ISSN 1990-519X, Cell and Tissue Biology, 2008, Vol. 2, No. 3, pp. 322–332. © Pleiades Publishing, Ltd., 2008.
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The Effect of Oxidized Glutathione and Its Pharmacological Analogue Glutoxim on Intracellular Ca^{2+} Concentration in Macrophages Ca^{2+}

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Abstract—Using Fura-2AM microfluorimetry, the effect of oxidized glutathione (GSSG) and its pharmacological analogue glutoxim on the intracellular Ca^{2+} concentration in rat peritoneal macrophages was investigated. It was shown that GSSG or glutoxim increase the intracellular Ca^{2+} concentration by inducing Ca^{2+} mobilization from thapsigargin-sensitive Ca^{2+} stores and subsequent Ca^{2+} entry from external medium. Dithiothreitol, which reduces S–S-bonds in proteins, completely prevents or reverses the increase of intracellular Ca^{2+} concentration induced by GSSG or glutoxim. This suggests that the increase of intracellular Ca^{2+} concentration induced by GSSG or glutoxim can be mediated by their interactions with functionally important SH-groups of proteins involved in Ca^{2+}-signaling.

Two structurally different tyrosine kinase inhibitors genistein and methyl-2,5-dihydroxycinnamate prevent or completely reverse the increase in the intracellular Ca^{2+} concentration induced by GSSG or glutoxim. On the contrary, tyrosine phosphatase inhibitor Na orthovanadate enhances the increase of intracellular Ca^{2+} concentration evoked by oxidizing agents. The data suggest that tyrosine kinases and tyrosine phosphatases are involved in the regulatory effect of GSSG and glutoxim on the intracellular Ca^{2+} concentration in macrophages.

Key words: peritoneal macrophages, glutoxim, oxidized glutathione, tyrosine kinases, tyrosine phosphatases.

DOI: 10.1134/S1990519X08030139

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; GSSG, oxidized glutathione.

Oxidation–reduction (redox) based regulation of signal transduction and gene expression is a fundamental regulatory mechanism in cell biology. Redox-sensitivity is caused by electrons transport on side chains of functional $–CH_2–SH$ groups of conservative residues of cysteine. The list of redox-sensitive signal transduction pathways steadily increases. The current data suggest that modulation of the cell redox state may prove to be an important strategy for the treatment of AIDS and some forms of cancer (Sen, 1998). A special role in redox signaling belongs to thioredoxin and glutathione systems.

Glutathione (γ-glutamylcysteinylglycine) is a universal tripeptide contained in the majority of plants, microorganisms, and in all mammal tissues. Glutathione exists in reduced (GSH) and oxidized (GSSG) forms (Meister, 1988; Lu, 1999; Sies, 1999). In eucaryotic cells, there are three main pools of GSH. It was found that 90% of cellular GSH is located in the cytosol; 10%, in mitochondria; and a small percent, in the endoplasmic reticulum (ER) (Hwang et al., 1992).

GSH is usually present in cells in high concentration (0.1–10 mM) and is the most widespread among cellular thiols and low-molecular-weight peptides. The functions of glutathione are widely diverse. One can say that GSH is one of the major factors in regulation of life, proliferation and death of the cell. The GSH functions in cells as a reducing agent and an antioxidant. GSH prevents the oxidation of SH-groups or reduces S–S-bonds induced by oxidative stress. The GSH inactivates free radicals involved in detoxication of xenobiotics (pharmaceutical agents, carcinogenes, etc.) (Hayes and Mc Lellan, 1999).

The GSH exists in the cell in an equilibrium with glutathione disulphide (GSSG), and their correlation is the measure of the redox-status of the cell. In a normal cell, the GSSG content is very low, less than 1% of the total GSH content (Meister, 1988; Lu, 1999). In mammalian cells, the ratio of concentrations of GSH/GSSG in the cytosol is ≥30 : 1, which allows maintaining very reduced redox potential –220 mV (Banhegyi et al., 1999). Conversely, the redox potential of the ER lumen is more oxidized (–180 mV), and is maintained by the ratio of [GSH]/[GSSG] from 3 : 1 to 1 : 1 (Hwang et al., 1992; Banhegyi et al., 1999). Thus, on the membrane of ER or sarcoplasmic reticulum (SR), a significant

(40–50 mV) difference of redox potentials does exist, and the reticulum lumen is more oxidized than the cytosol (Hwang et al., 1992; Banhegyi et al., 1999).

GSSG is capable to enter in thiol–disulphide exchange with SH-groups of proteins, resulting in the formation of a mixed disulphide complex, or may oxidize the endogenous SH-groups forming disulphides. At oxidative stress, the GSH is oxidized to GSSG, and accumulation of GSSG occurs in the cytosol. To prevent the shift of cellular redox-equilibrium, GSSG may actively exit from the cell or interact with SH-group of protein (PSH), forming the mixed disulphide (PSSG). Thus, under strong oxidative stress, the reduction of GSH intracellular concentration occurs (Lu, 1999).

The oxidation–reduction system GSH/GSSG takes part in the regulation of gene expression and signaling in norm and pathology in immunocompetent cells (Droge et al., 1994, 1998). In human and mouse macrophages, it was shown that GSH and S-nitrosoglutathione play a significant role in macrophage antimicrobial activity and are toxic for *Mycobacterium tuberculosis* (Venketaraman et al., 2003). Moreover, glutathione is one of the leading factors regulating apoptosis in macrophages (Boggs et al., 1998).

The GSSG content is increased in cells during different disturbances in cellular functions; therefore, GSSG was originally considered a biologically aggressive molecule (Kulinskiy and Kolesnichenko, 1990). However, the studies on the GSSG effect on cells in various concentrations, performed in a number of laboratories, showed that GSSG could have a receptor-mediated effect on cellular processes (Burova et al., 2005; Filomeni et al., 2002, 2005). In addition, the pharmaceutical agent glutoxim(*r*) (PHARMA-VAM, Moscow), a synthetic analogue of GSSG, has found clinical application as an immunomodulator and a hemostimulator in complex therapy in cases of bacterial and viral diseases (Zhukov et al., 2004), psoriasis (Korsunskaya et al., 2003; Chermoshentsev, 2003), and radio- and chemotherapy in oncology (Filatova et al., 2004).

The role of Ca^{2+} ions, a ubiquitous secondary messenger, in the regulatory effect of GSSG on cells had not been studied. Therefore, the purpose of the present research was to study the effect of GSSG and glutoxim on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and Ca-signals induced by the purinergic agonist ATP and the endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin in rat peritoneal macrophages.

MATERIALS AND METHODS

The experiments were performed on cultivated resident rat peritoneal macrophages. Quartz glasses with cells were placed in the experimental chamber, which was filled with saline containing, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES–NaOH (pH 7.3–7.4). Calcium-free medium contained 0 mM $CaCl_2$ and 1 mM EGTA. The detailed description of macrophages cultivation procedure was given earlier (Krutetskaya et al., 1997a).

The reagents used in experiments were from Sigma (United States). The stock solutions of thapsigargin (500 μM), nifedipine (20 mM), genistein (50 mM), and methyl-2,5-dihydroxycinnamate (25 mM) were prepared in DMSO. The stock solutions of ATP (100 mM), dithiothreitol (100 mM), sodium orthovanadate (10 mM), GSSG (50 and 5 mg/ml), glutoxim (50 and 5 mg/ml), verapamil (20 mM), chlorides of Ni^{2+} (1 M), La^{3+} (1 M) and Gd^{3+} (50 mM) were prepared in water. For measurement of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the fluorescent probe Fura-2AM was used. Macrophages were incubated for 45 min in saline containing 2 μM Fura-2AM, at a room temperature.

The glasses with stained cells were washed with saline and transferred to the experimental chamber disposed on the small table of the Lyumam-KF luminescence microscope (LOMO, St. Petersburg). The fluorescence of Fura-2AM was excited at 337 nm with the LGI-503 nitrogen laser. The laser was located from one side of the microscope at an angle of 30° to the experimental chamber, that allowed the laser beam to be directed towards the object. The fluorescence intensity was recorded with the SFN-10 spectrophotometer at 510 nm. The signal from FEU-79 was enhanced with the specially constructed amplifier and recorded with the IBM PC digital computer using the original software. In experiments, a lens 10X with the aperture 0.4 was used. Under such amplification, 40–50 cells were observed. To avoid photobleaching, the measurements were conducted every 20 s, irradiating the object within 2.5 s. After adding ATP, the cells were irradiated continuously until the maximum fluorescence was reached. $[Ca^{2+}]_i$ was calculated according to the Grynkiewicz equation (Grynkiewicz et al., 1985). The experiments were conducted at room temperature (22–24°C) on the second and third days of cell cultivation.

RESULTS AND DISCUSSION

The addition of 200 μM ATP to peritoneal macrophages induces a biphasic Ca^{2+}-signal: a transient peak caused by Ca^{2+} mobilization from the stores and a long-lasting plateau reflecting the Ca^{2+} entry from the external medium (Alonso-Torre, Trautmann, 1993) (Figs. 1a, 1b). Thapsigargin (0.5 μM), a specific inhibitor of endoplasmic Ca^{2+}-ATPase (Thastrup et al., 1989), also induces a biphasic Ca^{2+}-signal: a less pronounced peak caused by Ca^{2+} mobilization from the stores a and long-lasting phase reflecting the store-dependent Ca^{2+}-entry from the external medium (Figs. 1b, 1d).

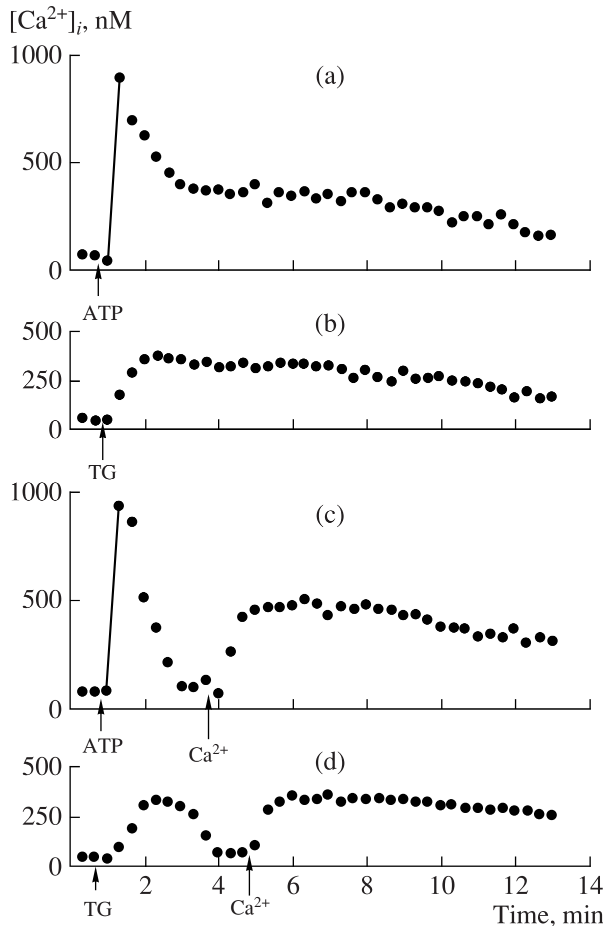

Fig. 1. Ca^{2+}-signals induced by ATP (a, c) and thapsigargin (TG; b, d), in peritoneal macrophages. Here and in Figs. 2–6, the abscissa axis shows time, min; the ordinate axis, Ca^{2+} concentration in the cytosol, nM. (a, b) Cells were stimulated with 200 μM ATP (a) or 0.5 μM TG (b) in normal saline. (c, d) Cells were stimulated with 200 μM ATP (c) or 0.5 μM TG (d) in nominally calcium-free medium; Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium.

The Effect of GSSG and Glutoxim on Ca^{2+}-Signals Induced by ATP and Thapsigargin in Macrophages

The effect of glutoxim and GSSG at concentrations of 10, 100, 200, 300, 400, 500, and 600 μg/ml was studied. Figure 2 shows the effect of glutoxim (100 μg/ml) on $[Ca^{2+}]_i$ in resting cells and Ca^{2+}-signals induced by 200 μM ATP (Figs. 2a, 2b) or 0.5 μM thapsigargin (Fig. 2c) in the macrophages incubated in a normal saline (a) or nominally calcium-free medium (Figs. 2b, 2c). It can be seen in Fig. 2 that preincubation of cells with glutoxim in the medium containing Ca^{2+} for 15 min before ATP addition induces a slight increase in the basal Ca^{2+} level and a decrease (by 20–30%). in the phase of Ca^{2+} mobilization from the stores induced by ATP. The $[Ca^{2+}]_i$ increase after the addition of glutoxim may be determined by both Ca^{2+} mobilization from the intracellular stores and Ca^{2+} entry from the external medium. To elucidate the mechanism of $[Ca^{2+}]_i$ increase induced by glutoxim, the experiments in the nominally calcium-free medium (Figs. 2b, 2c) were performed. It was found that, under such conditions, glutoxim induces a $[Ca^{2+}]_i$ increase and subsequent decrease (by 20–30%) in the phase of Ca^{2+} mobilization from the stores induced by ATP (Fig. 2b) or thapsigargin (Fig. 2c).This indicates that the $[Ca^{2+}]_i$ increase caused by glutoxim is determined by Ca^{2+}mobilization from the intracellular Ca^{2+} stores. A similar pattern was obtained in the presence of GSSG (Fig. 3).

It should be emphasized that we have not observed any dose-dependence of the effect of glutoxim or GSSG on $[Ca^{2+}]_i$. These agents at the studied concentrations had practically the same effect on $[Ca^{2+}]_i$ in resting cells and on Ca^{2+}-signals induced by ATP or thapsigargin in the macrophages. However, a more prolonged (30-min) incubation of the cells with glutoxim induced a more pronounced $[Ca^{2+}]_i$ increase.

The data shown in Fig. 4 confirm that the $[Ca^{2+}]_i$ increase induced by glutoxim or GSSG is determined by Ca^{2+} mobilization from stores. Addition of 2 mM Ca^{2+} to the external medium of the cells preincubated for 2 min with glutoxim (Fig. 4a) or for 16 min with GSSG (Fig. 4b) induced Ca^{2+} entry that was caused presumably by the Ca^{2+} release from the intracellular store. After the emptying of Ca^{2+}-stores by 0.5 μM thapsigargin, glutoxim induced no $[Ca^{2+}]_i$ increase (Fig. 4c). This suggests that glutoxim or GSSG induce Ca^{2+} mobilization from the thapsigargin-sensitive Ca^{2+}-stores.

Earlier, we described pharmacological properties of the store-dependent Ca^{2+} entry in rat peritoneal macrophages (Krutetskaya et al., 1997c, 2000). It was shown that store-dependent Ca^{2+} entry induced by emptying of Ca^{2+}-stores by 0.5 μM thapsigargin or 200 μM UTP is blocked by the following pharmaceutical agents: (1) two structurally different inhibitors of voltage-gated Ca^{2+}-channels, nifedipine (20 μM) or verapamil (40 μM); (2) ions Ni^{2+} (1 mM), La^{3+} (1 mM), and Gd^{3+} (10 μM); and (3) niflumic acid (100 μM), a blocker of non-selective cation channels.

Therefore, we studied the influence of these organic (nifedipine, verapamil) and inorganic (Ni^{2+}, La^{3+}, and Gd^{3+}) blockers of Ca^{2+}-channels on the Ca^{2+} entry induced by ATP or thapsigargin, after the treatment of the cells with glutoxim or GSSG. The data shown in Figs. 2–4 suggest that the agents studied change the pharmacological properties of Ca^{2+}-channels in macrophages. The channels become less sensitive to the blocking effect of Ca^{2+}-antagonists, such as nifedipine (Figs. 2, 3c) and verapamil (Figs. 2c, 3b) as well as the

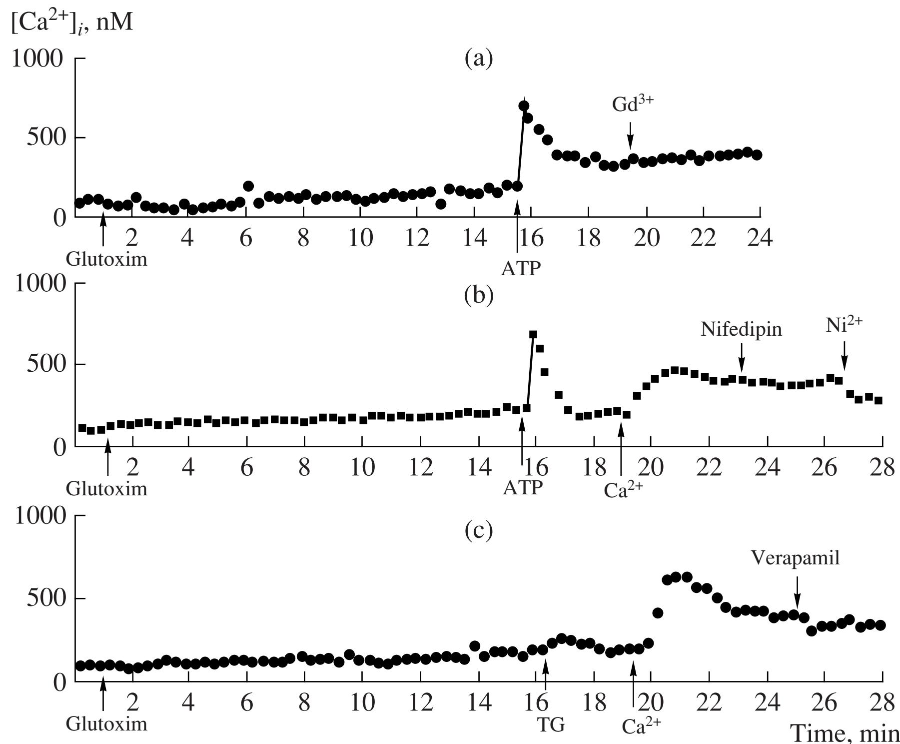

Fig. 2. Effect of glutoxim on the intracellular Ca^{2+} concentration and Ca^{2+}-signals induced by ATP or thapsigargin in macrophages. (a) Macrophages in normal saline were preincubated for 15 min in the presence of 100 μg/ml glutoxim and then stimulated with 200 μM ATP; 20 μM Gd^{3+} was added during the plateau phase of Ca^{2+}-signal. (b, c) Cells were incubated for 15 min in the presence of 100 μg/ml glutoxim in nominally calcium-free medium, then stimulated with 200 μM ATP (b) or 0.5 μM TG (c), after which Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium. During the developed Ca^{2+} entry, 80 μM nifedipine (b) or 80 μM verapamil (c) and then 2 mM Ni^{2+} (b) were added.

inorganic blockers La^{3+} (Fig. 3a) and Gd^{3+} (Fig. 2a). The only effective blocker was Ni^{2+} ions (Figs. 2b, 3b). Nifedipine and verapamil were ineffective even at a concentration of 80 μM. In some cases, after nifedipine addition, some paradoxical enhancement of Ca^{2+} entry was observed.

The fact that GSSG and glutoxim change the pharmacological properties of Ca^{2+} channels, making them less sensitive to the blocking effect of organic and inorganic Ca^{2+}-channel blockers, suggests that these oxidizing agents modify the functionally important SH-groups located in the outer mouth and/or in the pore of store-dependent Ca^{2+}-channels.

Dithiothreitol Influence on the Effect of Glutoxim and GSSG on $[Ca^{2+}]_i$

It is known that GSSG is able to enter in the thiol–disulphide exchange with active protein SH-groups with formation of a mixed disulphide complex or may oxidize the endogenous SH-groups with formation of disulphides (Meister, 1988; Lu, 1999). Therefore, we studied the influence of dithiothreitol (DTT), an agent reducing the S–S-bonds in proteins, on the effect of GSSG and glutoxim.

It was shown that the addition of 100 μg/ml glutoxim to macrophages in nominally calcium-free medium induced a significant $[Ca^{2+}]_i$ increase determined by Ca^{2+} mobilization from the intracellular Ca^{2+}-stores (Figs. 5a, 5c). The addition of 2 mM Ca^{2+} to the external medium caused a fast $[Ca^{2+}]_i$ increase reflecting Ca^{2+} entry from the external medium. The addition of 1 mM DTT during the phase of already developed Ca^{2+} entry induced by glutoxim completely reversed $[Ca^{2+}]_i$ increase and returned $[Ca^{2+}]_i$ to the basal level (Fig. 5c). Preincubation of macrophages for 10 min with 1 mM DTT in calcium-free medium almost completely prevented the glutoxim-induced $[Ca^{2+}]_i$ increase (Fig. 5b).

Thus, the reducing agent DTT prevents or completely reverses the $[Ca^{2+}]_i$ increase induced by glutoxim or GSSG in rat peritoneal macrophages.

The results are in agreement with the data obtained for permeabilized rat hepatocytes, according to which GSSG and cystine effect is reversed by DTT (Renard et al., 1992).

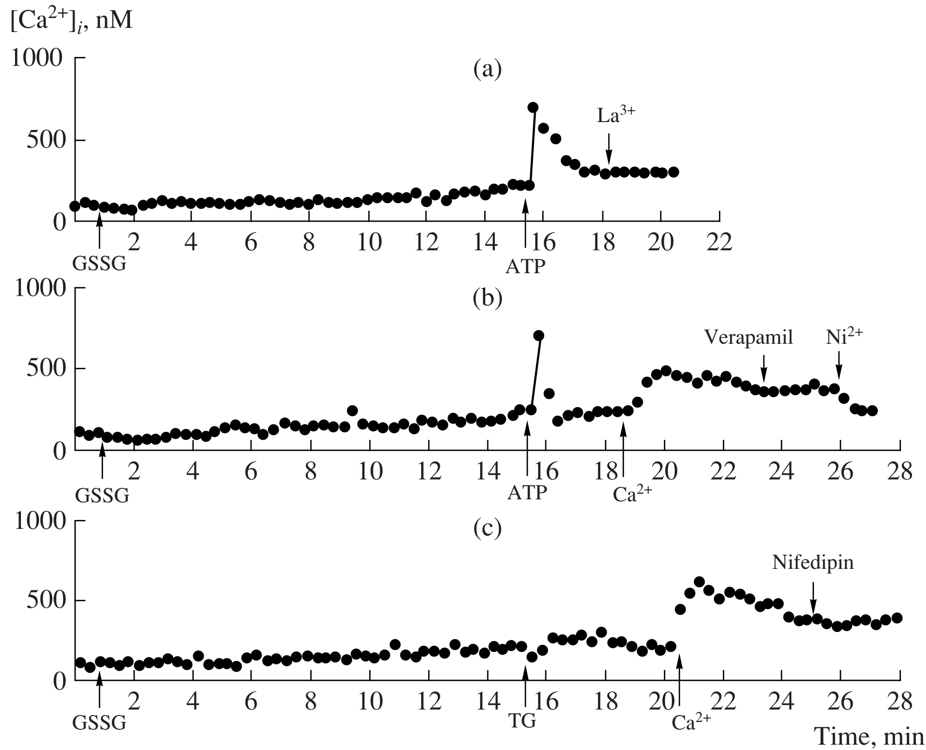

Fig. 3. Effect of oxidized glutathione (GSSG) on the intracellular Ca^{2+} concentration and ATP- or thapsigargin-induced Ca^{2+}-signals in macrophages. (a) Macrophages in normal saline were preincubated for 15 min in the presence of 100 μg/ml GSSG and then stimulated with 200 μM ATP; 1 mM La^{3+} was added during the plateau phase of Ca^{2+}-signal. (b, c) Cells were incubated for 15 min in the presence of 100 μg/ml GSSG in nominally calcium-free medium and then stimulated with 200 μM ATP (b) or 0.5 μM TG (c), after which Ca^{2+} entry was initiated by the addition of 2 mM Ca^{2+} to the external medium. During the developed Ca^{2+} entry, 80 μM verapamil (b) or 80 μM nifedipine (c) and then 2 mM Ni^{2+} (b) were added.

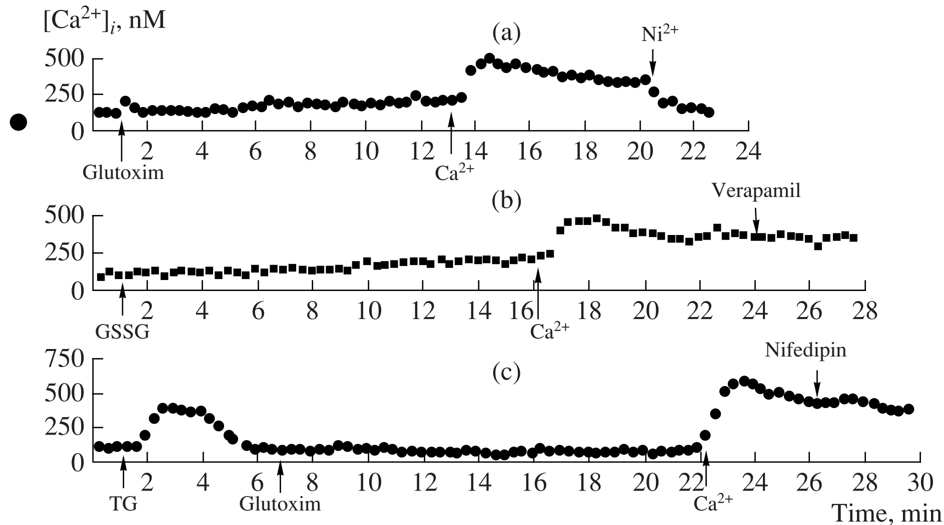

Fig. 4. Effect of glutoxim and GSSG on intracellular Ca^{2+} concentration in macrophages. (a, b) Cells were preincubated for 12 min (a) or 16 min (b) in the presence of 100 μg/ml glutoxim (a) or GSSG (b) in nominally calcium-free medium, after which Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium. During the developed Ca^{2+} entry, 2 mM Ni^{2+} (a) or 80 μM nifedipine (b) were added. (c) Macrophages were stimulated with 0.5 μM TG in nominally calcium-free medium; after the termination of the phase of Ca^{2+} mobilization from stores, 100 μg/ml glutoxim was added and 15 min later 2 mM Ca^{2+} was added to the external medium. During the developed Ca^{2+} entry, 80 μM nifedipine was added.

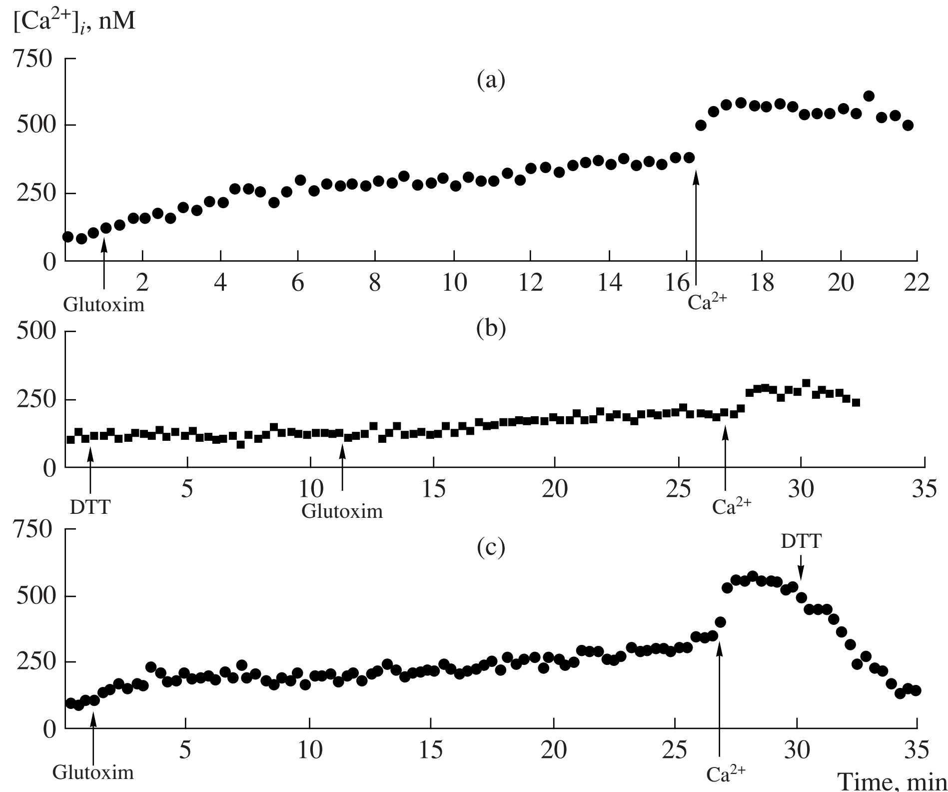

Fig. 5. Influence of dithiothreitol (DTT) on the effect of glutoxim on the intracellular Ca^{2+} concentration in macrophages. (a) Macrophages were incubated for 15 min with 100 μg/ml glutoxim in nominally calcium-free medium; Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium. (b) Cells were preincubated for 10 min in the presence of 1 mM DTT in calcium-free medium, after which 100 μg/ml glutoxim was added; 16 min later Ca^{2+} entry was induced by addition of 2 mM Ca^{2+} to the external medium. (c) Macrophages were incubated for 25 min in the presence of 100 μg/ml glutoxim in calcium-free medium, then Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium; 1 mM DTT was added during the developed Ca^{2+} entry.

Thus, it can be assumed that $[Ca^{2+}]_i$ increase induced by glutoxim or GSSG is mediated by their interaction with the functionally important SH-groups of proteins involved in the processes of Ca^{2+}-signalling.

Influence of Inhibitors of Tyrosine Kinases and Tyrosine Phosphatases on the Effect of GSSG and Glutoxim on $[Ca^{2+}]_i$ in Macrophages

Earlier, we have shown that phenylarsine oxide (PAO) causes a dose-dependent $[Ca^{2+}]_i$ increase connected with both Ca^{2+} mobilization from stores and Ca^{2+} entry from the external medium (Krutetskaya et al., 1997b; Krutetskaya and Lebedev, 1998). As a specific sulfhydryl reagent, PAO covalently binds to SH-groups of adjacent protein (Webb, 1966).

It was shown that $[Ca^{2+}]_i$ increase in macrophages induced by PAO (10–50 μM) is effectively blocked by tyrosine kinase inhibitors, 100 μM genistein and 25 μM methyl-2,5-dihydroxycinnamate (MDC).

Preincubation of the cells with 100 μM genistein or 25 μM MDC significantly reduces but does not prevent the Ca^{2+}-response to PAO (Krutetskaya et al., 1997b; Krutetskaya and Lebedev, 1998).

In addition, it was shown in human epidermoid carcinoma A431 cells that GSSG and glutoxim cause transactivation of the epidermal growth factor receptor and activation of its intrinsic tyrosine kinase activity (Burova et al., 2005; Vasilenko et al., 2006). In macrophages, including rat peritoneal macrophages, the receptors with intrinsic tyrosine kinase activity were also identified: receptors for the colony-stimulating factor 1 (CSF-1) (Yue et al., 1993; Correll et al., 2004); receptors for the hepatocyte growth factor (HGF) (Camussi et al., 1997); receptors for the epidermal growth factor (EGF) (Nolte et al., 1997); and receptors for the macrophage-stimulating protein (MSP, macrophage-stimulating protein) (Iwama et al., 1995; Wang et al., 2000; Zhou et al., 2002; Correll et al., 2004).

These receptor tyrosine kinases contain external cysteine-rich domains and may be also targets for GSSG and glutoxim.

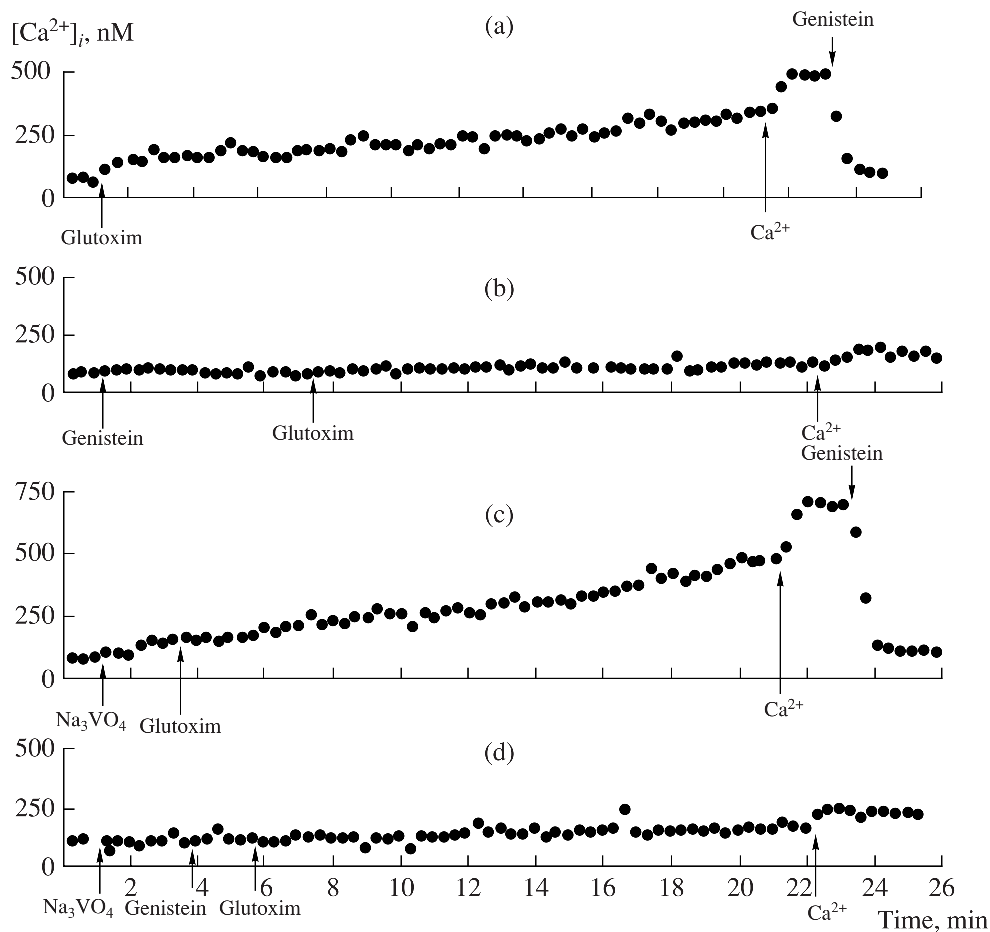

Fig. 6. Influence of genistein and sodium orthovanadate (Na_3VO_4) on glutoxim effect on intracellular Ca^{2+} concentration in macrophages. (a) Cells were incubated for 20 min in the presence of 100 μg/ml glutoxim in calcium-free medium, then Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium; 100 μM genistein was added during the developed Ca^{2+} entry. (b) Cells were preincubated for 6 min with 100 μM genistein in calcium-free medium, then 100 μg/ml glutoxim was added; 16 min later Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium. (c) Cells were incubated for 2 min in the presence of 50 μM Na_3VO_4 in calcium-free medium, then 100 μg/ml glutoxim was added, and 18 min later Ca^{2+} entry was initiated by addition of 2 mM Ca^{2+} to the external medium; during the developed Ca^{2+} entry, 100 μM genistein was added. (d) Macrophages were preincubated for 6 min in the presence of 50 μM Na_3VO_4 and 100 μM genistein in calcium-free medium, then 100 μg/ml glutoxim was added, and 16 min later Ca^{2+} entry was induced by addition of 2 mM Ca^{2+} to the external medium.

Moreover, it is known that oxidative stress and decrease in the GSH/GSSG ratio change the activity of redox-sensitive enzymes, first of all, tyrosine kinases and tyrosine phosphatases (Staal et al., 1994; Rao et al., 2000; Forman and Torres, 2002), which results in an increased phosphorylation of proteins at tyrosine residues.

To determine a possible role of tyrosine phosphorylation in the action of GSSG and glutoxim on $[Ca^{2+}]_i$ in macrophages, we studied the effect of two structurally different tyrosine kinase inhibitors genistein (Akiyama et al., 1987; Akiyama and Ogawara, 1991; Hidaka and Kobayashi, 1992) and MDC (Umezava et al., 1990; Casnellie, 1991) and the inhibitor of tyrosine phosphatases sodium orthovanadate on the Ca^{2+} response induced by GSSG or glutoxim (Gordon, 1991).

It is shown that the addition of 100 μM genistein (Fig. 6a) or 25 μM MDC (not shown) during an already developed Ca^{2+} entry induced by 100 μg/ml glutoxim completely suppressed the glutoxim-induced $[Ca^{2+}]_i$ increase and returned $[Ca^{2+}]_i$ to the basal level. In addition, the preincubation of the macrophages with 100 μM genistein for 7 min before the addition of 100 μg/ml glutoxim almost completely suppressed the $[Ca^{2+}]_i$ increase and the Ca^{2+} entry induced by glutoxim (Fig. 6b). The inhibition of the glutoxim-induced Ca^{2+} entry by genistein or MDC is in agreement with our

previous data that genistein and MDC inhibit the Ca^{2+} entry induced by ATP, UTP (uridine triphosphate), thapsigargin, and cyclopiazonic acid in rat peritoneal macrophages (Krutetskaya et al., 1997a).

The total level of protein phosphorylation on tyrosine residues in cells is controlled by an opposite action of tyrosine kinases and tyrosine phosphatases (Sun and Tonks, 1994). An increase in phosphorylation level may be determined by an increase in the tyrosine kinase activity or a decrease in the tyrosine phosphatase activity (Fischer et al., 1991; Hunter, 1995, 1996).

To determine a possible role of tyrosine phosphatases in the $[Ca^{2+}]_i$ increase induced by glutoxim or GSSG, we used sodium orthovanadate, which changes the balance between the activity of tyrosine kinases and tyrosine phosphatases. It was shown that the addition of 50 μM sodium orthovanadate 2 min before the addition of 100 μg/ml glutoxim enhanced the glutoxim-induced Ca^{2+}-response determined by Ca^{2+} release from the stores and subsequent Ca^{2+} entry from the external medium (Fig. 6c). Addition of genistein during the developed Ca^{2+} entry returned $[Ca^{2+}]_i$ to the basal level. Thus, the pretreatment of cells with sodium orthovanadate increased the glutoxim-induced Ca^{2+} entry but did not prevent subsequent inhibition of Ca^{2+} entry by genistein. This is in agreement with our previous data that the preincubation of cells with sodium orthovanadate enhanced the ATP-induced Ca^{2+} entry but did not prevent subsequent inhibition of Ca^{2+} entry by genistein (Krutetskaya et al., 1997a).

The enhancement of Ca^{2+} entry by sodium orthovanadate confirmed the importance of tyrosine kinase activation for stimulation of Ca^{2+} entry. This is in agreement with the data that the degree and duration of protein phosphorylation on tyrosine, mediated by tyrosine kinase receptors, could be considerably enhanced by treatment of cells with sodium orthovanadate or its peroxy derivatives (Gordon, 1991; Posner et al., 1994; Hunter, 1995).

Figure 6d shows the combined effect of genistein and sodium orthovanadate on $[Ca^{2+}]_i$ increase induced by glutoxim. The cells were incubated with 50 μM sodium orthovanadate and 100 μM genistein and then treated with 100 μg/ml glutoxim; 16 min later, 2 mM Ca^{2+} was added to the external medium. The suppression of both phases of the glutoxim-induced Ca^{2+} response was observed. Thus, sodium orthovanadate did not prevent the inhibition of Ca^{2+} entry by genistein even if both inhibitors were added before glutoxim. It is possible to assume that the stimulation of Ca^{2+} entry by glutoxim or GSSG is mediated by tyrosine kinase activation or increased tyrosine phosphorylation of the Ca^{2+}-channel in the plasma membrane or a regulatory protein related to the channel. It is also possible that the glutoxim- or GSSG-induced Ca^{2+} mobilization from stores is connected with phosphorylation on tyrosine and activation of the Ca^{2+}-release channel of IP_3-receptor in ER membrane (Jayaraman et al., 1996). This is in agreement with the data that the IP_3-receptor of type 1, identified in cerebellum cells, smooth muscle cells of the aorta, and T-lymphocytes of the human, contains two potential sites of phosphorylation on tyrosine (Tyr 482 and Tyr 2617). Tyr 482 is near the IP_3-binding site, and Tyr 2617 is located near the C-terminal domain of the receptor that forms the Ca^{2+}-release channel (Harnick et al., 1995; Marks, 1997). It was found that· upon activation of T-lymphocytes, the tyrosine phosphorylation of the IP_3-receptor occurs, which results in an increase of the open state probability of the Ca^{2+}-release channel (Harnick et al., 1995; Jayaraman et al., 1996).

Tyrosine phosphatases may be targets for covalent modification with GSSG or glutoxim. Two types of tyrosine kinases and tyrosine phosphatases (receptor and cytoplasmic) have been identified (Hunter, 1995, 1996). In the catalytic domain of all known tyrosine phosphatases, both cytoplasmic (Barford et al., 1994; Denu et al., 1996) and receptor ones (Fischer et al., 1991; Walton and Dixon, 1993), the functionally important cysteine residue was identified, which exhibits phosphatase activity only in the reduced state. The cytoplasmic tyrosine phosphatases (PTP1C and PTP1D) were detected, which contain two SH2-domains (src homology domains) interacting with phosphorylated tyrosine residues of proteins. These SH2-domains containing cysteine residues allow tyrosine phosphatase to bind to phosphorylated protein substrates and apparently ensure the specific localization of tyrosine phosphatase in the cell. The tyrosine phosphatase 1B (PTP1B) is also the target for redox-regulation in cells (Denu and Dixon, 1998; Barrett et al., 1999; Filomeni et al., 2002). Therefore, the cysteine residues in SH2-domains and the conservative cysteine residue in the catalytic domain of tyrosine phosphatase may be possible targets for covalent modification by GSSG or glutoxim.

Thus, this was the first study to show that GSSG and its pharmacological analogue glutoxim increase $[Ca^{2+}]_i$, causing Ca^{2+} mobilization from the thapsigargin-sensitive Ca^{2+}-stores and subsequent Ca^{2+} entry in rat peritoneal macrophages. With the use of tyrosine kinase and tyrosine phosphatase inhibitors, the involvement of tyrosine kinases and tyrosine phosphatases in the $[Ca^{2+}]_i$ increase induced by GSSG or glutoxim in macrophages was detected. Taking into account that genistein and MDC inhibit a broad spectrum of tyrosine kinases, it is possible to suggest that both receptor and cytoplasmic tyrosine kinases may be involved in the regulatory effect of glutoxim or GSSG on $[Ca^{2+}]_i$.

REFERENCES

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S.-I., Itoh, N., Shibuya, M., and Fukami, Y., Genistein, a Specific Inhibitor of Tyrosine-specific Protein Kinases, *J. Biol. Chem.,* 1987, vol. 262, pp. 5592–5595.

Akiyama, T. and Ogawara, H., Use and Specificity of Genistein as an Inhibitor of Protein Tyrosine Kinases, *Methods Enzymol.,* 1991, vol. 201, pp. 362–370.

Alonso-Torre, S.R. and Trautmann, A., Calcium Responses Elicited by Nucleotides in Macrophages. Interaction Between Two Receptor Subtypes, *J. Biol. Chem.*, 1993, vol. 268, pp. 18640–18647.

Banhegyi, G., Lusini, L., Puskas, F., Rossi, R., Fulceri, R., Braun, L., Mile, V., diSimplicio, P., Mandel, J., and Benedetti, Preferential Transport of Glutathione versus Glutathione Disulfide in Rat Liver Microsomal Vesicles, *J. Biol. Chem.*, 1999, vol. 274, pp. 12213–12216.

Barford, D., Flint, A.J., and Tonks, N.K., Crystal Structure of Human Protein Tyrosine Phosphatase 1B, *Science.*, 1994, vol. 263, pp. 1397–1404.

Barrett, W.C., DeGnore, J.P., Konig, S., Fales, H.M., Keng, Y.F., Zhang, Z.Y, Yi, M.B., and Chock, P.B., Regulation of PTP1B via Glutathionylation of the Active Site Cysteine 215, *Biochemistry*, 1999, vol. 38, pp. 6699–6705.

Boggs, S.E., McCormick, T.S., and Lapetina, E.G., Glutathione Levels Determine Apoptosis in Macrophages, *Biochem. Biophys. Res. Com.*, 1998, vol. 247, pp. 229–233.

Burova E.B., Vasilenko K.P., Antonov V.D., and Nikolskii, N.N., Transactivation of the Epidermal growth Factor Receptor by Oxidized Glutathione and Its Pharmacological Analogue Glutoxim in A431 Cells), *Dokl. Akad. Nauk*, 2005, vol. 404, no. 1, pp. 122–124 [*Dokl. Biol. Sci.*, 2005, vol. 404, no. 1, pp. 392–394].

Camussi, G., Montrucchio, H., Lupia, E., Soldi, R., Comoglio, P.M., and Bussolino, F., Angiogenesis Induced in Platelet-Activating Factor Syntheses From Macrophages, *J. Immonol.*, 1997, vol. 158, pp. 1302–1309.

Casnellie, J.E., Protein Kinase Inhibitors: Probes for the Functions of Protein Phosphorylation, *Adv. Pharmacol.*, 1991, vol. 22, pp. 167–205.

Chermoschentsev, A.A., The Eficiency of Glutoxim in Complex Therapy of Those Sick of Drop Form of Psoriasis, *Ros. Zh.. Kozhn. Vener.. Bol.*, 2003, vol. 1, pp. 38–41.

Correll, P.H., Morrison, A.C., and Lutz, M.A., Receptor Tyrosine Kinases and the Regulation of Macrophage Activation, *J. Leukocyte Biol.*, 2004, vol. 75, pp. 731–737.

Denu, J.M. and Dixon, J.E., Protein Tyrosine Phosphatases: Mechanisms of Catalysis and Regulation, *Curr. Opin. Chem. Biol.*, 1998, vol. 2, pp. 633–641.

Denu, J.M., Stuckey, J.A., Saper, M.A., and Dixon, J.E., Form and Function in Protein Dephosphorylation, *Cell*, 1996, vol. 87, pp. 361–364.

Droge, W., Hack, V., Breitkreutz, R., Holm, E., Shubinsky, G., Schmid, E., and Galter, D., Role of Cysteine and Glutathione in Signal Transduction, Immunopathology and Cachexia, *BioFactors.,* 1998, vol. 8, pp. 97–102.

Droge W., Schulze-Osthoff K., Mihm, S., Galter, D., Schenk, H., Eck, H.P., Roth, S., and Gmunder, H., Functions of Glutathione and Glutathione Disulfide in Immunology and Immunopathology, *FASEB J.,* 1994, vol. 8, pp. 1131–1138.

Filatova, E.I., Bylinskaya, E.N., and Alaberg, S.D., *Primenenie glutoksima v soprovozhdenii luchevoy terapii rasprostranennogo raka sheyki matki* (Glutoxim Application in Accompanying of Radiotherapy of Disseminated Cervical Carcinoma), *Tezisy III Syezda Onkologov i Radiologov SNG (Abstracts of Papers of III Congress of Oncologists and Radiologists of CIS)*, Vol. 2, Minsk, 2004, p. 354.

Filomeni, G., Aquilano, K., Civitareale, P., Rotilio, G., and Ciriolo, M.R., Activation of c-jun-N-termainal Kinase is Required for Apoptosis Triggered by Glutathione Disulfide in Neuroblastoma Cells, *Free Rad. Biol. Med.*, 2005, vol. 39, pp. 345–354.

Filomeni, G., Rotilio, G., and Ciriolo, M.R., Cell Signalling and the Glutathione Redox System, *Biochem. Pharmacol.*, 2002, vol. 64, pp. 1057–1064.

Fischer, E.H., Charbonneau, H., and Tonks, N.K., Protein Tyrosine Phosphatases: a Diverse Family of Intracellular and Transmembrane Enzymes, *Science*, 1991, vol. 253, pp. 401–406.

Forman, J.H. and Torres, M., Reactive Oxygen Species and Cell Signaling, *Am. J. Respir. Crit. Care Med.*, 2002, vol. 166, pp. S4–S8.

Gordon, J.A., Use of Vanadate as Protein-phosphotyrosine Phosphatase Inhibitor, *Methods Enzymol.*, 1991, vol. 201, pp. 477–482.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y., A New Generation of Ca^{2+} Indicators with Greatly Improved Fluorescence Properties, *J. Biol. Chem.,* 1985, vol. 260, pp. 3440 – 3450.

Harnick, D.J., Jayaraman, Th., Ma, Y., Mulieri, Ph., Go, L.O., and Marks, A.R., The Human Type 1 Iinositol 1,4,5-Trisphosphate Receptor from T lymphocytes. Structure, Localization, and Tyrosine Phosphorylation, *J. Biol. Chem.*, 1995, vol. 270, pp. 2833–2840.

Hayes, J.D. and Mc Lellan, L.I., Glutathione and Glutathione-dependent Enzymes Represent a Co-ordinately Regulated Defence Against Oxidative Stress, *Free Radical Res.*, 1999, vol. 31, pp. 273–300.

Hidaka, H. and Kobayashi, R., Pharmacology of Protein Kinase Inhibitors, *Annual Rev. Pharmacol. Toxicol.*, 1992, vol. 32, pp. 377–397.

Hunter, T., Protein Kinases and Phosphatases: the Yin and Yang of Protein Phosphorylation and Signaling, *Cell*, 1995, vol. 80, pp. 225–236.

Hunter, T., Tyrosine Phosphorylation: Past, Present and Future, *Biochem. Soc. Trans.*, 1996, vol. 24, pp. 307–327.

Hwang, C., Sinsky, A.J., and Lodish, H.F., Oxidized Redox State of Glutathione in the Endoplasmic Reticulum, *Science*, 1992, vol. 257, pp. 1496–1502.

Iwama, A., Wang, M.-H., Yamaguchi, N., Ohno, N., Okano, K., Sudo, T., Takeya, M., Gervais, F., Morissette, C., and Leanard, E.J., Terminal Differentiation of Murine Resident Peritoneal Macrophages Is Characterized by Expression of the STK Protein Tyrosine Kinase, a Receptor for Macrophage-stimulating Protein, *Blood,* 1995, vol. 86, pp. 3394–3403.

Jayaraman, T., Ondrias, K., Ondriasova, E., and Marks, A.R., Regulation of the Inositol 1,4,5-Trisphosphate Receptor by Tyrosine Phosphorylation, *Science,* 1996, vol. 272, pp. 1492–1494.

Korsunskaya, I.M., Reznikova, M.M., Putintsev, A.Yu., and Avetikyan, S.S., The Experience of Glutoxim Application in Dermatology, *Lechaschiy Vrach*, 2003, vol. 4, pp. 78–79.

Krutetskaya, Z.I. and Lebedev, O.E., *Rol' tirozinovogo fosforilirovaniya v regulyatsii aktivnosti ionnykh kanalov kletochnykh membran* (The Role of Tyrosine Phosphorylation in the Regulation of Activity of Ionic Channels of Cell Membranes), St. Petersburg, 1998.

Krutetskaya, Z.I., Lebedev, O.Ye., and Krutetskaya, N.I., The Mechanizms of Ca^{2+} Signaling in Peritoneal Macrophages, *Ros. Fiziol. Zh.. im. I.M. Sechenova*, 2000, vol. 86, no. 8, pp. 1030–1048.

Krutetskaya, Z.I., Lebedev, O.Ye., Krutetskaya, N.I., Butov, S.N., and Petrova, T.V., Phenylarsine Oxide, an Inhibitor of tyrosine Phosphatases, Induces the Increase of Intracellular Ca^{2+} Concentration in Rat Peritoneal Macrophages and in Human Fibroblasts, *Tsitologiya* (St. Petersburg), 1997, vol. 39 (12), pp. 1116–1130.

Krutetskaya, Z.I., Lebedev, O.Ye., Tyuschev, V.E., Krutetskaya, N.I., and Roschina, N.G., The Organic and Non-Organic Inhibitors of Potential-Dependent Ca^{2+}channels Inhibit the Store-Dependent Ca^{2+} Entry in Rat Peritoneal Macrophages, *Tsitologiya* (St. Petersburg), 1997c, vol. 39, no. 12, pp. 1131–1141.

Krutetskaya, Z.I., Lebedev, O.Ye., Tyushev, V.E., Krutetskaya, N.I., and Roschina, N.G., The Influence of Tyrosine Kinase and Tyrosine Phosphatase Inhibitors on ATP- and Thapsigargin-Induced Ca^{2+} Entry in Rat Peritoneal Macrophages, *Tsitologiya* (St. Petersburg), 1997, vol. 39 (2/3), pp. 164–176.

Kulinskiy, V.I. and Kolesnichenko, L.S., The Biological Role of Glutathione, *Usp. Sovr. Biol.*, 1990, vol. 110, no. 1, pp. 20–33.

Lu, S.C., Regulation of Hepatic Glutathione Synthesis: Current Concepts and Controversies, *FASEB J.*, 1999, vol. 13, pp. 1169–1183.

Marks, A.R., Intracellular Calcium-release Channels: Regulators of Cell Life and Death, *Amer. J. Physiol.*, 1997, vol. 272, pp. H597–H605.

Meister, A., Glutathione Metabolism and Its Selective Modification, *J. Biol. Chem.,* 1988, vol. 263, pp. 17205–17208.

Nolte, C., Kirchhoff. F., and Kettenmann, H., Epidermal Growth Factor Is a Motility Factor for Microglial Cells in Vitro: Evidence for EGF Receptor Expression, *Eur. J. Neurosci.,* 1997, vol. 9, pp. 1690–1698.

Posner, B.I., Faure, R., Burgess, J.W., Bevan, A.P., Lachance, D., Zhang-Sun, G., Fantus, I.G., Ng, J.B., Hall, D.A., and Lum, B.S., Peroxovanadium Compounds: a New Class of Potent Phosphotyrosine Phosphatase Inhibitors which Are Insulin Mimetics, *J. Biol. Chem.*, 1994, vol. 269, pp. 4596–4604.

Rao, R.K., Baker, L., Li., R.D., Baker, S.S., and Gipta, A., Glutathione Oxidation and PTPase Inhibition by Hydrogen Peroxide in Caco-2 Cell Monolayer, *Am. J. Physiol. Gastrointest. Liver Physiol.,* 2000, vol. 279, pp. G332–G340.

Renard, D.C., Seitz, M.B., and Thomas, A.P., Oxidized Glutathione Causes Sensitization of Calcium Release to Inositol 1,4,5-Trisphosphate in Permeabilized Hepatocytes, *Biochem. J.*, 1992, vol. 284, pp. 507–512.

Sen, C.K., Redox Signaling and the Emerging Therapeutic Potential of Thiol Antioxidants, *Biochem. Pharmacol.,* 1998, vol. 55, pp. 1747–1758.

Sies, H., Glutathione and Its Role in Cellular Functions, *Free Radical Biol. Med.*, 1999, vol. 27, pp. 916–921.

Staal, F.J.T., Anderson, M.T., Staal, G.E.J., Herzenberg, L.A., Gitler, C., and Herzenberg, L., Redox Regulation of Signal Transduction: Tyrosine Phosphorylation and Calcium Influx, *Proc. Natl. Acad. Sci. USA*, 1994, vol. 91, pp. 3619–3622.

Sun, H. and Tonks, N.K., The Coordinated Action of Protein Tyrosine Phosphatases and Kinases in Cell Signaling, *Trends Biochem. Sci.,* 1994, vol. 19, pp. 480–485.

Thastrup, O., Dawson, A.P., Scharff, O., Foder, B., Cullen, P.J., Drobak, B.K., Bjerrum, P.J., Christensen, S.B., and Hanley, M.R., Thapsigargin, a Novel Molecular Probe for Studying Intracellular Calcium Release and Storage, *Agents Actions*, 1989, vol. 27, pp. 17–23.

Umezawa, K., Hori, T., Tajima, H., Imoto, M., Isshiki, K., and Takeuchi, T., Inhibition of Epidermal Growth Factor-induced DNA Synthesis by Tyrosine Kinase Inhibitors, *FEBS Lett.*, 1990, vol. 260, pp. 198–200.

Vasilenko, K.P., Burova, Ye.B., Antonov, V.D., and Nikolskiy, N.N., Oxidized Glutathione Induces Activation of the Epidermal Growth Factor Receptor and MAP Kinases ERK 1.2, *Tsitologiya* (St. Petersburg), 2006, vol. 48, no. 6, pp. 500–507.

Venketaraman, V., Dayaram, Y.K., Amin, A.G., Ngo, R., Green, R.M., Talaue, M.T., Mann, J., and Connell, N.D., Role of Glutathione in Macrophage Control of Mycobacteria, *Infect. Immun.*, 2003, vol. 71, pp. 1864–1871.

Walton, K.M. and Dixon, J.E., Protein Tyrosine Phosphatases, *Annu. Rev. Biochem.*, 1993, vol. 62, pp. 101–120.

Wang, M.-H., Fung, H.-L., and Chen, Y.-Q., Regulation of the RON Receptor Tyrosine Kinase Expression in Macrophages: Blocking the RON Gene Transcription by Endotoxin-Induced Nitric Oxide, *J. Immunol.,* 2000, vol. 164, pp. 3815–3821.

Webb, J.L., *Arsenicals*, in *Enzymes and Metabolic Inhibitors*, New York: Academic, 1966, vol. 3, pp. 595–793.

Yue, X., Favot, P., Dunn, T.L., Cassady, A.I., and Hume, D.A., Expression of mRNA Encoding the Macrophage Colony-stimulating Factor Receptor (c-fms) Is Controlled by a Constitutive Promoter and Tissue-Specific Transcription Elongation *Mol. Cell. Biol.*, 1993, vol. 13, pp. 3191–3201.

Zhou, Y.-Q., Chen, Y.-Q., Fisher, J.H., and Wang, M.H., Activation of the RON Receptor Tyrosine Kinase by Macrophage-Stimulating Protein Inhibits Inducible Cyclooxygenase-2 Expression in Murine Macrophages, *J. Biol. Chem.,* 2002, vol. 277, pp. 38104–38110.

Zhukov, O.B. Zubarev, A.R., Mezentseva, M.V., Andryushkova, Yu.A., and Ose, I.V., Modern Aspects of Immunomodulating Therapy for Those Sick with Recurrent Infections of Sexual Transmission and with Antibiotic-Resistant Bacterial Prostatitis, *Vracheb. Sosl.*, 2004, vol. 5/6, pp. 51–56.