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CELL BIOLOGY

# Transactivation of the Epidermal Growth Factor Receptor by Oxidized Glutathione and Its Pharmacological Analogue Glutoxim® in A431 Cells

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γ-Glutamylcysteinylglycine (reduced and oxidized glutathione, GSH and GSSG, respectively) is a widespread peptide in cells of aerobic organisms, especially in the reduced form. Today it is generally believed that the system of oxidized and reduced glutathione is the most important known redox system for the regulation of many processes in the cell [1]. The intracellular GSH is a free-radical scavenger; as an antioxidant, it protects cell membranes from oxidative stress, DNA from radiation and UV irradiation, and cells from toxic xenobiotics (drugs, carcinogens, etc.) [2, 3]. The ability of GSH to form disulfide bonds with the cysteine residues in proteins underlies the phenomenon of S-glutathionylation, which is very important for the regulation of functions of proteins. In the course of protecting proteins from denaturation caused by oxidation of their thiol groups in stress, GSH is converted into glutathione disulfide (GSSG). It was also shown that changes in the redox state of the cell are required for the activation of a number of transcriptional factors (such as AP-1, NF-κB, or STAT) and protein tyrosine phosphatases (PTB-1B) [4, 5]. An interesting feature of GSH is its paradoxical ability to induce oxidative processes in cells [6].

Analysis of published literature shows that the majority of studies are devoted to the investigation of different aspects of action of reduced glutathione, whereas the mechanism of action of oxidized glutathione of cells has not been studied before. For this reason, out attention was focused on GSSG and its synthetic analogue Glutoxim® (FARMA-BAM, Moscow). Glutoxim® was permitted for therapeutic use as an immunostimulant. It was shown that Glutoxim® exhibits immunocorrective propoerties, stimulates hematopoiesis, enhances the resistance of the whole body and cells in local and generalized chromic infections, increases the effectiveness of chemotherapy with respect to intracellular infections, and removes the manifestation of the nonspecific syndrome of chronic diseases.

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Taking into account the aforementioned facts, studying the mechanism of action of oxidized glutathione and its pharmacological analogue is a topical problem whose solution would clarify the intracellular pathways of signal transduction from these agents (in particular, the presumable involvement of the epidermal growth factor receptor (EGFR) in this process). Today, the question on EGFR transactivation upon cell stimulation with agents that are not direct EGFR ligands is actively discussed. Transactivation means EGF-independent activation of EGFR induced by cytokines, G-protein-coupled receptors, and various stressors [7]. It is currently believed that EGFR transactivation plays a key role in cell response to different external effects. In this study, special attention is paid to the investigation of the EGFR transactivation induced by GSSG and Glutoxim®, as well as the activation of the EGFR-regulated transcription factors STAT1 and STAT3.

This study was performed with A431 human epidermoid carcinoma cells obtained form the Russian Collection of Cell Cultures, Institute of Cytology, Russian Academy of Sciences. Before experiment, cells were incubated for one day in a medium with a decreased serum content to remove growth factors and decrease the baseline level of activation of signal proteins. Cell lysates were prepared and electrophoresis and immunoblotting were performed as described in earlier [8, 9]. High-sensitivity method of enhanced chemiluminescence (ECL, Amersham) was used to detect antigens on immunoblotting membranes.

We found that oxidized glutathione added at a concentration of 1 or 10 μg/ml to A431 cells activated EGFR (Fig. 1a); Glutoxim® had a similar effect. A study of activation of EGFR tyrosine kinase in dependence on the duration of incubation with the preparations of interest showed that the degree of EGFR phosphorylation, which reflects the activity of its tyrosine kinase, was maximum after incubation for 5–10 min and 1 h. The time course of activation has a sinuous pattern: after 30 min of incubation with the preparations, the degree of EGFR activation decreased to the control value. Thus, this was the first study to demonstrate EGFR transactivation upon incubation with oxidized

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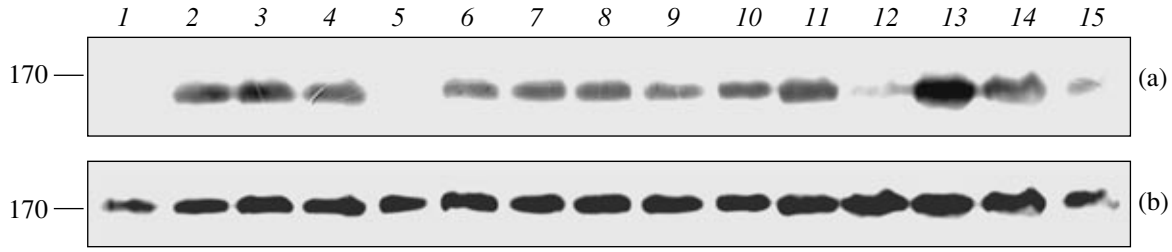


**Fig. 1.** Activation of EGFR in A431 cells incubated with oxidized glutathione and Glutoxim®. (a) Immunoblotting of electrophoretically separated total cell lysates was performed using antibodies to phosphotyrosine (PY20, Sigma, United States). (b) The same membrane after the removal of antibodies and subsequent detection of EGFR using specific antibodies (mAb 2760, Sigma). (*1*) Intact cells; (lanes *2–8*) cells were incubated with oxidized glutathione at a concentration of 1 μg/ml (lanes *2–5*) or 10 μg/ml (lanes *6–8*) for 5 min (lanes *2, 6*), 10 min (lanes *3, 7*), 30 min (lane *5*), and 1 h (lanes *4, 8*); (lanes *9–15*) Cells were incubated with Glutoxim® at a concentration of 1 μg/ml (lanes *9–12*) or 10 μg/ml (lanes *13–15*) for 5 min (lanes *9, 13*), 10 min (lanes *10, 14*), 30 min (lane *12*), and 1 h (lanes *11, 15*). Molecular weight of protein markers (kDa) in shown on the left.

glutathione and its pharmacological analogue Glutoxim®.

A comparison of phosphorylation sites of the cytoplasmic fragment of EGFR upon incubation of A431 cells with oxidized glutathione and EGF showed considerable differences in their activation (Fig. 2). In the control (untreated) cells, a low baseline level of phosphorylation was observed at the tyrosine residues 845 and 1068, and phosphorylation at positions 992 and 1045 was absent. Oxidized glutathione markedly increase the level of phosphorylation of the tyrosine residues at positions 845 and 1068 compared to the control level but did not stimulate phosphorylation at positions 992 and 1045. It is known that phosphorylation of Tyr 845 leads to additional activation of EGFR tyrosine kinase, and Tyr1068 serves as a binding site for the factor STAT3 and adaptor protein GRB2. Incubation with EGF significantly enhanced phosphorylation of the tyrosine residues 845, 1045, and 1068 and slightly stimulated phosphorylation of Tyr 992, which is consistent with the published data [10]. Therefore, the phosphorylation spectra of tyrosine residues in EGFR under exposure to its specific ligand EGF and oxidized glutathione are different.

Then, we studied the activation of the transcription factors STAT1 and STAT3, which are involved in signal transduction from EGFR. We discovered that incubation of A431 cells with oxidized glutathione and Glutoxim® led to the activation of the transcription factor STAT3, yet to a lesser extent than the incubation with EGF (Fig. 3a). However, by contrast to EGF, no activation of the transcription factor STAT1 in the presence of the preparations studied was observed (Fig. 3b). This result agrees with the results of analysis of EGFR phosphorylation: sites 992 and 1045 were not phosphorylated in the presence of oxidized glutathione (Fig. 2). It is known that these sites are required for the activation of the factor STAT1 under exposure to EGF. The discovered increase in the activation of the factor STAT3 under exposure to oxidized glutathione and Glutoxim® correlates well with the activation of site 1068, which ensures the interaction between EGFR and STAT3.

The results of this study demonstrate the possibility of activation of EGFR by the new stimulant, exogenous oxidized glutathione. The effect of the preparation Glutoxim® on the activation of EGFR and factor STAT3 did not differ from that of oxidized glutathione. However, the mechanism of activation of EGFR by these preparations requires further investigation. A comprehensive analysis of the role of activation of EGFR and EGFR-dependent signaling seems a promising direction to

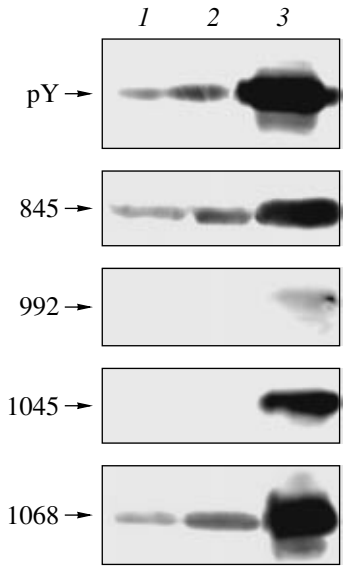


**Fig. 2.** Detection of tyrosine phosphorylation sites in EGFR under exposure to oxidized glutathione and EGF of (*1*) intact cells and cells incubated with (*2*) oxidized glutathione (10 μg/ml) for 5 min or (*3*) EGF (200 ng/ml) for 15 min. Immunoblotting was performed using antibodies to phosphotyrosine (PY20, Sigma) or antibodies to EGFR sites phosphorylated at tyrosine (phospho-EGF receptor antibody sampler kit, Cell Signaling), at positions 845, 992, 1045, and 1068 (shown on the left).

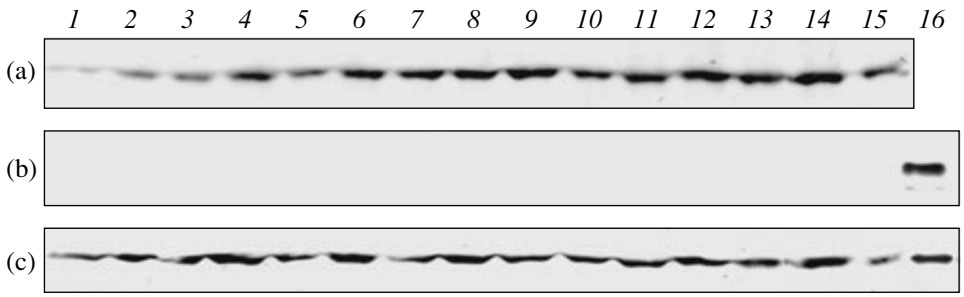


**Fig. 3.** Phosphorylation of transcription factors STAT3 and STAT1 under exposure to oxidized glutathione and Glutoxim®. (a) Immunoblotting was performed using specific antibodies to STAT3 phosphorylated at Tyr 705 (pTyr705 STAT3 pAb, Cell Signaling). Designations: (lanes *1–15*), the conditions of treatment of cells were the same as in Fig. 1; (lane *16*), cells were incubated with EGF at a concentration of 200 ng/ml for 15 min. (b) Immunoblotting performed with the use of specific antibodies to STAT1 phosphorylated at Tyr 701 (pTyr705 STAT1 pAb, Cell Signaling). (c) The same membrane after the removal of antibodies and subsequent detection of STAT1 using specific antibodies (STAT1 mAb, BD Translab).

account for the pharmacological efficacy of the preparation Glutoxim®.

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