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Forum Review

Oxidants and Tyrosine Phosphorylation: Role of Acute and Chronic Oxidative Stress in T- and B-Lymphocyte Signaling

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ABSTRACT

The cellular response to an extracellular signal starts with the induction of a signaling cascade that transmits the signal through the cytoplasm to the nucleus, resulting in the activation of transcription factors that activate specific target genes. The signaling cascade involves a series of biochemical modifications that include phosphorylation events on tyrosine residues due to the activation of specific protein kinases. Recently, evidence accumulated that reactive oxygen species, including hydrogen peroxide, superoxide, and the hydroxyl radical, are important chemical mediators that regulate the transduction of signals from the membrane to the nucleus by modulating the protein activity by oxidation and reduction. In this report, the redox regulation of signaling involving protein tyrosine kinase activity, in particular in T- and B-lymphocyte signaling, is reviewed. *Antioxid. Redox Signal.* 4, 543–551.

OXIDANTS STIMULATE PROTEIN TYROSINE PHOSPHORYLATION

TYROSINE PHOSPHORYLATION is one of the key modifications in signal transduction. The enzymes that carry out these modifications are the protein tyrosine kinases (PTKs). The PTKs can be subdivided in two classes: the transmembrane receptor PTKs (RTKs) and the nonreceptor PTKs (NRTKs). Activation of PTKs is one of the earliest events upon extracellular stimulation of cells. PTKs transfer γ-phosphate to tyrosine residues, thereby modulating enzyme activity and generating binding sites for recruitment of signaling proteins that lead to downstream transmission of signaling cascades such as the mitogen-activated protein (MAP) kinase and phosphatidylinositol (PI) 3-kinase pathways.

RTKs include the receptors for insulin and growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and nerve growth factor. Binding of insulin to its receptor results in receptor autophosphorylation, and recruitment and tyrosine phosphorylation of the insulin receptor substrate-1 adapter protein (IRS-1) (26). Subsequently, phosphorylated IRS-1 binds signaling molecules that contain Src homology binding domains (SH2), including PI 3-kinase, SHP2 (Syp), Nck, Crk, and Grb2. Evidence accumulated that reactive oxygen species (ROS) could also regulate RTK signal transduction by interference with PTK activity (40, 41, 43, 44, 60). Vanadate, an inhibitor of protein tyrosine phosphatase (PTPase) activity, H_2O_2, and pervanadate (the combination of H_2O_2 and vanadate) are known to have insulin mimetic effects (11, 22, 24, 25, 29, 31). The agents alone or in combination stimulate tyrosine phosphorylation of the insulin receptor and IRS-1. Results suggest that H_2O_2/vanadate augments the autophosphorylation of tyrosines 1,162 and 1,163 of the insulin receptor kinase leading to its activation. However, although pervanadate mimics many of insulin actions, pervanadate treatment does not appear to induce insulin receptor/IRS-1 association (66). In analogy to the insulin receptor, oxidants induce ligand-independent activation of the EGF, PDGF, and FGF receptors. Induction of tyrosine phosphorylation of the EGF and FGF receptor type I was demonstrated to result in complex formation with Shc-Grb2-Sos, resulting in downstream signaling (49, 50). For the EGF receptor, it is demonstrated that H_2O_2 induced a selective phosphorylation pattern that was different from that induced by EGF (15).

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As a common principle, H_2O_2 like the sulfhydryl-alkylating agent iodoacetamide inhibits tyrosine dephosphorylation in a thiol-sensitive manner (13). Hence, the primary target of ROS may be a sulfhydryl group on cysteine residues that, when easy accessible like the cysteine in the catalytic domain of PTPases, is oxidized. It is believed that the tyrosine phosphorylation capacity of H_2O_2 involves the inhibitory effects on multiple intracellular PTPases (11, 19). Because the level of tyrosine phosphorylation of cellular proteins is determined by the balance of PTK and PTPase activity, oxidant-induced inactivation of PTPases results in an apparent enhancement of tyrosine phosphorylation.

The subfamily of NRTKs involves the Jak, Syk, and Src kinases. These kinases have a pivotal role in the regulation of the immunological response. Src family members have multiple substrates, including the PDGF and EGF receptor. The Jak kinases, which are associated with the intracellular domain of cytokine receptors, are trans-activated upon ligand-induced receptor oligomerization. Subsequently, activated Jaks phosphorylate the receptor, which leads to recruitment and phosphorylation of the STAT (signal transducers and activators of transcription) family of transcription factors. Phosphorylated STAT factors dimerize and translocate to the nucleus, where they activate gene transcription. Members of the STAT family of transcription factors, including STAT1 and STAT3, are activated in fibroblasts and A-431 carcinoma cells in response to H_2O_2. STAT activation in these cell lines is oxidant-specific and does not occur in response to superoxide- or nitric oxide-generating stimuli. Treatment of cells with DL-buthionine (*S,R*)-sulfoximine (BSO), an inhibitor of γ-glutamyl-cysteine synthetase, an essential enzyme for glutathione (GSH) synthesis, which depletes intracellular GSH, also activates the STAT pathway. Moreover, H_2O_2 stimulates the activity of the known STAT kinases JAK2 and TYK2. Activation of STATs by PDGF is significantly inhibited by *N*-acetyl-L-cysteine (NAC), an antioxidant, indicating that ROS production contributes to STAT activation in response to PDGF. These findings indicate that the JAK–STAT pathway responds to intracellular ROS and that PDGF uses ROS as a second messenger to regulate STAT activation (58).

Besides an effect of ROS as an extracellular source of oxidative stress, endogenous ROS have also been postulated to induce tyrosine phosphorylation (12). Neutrophils produce large amounts of ROS in response to invading microorganisms, by assembly and activation of a multicomponent enzyme complex, the NADPH oxidase. In accordance, ROS were shown to activate Lyn, Fgr, Hck, Syk, and Bruton's tyrosine kinase (Btk) (7, 12, 67). These data indicate a role for endogenously produced ROS by adherent neutrophils in the regulation of Src family kinase activity and in the cross-talk between reorganization of the cytoskeleton, production of ROS, and Src family tyrosine kinase activities in signaling by adhesion (67).

ANTIGEN RECEPTOR INDUCED SIGNALING IN T- AND B-LYMPHOCYTES

In T and B lymphocytes, NRTKs play an essential role in the transmission of signals generated upon recognition of foreign antigen through antigen specific receptors on their surface [T-cell receptor (TCR) and B-cell receptor (BCR), respectively]. These receptors, which lack intrinsic tyrosine kinase activity, stimulate tyrosine phosphorylation through association with Src family kinases.

Upon TCR activation (Fig. 1), the TCR ζ-chain is phosphorylated on tyrosine by the action of the Src kinases Lck and Fyn, which are attached to the innerside of the membrane through myristylation and palmitylation of their N-terminal portion. The phosphorylated TCR-ζ immunoreceptor tyrosine-based activation motifs (ITAMs) serve as docking site for ZAP-70 (ζ-associated protein kinase of 70 kDa), which becomes phosphorylated and is directly responsible for the phosphorylation of protein Linker for Activation of T cells (LAT) on several tyrosine residues. LAT is an integral membrane that serves as a central adapter protein in the TCR-mediated signaling pathway. LAT interacts with other signaling proteins exclusively through these phosphorylated tyrosine residues (10, 51, 70), thereby mediating the assembly of multiprotein signaling complexes that serve to amplify and diverge the TCR-mediated signal and initiate the downstream signaling cascades. Signaling molecules that associate with LAT after phosphorylation, either directly or indirectly, include the adapter proteins Grb2 (growth factor receptor bound protein 2), SLP76 (SH2 domain-containing leukocyte protein of 76 kDa), Gads (Grb2-related adapter downstream of Shc), Grap (Grb2-related adapter protein), Shb, SLAP (Src-like adapter protein), a phospholipase, PLCγ1, the lipid kinase PI 3-kinase, and the Tec kinase Itk (inducible T-cell kinase) (Fig. 1) (4, 5, 9, 33–35, 57, 61, 70). The association of PLCγ1 with LAT couples the TCR-induced signal to the Ca^{2+}-dependent activation of calcineurin, while the association of the Grb2/Sos complex with LAT results in the activation of the Ras/Raf1/ERK (extracellular signal-regulated kinase) pathway, and the association of an SLP-76/Vav complex activates the MAP kinase p38/Mpk2 through a Rac-1-dependent signaling route.

The PTPase CD45 is essential for signaling in both T and B cells, acting at least in part by dephosphorylation of the negative regulatory C-terminal phosphorylation site in Src family kinases, permitting them to be activated (28, 65).

The BCR-mediated signaling pathway (Fig. 2) shows many similarities to those mediated through the TCR/CD3 complex. As in T cells, the Src family kinases are believed to act early followed by the Syk family kinase Syk in B cells instead of ZAP-70 in T cells. Moreover, B cells do not express LAT. The central role in the BCR-mediated signaling pathways is played by the adapter protein BLNK. B-cell linker protein (BLNK) shows homology with SLP-76 and has integrated the functions of both SLP-76 and LAT, although BLNK is not inserted in the membrane (27).

OXIDATIVE SIGNALING IN T- AND B-LYMPHOCYTES

In T cells, H_2O_2 and pervanadate were shown to induce tyrosine phosphorylation of Lck, Fyn, and ZAP-70 (Table 1, Fig. 1A (20, 21, 42, 54, 55, 59, 69). This effect is likely explained by the inhibitory effects on PTPase activity. Although

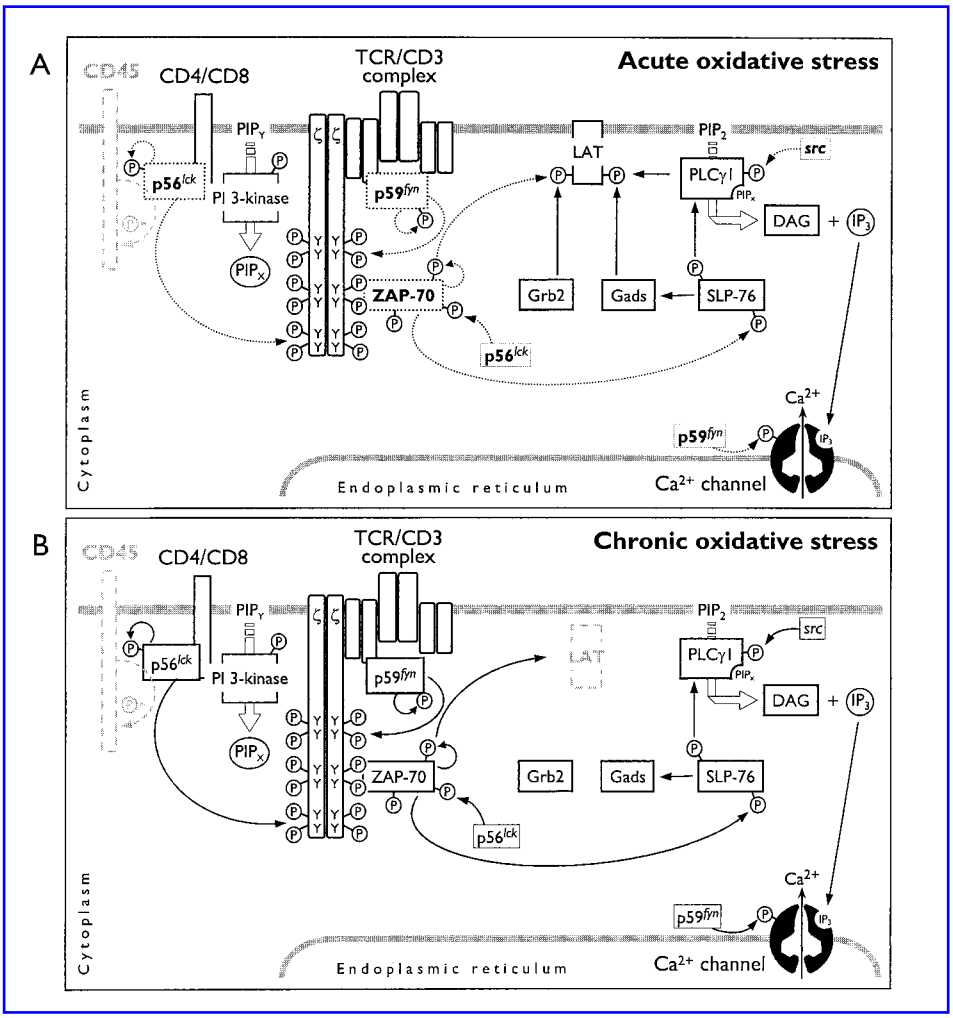

FIG. 1. Redox regulation of TCR signaling. The gray-labeled molecules and dashed lines indicate inhibitory events, whereas bold molecules and dotted lines refer to activation events upon TCR signaling under conditions of acute oxidative stress (**A**) and under conditions of chronic oxidative stress (**B**).

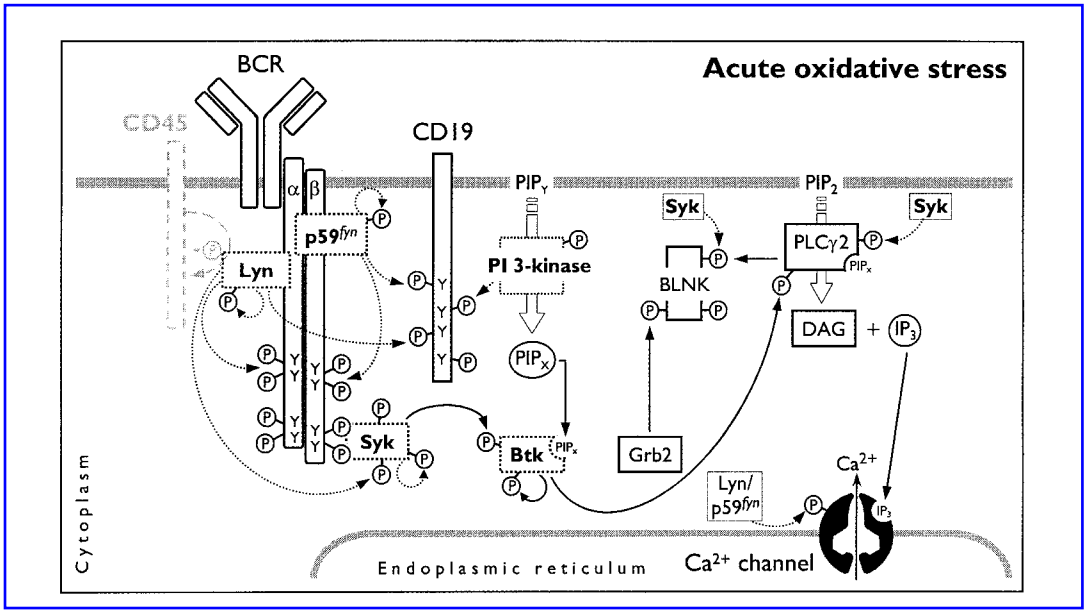

FIG. 2. Redox regulation of BCR signaling. The gray-labeled molecules and dashed lines indicate inhibitory events, whereas bold molecules and dotted lines refer to activation events upon BCR signaling under conditions of acute oxidative stress.

TABLE 1. MODULATION OF PROTEIN TYROSINE REGULATED MOLECULES BY ACUTE AND CHRONIC OXIDATIVE STRESS

Signaling molecule	*Acute*	*Chronic*
RTKs: insulin, EGF, and PDGF receptors	Activation	ND
NRTKs:		
Src, Lck, Fyn, ZAP-70, Syk, Lyn, Fgr, Hck, Btk, Ltk	Activation	Neutral*
Jak2, Tyk2	Activation	Activation
Adapter molecules: LAT	Neutral	Inactivation
PTPase	Inactivation	ND

ND, not determined.
*This was studied for the T-cell PTKs only.

the stimulatory effects of pervanadate on PTR-mediated signaling events were accompanied by marked inhibition of CD45-associated PTPase activity, the ability of pervanadate to stimulate tyrosine phosphorylation in CD45-negative Jurkat cells suggests that PTPases other than CD45 are important intracellular targets for pervanadate. In addition, evidence exists that an as yet unidentified oxidant-induced tyrosine kinase may function as an activator of PTKs (8, 20). Moreover, a regulatory mechanism is proposed in which the H_2O_2-induced activation of Src kinases is triggered by oxidation of specific cysteines (44), *e.g.,* the activity of Lck is regulated by the phosphorylation status of tyrosine residues at positions 394 and 505. Lck is a typical Src-like kinase in that its activity is stimulated by phosphorylation of a tyrosine (for Lck Tyr^{394}) and inhibited by phosphorylation of a highly conserved C-terminal tyrosine (Tyr^{505}) (3, 42). The inhibition of kinase activity by phosphorylation of Tyr^{505} is believed to be a consequence of destabilization of the kinase structure necessary for activation. H_2O_2 will almost certainly inhibit the phosphatase(s) responsible for dephosphorylation of Tyr^{394}, thereby also affecting Tyr^{505}. It is believed that a kinase other than Lck itself is capable of phosphorylating Lck at Tyr^{394}, thereby functioning as an oxidant-induced activator of Lck (35, 40). The compensation for the defect in dephosphorylation of the C-terminal Tyr^{505} may be explained by an oxidant-induced conformational change of the kinase that is critical for its enzymatic function. It was demonstrated that two cysteines at positions 464 and 475 that are highly conserved throughout the Src family are critical for its kinase activity (44). Alkylation of these residues results in a loss of enzymatic activity, whereas oxidation causes enzyme activation (2, 36, 45, 56, 62, 63). These cysteines are thought to play important roles in catalysis and/or substrate recognition by stabilization of the kinase structure for activation. Indicative of the complexity in the way Src kinases are regulated is the finding that activation of Hck by peroxynitrite, a strong oxidant presumed to be formed *in vivo* under intense oxidative stress, or by H_2O_2 could be explained by reversible sulfhydryl redox changes, whereas Lyn was unaffected by H_2O_2 and its direct activation by peroxynitrite occurred through a still unknown modification not reverted by sulfhydryl reduction or inhibited by sulfhydryl alkylation (36). Given these results, it is conceivable that oxidant regulation of tyrosine kinase activity involves, besides inhibition of phosphatases and induction of kinase activity, "sulfhydryl switches" and additional unknown modifications.

Ionizing radiation generates ROS that have a similar effect on the tyrosine phosphorylation status of these kinases. However, whereas the activation of ZAP-70 by UV light requires the presence of the CD3 component and the PTPase CD45, the latter was not required for H_2O_2-induced activation of ZAP-70. This finding indicates a difference in the activation mechanisms induced via H_2O_2 and UV light.

H_2O_2 treated B cells have been shown to exhibit PTK-dependent inositol 1,4,5-trisphosphate (IP_3) generation and Ca^{2+} release (46). Moreover, it was found that Syk is rapidly activated after H_2O_2 treatment as well as in response to BCR activation (Fig. 2) (4, 46, 47, 52, 53). Comparative studies using Syk- and Lyn-deficient chicken B cells revealed that both Syk and Lyn regulate Ca^{2+} mobilization and IP_3 production in B cells in response to oxidative stress, most likely through tyrosine phosphorylation of PLCγ2 (48).

EFFECTS OF CHRONIC OXIDANTS STIMULATION ON T-LYMPHOCYTE ANTIGEN RECEPTOR SIGNALING

In contrast to acute oxidative stress, mimicked by, *e.g.,* H_2O_2 treatment, which often results in activation of signaling pathways and ultimately of transcription factors (1, 6, 16, 70), chronic oxidative stress is disabling to a cell because it interferes with the overall oxidation status, and therefore the functioning of proteins containing a sulfhydryl group (23, 30, 32).

Longitudinal exposure of human T cells to an extended period of weak oxidative stress suppresses transmembrane and nuclear signal transduction(14). Treatment of T cells with products of polyamine oxidase during a period of 2–3 days suppressed both TCR/CD3-induced tyrosine phosphorylation and the interleukin-2 production. Polyamine oxidase activity also caused a reduction in intracellular Ca^{2+} mobilization after mitogenic stimulation. In accordance with oxidant dependence of this suppressive mechanism, NAC significantly reversed the polyamine oxidase effects on signal transduction and cytokine production.

Treatment of peripheral blood (PB) T cells, which normally express LAT in the membrane (Fig. 3A), with BSO leads to a

decrease in the intracellular GSH concentrations, thereby mimicking effects of chronic oxidative stress. These conditions led to displacement of LAT from the plasma membrane (Table 1, Fig. 3C), which as a consequence remained unphosphorylated upon stimulation of the T cells through the TCR/CD3 complex, thus blocking the progression of the TCR-mediated signaling pathways (17). The TCR-mediated signaling cascade leading to LAT phosphorylation, *i.e.,* the successive activation of the Src tyrosine kinase $p56^{lck}$, the recruitment of ZAP-70 to the phosphorylated ITAMs of the ζ-chain of the TCR/CD3 complex, and the activation through phosphorylation of ZAP-70, is intact in cells with lowered intracellular levels of GSH (17) as a hallmark of chronic oxidative stress (Table 1). Since the membrane localization of LAT is crucial for its function, the hyporesponsiveness of T lymphocytes due to chronic oxidative stress is believed to be primarily the result of the membrane displacement of LAT, which abrogates the TCR-induced signaling cascade.

The hyporesponsiveness of T cells in the synovial fluid (SF) of inflamed joints of patients with the chronic inflammatory disease rheumatoid arthritis (RA) is believed to be the consequence of dysfunctional TCR signaling as a result of chronic oxidative stress (37, 38). These cells lack the capacity to tyrosine-phosphorylate LAT, which is displaced from the membrane (Fig. 3B), and have significant decreased levels of the protective antioxidant GSH. Restoration of intracellular GSH concentrations in SF T cells by treatment with NAC, leads to expression of LAT in the membrane (Fig. 3D). The decrease in GSH concentration in these cells is likely a result of the excessive production of ROS, which deplete the antioxidant capacity of the T cells in the inflamed joint. The decreased intracellular concentrations of GSH go together with a marked increase in the concentrations of the antioxidant thioredoxin in the SF (17, 37–39, 68).

Preliminary observations indicate that besides extracellular ROS produced by phagocytic cells as source for oxidative radical stress, ROS accumulate in SF T cells of RA patients as a consequence of deregulated signaling of the GTPase family members Ras and Rap1 (P. Remans, K. Reedquist, J.L. Bos, C.L. Verweij, and S.I. Gringhuis, unpublished observations). In contrast to normal PB T cells, Ras is constitutively active in SF T cells, whereas Rap1 cannot be activated via TCR signaling. As Rap1 was shown to inhibit Ras-induced ROS production in PB T cells, the deregulation of Ras and Rap1 is thought to contribute to excessive intracellular oxidative stress.

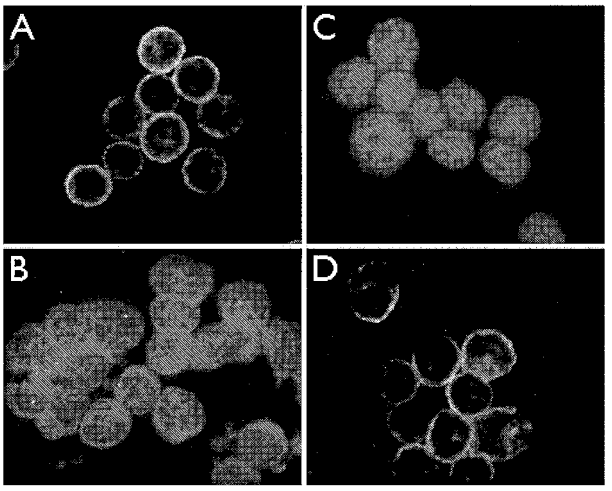

FIG. 3. Subcellular localization of LAT in peripheral blood (PB) and synovial fluid (SF) T cells in the absence and presence of agents that modulate the intracellular redox balance. T cells were isolated and, after being placed in culture, fixed, permeabilized, and incubated with specific antibodies directed against LAT. Subsequently, bound anti-LAT antibodies were detected with fluorescein isothiocyanate-conjugated swine anti-rabbit antibodies. PB T cells from a healthy individual were cultured in medium for 72 h in the absence (**A**) and presence of 200 μ*M* BSO (**C**). SF T cells of a patient with RA were cultured in medium for 48 h in the absence (**B**) and presence of 5 m*M* NAC (**D**).

CHRONIC OXIDATIVE STRESS AFFECTS CONFORMATION OF LAT

GSH can affect the conformation of proteins by altering the oxidation status of their sulfhydryl groups. Recent evidence shows that chronic oxidative stress results in the membrane displacement of LAT by inducing a conformational

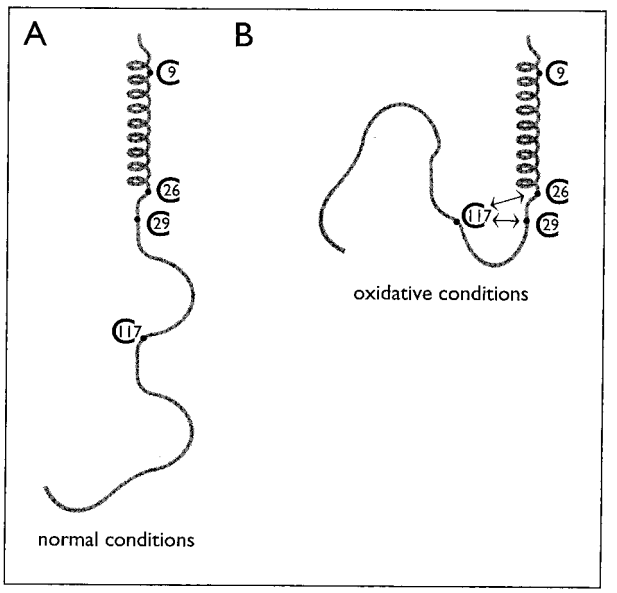

FIG. 4. Hypothetical model for the membrane displacement of LAT under conditions of chronic oxidative stress. (**A**) Under normal conditions, the N-terminal α helix of LAT is inserted into the lipid bilayer of the cellular membrane. (**B**) Under oxidative conditions, the sulfhydryl group at cysteine residue C117 in the cytoplasmic tail of LAT forms an intramolecular or an intermolecular disulfide bond with the sulfhydryl group of either cysteine C26 or C29, which are present at the end of the α helix or just proximal to the α helix, respectively. The induced conformational change causes steric hindrance, thus preventing the α helix from becoming integrated into the plasma membrane and localizing LAT in the cytoplasm, consequently causing the hyporesponsiveness of T cells upon antigenic stimulation.

change of the protein that might interfere with the insertion of the α-helical structure into the plasma membrane (18). Human LAT contains four cysteine residues, which contain sulfhydryl groups, at positions C9 and C26, in the transmembrane α-helix, at position C29, intracellular and just proximal to the α-helix, and at position C117, approximately halfway in the cytoplasmic tail (64, 70). The expression of redox-insensitive cysteine-to-serine mutants at positions C117 and C26/C29 allows for a partial restoration of the TCR signaling pathways under conditions of chronic oxidative stress, leading to the transcriptional activation of the interleukin-2 gene expression. From these experiments, it is hypothesized that oxidative conditions lead to formation of an intramolecular disulfide bond between residue cysteine C117 and either cysteine C26 or C29 (Fig. 4). These results are indicative that LAT plays a role as a crucial intermediate in the sensitivity of TCR signaling, and hence T-cell function toward chronic oxidative stress.

CONCLUDING REMARKS

ROS have emerged as physiological mediators of cellular responses. When exogenous H_2O_2 is applied to cells as one form of ROS, it leads to an increase in tyrosine-phosphorylated proteins that might derive from the activation of RTKs and NRTKs and/or inhibition of PTPases. Furthermore, H_2O_2-stimulated Ca^{2+} mobilization and tyrosine phosphorylation patterns in lymphocytes are similar to those observed following antigen receptor activation. In contrast to this form of acute oxidative stress, high doses of ROS or chronic low-level oxidative stress may shift the redox balance into an oxidative mode affecting the function of proteins susceptible to oxidation. Indeed chronic oxidative stress suppresses mitogen-induced protein tyrosine phosphorylation and Ca^{2+} mobilization in T cells. Furthermore, moderate levels of ROS block the cell cycle, whereas low-dose ROS exert mitogenic effects. These effects of chronic oxidative stress could have severe effects on the functional status of immune cells at the site of a chronic inflammation. Thus, the dosage and the duration of the redox signal are important aspects in the redox regulation of cell function.

ABBREVIATIONS

BCR, B-cell receptor; BLNK, B-cell linker protein; BSO, DL-buthionine-(*S,R*)-sulfoximine; Btk, Bruton's tyrosine kinase; EGF, epidermal growth factor; FGF, fibroblast growth factor; Gads, Grb2-related adapter downstream of Shc; Grb2, growth factor receptor bound protein 2; GSH, glutathione; H_2O_2, hydrogen peroxide; IP_3, inositol 1,4,5-trisphosphate; IRS-1, insulin receptor substrate-1; ITAM, immune receptor tyrosine-based motif; LAT, linker for activation of T cells; MAP, mitogen-activated protein; NAC, *N*-acetyl-L-cysteine; NRTK, nonreceptor protein tyrosine kinase; PB, peripheral blood; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PLC, phospholipase C; PTK, protein tyrosine kinase; PTPase, phosphatase; RA, rheumatoid arthritis; ROS, reactive oxygen species; RTK, receptor protein tyrosine kinase; SF, synovial fluid; SH2, Src homology-2; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; STAT, signal transducers and activators of transcription; TCR, T-cell receptor; ZAP-70, ζ-associated protein kinase of 70 kDa.

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