Role of Thiol-Disulfide System in Mechanisms of Functional Changes in Neutrophils under Conditions of Oxidative Stress

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We studied the state of the thiol-disulfide system (contents of reduced and oxidized glutathione, their ratio, and concentrations of protein SH-groups and protein-bound glutathione) and functional properties of neutrophils (production of hydroxyl radicals, IL-8, and TNF-α and myeloperoxidase activity) from healthy donors under conditions of oxidative stress *in vitro* induced by H_2O_2 in a final concentration of 200 μM and from patients with community-acquired pneumonia. We evaluated the role of reduced and protein-bound glutathione in the regulation of functional state of blood neutrophils from patients with community-acquired pneumonia and during oxidative stress *in vitro* under conditions cell incubation with N-ethylmaleimide or 1,4-dithioerythritolsulfhydryl, the blocker and protector of sulfhydryl groups, respectively.

Key Words: *thiol-disulfide system, neutrophils; interleukins; oxidative stress*

Oxidative stress (OS) accompanying inflammation is characterized by excessive accumulation of reactive oxygen species (ROS) in tissues. Neutrophil leukocytes are the main producers of ROS essential for elimination of the phlogogen. However, this simultaneously leads to exhaustion of antioxidant defense reserves in neutrophils, prooxidant shift of the redox status, and dysregulation of intra- and extracellular redox-dependent signal transduction pathways [4,5,6]. The molecular mechanisms of ROS interaction with the phlogogen and tissues are studied in detail [2,4,5], but little is known about the effect of free radicals directly on the redox state of neutrophils. Due to their high reaction capacity, ROS induce oxidative modification of macromolecules in the target cells and neutrophils, which modulates the functional state of phagocytizing cells and induces their apoptosis [4,6,14]. An essential role in the maintenance of cell redox-potential determining the efficiency of their functioning is played by the thiol-disulfide system. SH-groups of proteins (protein-SH) and reduced glutathione (GSH) act as acceptors of hydroxyl radicals ($HO^{\bullet}$) and singlet oxygen, thus attenuating the destructive and cytotoxic effects of ROS [15]. GSH maintains the antioxidant and detoxification potential of glutathione-dependent enzymes [2,4] and participates in the expression of redox-dependent genes, regulation of intracellular signaling, and other processes [3,11,12,13].

Here we studied the effect of thiol-disulfide system on functional state of neutrophil leukocytes of the blood under conditions of oxidative stress.

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MATERIALS AND METHODS

We analyzed venous blood obtained from 27 healthy donors (10 men and 17 women) aging 18-50 years (mean age 33±6 years) and 48 emergency patients with community-acquired pneumonia (CAP, 23 men and 25 women, 36±4 years). The diagnosis corresponded to modern standard of CAP diagnostics [7]. The blood was taken before therapy using BD VACUTAINER™ (Greiner-bio-one) with lithium heparin.

Neutrophil leukocytes were isolated by centrifugation in double Ficoll-Paque density gradient (1.077 and 1.093 g/cm^3, Pharmacia), washed three times with RPMI-1640 (Vektor-Best), and a suspension with cell concentration of 2×10^6/ml was prepared. Cell viability of isolated neutrophils was evaluated in the test with 0.5% trypan blue (Serva). The relative content of viable cells not containing the dye was 95%. The cells were cultured in complete nutrient medium containing 90% RPMI-1640, 10% heat-inactivated (30 min at 56°C) FCS (Biolot), 0.3 mg/ml L-glutamine, 100 μg/ml gentamicin, and 2 μmol/ml HEPES (Flow). For respiratory burst modeling, H_2O_2 (final concentration of 200 μM) was added to the incubation medium for neutrophils from healthy donors [12]. For evaluation of the role of glutathione and the thiol-disulfide system in the maintenance of functional activity of cells under conditions of OS *in vitro* and in CAP, neutrophils were incubated with SH-group blocker N-ethylmaleimide (Sigma) [13] of SH-group protector 1,4-dithioerythritol (DTE, Sigma) [8] in a final concentration of 5 mM.

The concentration of TNF-α and IL-8 in the incubation medium was measured using EIA test systems (Vector-Best); the results were expressed in pg/ml. HO$^\bullet$ production by cells was assessed by the content of degradation products of the model substrate 2-deoxy-D-ribose (MP, 15 mM) and expressed in nmol/mg protein [1]. An aliquot of neutrophils was resuspended (standard concentration of cells) and a lysate was prepared by adding 1% triton X-100. In the neutrophil lysate, activity of myeloperoxidase was determined by conversion of *o*-phenylenediamine (MP) catalyzed by this enzyme using calibration plot constructed by using horseradish peroxidase within the range from 0.01 to 2 μg/ml [1]; the contents of protein-SH and protein-bound glutathione (protein-SSG) were measured as described elsewhere [9] using the capacity of 1% sodium borohydride (Sigma) to release GSH from its complexes with proteins. Cell lysate was deproteinated with 5% sulfosalicylic acid, the content of GSH and its oxidized from GSSG was evaluated by the method [11] based on enzyme-catalyzed reaction of recycling and blockade of SH-groups of GSH with vinylpyridine (Wako); calibration plot with GSH (MP) concentration 3-100 μM was constructed. Protein content in samples was determined by the method of Bradford based on protein staining with Coomassie blue G-250; calibration curve was constructed using BSA solutions with concentrations of 1-10 μg per 100 ml.

The data were processed by methods of variation statistics. The data are presented as median (Me) and upper and lower quartiles (Q_1-Q_3). Distribution normality was verified using Shapiro–Wilk test. The significance of differences between independent and dependent samples was evaluated using Mann–Whitney rank test and nonparametric Wilcoxon test. Nonparametric Spearman correlation test was used for evaluation of the relationship between the parameters and correlation strength and direction. The differences were significant at $p<0.05$.

RESULTS

Evaluation of the status of the thiol-disulfide system of blood neutrophils in OS revealed the following regularities. The concentration of GSH in neutrophils from healthy donors during cell incubation with 200 μM H_2O_2 and in neutrophils from patients with CAP was reduced by 2.7 and 3 times, respectively, compared to the control value ($p<0.05$), which suggests its intensive utilization in antioxidant processes compensating activation of free-radical oxidation. GSH is used in redox reaction as a donator of SH-groups protecting the cell from HO$^\bullet$ and other ROS. Under conditions of OS, the oxidative changes primarily involve redox-sensitive elements, first of all, functional thiol groups of proteins and peptides [14]. The content of protein-SH in blood neutrophils during *in vitro* OS modeling and in patients with CAP decreased by 1.3 and 1.9 times, respectively; in parallel, the content of GSSG and protein SSG increased compared to the corresponding control values ($p<0.05$; Table 1). Hence, the decrease in intracellular pool of GSH observed by us in OS was largely determined by its utilization in reversible binding of protein SH-groups. The results obtained in experimental OS were comparable with the corresponding findings in patients with CAP ($p>0.05$).

Myeloperoxidase activity in neutrophils and secretion of HO$^\bullet$ in patients with CAP increased by 1.2 and 1.8 times, respectively, compared to the corresponding parameters in the control group ($p<0.05$, Table 2). The increase in ROS production is determined by functional changes in neutrophils related to their protective role as inflammation effector cells (elimination of phlogogen). We observed no significant differences in myeloperoxidase activity and HO$^\bullet$ production in OS *in vitro* and in patients with CAP ($p>0.05$). On the whole, our findings suggest that H_2O_2 concentration of

200 μM is optimal for induction of experimental OS in neutrophils, because the values of the test parameters in this exposure approximate those observed in acute inflammation.

The content of GSH and protein free thiol groups and GSH/GSSG ratio during incubation of blood neutrophils exposed to *in vitro* OS or isolated from patients with CAP with N-ethylmaleimide (SH-group blocker) were significantly lower than in cell culture incubated with 200 μM H_2O_2 or intact neutrophils from patients with CAP ($p<0.05$). N-ethylmaleimide can enter the cell and bind free SH-groups in peptides and proteins, it also interacts with glutathione with the formation of stable glutathione–N-ethylmaleimide complexes without GSH oxidation into disulfide state [10].

Addition of N-ethylmaleimide to neutrophils exposed to OS *in vitro* or to cells isolated from patients with CAP reduced the production of IL-8, TNF-α, and $HO^{\bullet}$ and myeloperoxidase activity compared to the corresponding parameters in neutrophils incubated in the absence of SH-groups ($p<0.05$, Table 2). Taking into account the decrease in GSH content in neutrophil leukocytes, addition of N-ethylmaleimide can promote oxidative modification of proteins, damage to active centers of myeloperoxidase and NADPH oxidase, and inhibition of their activity. This assumption is confirmed by the existence of a positive correlation between the content of GSH and myeloperoxidase activity in OS *in vitro* in the presence of N-ethylmaleimide ($r=0.70$; $p<0.05$). GSH deficit against the background of N-ethylmaleimide promoted the decrease in the production of inflammatory cytokines. It is known that low level of glutathione in cells affects binding of NF-κB nuclear factor with DNA, because activation of transcription factors with regulatory DNA sites is controlled by redox-homeostasis, in particular, by thiol-disulfide balance (ratio of reduced and oxidized SH-groups) [4]. The presence of reduced equivalents is required for binding of NF-κB with DNA in the nucleus, while oxidation of cysteine in DNA-binding domain is accompanied by the formation of protein-SSG and inhibition of DNA-binding activity [3,5], which probably takes place during treatment with N-ethylmaleimide.

The presence of SH-group protector DTE in neutrophil culture under conditions of OS *in vitro* led to a decrease in the content of GSSG and protein-SSG in cells (by 1.3 and 2.0 times, respectively) against the background of the increase in GSH level and GSH/GSSG ratio (by 1.6 and 2.7 times, respectively)

TABLE 1. Effect of SH-Group Blocker N-Ethylmaleimide (NEM) and SH-Group Protector DTE on Thiol-Disulfide System of Blood Neutrophils from Healthy Donors under Conditions of OS *in Vitro* and in Patients with CAP

Group	GSH, nmol/mg protein	GSSG, nmol/mg protein	GSH/GSSG	Protein-SH, nmol/mg protein	Protein-SSG, nmol/mg protein
Healthy donors					
intact neutrophils (control)	4.84	0.27	18.57	3.15	0.05
	(4.64-5.39)	(0.25-0.31)	(16.72-19.37)	(2.83-3.39)	(0.03-0.08)
incubation with H_2O_2	1.97*	055*	3.0*	2.45*	0.32*
	(1.45-2.40)	(0.51-0.59)	(2.84-4.29)	(2.28-2.85)	(0.11-0.62)
incubation with H_2O_2 and NEM	1.04*+	0.57*	1.86*+	0.35*+	0.37*
	(1.02-1.12)	(0.52-0.64)	(1.74-1.98)	(0.33-0.36)	(0.35-0.38)
incubation with H_2O_2 and DTE	3.18*+	0.41*+	8.06*+	2.61	0.16*+
	(3.06-3.40)	(0.38-0.43)	(7.52-8.26)	(2.29-3.12)	(0.12-0.17)
Patients with CAP					
intact neutrophils (control)	1.62*	0.47*	2.84*	1.69*+	0.58*+
	(1.23-1.92)	(0.37-0.66)	(2.45-4.67)	(1.50-1.93)	(0.47-0.70)
incubation with NEM	1.11*o	0.55*o	1.83*o	0.45*o	0.77*o
	(0.92-1.47)	(0.52-0.72)	(1.67-2.34)	(0.35-0.51)	(0.59-0.87)
incubation with DTE	1.80*	0.49*	2.74*	1.35*	0.55*
	(1.44-1.96)	(0.40-0.62)	(2.25-5.66)	(1.06-1.90)	(0.25-0.66)

Note. Here and in Table 2: $p<0.05$ compared to: *control, +neutrophils incubated with H_2O_2, ointact neutrophils from patients with CAP.

TABLE 2. Effect of SH-Group Blocker N-Ethylmaleimide (NEM) and SH-Group Protector DTE on Functional State of Blood Neutrophils from Healthy Donors under Conditions of OS *in Vitro* and in Patients with CAP ($Me(Q_1-Q_3)$)

Group	HO•, nmol/mg	Myeloperoxidase, arb. units/mg	IL-8, pg/ml	TNF-α, pg/ml
Healthy donors				
intact neutrophils (control)	25.32	715.80	281.10	124.52
	(19.87-27.60)	(669.80-741.10)	(273.71-304.10)	(89.02-156.00)
incubation with H_2O_2	45.38*	846.70*	334.10*	170.40*
	(38.21-70.31)	(806.50-986.60)	(311.82-352.00)	(119.91-188.60)
incubation with H_2O_2 and NEM	24.87+	636.35*+	92.95*+	56.45*+
	(10.36-46.86)	(578.60-673.60)	(77.63-116.01)	(36.20-68.11)
incubation with H_2O_2 and DTE	23.46+	697.05+	105.90*+	
	(17.6-32.28)	(616.80-772.95)	(68.05-126.30)	
Patients with CAP				
intact neutrophils (control)	46.79*	832.00*	325.91*	168.31*
	(40.64-52.74)	(747.80-924.01)	(312.91-335.40)	(153.27-171.47)
incubation with NEM	31.1+o	694.33*o	255.97*o	103.67o
	(22.44-33.95)	(609.40-831.50)	(241.40-280.20)	(81.61-131.33)
incubation with DTE	37.63*o	769.50	305.51	145.65*o
	(33.72-40.02)	(645.12-913.49)	(270.73-371.30)	(125.23-164.75)

compared to the corresponding values in cells incubated with 200 μM H_2O_2 ($p<0.05$; Table 1). The maintenance of SH-groups in the reduced state with DTE under conditions of experimental OS produced more pronounced protective effect on the processes of oxidative modification of neutrophil proteins, while during the acute period of CAP the reducing potential of DTE was rapidly exhausted in antioxidant defense reactions, because the level of GSH, GSSG, and protein-bound glutathione, and GSH/GSSG ratio did not significantly differ ($p>0.05$) from the corresponding parameters in cells incubated without SH-group protectors (Table 1).

In the presence of DTE, the production of TNF-α and HO• during OS *in vitro* and in patients with CAP was reduced compared to the corresponding parameters in cells not exposed to the reducing potential of DTE ($p<0.05$; Table 2). Similarly to SH-containing protein, GSH acts as ROS inhibitor and membrane stabilizer, because it effectively binds copper ions and prevents their reduction in Fenton reaction leading to the formation of HO• [15]. Myeloperoxidase activity in the presence of DTE was reduced only under conditions of OS *in vitro* ($p<0.05$), but not in patients with CAP, which confirmed the protective effect of SH-groups ($r=0.90$; $p<0.05$, coefficient of correlation between the content of SH-groups and myeloperoxidase activity in patients with CAP during cell incubation with DTE).

Thus, dysfunction of blood neutrophils (enhanced production of HO•, IL-8, and TNF-α and activation of myeloperoxidase) in OS is accompanied by imbalance in cell redox-status. The mechanisms of dysregulation of redox-dependent signal-transduction systems in neutrophils in OS *in vitro* induced by 200 μM H_2O_2 and in patients with CAP are associated with imbalance of the thiol-disulfide system (decreased content of GSH, thiol groups in proteins, and accumulation of oxidized and protein-bound forms of glutathione). Deficit of GSH and free sulfhydryl groups of proteins in blood neutrophils caused by blockade of SH-groups with N-ethylmaleimide under conditions of OS is associated with a decrease in functional activity of cells (production of HO•, IL-8, and TNF-α and activity of myeloperoxidase).

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