

Redox regulation of signal transduction: Tyrosine phosphorylation and calcium influx

(inflammatory cytokines/human immunodeficiency virus infection)

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Contributed by Leonard A. Herzenberg, December 15, 1993

ABSTRACT Studies presented here show that altering the intracellular redox balance by decreasing glutathione levels profoundly affects early signal transduction events in human T cells. In a T-cell receptor (TCR) signaling model, short-term pretreatment with buthionine sulfoximine, which specifically decreases intracellular glutathione, essentially abrogates the stimulation of calcium influx by anti-CD3 antibodies without significantly impairing other aspects of TCR-initiated signal transduction, such as overall levels of TCR-stimulated tyrosine phosphorylation. In an inflammatory-cytokine signaling model, the failure of tumor necrosis factor α to stimulate more than minimal tyrosine phosphorylation in lymphocytes is overcome by buthionine sulfoximine pretreatment—i.e., tumor necrosis factor α stimulates extensive tyrosine phosphorylation in glutathione-depleted lymphocytes. These redox-dependent changes in T-cell responsiveness suggest that the glutathione deficiency that we and others have demonstrated in human immunodeficiency virus-infected individuals may contribute significantly to the immunodeficiency and the increased inflammatory reactions in these individuals.

Glutathione (GSH) participates in a wide variety of intracellular processes, including amino acid transport, synthesis of deoxyribonucleoside triphosphates, and defense against reactive oxidative intermediates produced by normal metabolic processes. It is the major intracellular redox buffer in almost all cell types (1). Furthermore, it has recently been implicated in protection against the induction of apoptotic and necrotic cell death in a variety of cell types (2–4).

The intracellular redox state, which is principally buffered by GSH, is coupled to the oxidation state of cysteine residues in proteins (5) by complex thiol/disulfide exchange mechanisms through which redox status influences the activity of a variety of enzymes (6). For example, all protein-tyrosine-phosphatases (PTPs) have a reactive cysteine residue in their active site, which must be in the reduced form for catalytic activity (7). Thus, decreases in intracellular GSH may lead to alterations in the activity of redox-sensitive enzymes, including protein-tyrosine kinases (PTKs) and PTPs (8, 9).

In this study, we document the impact of changes in intracellular GSH on signal transduction in T lymphocytes. Ultimately, T-cell antigen receptor (TCR)-stimulated signal transduction results in the activation of transcription factors that enable the expression of genes involved in the activation of T cells. We and others have already shown that the induction/activation of some of these transcription factors is redox regulated—e.g., NF- κ B (10–12) and AP-1 (13, 14). In addition, several investigators have shown that the culmination of the receptor-triggered T-cell activation process—

proliferation and function—is markedly influenced by the redox state of the cell (reviewed in ref. 15). Data presented here, which identify a central mechanism underlying the GSH dependency of these T-cell responses, show that alterations in intracellular GSH levels change the course of TCR- and cytokine-stimulated signal transduction.

MATERIALS AND METHODS

Cells. Peripheral blood lymphocytes (PBLs) were obtained by Ficoll/Hypaque density centrifugation of whole blood from healthy individuals and were depleted from monocytes by adherence. These PBLs contained >80% T lymphocytes. PBLs and Jurkat T cells were maintained and stimulated in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum, L-glutamine (290 μ g/ml); penicillin (100 units/ml), and streptomycin (70 μ g/ml).

Stimulations and Western Blotting. Jurkat T cells were stimulated for 3 min with tumor necrosis factor α (TNF α , 20 ng/ml), diamide (1 mM), phenylarsine oxide (PAO, 10 μ M), or antibody G19-4 (anti-CD3, 10 μ g/ml). After stimulation, cells were lysed for 30 min on ice in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris Cl, pH 7.5/150 mM NaCl/1% (vol/vol) Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS] containing 50 mM NaF, 100 μ M Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μ g/ml), and antipain (2 μ g/ml). Equal amounts of protein as determined by the DC protein assay (Bio-Rad) were separated by SDS/10% PAGE and transferred to nitrocellulose membranes. Phosphotyrosine-containing proteins were identified by incubating the blot with horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC-20 (Signal Transduction Laboratories). The membrane was developed with the enhanced chemiluminescence (ECL) detection system (Amersham).

Measurement of Intracellular GSH and Calcium Flux. Fluorescence-activated cell sorter (FACStarPlus, Becton Dickinson) measurements of intracellular GSH were done as described (16). Intracellular calcium was measured with indo-1 (Molecular Probes) (17). Baseline calcium levels were recorded, cells were stimulated as indicated in the text, and the ratio of the fluorescence intensities at 480 nm and 400 nm was then continuously recorded during a 6- to 7-min period (60,000–100,000 cells). Fiftieth percentile (median) of the 400/480 ratio for the total cell population was determined at 20-sec intervals (with FACS/DESK software) and plotted as a

Abbreviations: BSO, buthionine sulfoximine; GSH, glutathione; PAO, phenylarsine oxide; PBL, peripheral blood lymphocyte; PTK, protein-tyrosine kinase; PTP, protein-tyrosine-phosphatase; TCR, T-cell antigen receptor; TNF, tumor necrosis factor.

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function of time. Peak calcium values indicate the highest median intracellular calcium level achieved in a given sample.

RESULTS

Lowering Intracellular GSH Selectively Increases the Stimulation of Tyrosine Phosphorylation by Inflammatory Cytokines. Stimulation of T cells through the TCR/CD3 complex (with anti-CD3) leads initially to the tyrosine phosphorylation of a number of proteins (18, 19). Lowering intracellular GSH levels to <20% of the control [by treatment with 100 μ M buthionine sulfoximine (BSO), which specifically inhibits γ -glutamylcysteinyl synthetase, an essential enzyme in GSH synthesis (20)] does not markedly change the basal phosphorylation level. Furthermore, it does not decrease (and occasionally increases) the overall tyrosine phosphorylation levels stimulated by anti-CD3. However, decreasing intracellular GSH to this level dramatically *increases* the levels of tyrosine phosphorylation stimulated by TNF α (Fig. 1).

Inflammatory cytokines such as TNF α are known to stimulate protein tyrosine phosphorylation in macrophages and certain other cell types (21). However, to our knowledge, this is the first demonstration that conditions can be found under which TNF α induces tyrosine phosphorylation in lymphocytes. In fact, as Fig. 1 shows, TNF α is as effective as anti-CD3 in stimulating tyrosine phosphorylation in T cells, once intracellular GSH has been depleted. Thus, taken together, these findings demonstrate that the stimulation of tyrosine phosphorylation by various agents is selectively regulated by intracellular GSH levels.

Oxidants Induce Tyrosine Phosphorylation. The overall level of tyrosine phosphorylation in the cell is controlled by the opposing actions of PTKs and PTPs. Increases in net tyrosine phosphorylation can be caused either by increased PTK activity or by decreased PTP activity. PAO is a PTP inhibitor that reacts with a conserved cysteine residue in the active site of PTPs (22, 23). Treatment of Jurkat T cells (Fig. 1) with PAO leads to increased net tyrosine phosphorylation (most likely by decreasing PTP activity). A similar increase in tyrosine phosphorylation is observed after treatment with diamide, an oxidant that reacts with thiols and therefore depletes GSH. Although PAO and diamide may have additional targets, these findings suggest that oxidant-induced

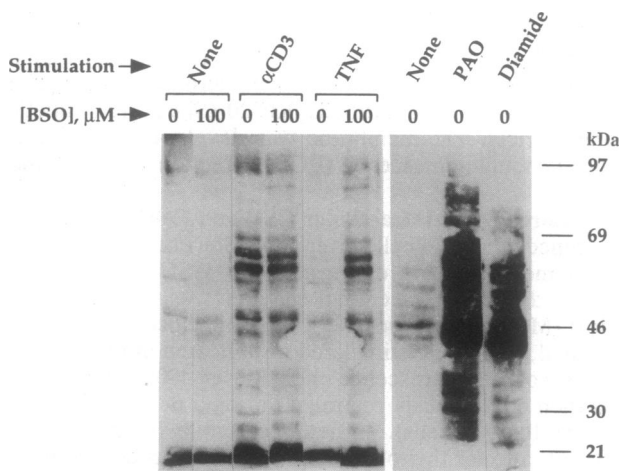


FIG. 1. Redox regulation of tyrosine phosphorylation in Jurkat T cells. (Left) Cells were cultured with either 0 or 100 μ M BSO for 24 hr and then stimulated with anti-CD3 antibody (α CD3, 10 μ g/ml) or TNF α (20 ng/ml). (Right) Cells were treated with the oxidants diamide (1 mM) or PAO (10 μ M) for 3 min. The tyrosine-phosphorylated proteins were analyzed by Western analysis with the RC-20 anti-phosphotyrosine antibody. Positions of molecular size markers are indicated.

increases in tyrosine phosphorylation may be partly caused by decreased PTP activity.

GSH Depletion Does Not Interfere with Signal Transduction Mediated by Serine/Threonine Phosphorylation Events. The idea that GSH regulates T-cell responses by specifically controlling tyrosine phosphorylation events is supported by data showing that BSO treatment does not interfere with T-cell stimulation by phorbol 12-myristate 13-acetate (PMA), which activates the serine/threonine kinase protein kinase C (data not shown). Pretreating Jurkat T cells for 24 hr with BSO (10 or 100 μ M) and then stimulating with PMA for 24 hr (in the continuous presence of BSO) does not alter the PMA-induced surface expression of CD25 (interleukin 2 receptor) and CD69. In fact, both CD25 and CD69 expression are slightly increased in BSO-treated cells after stimulation with PMA.

Lowering Intracellular GSH Decreases Anti-CD3-Stimulated Calcium Flux. A transient increase in free intracellular calcium occurs shortly after the tyrosine phosphorylation events described above (24, 25). Treating cells with a range of BSO concentrations that progressively decreases GSH correspondingly decreases the anti-CD3-induced calcium flux (Fig. 2 Top). This decrease is observed regardless of the calcium flux parameter measured: peak TCR-stimulated intracellular calcium concentrations decrease, the peak appears later, and progressively fewer cells show a detectable flux as GSH levels decrease (data not shown).

GSH levels regulate TCR-stimulated calcium flux in both transformed (Jurkat cell line) and primary (from PBLs) T cells; however, the primary cells show a far greater sensitivity to decreases in GSH (Fig. 3). A decrease of 10–30% in GSH in PBLs is sufficient to completely block the TCR-stimulated calcium flux, whereas a nearly 80–90% decrease in GSH is required to abrogate the flux in Jurkat cells. Thus even small decreases in intracellular GSH in peripheral T cells may have significant physiological consequences.

Lowering intracellular GSH levels by a variety of methods (e.g., growing cells on cysteine-deficient medium or at different cell densities; treating cells for different lengths of time with a single BSO concentration; treating cells with various BSO concentrations for a constant time period) all decrease calcium flux in proportion to the decrease in GSH concentration (Fig. 2 Top). Furthermore, restoring GSH levels (by growing GSH-depleted cells in normal growth medium) restores the calcium flux in proportion to the restoration of GSH (Fig. 2 Middle). The collective display of data from these experiments shows that the magnitude of the TCR-stimulated calcium flux is determined only by the intracellular GSH level, regardless of the means used to achieve that level (Fig. 2 Bottom).

The decrease in calcium flux in GSH-depleted cells is not due to nonspecific interference with physiological function or the overall ability to "flux" calcium. BSO treatment does not alter the CD3 expression, viability, or growth rate over a 2-day period in these cells (data not shown). Furthermore, it does not interfere with calcium flux stimulated by the ionophore ionomycin (data not shown) or by thapsigargin, which triggers the release of internal calcium stores required for initiation of calcium influx (26) (Fig. 4). Thus, the redox-sensitive step is located upstream of calcium release from internal stores.

Aluminum fluoride (AlF_4^-) treatment, like TCR stimulation, leads to the production of inositol trisphosphate and diacylglycerol from phosphatidylinositol. AlF_4^- interacts with guanine nucleotide-binding regulatory proteins (G proteins) that activate phospholipase C- β in the absence of tyrosine phosphorylation. Therefore, both AlF_4^- and CD3 use a similar pathway to stimulate a calcium flux in that both use an isoenzyme of phospholipase C (either β or γ). However, stimulation with AlF_4^- specifically bypasses the requirement

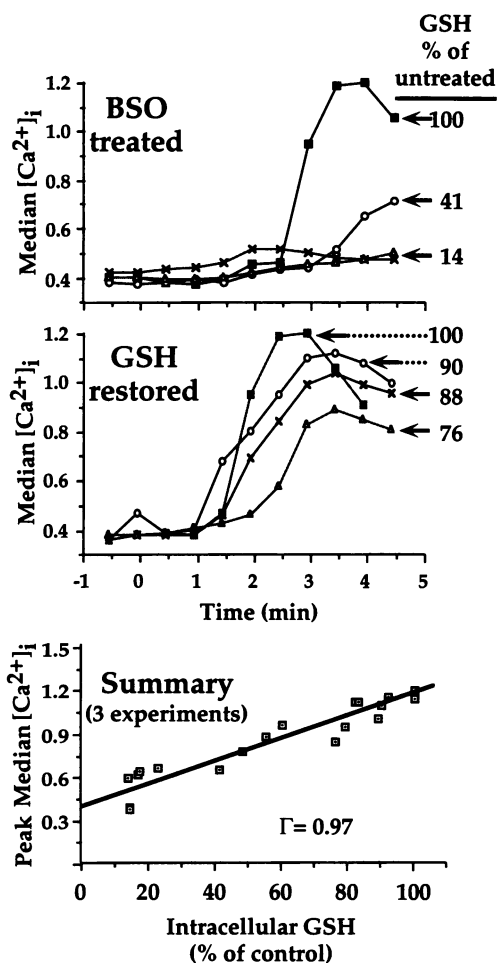


FIG. 2. GSH levels regulate the anti-CD3-induced calcium flux in Jurkat T cells. Anti-CD3-induced calcium flux is decreased in BSO-treated Jurkat T cells. (Top) Cells were treated with 0 μM (\blacksquare) 10 μM (\circ), 40 μM (\times), or 160 μM (\triangle) BSO for 24 hr, loaded with indo-1, and stimulated with anti-CD3. $[\text{Ca}^{2+}]_i$, intracellular concentration of free calcium is revealed by the indo-1 ratio (see Methods). (Middle) Restoration of GSH levels restores the anti-CD3-induced calcium flux. Jurkat T cells were treated for 24 hr with the indicated BSO concentrations (symbols as above), washed, and then cultured in medium without BSO for 12 hr to restore GSH levels, after which GSH levels and calcium flux after anti-CD3 stimulation were measured. (Bottom) Relationship between peak intracellular calcium (after anti-CD3 stimulation) and intracellular GSH levels. Data from several experiments using BSO to deplete GSH in Jurkat T cells are summarized. There is an apparent linear correlation between GSH levels and peak calcium concentration; the significance of this dependence is unknown.

for the tyrosine phosphorylation events occurring after TCR stimulation (27). The flux induced by AlF_4^- is virtually unchanged in BSO-treated cells. Therefore, the signal transduction events downstream of the production of inositol trisphosphate are relatively unaffected by decreased GSH levels (Fig. 4).

We have not yet identified the step through which GSH regulates the TCR signaling cascade. Depletion of GSH inhibits the TCR-stimulated calcium influx, whereas TCR-triggered tyrosine phosphorylation is maintained or increased (24, 25) (Fig. 1). However, it is important to recognize that the phosphorylation of tyrosine residues on proteins that are specifically important to the generation of calcium flux may be unaffected or may be selectively decreased regardless of the overall tyrosine phosphorylation level. In fact, examination of the complex signal transduction pathway triggered by anti-CD3 stimulation indicates that *increases* in tyrosine

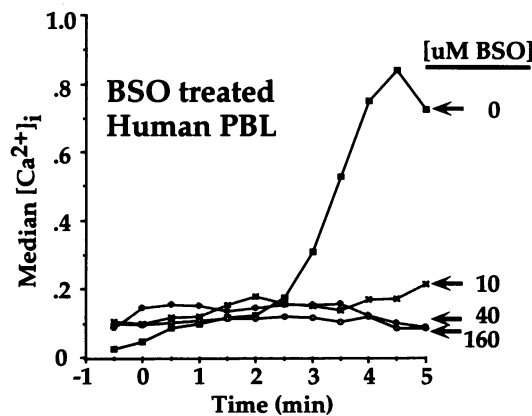


FIG. 3. GSH regulates the anti-CD3 induced calcium flux in primary peripheral blood lymphocytes. Primary T cells were treated with the indicated concentrations of BSO for 2 days, loaded with indo-1, and stimulated with anti-CD3. Median intracellular level of free calcium ($[\text{Ca}^{2+}]_i$) was determined.

phosphorylation of inhibitory sites of Fyn and Lck will repress the activities of these enzymes and result in the *decreased* phosphorylation of other proteins (e.g., phospholipase C- γ 1; ref. 28). Therefore, regulation of the phosphorylation of particular tyrosine residues may mediate the redox sensitivity, as suggested by the studies of Kavanagh *et al.* (29). This hypothesis is supported by immunoprecipitation data from those studies (29), which show that anti-CD3 stimulated tyrosine phosphorylation of phospholipase C- γ 1 is decreased in T cells depleted of GSH by treatment with the oxidant chloro-2,4-dinitrobenzene. Chloro-2,4-dinitrobenzene is less specific than BSO in its action upon GSH levels, since it also reacts spontaneously with protein thiols (30). There-

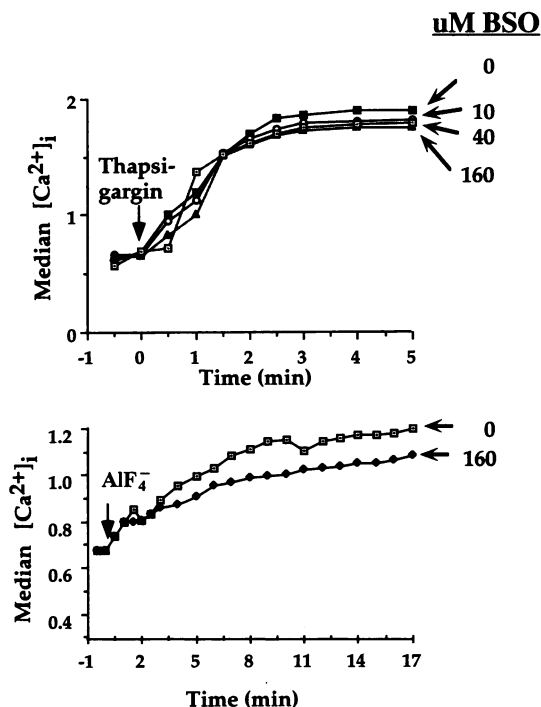


FIG. 4. GSH does not regulate the thapsigargin or the AlF_4^- -induced calcium flux. Jurkat T cells were treated with a variety of BSO concentrations for 24 hr and were then stimulated with either 1 μM thapsigargin (Upper) or 30 mM AlCl_3 plus 25 μM NaF (Lower) at zero time, and then the median intracellular concentration of free calcium ($[\text{Ca}^{2+}]_i$) was determined.

fore these studies need to be confirmed with the more specific agent BSO.

DISCUSSION

The intracellular GSH concentration in T cells provides a central mechanism for modulating responses elicited by antigen and cytokine signals. At higher concentrations of GSH, proliferation and mitogenic stimulations will be favored ("antigenic mode"); whereas at lower GSH concentrations, inflammatory-type responses that involve the induction of transcription factors NF- κ B and AP-1 are more likely to occur ("inflammatory mode"). That is, when GSH levels are high, T cells respond poorly to stimulation with TNF α and interleukin 1 but respond well to TCR stimulation by producing a strong calcium flux (Fig. 3) and actively proliferating. In contrast, when GSH levels are low, T cells fail to flux calcium (Fig. 3) or to proliferate in response to TCR stimulation. Instead, they respond strongly to inflammatory cytokine stimulation with changes in tyrosine phosphorylation pattern (Fig. 1) and the induction of particular transcription factors, such as AP-1 and NF- κ B (10, 14, 31, 32).

These GSH-regulated functional differences could underlie major aspects of the immunodeficiency in individuals infected with human immunodeficiency virus. Such individuals tend to have low levels of extracellular thiols (33), substantial decreases in median intracellular GSH levels in both CD4⁺ and CD8⁺ T cells (ref. 34; reviewed in ref. 15), and increased apoptotic activity (35). As we have shown, lowering median GSH levels in normal PBLs by as little as 10% significantly decreased the anti-CD3-stimulated calcium flux (Fig. 3). Furthermore, lowering median GSH levels by 30% completely abrogated the calcium flux (Fig. 3) and markedly increased overall tyrosine phosphorylation (Fig. 2). These findings suggest that the defective T-cell function in human immunodeficiency virus-infected individuals is due, at least in part, to GSH-regulated changes in the signals transduced following cytokine and antigenic stimulation (36).

F.J.T.S. and M.T.A. contributed equally to this work. We thank Dr. J. Ledbetter for the gift of the G19-4 anti-CD3 antibody and to Becton Dickinson Immune Systems and PharMingen for gifts of antibodies recognizing human cell surface antigens. We are grateful to Drs. R. Lewis, M. Roederer, G. Rijksen, and S. W. Ela for useful and encouraging discussions. F.J.T.S. is supported by Department of Genetics funds and M.T.A. by a University-wide AIDS Research Program fellowship (F90ST019). The overall project is supported in part by National Institutes of Health Grants CA42509 and AI31770.

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