8 | 6.9 ± 1.9 | 7.3 ± 1.6 |
| CAT x $10^6$ U/ml cells | 1.9 ± 0.4 | 2.2 ± 0.1 | 2.1 ± 0.4 | 2.0 ± 0.8 | 2.1 ± 0.1 | 2.4 ± 0.4* |
| GSH-Px x $10^3$ U/ml cells | 12.2 ± 3.7 | 17.8 ± 4.6 | 13.6 ± 2.1 | 15.4 ± 3.4 | 11.0 ± 2.2 | 16.9 ± 5.2 |
| GR x $10^5$ U/ml cells | 16.1 ± 5.9 | 26.9 ± 6.7 | 23.2 ± 4.7 | 14.8 ± 8.4 | 23.6 ± 3.6 | 20.5 ± 8.4 |
| GST x $10^3$ U/ml cells | 2.0 ± 0.6 | 1.6 ± 0.6 | 2.1 ± 1.1 | 1.5 ± 0.8 | 1.6 ± 0.8 | 2.1 ± 1.1 |

Values represent mean ± SEM for 4 experiments
*p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

In rat reticulocytes high doses of SIN-1 induced decrease of Vit C and increase of Vit E concentrations (Table 3). These alterations were classical example of antioxidative network of interaction of Vit E and Vit C redox cycle (PACKER *et al.*, 2001) and defence role of antioxidative vitamins. Level of GSH redox couple is not changed.

*Table 3. - Effects of SIN-1 on Vit C, Vit E, GSH and GSSG concentrations in rat reticulocytes*

| | SIN-1 (mM) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.1 | 0.25 | 0.5 | 1.0 | 1.5 |
| Vit C Mmol/ml cells | 159 ± 33.9 | 156.7 ± 37.7 | 127.5 ± 25.5 | 206.3 ± 52.5 | 231.9 ± 38.7 | 83.3 ± 26.2 |
| Vit E µg/ml cells | 2.5 ± 0.7 | 2.1 ± 0.6 | 2.1 ± 0.1 | 2.6 ± 0.7 | 2.9 ±1.4 | 4.0 ± 0.8* |
| GSH Mmol/ml cells | 5.1 ± 1.9 | 5.4 ± 2.1 | 5.4 ± 2.2 | 6.3 ± 3.0 | 5.4 ± 2.1 | 4.9 ± 1.9 |
| GSSG nmol/ml cells | 0.9 ± 0.1 | 0.9 ± 0.1* | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.03 | 0.8 ± 0.2 |
| GSSG/GSH x $10^{-3}$ | 0.10 | 0.08 | 0.09 | 0.07 | 0.09 | 0.09 |

Values represent mean ± SEM for 4 experiments
*p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

As presented in Fig. 1. in rat reticulocytes SIN-1 inhibited the activity of Mn SOD which was followed by stimulation of CuZn SOD activity. Data of NIKETIĆ *et al.* (1999) showed that NO did not affect CuZn SOD activity, while it inhibited Mn SOD activity with liberation of and $NO^-$ ions.

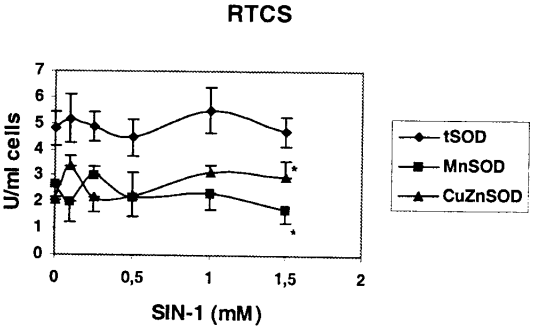


Fig. 1. - Effects of SIN-1 on SOD activity in rat reticulocytes. Values represent mean ± SEM for 4 experiments. *p < 0.05, control (0 mM SIN-1), versus SIN-1 (other concentrations)

However, our results showed SIN-1-induced activation of CuZn SOD activity, which was probably consequence of feedback induction of Mn SOD and CuZn SOD enzymes. SIN-1 did not alter activities of other AOS enzymes in rat reticulocytes (Table 4).

In conclusion, our results suggest that SIN-1 induce oxidative stress in rat red blood cells and mobilized non-enzymatic components of AOS. In reticulocytes SIN-1 inhibited Mn SOD, activity which was followed by stimulation the activity of CuZn SOD.

*Table 4. - Effects of SIN-1 on the activities of AOS enzyme in rat reticulocytes*

| | SIN-1 (mM) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.1 | 0.25 | 0.5 | 1.0 | 1.5 |
| CAT x $10^6$ U/ml cells | 2.5 ± 0.2 | 2.3 ± 0.3 | 2.3 ± 0.2 | 2.4 ± 0.3 | 2.2 ± 0.4 | 1.9 ± 0.3 |
| GSH-Px $^3$ x 10 U/ml cells | 8.4 ± 4.8 | 6.9 ± 3.3 | 9.3 ± 3.2 | 8.1 ± 2.5 | 6.8 ±1.7 | 11.3 ± 4.2 |
| GRU/ml cells | 23.5 ± 0.9 | 24.1 ± 4.6 | 14.4 ± 1.9 | 22.1 ±4.4 | 18.8 ± 2.0 | 19.9 ± 0.9 |
| GST x $10^3$ U/ml cells | 1.6 ± 0.8 | 1.3 ± 0.7 | 1.5 ± 0.5 | 1.3 ± 0.5 | 1.5 ± 0.6 | 2.0 ± 0.8 |

Values represent mean ± SEM for 4 experiments

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

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# EFEKTI SIN-1 NA ANTIOKSIDACIONI ZAŠTITNI SISTEM U CRVENIM KRVNIM ĆELIJAMA PACOVA

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Izvod

Cilj ovog istraživanja bio je da se objasni efekat 3-morfolino-sidnonimin-hidrohlorida (SIN-1) na antioksidacioni zaštitni sistem (AOS) u crvenim krvnim ćelijama pacova. Suspenzije eritrocita i crvenih krvnih ćelija bogate retikulocitima aerobno su inkubirane bez (kontrola) ili u prisustvu različitih koncentracija SIN-1 (0.1, 0.25, 0.5, 1.0 i 1.5 mM). Koncentracije neenzimskih komponenti AOS i aktivnost enzima AOS određivane su nakon inkubacije crvenih krvnih ćelija. U eritrocitima pacova SIN-1 ne menja Vit C, dok povećava koncentraciju Vit E. Nepromenjena koncentracija redukovanog glutationa (GSH) praćena je smanjenjem nivoa oksidovanog glutationa (GSSG) rezultujući smanjenjem redukcionog potencijala ovog redoks para (smanjenje GSSG/2GSH odnosa). Ovi podaci pokazuju da je GSH redoks par uključen u odbranu eritrocita od oksidacionih oštećenja. Aktivnost svih enzima AOS-a nisu bile promenjene u prisustvu SIN-1 u eritrocitima pacova. U retikulocitima pacova SIN-1 u visokim dozama izaziva smanjenje Vit C i povećanje Vit E koncentracije što ukazuje na zaštitnu ulogu antioksidacionih vitamina. Nivo GSH redoks para nije promenjen. SIN-1 inhibira aktivnost Mn SOD što je praćeno stimulacijom aktivnosti CuZn SOD. SIN-1 ne menja aktivnost ostalih enzima AOS-a u retikulocitima pacova.

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