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THE EFFECTS OF HYDROGEN–PEROXIDE ON RAT RETICULOCYTE ENERGY METABOLISM

Snežana D. Maletić, Ljiljana M. Dragićević – Đoković, Ivana Banić, Slađan Z. Pavlović*, Radoslav V. Žikić* and Milosav M. Kostić

Faculty of Medicine, P. O. Box 124, 34000 Kragujevac, Yugoslavia
**Faculty of Science, P. O. Box 60, 34000 Kragujevac, Yugoslavia*

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ABSTRACT. Exogenous hydrogen–peroxide (H_2O_2) in concentrations of 2 and 5 *mmol /l*, but not 0.25 and 1 *mmol/l*, induces significant changes of energy metabolism in reticulocytes of rats. It induces reduction of total and coupled oxygen consumption accompanied by stimulation of glycolysis and increased degradation of adenine nucleotides. Due to inhibition of mitochondrial oxidative phosphorylation appears a significant decline of ATP production. However, simultaneous stimulation of glycolysis (Pasteur effect) is not sufficient to prevent the decline of ATP concentration. The loss of ATP is not compensated with an expected increase of ADP and AMP concentrations, but with an increase of hypoxanthine concentrations. Despite reduced overall ATP production and ATP concentrations, average energy turnover was not significantly changed. Activity of oxidative pentose phosphate shunt, metabolic pathway serving primarily antioxidative function in reticulocytes, was also unchanged under the influence of H_2O_2.

INTRODUCTION

Free radicals are very reactive atoms, ions or molecules, which contain one or more unpaired electrons in exterior orbit. Among free radicals so–called oxygen free radicals, such as superoxide anion radicals ($O_2^{\cdot -}$), hydrigen–peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($1/2\ O_2$) are of particular importance. They are generated in every cell. In erythrocytes and reticulocytes processes of oxidoreduction of haemoglobin, methemoglobin as well as of other iron containing molecules and iron itself represent a permanent source of these reactive species.

Hydrogen–peroxide may be generated by dismutation of superoxide anion catalyzed by superoxide dismutase [1]. Enzymes such as urate–oxidase and L–amino–acid oxidase also generate H_2O_2. In addition, autooxidation of ascorbic acid, glutathione, catecholamines as well as of thiols is accompanied by H_2O_2 production [2,3]. Reduction of H_2O_2 is performed by a series of enzymes like catalase, glutathione peroxidase, cytochrome P_{450} and cytochrome oxidase [4]. In erythrocytes and reticulocytes inactivation of hydrogen–peroxide is regulated mainly by catalase and glutathione–peroxidase. Since H_2O_2 is very potent oxidant, increase of its concentration causes a many harmful effects. In red blood cells increased oxidation of haemoglobin and stimulated formation of methemoglobin, increased gluhathione (GSH) oxidation, stimulation of lipid peroxidation and premature hemolysis in the presence of H_2O_2 have been reported [5].

Processes of energy production, especially oxidative phosphorylation in mitochondria, are usually associated with simultaneous generation of oxygen free radicals [6,7]. However, once formed oxygen free radicals may affect energy production, as well as energy consumption. In endothelial cells and macrophagelike tumor cell line $P388D1$ H_2O_2 decreases NAD and ATP levels [8], increases intracellular concentration of Ca^{2+} (due to Ca, $Mg-ATP$ase inhibition) [9], stimulates oxidative pentose phosphate pathway [10] and decreases phosphorylation of ADP in glycolysis as well as in oxidative phosphorylation [11]. However, about metabolic effects of H_2O_2 in reticulocytes and erythrocytes there are no data. Hence, the aim of this study was to investigate effects of exogenous H_2O_2 on energy metabolism, primarily on energy production in rat reticulocytes.

Materials and methods

In this study reticulocyte–rich red blood cell suspensions of rats were used. Reticulocytosis was induced by phenylhydrazine hydrochloride treatment (35 mg/kg body weight during three days) [12]. After $7-8$ days, when blood was taken by exsanguination, reticulocytosis amounted to $60-80\%$. Three times washed red blood cells were resuspend in incubation buffer containing: 50 $mmol/l$ $Hepes$, 100 $mmol/l$ $NaCl$, 5 $mmol/l$ KCl, 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 ^0C [12]. Cell suspensions (final haematocrit value about 0.20) were incubated aerobically without (control) or in the presence different concentrations of hydrogen–peroxide ($0.25, 1, 2$ and 5 $mmol/l$).

Oxygen consumption was measured by Warburg technique [13]. 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 $\mu mol/l$) resistant oxygen consumption [14].

The aliquots of red blood cells suspensions for extraction of glucose, lactate, adenine nucleotides and hypoxanthine taken at the start and after two hours of aerobic incubation. In neutralized perchloric acid extracts glucose, lactate, ATP, ADP, AMP and hypoxanthine were determined enzymatically by means of spectrophotometric technique [15,16,17,18,19].

Metabolic flux through oxidative pentose phosphate pathway (OPP) was performed as we previously described in details [20].

All values are expressed as mean $\pm$ SEM. Statistical evaluation was performed

by paired Student's-test. The value of $p < 0.05$ was taken as the least degree of significance.

Results

Results of our investigations show that total and coupled oxygen consumption in reticulocyte–rich red blood cell suspensions of rats amount to 16.90 ± 1.10 and 13.05 ± 0.60 $\mu mol/ml\ rtcs/h$, respectively (Tab. 1), which is in accordance with our previous results [12]. Both of them are changed in the presence of H_2O_2 (Tab. 1, Fig. 1). Effects appear to be dependent on the time of incubation and dose of H_2O_2: significant reduction of the total, as well as coupled oxygen consumption occurs after 60 min of incubation and in the presence of 2 and 5 $mmol/l$ H_2O_2, but not with 0.25 and 1.0 $mmol/l$ H_2O_2. Uncoupled oxygen consumption was not significantly altered in the presence of H_2O_2 (Fig. 1).

Table 1. Effects of H_2O_2 on oxygen consumption (ΔO_2) in rat reticulocytes.

H_2O_2 mol/l	Total ΔO_2 ($\mu mol/ml\ rtcs$) 60 min	120 min	Coupled ΔO_2 ($\mu mol/ml\ rtcs$) 60 min	120 min
0.00	16.90 ± 1.10	32.77 ± 1.53	13.05 ± 0.60	23.32 ± 1.11
0.25	16.51 ± 1.50	29.15 ± 2.16	13.09 ± 0.80	22.22 ± 1.58
1.00	15.55 ± 1.48	29.16 ± 2.60	11.87 ± 0.79	23.39 ± 2.06
2.00	14.69 ± 0.80	$29.66 \pm 0.73^*$	$11.37 \pm 0.65^{**}$	$20.98 \pm 0.58^*$
5.00	$11.87 \pm 1.09^{***}$	$26.27 \pm 1.32^{***}$	$8.59 \pm 0.71^{***}$	$18.19 \pm 0.77^{***}$

Means $\pm SEM$ for 4 paired experiments.
$^*p < 0.05$; $^{**}p < 0.025$; $^{***}p < 0.01$.

Glucose consumption and lactate accumulation measured after 2 hours of aerobic incubation of reticulocyte–rich red blood cells suspensions amounted to 8.40 ± 1.21 and 9.54 ± 0.95 $\mu mol/ml$ cells, respectively. Fig. 2 indicates that H_2O_2, in concentrations of $1, 2$ and 5 $mmol/l$, induces significant stimulation of glucose consumption and lactate accumulation in rat reticulocytes. Mean glucose consumption was increased for $8 - 9$ %, while lactate accumulation for $18 - 26$ %, as compared to the control incubations.

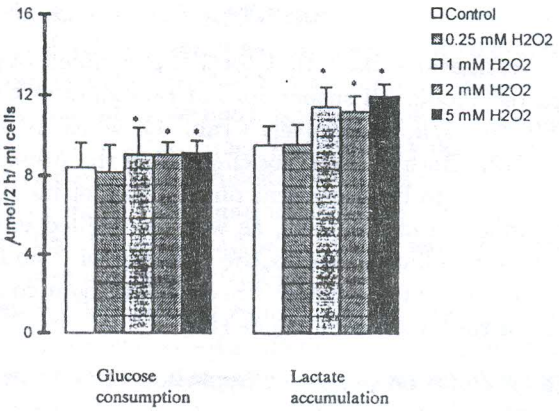

FIG. 1. Effects of H_2O_2 on total, coupled and uncoumpled oxygen consumption in rat reticulocytes after 2 hours of aerobic incubation at 37^0C.
Means $\pm SEM$ from 4 paired experiments; $p < 0.05$; $^{**}p < 0.01$ (as compared to the control) .

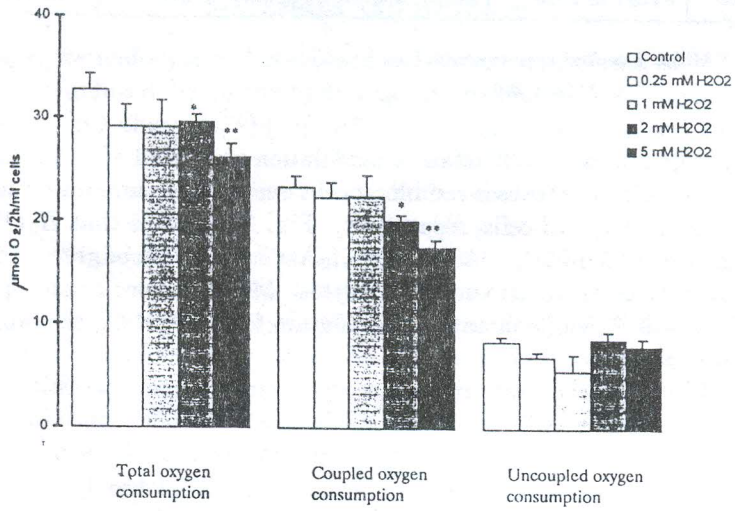

FIG. 2. Effects of H_2O_2 on glucose consumption and lactate accumulation in rat reticulocytes after 2 hours of aerobic incubation at 37^0C.
Means $\pm SEM$ from 5 paired experiments; $^{*}p < 0.05$ (as compared to the control).

Beside reduction of cell respiration and stimulation of glycolysis, H_2O_2 induces marked alterations of energy status of reticulocytes (Fig. 3). Concentration of ATP was decreased for 8 %, 14 % and 24 % in the presence of $1, 2, 5$ $mmol/l$ H_2O_2, respectively. However, significant differences in ADP and AMP levels, except in the presence 5 $mmol/l$ of H_2O_2, were not found. Hence, concentration of total adenine nucleotides (TAN) after 2 hours of incubation was significantly decreased in the presence of 2 and 5 $mmol/l$ H_2O_2 (Fig. 4).

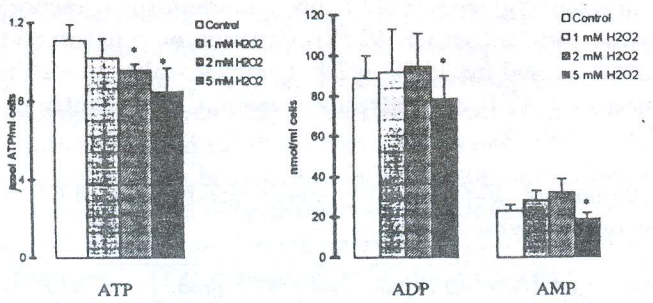

FIG. 3. Effects of H_2O_2 on ATP, ADP and AMP levels in rat reticulocytes.
Means $\pm SEM$ from 5 paired experiments; $^*p < 0.05$ (as compared to the control).

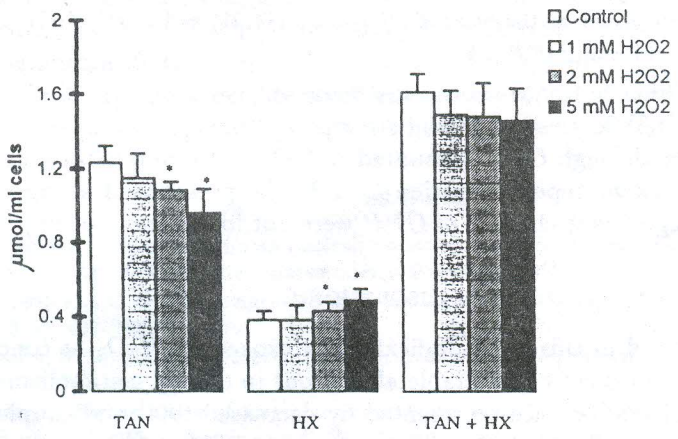

FIG. 4. Effects of H_2O_2 on amount of total adenine nucleotides (TAN, e. g. $ATP + ADP + AMP$), hypoxanthine (HX) and $TAN + HX$ levels in rat reticulocytes.
Means $\pm SEM$ from 5 paired experiments; $^*p < 0.05$ (as compared to the control).

Because H_2O_2–induced decrease of ATP and TAN levels can be the consequence of increased catabolism of adenine nucleotides, we determined concentration of hypoxathine, final product of adenine nucleotide catabolism in red blood cells [23]. Results in Fig. 4 show that concentration of hypoxanthine increases in dose–dependent manner in the presence of H_2O_2. This elevation is sufficient to compensate decrease of TAN level.

Due to decrease of coupled oxygen consumption, ATP production via oxidative phosphorylation was significantly diminished. Results in the Tab. 2 show that simultaneous increase of glycolytic ATP production in the presence of H_2O_2 is not enough to provide constant either ATP production or concentration. Total ATP production was decreased for 19 %. ATP–turnover, calculed on the basis of total ATP production and ATP concentration, was not significantly prolonged in the presence of H_2O_2 (Tab. 2).

TABLE 2. Influence of H_2O_2 on the energy production, ATP level and ATP turnover in rat reticulocytes.

H_2O_2 $mmol/l$	$OxP-ATP$ prod. $\mu mol/ml\ cells/2h$	Glyc–ATP prod. $\mu mol/ml\ cells/2h$	total ATP prod. $\mu mol/ml\ cells/2h$	ATP $\mu mol/ml\ cells/2h$	ATP turnover $times/h$	min
0	116.60 ± 5.55	9.54 ± 0.95	126.14 ± 3.25	1.11 ± 0.08	56.82	1.05
1	116.95 ± 10.3	$11.46 \pm 0.97^*$	128.41 ± 5.63	1.02 ± 0.10	62.94	0.95
2	$104.90 \pm 2.90^*$	$11.24 \pm 0.78^*$	$116.14 \pm 1.84^*$	$0.96 \pm 0.03^*$	60.49	0.99
5	$90.95 \pm 3.85^{**}$	$12.02 \pm 0.60^*$	$102.97 \pm 2.22^{**}$	$0.85 \pm 0.12^*$	60.57	0.99

Means $\pm SEM$ for 4 paired experiments. ATP production in oxidative phosphorylation ($OxP - ATP$ prod.) was calculated on the bases of $P/O = 2.5$ (14, 21) and in glycolysis ($glyc - ATP$ prod.) on the basis of lactate/$ATP = 1$.
$^*p < 0.05$; $^{**}p < 0.01$.

Metabolic flux through OPP amounted to $230 \pm 10\ nmol/h/ml\ rtcs$, which is almost the same as we reported earlier (20). In the presence of all applied H_2O_2 concentrations significant changes of OPP were not found (not shown).

DISCUSSION

Results presented in this study indicate that exogenous H_2O_2 at concentration of 2 and 5 $mmol/l$ causes considerable alterations in energy metabolism of reticulocytes. These alterations are represented by decreased total and coupled oxygen consumption, increased rate of glycolysis and stimulated catabolism of adenine nucleotides. Earlier investigation of Hyslop et al. [11] show that H_2O_2 decreases total and coupled (oligomycin sensitive) oxygen consumption in endothelial and tumor $P388D_1$ cells. Under the same experimental conditions uncoupled (oligomycin resistant) oxygen consumption decreased, while our results show its unsignificant changes (Fig. 1).

Due to decrease of coupled oxygen consumption ATP production via oxidative phosphorylation was lower for 22 % ($p < 0.01$) in the presence of 5 $mmol/l$ H_2O_2

(Tab. 2). According to literature data this decrease of energy production is more probably due to inactivation of ATP–synthase activity, then due to decreased capacity of respiratory chain for coupled transport of electrons [11]. Decrease of oxidative phosphorylation is usually associated with simultaneous increase glycolytic rate, which represents Pasteur effect [21]. Our results show that H_2O_2 stimulates glycolysis (Fig. 2, Tab. 2), but it is not sufficient to provide constancy of ATP production either ATP concentration. Namely, in the presence of H_2O_2 there is decrease of ATP concentration, which is not ADP and AMP concentration (Fig. 3), as found in some other cells [22]. However, our results indicate that H_2O_2 induces increase in concentration of hypoxanthine, which is sufficient to preserve the total amount of purines in reticulocytes (Fig. 4). Similar changes were found in platelets exposed to the exogenous H_2O_2 [23]. Detected elevation in concentration of hypoxanthine indicates increased catabolism of adenine nucleotides was also found in reticulocytes incubated with phenylhydrazine hydrochloride, which is known as an agent acting by stimulation of oxygen free radicals production [24,25].

Decrease of overall ATP production in reticulocytes incubated with H_2O_2 is indicative for decreased ATP consumption under such conditions [14,21]. As a matter of fact, in the previous works reduction of energy consumption due to H_2O_2–induced inhibition of Na, K–ATPase [26] as well as of Ca, Mg–ATPase activity [9] was demonstrated. However, all in all energy turn–over was not significantly altered in reticulocytes incubated with H_2O_2 (Tab. 2).

Oxidative pentose phosphate pathway is also not significantly changed under the influence of such high H_2O_2 concentrations (see Results). This metabolic pathway is very often altered in situations of increased oxidative stress [20]. Unaltered metabolic flux through the OPP in the presence of H_2O_2, as well as unchanged levels of GSH and lipid peroxidation (S. D. Maletić, Lj. M. Đoković, M. M. Kostić, unpublished results) indicate that reticulocytes posses efficient protective mechanism to prevent significant H_2O_2–induced alterations. There is no doubt that energy metabolism of reticulocytes, despite some alterations reported here, also contributes significantly in efficient protection against harmful effects of H_2O_2.

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EFEKTI VODONIK–PEROKSIDA NA ENERGETSKI METABOLIZAM RETIKULOCITA PACOVA

Snežana D. Maletić, Ljiljana M. Dragićević – Đoković, Ivana Banić, Slađan Z. Pavlović*, Radoslav V. Žikić* i Milosav M. Kostić

Institut za fiziologiju, Medicinski fakultet, 34000 Kragujevac
**Prirodno–matematički fakultet, p.p. 60, 34000 Kragujevac*

I Z V O D

Egzogeni H_2O_2 u koncetracijama od 2 i 5 $mmol/l$, ali ne 0.25 i 1 $mmol/l$, izaziva značajne promene u energetskom metabolizmu retikulocita pacova. Te promene se manifestuju smanjenjem ukupne i kuplovane potrošnje O_2, povećanjem brzine glikoze i stimulacijom katabolizma adenin nukleotida. Usled inhibicije oksidativne fosforilacije u respiratornom lancu, pod uticajem H_2O_2 dolazi do smanjenja produkcije ATP. Inhibicija ćelijskog disanja udružena je sa porastom brzine glikoze (Pasteurov efekat) čiji intezitet nije dovoljan da obezbedi stalnost koncetracije ATP. Smanjenje koncetracije ATP u prisustvu H_2O_2 nije praćeno povećanjem koncetracije ADP i AMP, ali se javlja povećanje koncetracije hipoksantina, koje kompenzuje gubitak adenin nukleotida. Međutim, u prosečnoj brzini metaboličkog turnovera nema značajnijih promena. Isto tako, ne menja se ni intezitet metaboličkog fluksa kroz oksidativni pentozo–fosfatni šant.