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INVESTIGATION OF REACTIVE OXYGEN SPECIES (ROS) AS THE SIGNALING MEDIATORS OF IMMUNOSTIMULATION

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ABSTRACT

INVESTIGATION OF REACTIVE OXYGEN SPECIES (ROS) AS THE SIGNALING MEDIATORS OF IMMUNOSTIMULATION

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Reactive oxygen species (ROS) are known to influence various functions of cells including proliferation and apoptosis. When Jurkat T cells were stimulated by H_2O_2, activation-associated phosphorylation of mitogen-activated protein kinases (MAPKs) was induced. H_2O_2 inhibited activities of protein tyrosine phosphatases (PTPs) that were immunoprecipitated from cells. Matrix-assisted laser desorption ionization (MALDI) analysis showed that H_2O_2 treatment of recombinant CD45 modified active site cysteine residue. Ectopic expression of different PTPs inhibited H_2O_2-induced phosphorylation of specific members of MAPKs. N-acetylcysteine inhibited 1-chloro-2,4-dinitrobenzene (DNCB)-induced ROS production, CD45 inhibition, Lck activation, and c-Jun NH_2-terminal kinase (JNK) and p38 phosphorylation. Curcumin, but not resveratrol, inhibited phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT3 induced by interferon-α and Concanavalin A (Con A). Both curcumin and resveratrol inhibited Con A-induced ROS production, JNK phosphorylation and IL-2 mRNA expression. On the other hand, curcumin, but not resveratrol, ablated Con A-induced c-Fos expression.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Reactive Oxygen Species (ROS) are generated by the incomplete reduction of oxygen during various biological processes. It has been known that ROS mediate diverse effects on the function of the cells. Because ROS can be generated rapidly in response to extracellular stimuli and can be degraded efficiently, they have been regarded as potential second messengers. Supporting this concept, various growth factor receptors, cytokine receptors, and GPCRs (G-protein coupled receptors) have been shown to produce ROS including H_2O_2 when cognate ligand binds the receptor (1).

Generation of ROS

The superoxide anion ($O_2^{\cdot -}$) is formed by the univalent reduction of oxygen molecule (O_2). In a cellular system, superoxide anion is produced enzymatically by NADPH oxidase or xanthine oxidase or non-enzymatically by semi-ubiquione in the repiratory chain of mitochondria. Superoxide is converted to H_2O_2 spontaneously or by superoxide dismutase. In the presence of reduced transition metals, H_2O_2 can be converted to the highly reactive hydroxyl radical ($OH^{\cdot}$).

Mithochondrial respiratory chain

ROS are continually produced from the mitochondrial respiratory chain in living cells. The major sites of ROS production in the respiratory chain lay within complexes I (NADH dehydrogenase) and III (ubiquinone-cytochrome c reductase) (2). Ceramide and arachidonic acid produce ROS formation from

mitochondria by direct interaction with the components of respiratory chain (3, 4). ROS production from mitochondria has been linked to the propagation of apoptotic signals and JNK activation. The importance of regulating ROS production from mitochondria was observed in the development of oxidative stress in mice deficient in mitochondrial SOD (Mn-SOD/SOD2) (5) or in mitochondrial glutathione-peroxidase-1 (GPx1) (6).

NADPH oxidase

Professional phagocytes such as neutrophils, macrophages and eosinohils produce high amount of ROS in the context of pathogen killing. Although originally termed respiratory burst because the process accompanies the consumption of O_2, it has been identified that NADPH oxidase, not mitochodrial respiratory burst, is responsible for the process. The components of NADPH oxidase complex are separated between the cytosol and the plama membrane in nonstimulated cells. The cytosolic complex composed of $p47^{phox}$/$p67^{phox}$/$p40^{phox}$ (phox for phagocyte oxidase) and the small GTPase Rac move to the plasma membrane on stimulation and combine with the membrane-bound flavocytochrome b558 (a heterodimer containing $gp91^{phox}$ and $p22^{phox}$) to form the active oxidase. Chronic granulomatous disease (CGD) is caused by a defect in any of the genes encoding $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$ or $p22^{phox}$. The genetic disorder results in the lack of ROS production and poor clearance of many bacterial and fungal pathogens (7). However, it has been questioned that ROS produced by oxidative burst are the major device for directly killing of bacteria. Proteases may be more important than ROS as bactericidal agents

because macrophages from mice deficient in cathepsin G protease can no longer kill bacteria, although their oxidative burst is not impaired (8). The immunodeficiency caused by CGD may not be primarily due to a defect in bacterial killing but rather due to a defect in macrophage and lymphocyte activation (9). Mice deficient in $p47^{phox}$ expression showed impaired NF-κB activation in response to lipopolysaccharide (10). ERK1/2 activation was inhibited by catalase when the respiratory burst in rat alveolar macrophage was stimulated by zymosan-activated serum (11).

Albeit a smaller amount compared to phagocytes, generation of ROS by NADPH oxidase has been also observed in lymphocytes, fibroblasts (12), vascular smooth muscle cells (13), endothelial cells (14), carotid body (15), lung (16) and kidney (17). In parallel to the demonstration, a number of cDNAs encoding homologues of gp91phox have been cloned and named Nox (for NADPH oxidase) or Duox (for Dual oxidase). Nox1 is highly expressed in colon epithelial cells (18). Nox2 designates the classical gp91phox in phagocytic cells. Nox3 was cloned from fetal kidney (19). Nox4 was found in kidney (20) and in osteoclasts (21). NOX5 is highly expressed in lymphocytes in spleen and lymph nodes (22), suggesting that the enzyme may have a role in lymphocyte function. Duox1 and 2 contain peroxidase domain in the N-terminal region and are expressed in thyroid gland (23).

Xanthine oxidase

Xanthine oxidase has been suggested to have a major role for ROS production during ischemic/reperfusion. In addition, levels of circulating xantine

oxidase have been shown to be increased in inflammatory diseases such as arthritis (24), artherosclerosis (25) and septoc shock (26). Xanthine oxidase is able to bind to glycosaminoglycans on the surface of vascular endothelial cells (27). Endothelium-bound xanthine oxidase is increased as a result of tissue inflammation (28). Pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ increased the activity of xanthine oxidase in mammary epithelial cells (29). A major role of ROS produced by xanthine oxidase in inflammation may be the increase of the adhesion of neutrophils to endothelial cells (30, 31). Interestingly, xanthine oxidase is capable of producing NO (32) and the activity of the enzyme is inhibited by NO (33).

Myeloperoxidase

Although microbicidal functions of MPO are well established in vitro, humans deficient in MPO expression generally do not have a higher risk of infection (34). Using an antibody detecting HOCl-modified protein, the generation of HOCl in vivo was demonstrated in human atherosclerotic plaques (35), which myeloperoxidase co-localized (36). HOCl activates MMP-7 in atherosclerotic plaques (37). MPO also inhibits endothelium-dependent vascular relaxation by consuming NO (38).

NO and ROS

Nitric oxide (NO) has been recognized as an important signaling molecule in vessel and neurons (39), although it may function as the agents damaging pathogens in pathological situations (40). Nitric Oxide (NO) activates guanylate

cyclase by binding to the heme moiety of the enzyme. The production of cGMP by guanylate cyclase has been shown to regulate the relaxation of smooth muscle cells and the inhibition of platelet adhesion (41).

By analogy with NO, ROS have both signaling and bactericidal functions depending on the circumstances. The important aspect is that ROS and NO influence the production and function of the other species. H2O2 may participate in the activation of guanylate cyclase activation by stimulating NOS (42) or by interaction of the cyclase with oxidized form of catalase called compound I (43). The interaction of NO with ROS is regarded to be responsible for the generation of RNS such as nitrosonium cation (NO+), nitroxyl anion (NO-), and peroxynitrite (ONOO-) (44). . It has been shown that NOS is capable of producing ROS when the substrate is limited (45). ROS production from NOS was observed in diabetic rats (46). NOS expressed in mitochondria was identified (47). NO production in mitochondria can contribute to the formation of ROS from respiratory chain by building up semi-ubiquinone (48), by enhancing oxygen consumption (49), or by inhibiting cytochrome oxidase (50). NO-induced cell death in human osteoarthritic synoviocytes is mediated by tyrosine kinase activation and ROS formation (51).

Physiological Functions of ROS

Various physiological functions involving ROS have been described as follows (reviewed in (52)): 1) regulation of vascular tone, 2) sensing of oxygen

tension, 3) enhancement of signal transduction, and 4) the maintenance of redox homeostasis

Regulation of vascular tone

H_2O_2 induced a relaxation of the aorta (53). Endothelial cells produce H2O2 that acts as an EDHF (endothelium-derived hyperpolarizing factor) and mediates the relaxation of smooth muscle cells in response to shear stress (54) or bradykinin (55). H2O2 may promote vascular relaxation by stimulating guanyl cyclase activity (56). Interestingly, without involvement of endothelial cells, H2O2 mediates the contraction of smooth muscle cells induced by angiotensin II (57). ROS mediate stretch-induced contraction of bovine coronary artery via activation of EGFR (58). Relaxation of the carotid artery in response to acetylcholine was impaired in CuZnSOD-deficient mice (59).

Sensing of oxygen tension

When arterial oxygen tension decreases, chemoreceptor cells of the carotid body release neurotransmitters that activate the sensory nerve endings of the carotid sinus nerve. Integration of the carotid sinus nerve input in the respiratory control centers of the brain stem results in an increased activity of the respiratory muscle with increased ventilation (60). EPO-producing cells, located mainly in the kidney in adults, release EPO in response to hypoxia. EPO reaches the bone marrow and activates erythropoiesis. Production of prostaglandins and other eicosanoid mediators is also influenced by oxygen tension because O_2 is the substrate for the oxygenation of arachidonic acid and

the hydroperoxy radical is required for the activation of cyclooxygenase and lipoxygenase (61).

Enhancement of signal transduction

It has been shown that various growth factors: (e.g. PDGF, EGF, VEGF), cytokines (TNF-α, IL-1β), and hormones (insulin, leptin) produce ROS upon cognate receptor activation. The attenuation of ROS production by antioxidants or ROS-scavenging enzymes inhibits signaling pathways activated by those stimuli. In addition, transactivation of EGF and PDGF receptors by angiotensin II is mediated by ROS (62, 63).

Maintenance of redox homeostasis

The effects of ROS in signaling have often been attributed to the shift in the redox potential of the cells. The ratio of glutathione disulfide (GSSG)/ glutathione (GSH) is a good indicator of the cellular redox state (64). Cells contain systems to control the amount of ROS. The system is regulated by the redox potential of the cells. The importance of the system is manifested by the dysregulation of the inflammatory process when the system was altered.

ROS in Inflammation

Inflammation is a complex set of interactions among soluble factors and cells that can arise in response to traumatic, infectious, or autoimmune injury (65). Immune cells infiltrate to the inflammatory sites and are activated to protect wounds from invading pathogens. However, excessive and/or sustained activation of immune cells may inhibit the healing procedure of wound (66) and

become a source for the inflammatory diseases (see section 6). ROS have been involved in the various stages of inflammation from initiation to resolution (67). H_2O_2 is present in exhaled air and the amount is increased in patients with inflammatory lung diseases (68, 69).

Chemotaxis

ROS induce chemotactic migration of various types of cells to the inflammatory sites by increasing chemokine expression in macrophages (70). Lysyl oxidase plays a critical role in the formation and repair of extracellular matrix by oxidizing lysine residues in elastin and collagen (71). It has been shown that H_2O_2 produced by the activity of lysyl oxidase mediates chemotaxis for smooth muscle cells (72).

Adhesion

ROS induce the adhesion of leukocytes to endothelial cells (73). H_2O_2 enhances eosionphil adhesion to endothelial cells by inducing the expression of β2 integrin (74). Firm adhesion of leukocytes, which is mediated by β_2-integrins (CD11/CD18), is initiated by engagement of activated complement, LTB_4 and PAF with their receptors on rolling leukocytes. The enhanced generation of oxidants results in the activation and deposition of complement, and phospholipase A_2-mediated production of LTB_4 and PAF in endothelial cells. Oxidants also mediate the initial expression of P-selectin by mobilizing the leukocyte rolling receptor from its preformed pool (Weibel-Palade bodies) in endothelial cells. Sustained rolling and adhesion of leukocytes on endothelial cells are ensured by an oxidant-dependent synthesis of endothelial cell adhesion

molecules, such as E-selectin and ICAM-1 (30). Oxidants, derived from either endothelial cells or leukocytes, elicit those responses by activating specific nuclear transcription factors (e.g., NF-κB).

Cytokine expression and activation

H_2O_2 induces gene expression of TGF-β1 (75) and connective tissue growth factor (76). ROS also activate latent form of TGF-β1 (77). In turn, TGF-β1 induces IL-6 expression in fibroblasts by ROS production (78, 79). Therefore, a feed-forward mechanism is established in which ROS induce the generation of ROS (80). This mechanism may contribute to the amplification of inflammatory signals.

Angiogenesis

Exposure of endothelial cells to H_2O_2 induces angiogenesis (81). NF-kB antisense nucleotide inhibited H_2O_2-induced angiogenesis in endothelial cells (82). H_2O_2 induces VEGF expression in endothelial cells (83) and in keratinocytes (84). ROS scavengers inhibit the angiogenesis induced by lymphocytes (85) and macrophages (86).

Remodeling of Extracellular Matrix

Matrix metalloproteinases (MMPs) are proteases important for cellular migration and tissue remodeling (87). Oxidants increase the activity of metalloproteinases (MMPs) (37, 88) and mediate the induction of the MMP gene expression (89). Lipid peroxidation products induce the expression of fibrogenic cytokines and collagen (90). ROS degrade hyaluronic acid in the extracellular matrix, which amplifies pro-inflammatory signals (91). ROS mediate the collagen

expression induced by TGF-β1 (92). ROS are involved in collagen-induced platelet aggregation and activation (93).

Generation of Autoimmunity

Increased expression of heat shock protein 65 coincides with a population of infiltrating T lymphocytes in atherosclerotic lesions of rabbits specifically responding to heat shock protein 65 (94). Lox-1 was identified as a receptor that mediated the presentation of HSP as an antigen by dendritic cells (95). ROS increase the level of Lox-1 mRNA in aortic endothelial cells (96).

Enhancement of Immune Response

Injury and inflammation lead to hypoxia and elevate lactate in wounds. Lactate enhances angiogenic activity in macrophages (97). Lactate decreases intracellular gluathione levels and enhances IL-2 production in T lymphocytes (98).

ROS in Diseases

Atherosclerosis

Elevated levels of cholestrol were shown to promote leukocyte-endothelial cell adhesion via ROS production from NADPH oxidase (99). A high level of homocysteine that was associated with the high incidence of atherosclerosis was shown to produce ROS in coronary artery via TNF-α production (100). Homocysteine also induced the expression of lox-1, receptor for oxLDL, in endothelium (96) and enhances T cell proliferation (101). SOD is expressed at higher levels in endothelial cells that are exposed laminar flow shear stress and

show the lower susceptibility to develop atherogenesis (102). The level of xanthine oxidase as well as anti-xanthine oxidase antibodies is elevated in patients with coronary heart diseases (103). The interaction of advanced glycation end products with corresponding cell surface receptor (RAGE) produces ROS and decreases GSH levels (104). ROS are involved in AGE-mediated vascular dysfunction through vascular endothelial growth factor (VEGF) production (105).

Diabetes

ROS provide a multi-faceted role in the vascular and neuronal complication caused by hyperglycemia in diabetes (reviewed in (106)). Increased glycolysis generates high level of NADH that provides a source of ROS by NADH oxidase and mitochondrial respiratory chain (107). Excess glucose is consumed by polyol pathway that depletes NADPH and thereby decreases cellular reducing capacity. High level of glucose may generate the non-enzymatic glycation of proteins yielding AGEs that have an important role in the complications of the inflammation. Gene transfer of SOD reverses endothelial dysfuction in aorta of diabetic rats (108).

A high level of glucose was shown to induce the rearragement of actin cytoskeleton in mesangial cells through ROS production (109). Hyperglycemia activates MMP-9 in a ROS-dependent manner in bovine aortic endothelial cells (110). Leptin, a hormone that controls body weight, induces production of ROS from mitochondria and oxidation of fatty acids (111). Hyperglycemia-induced mitochondrial ROS production induces plasminogen activator inhibitor-1 by

increasing Sp1 glycosylation (112). Interestingly, placental vessels from gestational diabetic patients showed a loss of relaxation response to exogenous and endogenous (lactate-derived) H2O2 (113). Lox-1 expression was upregulated in vascular endothelial cells of streptozotocin-induced diabetic rats (114).

Rheumatoid Arthritis

The rheumatoid synovial microenvironment is relatively ischemic and hypoxic (115). Hypoxia/reoxygenation induces NF-κB activation and ICAM-1 expression in synovial fibroblasts with the enhanced adhesion of lymphocytes (116). Xanthine oxidase mediates bone resorption induced by TNF-α and IL-1β (117). TNF-α is a major cytokine for inflammatory arthritis such as rheumatoid arthritis where it stimulates synovial hyperplasia and cartilage destruction. It has been shown that TNF-α induces c-fos gene expression in chondrocytes by NADPH-dependent ROS production (118). Antioxidants inhibit TNF-α-induced IL-8, MCP-1, and collagenase expression in synovial cells (119). Integrin induced MMP-1 gene expression by ROS-dependent IL-1α induction in rabbit synovial fibroblasts (120). IL-1β also induces c-fos and collagenase expression in chondrocytes in ROS-dependent manner (121). The risk of rheumatoid arthritis is increased in patients with low levels of serum antioxidants such as α-tocopherol and β-carotene (122). The transfer of SOD and catalase genes ameliorated antigen-induced arthritis in rats (123). N-acetylcystein also alleviated collagen-induced arthritis in mice (124).

Ischemia/Reperfusion

Xanthine oxidase and NADPH oxidase have been shown to produce high amount of ROS during ischemia/reperfusion injury (125, 126). SOD gene transfer ameliorated tissue damage caused by ischemia/reperfusion (127, 128). Conversely, glutathione peroxidase knockout mice are susceptible to myocardial ischemia reperfusion (129).

Neurodegenerative Diseases

ROS generated by oxidation of dopamine has been implicated in the destruction of dopaminergic neurons in Parkinson's disease (130). Amyloid β-protein activates NADPH oxidase in microglial cells, which contributes to ROS-mediated inflammatory process in Alzheimer's disease (131). It has been shown that amyloid β activates cells by engaging RAGE (132). The intracellular redox state modulates the balance between proliferation and differentiation in glial precursor cells (133). The level of uric acid, a general antioxidant in the plasma, was significantly reduced in the brains of patients with Parkinson's (134) and Alzheimer's disease (135). The decrease of cytochrome oxidase activity by mutations of mitochondrial DNA was associated with late-onset Alzheimer's disease (136).

Cancer

Cancer cells produce high amount of ROS (137). When fibroblasts were transfected with oncogenic form of Ras, the proliferation capacity of the cells was correlated with the amount of ROS produced upon transfection (138). ROS production by tumor cells has been suggested to induce proliferation and angiogenesis. Humans with MPO deficiency show a high incidence of malignant

tumors as MPO-deficient leukocytes exhibit a poor lytic action against malignant cells (34). Ectopic expression of Nox1, an isoform of nonphagoctyic NADPH oxidase, in fibroblasts induced malignant transformation that was reversed by catalase coexpression (139). Transfection of cancer cells with 12-LOX or 15-LOX enhances their tumorgenic potential (140, 141).

HIV infection

A massive loss of glutathione in plasma as well as in pheripheral blood lymphoctes is observed in HIV infection (142). NAC is regarded to be a useful treatment for HIV infection by replenishing GSH pool (143, 144).

ROS in T Cells

Functional activation of T cells has been shown to be enhanced by the exposure of ROS and by the shift of the intracellular redox status (145-147). The enhancement of T cell function by ROS may have important consequences when the ligand concentration for TCR or costimulatory receptors is suboptimal. ROS in the inflammatory condition may lower the threshold for triggering T cell activation. According to this model, simultaneous injection of glutathione was found to inhibit in vivo immunization by small amounts of antigen (148). There is evidence that phagocytes release ROS in a quantal manner in the immunological synapse created by the interaction of T cell receptor with MHC-bound peptide (149). Helper T cells are not properly activated in p47phox-deficient mice (150). The profile of cytokine production by antigen-presenting cells is influenced by the

intracellular redox status (151), thereby regulating the balance between Th1 and Th2 cell types (152).

ROS-induced Signaling pathways

Activation of Mitogen-Activated Protein Kinase (MAPK)

One well documented result of cell stimulation with H_2O_2 is MAPK activation. The MAPK family members considered in this report include ERK, p38, and JNK. ERK activation has been mainly implicated in proliferation in response to growth factors, whereas p38 and JNK activation are more important to stress responses, such as in inflammation. It has been suggested that the combination of the magnitude and kinetics of activation of each member of MAPK family determines the appropriate response of the cell according to the specific stimulus (153). In the case of T cells, the distinct activation profile of three members of the MAPK family has been shown to influence the specific stages of thymocyte development as well as the precise effector function of mature T cells (154).

Inhibition of Protein Tyrosine Phosphatase (PTP)

One of the potential molecules directly affected by ROS are the protein tyrosine phosphatases (PTPs). All PTP contain a conserved cysteine residue that is located in the motif HC(X5)RS/T (155), and the mutation of the cys resulted in the loss of activity. The cysteine acts as a nucleophile, forming a thiophosphoenzyme intermediate during hydrolysis. The positively charged electrostatic field provided by nearby amino acid residues such as Arg and His

maintains the cysteine residue in the form of thiolate anion (-S-) that was more susceptible to the oxidation by ROS compared to other cysteine residues. Supporting this concept, treatment of purified PTPs with H_2O_2 inhibits the activity of the enzyme (156) and exposure of cells to H_2O_2 also leads to the downregulation of PTP activity (157, 158). Hyperglycemia has been reported to decrease PTP activity in platelets in a ROS-dependent manner (159). Stimulation of cells with insulin or EGF inhibits PTP1B activity with endogenous production of ROS (160, 161). PDGF increases intracellular ROS and inactivates LMW PTP and SHP-2 by oxidation (162, 163). These results suggest that PTP inhibition by ROS has physiological significance during cell stimulation.

Conclusion

Cells have the ability to adapt to the change in the redox status occurred inside the cells as well as outside the cells. Generation of ROS from mitochondrial respiratory chain intrinsically suggests that cells need to cope with ROS. Generation of ROS at the inflammatory sites indicates that cells need to fulfill their functions during exposure to ROS. It is conceived that cellular adaptation to the oxidative environment occurs through the activation of signaling pathways. Moreover, ROS production itself is a regulated process, as evidenced by the modulation of the activity of ROS-producing enzymes by cytokines and growth factors. Therefore, it is important to understand how cellular signaling pathways are activated by ROS.

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CHAPTER TWO

CAMP POTENTIATES H_2O_2-INDUCED ERK1/2 PHOSPHORYLATION WITHOUT THE REQUIREMENT FOR MEK1/2 PHOSPHORYLATION

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ABSTRACT

In Jurkat T lymphocytes, hydrogen peroxide (H_2O_2) potentiates the phosphorylation level of extracellular signal regulated kinase 1 and 2 (ERK1/2) caused by T cell receptor (TCR) stimulation with anti-CD3 and anti-CD28 or anti-CD3 alone. Submillimolar concentrations of H_2O_2 induced phosphorylation of ERK1/2 and MAP/ERK kinase 1 and 2 (MEK1/2) without antigenic stimulation. H_2O_2 also induced the electrophoretic mobility shift of Lck from 56 kDa to 60 kDa. The MEK inhibitor, PD98059 attenuated ERK1/2 and MEK1/2 phosphorylation as well as the migration shift of Lck induced by H_2O_2. The phospholipase C (PLC) inhibitor, U73122, and EGTA reduced the phosphorylation of both ERK1/2 and MEK1/2 induced by H_2O_2. Interestingly, an increase of intracellular cAMP level with forskolin or 8-(4-chlorophenylthio)-cAMP augmented ERK1/2 phosphorylation by H_2O_2, while inhibiting MEK1/2 phosphorylation by H_2O_2. These results demonstrate an alternative pathway that results in augmentation of ERK1/2 phosphorylation without concomitant MEK1/2 phosphorylation in T cells.

INTRODUCTION

Both exogenous treatment and endogenously produced hydrogen peroxide (H_2O_2) has been shown to affect a number of cellular functions including gene activation, proliferation, and apoptosis [1]. H_2O_2 is produced from mitochondria in the reduction of O_2 to H_2O during respiration [2]. Substantial amount of H_2O_2 is produced and secreted by activated phagocytes through NADPH oxidase system at sites of inflammation [3]. In both cases, superoxide ($O_2^{\cdot-}$) may be converted to H_2O_2 spontaneously or by the action of superoxide dismutase [4]. There is increasing evidence to suggest that H_2O_2 generated at low levels during normal cell signaling may act as a second messenger [5]. Various growth factor receptors, cytokine receptors, and G-protein coupled receptors have been shown to generate H_2O_2 following ligand activation (reviewed in [6]). It is not clear whether H_2O_2 production by these receptors is linked to the activation of NADPH oxidase, although several examples of NADPH oxidase system similar to that in phagocytes have been found in nonphagocytic cells [7,8].

H_2O_2 is capable of affecting the function of proteins by oxidizing thiol groups in Cys amino acid residues or in cofactor molecules. Transcription factors such as AP-1 and NF-κB respond to redox change caused by H_2O_2 [9]. In addition the Cys118 residue of Ras was shown to be sensitive to oxidants such as H_2O_2 and nitric oxide (NO) [10]. The most well documented effect of H_2O_2 on signaling is due to the fact that H_2O_2 functions as a general inhibitor of protein tyrosine phosphatases (PTPs) by oxidizing the essential catalytic Cys residue to a sulfenic acid (Cys-SOH) [11]. The catalytic Cys residue of PTPs is

vulnerable to H_2O_2 because the pKa of the residue is about 5.5 as compared to a pKa of about 8 for most other protein Cys residues [6]. This low pKa is maintained by basic amino acid residues in the active site of the enzyme [12]. It has been proposed that, because H_2O_2 is a mild oxidant compared to other reactive oxygen species, the inhibition of PTPs would be the predominant cellular effect at low concentrations of H_2O_2 [13]. This is supported by the rapid increase of tyrosine phosphorylation in cells treated with H_2O_2. Inhibition of PTPs by H_2O_2 leads to unopposed action of protein tyrosine kinases which activate phosphorylation cascades leading to downstream signals such as phosphorylation of Mitogen-Activated Protein Kinase (MAPK) [14].

Certain lymphocytes, including Jurkat cells, produce reactive oxygen species (ROS) responding to anti-Fas antibody (Ab) [15], and activation of the T cell receptor (TCR) by anti-CD3 Ab has been proposed to produce ROS including H_2O_2 [16]. CD28 costimulation produces ROS by a lipoxygenase, resulting IL-2 gene expression through NF-κB activation [17]. Moreover, H_2O_2 may be supplied to T cells at the site of inflammation where activated granulocytes release large amounts of H_2O_2 leading to relatively large local concentrations (estimated at 10 to 100 μM) [18-20]. Treatment of cells with micromolar concentrations of H_2O_2 was previously shown to enhance IL-2 gene expression after TCR activation as well as mitogenically induced T cell proliferation [20,21].

P44/42 MAPK (ERK1/2) is activated by a variety of extracellular signals including growth factors, cytokines, T cell antigens, phorbol esters, and

hormones [22]. The enzyme is activated by phosphorylation at a tyrosine and a threonine residue located in the "activation loop" [23]. These residues are phosphorylated by the dual-specificity kinases, MEK1 and 2 [24]. The activated form of ERK1/2 not only phosphorylates cytosolic substrates such as RSK, but also translocates to the nucleus, phosphorylating and thereby activating transcription factors such as Elk-1 [25]. Activation of MEK1/2 is also controlled by phosphorylation of serine residues [26]. MAPK kinase kinases (MAPKKK) such as Raf-1 are responsible for phosphorylating MEK1/2 [27].

There is a complex interaction between cAMP and the ERK signaling pathway [28]. cAMP activation of protein kinase A (PKA) is known to disturb the Raf-1→ MEK1/2 → ERK1/2 cascade by phosphorylating Raf-1, an event which inhibits its activation by the Ras G-protein [29,30]. Besides inhibiting Raf-1, cAMP can also lead to ERK activation, changing the components in ERK cascade by activating Rap1 in B-raf (a member of MAPKKK family) expressing PC12 cells [31]. Interestingly, ERK activation by PKA can also be achieved by phosphorylating and inhibiting PTPs that dephosphorylate tyrosine residue in ERK [32,33]. The inhibition of these PTPs by PKA has a positive role in the maintenance of ERK activation. In a feedback loop, ERK can affect the level of cAMP by phosphorylating a family of cAMP-specific phosphodiesterases (PDE4D) [34]. For instance, a short isoform of PDE4D, PDE4D1, is activated by ERK phosphorylation, whereas the long isoforms are inhibited by ERK phosphorylation. In the case of long PDE4D isoforms, the inhibitory effect of ERK phosphorylation is ablated when PKA phosphorylates the

phosphodiesterases [35,36]. These complex cross talk mechanisms are proposed to control the amplitude and duration of ERK1/2 phosphorylation, thereby modulating the specific outcome depending on the stimuli [37].

We have addressed one aspect of the cross talk affecting ERK1/2 by treating cells with H_2O_2 and appropriate inhibitors and activators. Treatment of T cells with H_2O_2 in the micromolar range reduced the threshold for effective T cell signaling as assessed by phosphorylation of ERK1/2. H_2O_2 induced the migration shift of Lck from p56 to p60, an event relevant to ERK1/2 activation and T cell activation. We found that cAMP had an unanticipated role in H_2O_2-induced ERK1/2 phosphorylation in T cells. When intracellular cAMP levels were increased, the H_2O_2-induced ERK1/2 MAPK phosphorylation was potentiated, whereas upstream MAPK Kinase (MAPKK), MEK1/2, phosphorylation was inhibited. Thus we conclude that H_2O_2 can affect the cross talk between ERK MAPK cascade and cAMP signaling in T cells. These results illustrate an alternative pathway that results in augmentation of ERK1/2 phosphorylation without concomitant MEK1/2 phosphorylation in T cells.

MATERIALS AND METHODS

Reagents and Antibodies

H_2O_2, PD98059, ethylene glycol-bis[beta-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA), forskolin, 8-(4-chlorophenylthio)adenosine-3`:5`-cyclic monophosphate and 8-(4-chlorophenylthio)guanosine-3`:5`-cyclic monophosphate were purchased from Sigma (St. Louis, MO). U73122 was purchased from Calbiochem (La Jolla, CA). Monoclonal anti-CD3 clone 235 (IgM type) and anti-CD28 clone NE51 (IgG type) were generously provided by Dr. Shu Man Fu (University of Virginia, Charlottesville, VA). Phospho-p44/42 (ERK1/2), phospho-MEK1/2, and p44/42 (ERK1/2) antibody were purchased from New England Biolabs (Beverly, MA). Lck and ERK2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibody were purchased from Bio-Rad (Hercules, CA). Horseradish peroxidase-conjugated rabbit anti-goat antibody was purchased from Calbiochem.

Cell culture

Jurkat cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc. Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), streptomycin/penicillin (100 units/ml; Life Technologies), and 50 μM beta-mercaptoethanol (Sigma). Cells were maintained in an exponential growth state (1.0-5.0 × 10^5 cells/ml).

Stimulation and activation of cells

Cells were reconstituted in the concentration of 1 X 10^6 cells/ml media before stimulation. Because PD98059, forskolin and U73122 were dissolved in dimethyl sulfoxide (DMSO), the same volume of DMSO was included in the control sample whenever these reagents were used. All antibody stimulation (anti-CD3 & anti-CD28) and H_2O_2 stimulation were performed for 10 min.

Cell lysis

Cells were washed twice with ice-cold phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4) and then lysed in an appropriate volume (3×10^7 cells/ml) of lysis buffer (1% Nonidet P-40 (Pierce), 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.23 units/ml aprotinin, 0.5 μg/ml leupeptin, 10 μg/ml DNase I, and 0.2 mM sodium orthovanadate) for 30 min on ice. Cell nuclei were pelleted by centrifugation at 10,000 rpm at 4°C for 10 min.

Immunoblotting

Lysates (30 μg) were separated with 10% SDS-PAGE gel and proteins were transferred to nitrocellulose (Bio-Rad). The membrane was blocked in 5% nonfat dry milk in TBS/T (80 mM Tris, pH 8.0, 3.6% (w/v) NaCl, 0.1% Tween-20 (Bio-Rad)) for 1 hr and incubated in primary antibody at 4°C overnight, followed by incubation in secondary antibody for 1 hr. Between treatments, the membrane was washed five times with TBS/T solution. Bands were visualized with chemiluminescence (Amersham, Arlington Heights, IL).

RESULTS

H_2O_2 enhances TCR-induced ERK1/2 phosphorylation.

We determined whether treatment of Jurkat T cells with H_2O_2 and with anti-CD3/CD28 antibodies would reduce the threshold of T cell activation at the level of ERK1/2 phosphorylation. As shown in Fig. 1, 50 μM of H_2O_2 enhanced ERK1/2 phosphorylation by TCR stimulation with anti-CD3/CD28 and anti-CD3. Anti-CD28 stimulation alone or together with 50 μM H_2O_2 was not able to induce ERK1/2 phosphorylation. Treatment with H_2O_2 alone at this concentration did not increase ERK1/2 phosphorylation. Anti-CD3/CD28 was more potent than anti-CD3 alone in the induction of ERK1/2 phosphorylation, and anti-CD3 with H_2O_2 increased ERK1/2 phosphorylation up to the level induced by anti-CD3/CD28. Phosphorylated ERK1/2 was detected using a phospho-p42/44 MAPK (ERK1/2) antibody which binds preferentially to the doubly phosphorylated form of ERK1/2 (phosphorylated at both tyrosine and threonine residues).

H_2O_2 treatment increases ERK1/2 and MEK1/2 phosphorylation.

To define the pathway leading to the enhancement of ERK1/2 phosphorylation by H_2O_2, we first determined the concentration of H_2O_2 required to induce phosphorylation ERK1/2. As shown in Fig. 2A (middle panel), 0.1 mM H_2O_2 was the minimum amount able to increase ERK1/2 phosphorylation without other TCR stimulation. Treatment with increasing amount of H_2O_2 showed that ERK1 (p44) was phosphorylated at low H_2O_2 concentrations (~0.1 mM), followed by the phosphorylation of ERK2 (p42) at higher concentrations (~0.5 mM). This

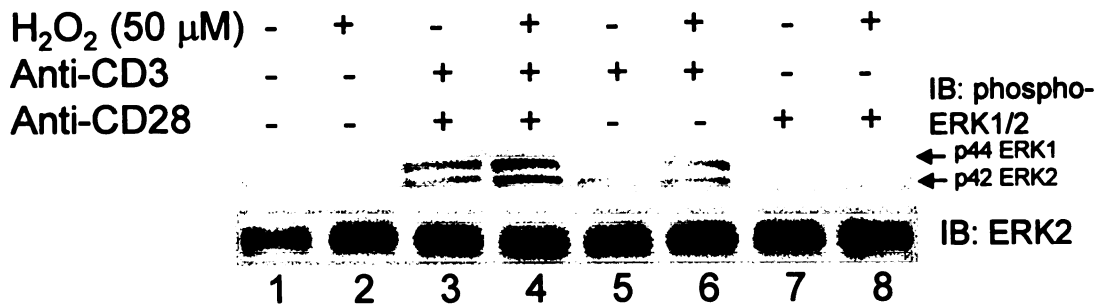
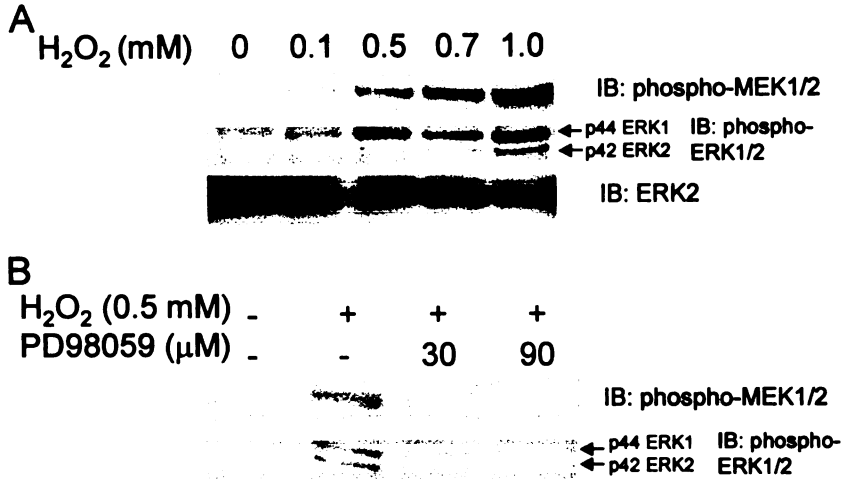
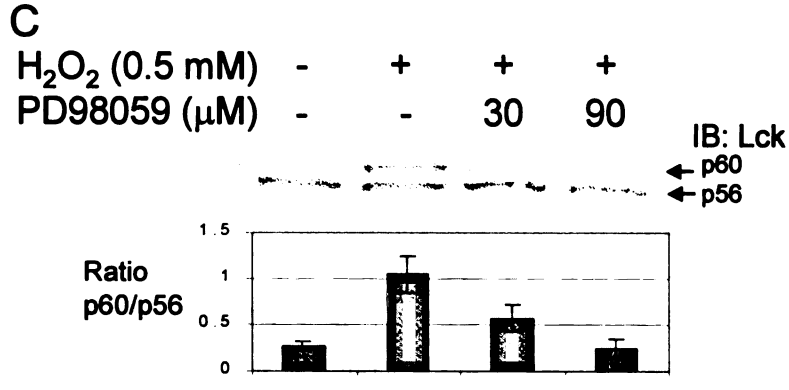


FIGURE 1. H_2O_2 potentiates TCR-induced ERK phosphorylation. Anti-CD3 (clone 235 1/500) and anti-CD28 (clone NE51, 1/500) were treated for 10 min with or without 50 μM H_2O_2, as indicated. After blotting with phospho–ERK1/2 Ab, the membrane was reprobed with ERK2 Ab to show that similar amounts of lysates were present in each lane. Result is representative of triplicate experiments.

FIGURE 2. H_2O_2 induces MEK1/2 and ERK1/2 phosphorylation and Lck mobility shift. (A) H_2O_2 induces ERK1/2 and MEK1/2 phosphorylation. Cells were treated with the Indicated amount of H_2O_2 for 10 min. After blotting with phospho-MEK1/2 and phospho-ERK1/2 Ab, the membrane was reprobed with ERK2 Ab as a loading control. (B) PD98059 reduces H_2O_2–induced ERK1/2 and MEK1/2 phosphorylation. Cells were pretreated with PD98059 for 30 min at the indicated concentration, followed by stimulation with 0.5 mM H_2O_2 for 10 min. (C) H_2O_2 induces the migration shift of Lck, which is blocked by PD98059. PD98059 was pretreated for 30 min at the indicated concentration, and cells were stimulated by 0.5 mM H_2O_2 for 10 min subsequently. The ratio of band intensity of p60 over that of p56 is plotted under the blot. Results are representative of triplicate experiments (±SD).


A
H_2O_2 (mM) 0 0.1 0.5 0.7 1.0
IB: phospho-MEK1/2
p44 ERK1
p42 ERK2
IB: phospho-ERK1/2
IB: ERK2
B
H_2O_2 (0.5 mM) - + + +
PD98059 (μM) - - 30 90
IB: phospho-MEK1/2
p44 ERK1
p42 ERK2
IB: phospho-ERK1/2


C
H_2O_2 (0.5 mM) - + + +
PD98059 (μM) - - 30 90
IB: Lck
p60
p56
Ratio p60/p56
1.5
1
0.5
0

suggests that ERK1 is more responsive to H_2O_2 than ERK2 at low H_2O_2 concentrations.

An increase in the phosphorylation of MEK1/2 was also detected after treatment with 0.1 mM H_2O_2 (Fig. 2A, upper panel). MEK1/2 phosphorylation was detected using a phospho-MEK1/2 antibody specific for the doubly phosphorylated form of MEK1/2 (two serine residues). Because the molecular weights (~46 KDa) of MEK1 and 2 isoforms are very close, only one band was detected. The increase of MEK1/2 phosphorylation indicates that, under these conditions, H_2O_2 acts by activating upstream kinases such as Raf-1. Ser/Thr phosphatases, such as those which dephosphorylate MEK1/2, are likely to be resistant to the inhibitory effect of H_2O_2 at submillimolar concentrations [11].

MEK inhibitor suppresses H_2O_2-induced phosphorylation of ERK1/2 and MEK1/2.

To determine whether MEK1/2 mediates ERK1/2 phosphorylation by H_2O_2, we treated the cells with the MEK inhibitor, PD98059. PD98059 inhibited both ERK1/2 and MEK1/2 phosphorylation by H_2O_2 (Fig 2B), suggesting that MEK1/2 is involved in the signaling pathway leading to ERK1/2 phosphorylation.

H_2O_2 induced-migration shift of Lck from p56 to p60 is blocked by MEK inhibitor.

The MAPK-mediated phosphorylation of Ser-59 in Lck correlates with the electrophoretic mobility shift of Lck from 56 kDa to 60 kDa [38]. The shift is relevant to T cell activation, because anti-CD3 [39], phorbol 12-myristate 13-acetate (PMA) [40], and ionomycin [41] were shown to induce this migration shift.

ERK binding to Lck has been proposed to have a positive role in T cell activation by excluding SHP-1 recruitment to Lck [42]. We found that H_2O_2 induced the migration shift of Lck, and the MEK inhibitor PD98059 in a dose dependent fashion blocked the Lck shift by H_2O_2 (Fig. 2C), suggesting that ERK is responsible for the H_2O_2–induced Lck shift.

U73122 and EGTA reduce H_2O_2-induced ERK1/2 and MEK1/2 phosphorylation.

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which activate protein kinase C (PKC) and increase intracellular Ca^{2+} level, respectively [43]. Treatment of Jurkat cells with the PKC activator, PMA [44] and the calcium ionophore, ionomycin [45] were independently shown to result in phosphorylation of ERK1/2. H_2O_2 and pervanadate were shown to induce tyrosine phosphorylation of PLCγ and production of inositol phosphates [46]. We used the PLC inhibitor, U73122, to determine whether phospholipase C was involved in ERK1/2 phosphorylation by H_2O_2. As shown in Fig 3A, U73122 reduced the level of phosphorylation in both ERK1/2 and MEK1/2 by H_2O_2 in a dose-dependent manner. This suggests that part of the H_2O_2 signaling pathway leading to ERK1/2 phosphorylation involves phosphoinositide turnover caused by PLC.

H_2O_2 is known to activate calcium channels in the plasma membrane of several types of plant cells [47,48], and H_2O_2 produced by EGF receptor has an important role in calcium influx from extracellular space [49]. Exogenously added

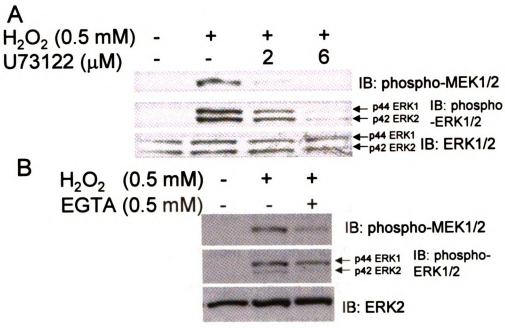


FIGURE 3. U73122 and EGTA inhibit MEK1/2 and ERK1/2 phosphorylation by H_2O_2. (A) U73122 reduces H_2O_2–induced MEK1/2 and ERK1/2 phosphorylation. Cells were pretreated with U73122 for 10 min at the indicated concentration, followed by stimulation with 0.5 mM H_2O_2 for 10 min. After blotting with phospho-MEK1/2 and phospho-ERK1/2 Ab, the membrane was reprobed with ERK1/2 Ab as a loading control. (B) EGTA attenuates H_2O_2–induced ERK1/2 and MEK1/2 phosphorylation. Cells were incubated with 0.5 mM EGTA for 30 min, followed by stimulation with 0.5 mM H_2O_2 for 10 min. After blotting with phospho-MEK1/2 and phospho-ERK1/2 Ab, the membrane was reprobed with ERK2 Ab as a loading control. Results are representative of three independent experiments.

H_2O_2 has been shown to increase the intracellular calcium level in lymphocytes including Jurkat T cells [50]. We determined whether calcium influx participated in H_2O_2-induced ERK1/2 phosphorylation by incubating cells with EGTA before H_2O_2 stimulation. As shown in Fig. 3B, extracelluar calcium depletion by 0.5 mM EGTA reduced H_2O_2- induced ERK1/2 and MEK1/2 phosphorylation. These results (Fig. 3) suggest that the H_2O_2-induced signaling pathways leading to ERK1/2 phosphorylation include PLC activation and calcium influx. MEK1/2 appeared to mediate the signals from phosphoinositide hydrolysis and calcium influx, because the level of phosphorylation in ERK1/2 was modulated by U73122 and EGTA in parallel with that in MEK1/2. We conclude that U73122 and EGTA inhibit H_2O_2- induced MEK1/2 phosphorylation, and reduced activity of MEK1/2 subsequently resulted in decreased phosphorylation level of ERK1/2.

Cyclic AMP potentiates H_2O_2-induced ERK1/2 phosphorylation while inhibiting MEK1/2 phosphorylation.

An elevated intracellular cAMP inhibits calcium influx and phosphatidylinositol turnover caused by TCR activation in some T cells [51,52]. Upregulation of intracellular cAMP level inhibits PLCγ-1 tyrosine phosphorylation caused by CD3 stimulation [53]. Because our results suggested that PLC inhibitor and EGTA reduced ERK1/2 phosphorylation by H_2O_2 (Fig. 3A), inhibition of phosphatidylinositol hydrolysis and calcium influx by cAMP would be expected to negatively affect H_2O_2-induced ERK1/2 phosphorylation. In addition, PKA has been shown to interfere with ERK MAPK pathway by phosphorylating Raf-1 in Jurkat T lymphocytes [54]. In contrast, ERK1/2 shows much less sensitivity than

JNK, another member of MAPK family, to cAMP-mediated inhibition in T cells [55]. In addition, a high concentration (0.5 mM) of the cell-permeable cAMP analogue was shown to induce ERK1/2 phosphorylation without antigenic stimulation in Jurkat T lymphocytes [33]. When we determined the role of cAMP in ERK1/2 phosphorylation by H_2O_2, unexpected results were observed. As shown in Fig. 4A, the level of H_2O_2-induced phosphorylation in ERK1/2 was upregulated by forskolin (an adenlyate cyclase activator), whereas H_2O_2-induced MEK1/2 phosphorylation was downregulated by the same reagent. The concentration of forskolin used in our study (30 μM) did not cause ERK1/2 phosphorylation or MEK1/2 phosphorylation without H_2O_2 (Fig. 4A, lane 2). Interestingly, ERK2 (p42) phosphorylation was highly upregulated by forskolin as compared to ERK1 (p44) phosphorylation. To confirm that the effect of forskolin was mediated by an increase in intracellular cAMP levels, we treated cells with 8-(4-chlorophenylthio)-cAMP, a membrane permeable cAMP analogue, before H_2O_2 stimulation. As shown in Fig. 4B, treatment with 50 μM 8-(4-chlorophenylthio)-cAMP also potentiated ERK1/2 phosphorylation, while inhibiting MEK1/2 phosphorylation by H_2O_2. This concentration of the cAMP analogue did not increase either ERK1/2 phosphorylation or MEK1/2 phosphorylation without H_2O_2 stimulation. Like forskolin, ERK2 (p42) was more sensitive to the amplifying effect by the cAMP analogue than the ERK1 (p44) isoform. The same concentration (50 μM) of 8-(4-chlorophenylthio)-cGMP had no effect on H_2O_2-induced MEK and ERK phosphorylation, further indicating the specificity of the action of cAMP (data not shown).

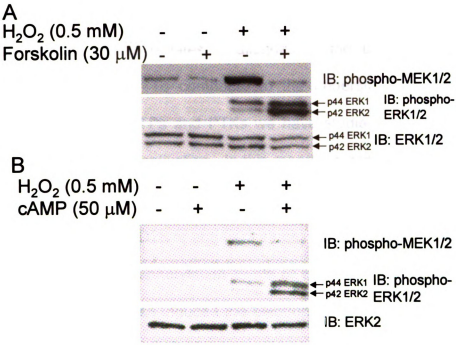


FIGURE 4. Effect of cAMP on H_2O_2-induced ERK1/2 and MEK1/2 phosphorylation. (A) Forskolin potentiates H_2O_2–induced ERK1/2 phosphorylation while inhibiting H_2O_2–induced MEK1/2 phosphorylation. Cells were treated with 30 μM forskolin for 30 min and stimulated by H_2O_2 for 10 min. After blotting with phospho-ERK1/2 and phospho-MEK1/2 Ab, the membrane was reprobed with ERK1/2 Ab as a loading control. (B) 8-(4-chlorophenylthio) cAMP enhances H_2O_2–induced ERK1/2 phosphorylation while inhibiting H_2O_2–induced MEK1/2 phosphorylation. Cells were pretreated with 50 μM 8-(4-chlorophenylthio) cAMP for 20 min, and subsequently stimulated by 0.5 mM H_2O_2 for 10 min. After blotting with phospho–ERK1/2 and phospho-MEK1/2 Ab, the membrane was reprobed with ERK2 Ab as a loading control. Results are representative of triplicate experiments.

DISCUSSION

The complex relationship and cross talk between signaling molecules has represented one of the greatest challenges to the understanding of signal transduction pathways. To contribute to the understanding of the complex relationships between PTPs and PTKs we have studied the effect of the PTP inhibitor, H_2O_2, on T cell activation. In this report we demonstrate that treatment with 50 μM H_2O_2 potentiated ERK1/2 phosphorylation caused by TCR stimulation with anti-CD3 and anti-CD28 or anti-CD3 alone. Submillimolar concentrations of H_2O_2 increased the phosphorylation levels of ERK1/2 and MEK1/2 in Jurkat T cells. H_2O_2 treatment also induced the electrophoretic mobility shift of Lck from 56 kDa to 60 kDa. This shift of Lck was blocked by PD98059 (MEK inhibitor), supporting the notion that ERK1/2 phosphorylation by H_2O_2 is responsible for the migration shift of Lck.

Because the MEK inhibitor (PD98059), the PLC inhibitor (U73122) and EGTA decreased the 0.5 mM H_2O_2 induced-phosphorylation level of both ERK1/2 and MEK1/2, we conclude, that at this concentration, H_2O_2 probably resulted in phosphorylation of ERK1/2 via upstream signaling pathways which activate MEK1/2. The activation of upstream signaling pathway is believed to involve H_2O_2-induced inhibition of proximal PTPs such as CD45 and SHP-1, which regulate members of Src and Syk tyrosine kinase families. In support of this view, millimolar concentration of H_2O_2 did not increase the general tyrosine phosphorylation level in a Lck deficient Jurkat cell variant (J.CaM1.6) [56] and did

not cause ERK1/2 phosphorylation in ZAP70 deficient (p116) Jurkat cell line variant [57].

In contrast, the effect of cAMP on H_2O_2-induced MEK1/2 and ERK1/2 phosphorylation suggests that H_2O_2 may activate other signaling pathways, which increase ERK1/2 phosphorylation without activating MEK1/2. It has previously been suggested that sustained activation of ERK1/2 may be independent of MEK activation [58]. In our experiments, involving only 10 minutes of stimulation, cAMP may have promoted transient MEK1/2 phosphorylation while promoting sustained ERK1/2 phosphorylation. Although this difference of kinetics may explain why MEK1/2 phosphorylation seems to be suppressed by cAMP, it does not completely explain the effect of cAMP on ERK1/2 phosphorylation by H_2O_2. Because cAMP actually amplifies the level of H_2O_2-induced ERK1/2 phosphorylation, rather than maintaining ERK1/2 phosphorylation at the same level, it seems to be reasonable to assume that another pathway leading to increased ERK1/2 phosphorylation is being activated by H_2O_2 and that this pathway is potentiated by cAMP. It is known that cAMP can activate Rap1 in a PKA-dependent manner [31] or via cAMP-activated GDP/GTP exchange factor [59,60]. In the presence of B-Raf MAPKKK, activated Rap1 can lead to ERK activation [31]. However, if cAMP enhances H_2O_2-induced ERK1/2 phosphorylation via B-raf, cAMP should also upregulate H_2O_2-induced MEK1/2 MAPKK phosphorylation concomitant with ERK1/2 phosphorylation, which was not the case in our experiments. Another consideration is that cAMP and H_2O_2 may activate other MAPK kinases (MAPKK)

that can phosphorylate ERK1/2. However, this seems unlikely since dual-specificity kinases that phosphorylate ERK1/2 other than MEK1/2 have not been reported and the specificity of the MAPK signaling cascade is believed to be tightly controlled between MAPKK and MAPK [61].

A more attractive possibility is that the increased phosphorylation of ERK1/2 is due to the inhibition of a phosphatase, thus allowing upstream kinases to act unopposed. Evidence for this has come from experiments as follows. PKA was shown to phosphorylate serine residues in He-PTP [33], a hematopoietic-specific PTP [62]. The serine residue is located in the KIM domain of the phosphatase, which interacts with ERK1/2 [63]. When this residue was phosphorylated by PKA, the interaction between He-PTP and ERK1/2 was inhibited, thereby leading to the loss of He-PTP activity towards the tyrosine residue in the activating loop of ERK1/2 [33]. It is possible that He-PTP becomes more sensitive to H_2O_2-induced inhibition when the complex of the phosphatase and ERK1/2 is dissociated by cAMP (Fig. 5). Maintenance of phosphorylated Tyr residue in ERK1/2 may make it a better substrate for subsequent Thr phosphorylation by MEK1/2. The low level of MEK1/2 activity is probably sufficient to increase the phosphorylation level of both Tyr and Thr residues in ERK1/2.

Although ERK1 and 2 seem to be functionally equivalent, it is not clear why two isoforms of ERK exist and whether there are signaling pathways differentially regulating ERK1 and ERK2 isoforms [64]. Scaffolding proteins such as MP1 may have such a role, favoring the activation of ERK1 over ERK2 by

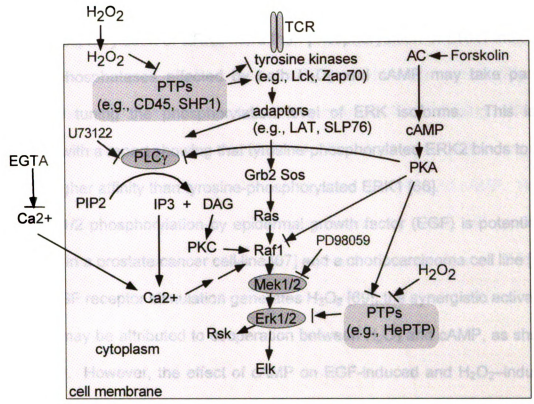


Figure 5. A model of cooperation between H_2O_2 and cAMP in ERK1/2 phosphorylation. Cyclic AMP inhibits upstream signaling pathways activated by H_2O_2, resulting in decrease of H_2O_2-induced MEK1/2 phosphorylation. H_2O_2 also inhibits ERK-dephosphorylating PTPs (e.g. He-PTP) that may be dissociated from ERK1/2 by PKA phosphorylation, leading to overall enhancement of ERK1/2 phosphorylation by cAMP. Arrows indicate activation pathways, whereas the blunt arrows indicate downregulation. Broken lines indicate that the components of the pathway are not clearly defined.

linking MEK1 with ERK1 [65]. Our observation that cAMP amplifies the effect of H_2O_2 on phosphorylation of ERK2 more than phosphorylation of ERK1 indicates that ERK phosphatases affected by both H_2O_2 and cAMP may take part in differentially tuning the phosphorylation level of ERK isoforms. This is in agreement with a report showing that tyrosine-phosphorylated ERK2 binds to He-PTP with higher affinity than tyrosine-phosphorylated ERK1 [66].

ERK1/2 phosphorylation by epidermal growth factor (EGF) is potentiated by forskolin in a prostate cancer cell line [67] and a choriocarcinoma cell line [68]. Because EGF receptor stimulation generates H_2O_2 [69], the synergistic activation of ERK1/2 may be attributed to cooperation between H_2O_2 and cAMP, as shown in our study. However, the effect of cAMP on EGF-induced and H_2O_2–induced ERK1/2 phosphorylation shows high cell-type specificity. cAMP or forskolin are known to attenuate EGF-induced ERK activation in hepatocytes [70], fibroblasts [29], and adenocarcinoma cells [71]. cAMP weakens H_2O_2–induced ERK1/2 phosphorylation in smooth muscle cells [72] and myofibroblasts [73]. The effect combined effect of H_2O_2 and cAMP may be also applied to the cross talk between other receptors generating cAMP and producing H_2O_2.

The results in this study suggest that there are at least two signaling pathways regulating ERK1/2 phosphorylation in T cells. One is the classical MAPK pathway involving the sequential activation through MAPKKK →MAPKK →MAPK by phosphorylation. The other is the pathway mediated by ERK1/2 phosphatases such as He-PTP. H_2O_2 seems to utilize both pathways to induce ERK1/2 phosphorylation. When the cAMP level was increased, the inhibition of

the H_2O_2–induced ERK MAPK cascade resulted in the decreased level of MEK1/2 phosphorylation, whereas the synergistic inhibition of ERK phosphatases led to the enhancement of ERK1/2 phosphorylation (Fig. 5). Although the ERK MAPK cascade is necessary for inducing ERK1/2 phosphorylation in T cells, the amplitude of ERK1/2 phosphorylation may be modulated by a different pathway, which is regulated by H_2O_2 and cAMP. When the environment provides these signaling molecules by other cell types or cytokines, this pathway may affect the amplitude and duration of ERK1/2 phosphorylation, which may lead to different outcomes of TCR stimulation.

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CHAPTER THREE

INHIBITION OF PTPS BY H_2O_2 REGULATES THE ACTIVATION OF DISTINCT MAPK PATHWAYS

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ABSTRACT

It has been shown that endogenous production of reactive oxygen species (ROS) during T cell activation regulates signaling events including MAPK activation. Protein tyrosine phosphatases (PTPs) have been regarded as targets of ROS including which modify the catalytic cysteine residues of the enzymes. We have analyzed the interplay between the inhibition of PTPs and the activation of MAPK by H_2O_2. Stimulation of Jurkat T cells with H_2O_2 induces the phosphorylation of ERK, p38, and JNK members of MAPK family. H_2O_2 stimulation of T cells was found to inhibit the PTP activity of CD45, SHP-1, and HePTP. Transfection of cells with wtSHP-1 decreased H_2O_2-induced ERK and JNK phosphorylation without affecting p38 phosphorylation. Transfection with wtHePTP inhibited H_2O_2-induced ERK and p38 phosphorylation without inhibiting JNK phosphorylation. The Src-family kinase inhibitor, PP2, inhibited the H_2O_2-induced phosphorylation of ERK, p38 and JNK. The phospholipase C (PLC) inhibitor, U73122, or the protein kinase C (PKC) inhibitor, Ro-31-8425, blocked H_2O_2-induced ERK phosphorylation, whereas the same treatment did not inhibit p38 or JNK phosphorylation. Taken together, these results suggest that inhibition of PTPs by H_2O_2 contributes to the induction of distinct MAPK activation profiles via differential signaling pathways.

INTRODUCTION

Reactive Oxygen Species (ROS) are generated by the incomplete reduction of oxygen during various biological processes [1]. It has been known that ROS mediate diverse effects on the function of the cells [2]. Because ROS can be generated rapidly in response to extracellular stimuli and can be degraded efficiently, they have been regarded as potential second messengers [3]. Supporting this concept, various growth factor receptors, cytokine receptors, and GPCRs (G-protein coupled receptors) have been shown to produce ROS including H_2O_2 when cognate ligand binds the receptor [4].

It has been shown that T cells produce endogenous ROS in response to various physiological stimuli. ROS production in thymocytes stimulated with Concanavalin A has been suggested to modulate JNK activation [5]. CD28 costimulation has been shown to produce ROS by a lipoxygenase, resulting in IL-2 gene transcription through NF-κB activation [6]. Recently, it has been demonstrated that endogenously produced ROS during T cell receptor (TCR) activation regulate ERK activation and Fas ligand expression [7]. In addition to the endogenous production of ROS, exogenously provided ROS have been shown to affect T cell function at inflammatory sites, where activated phagocytes release high amount of ROS through the NADPH oxidase system [8]. Amine oxidase in the endothelial cells has also been proposed as a source of H_2O_2 during T cell migration [9]. Abnormal regulation of T cell function in complex diseases such as rheumatoid arthritis [10], atherosclerosis [11], AIDS [12], and cancer [13] has been attributed to the oxidative environment in pathological

situations. Although it is clear that endogenously or exogenously produced ROS affect T cell signaling, the target molecules of ROS and the mechanism of ROS regulation have not been precisely defined.

Compared to the other members of ROS, H_2O_2 is more stable and membrane permeable leading to the proposal that H_2O_2 can function as a second messenger. Both exogenous treatment and endogenous production of H_2O_2 has been suggested to contribute cellular signaling by inhibiting protein tyrosine phosphatases (PTPs) [14]. PTPs contain an essential catalytic cysteine residue in their active sites with a pKa (~5.5) as compared to the pKa (~8) of other cysteine residues in most proteins [15]. The low pKa makes the thiolate anion especially susceptible to the inhibitory action of H_2O_2. H_2O_2 has been shown to inhibit PTPs *in vitro* [16] as well as inside cells [17]. For example, it has been shown that H_2O_2 treatment substantially reduces total cellular PTP activity in the MO7e [18] and HER14 cells [19]. Specifically, H_2O_2 was found to inhibit PTP activity of CD45 in Jurkat T lymphocytes [20] and neutrophils [21]. PTP activity of SHP-1 was also found to be inhibited by H_2O_2 in SHP-1 transfected HELA cells [22]. Recent reports have shown that endogenously produced ROS inactivate PTP1B when insulin receptor [23] or EGF receptor [24] were triggered, suggesting that inhibition of PTPs by H_2O_2 may be a physiologically important event during cell signaling.

CD45, SHP-1, and HePTP are PTPs predominantly expressed in T cells and it has been known that the activities of these PTPs are important in T cell activation. CD45 is the most abundant PTP in T cell, accounting for about 75%

PTP activity in T cell membrane [25]. CD45 activity is critical for T cell activation and T cells deficient in CD45 expression failed to generate signals responding to TCR-engaging antibodies [26-28]. One requirement for CD45 in T cell activation has been understood to be the dephosphorylation of the inhibitory tyrosine residue of Src-family kinases such as Lck and Fyn [29]. However, it has been reported that CD45 may also dephosphorylate the activation loop tyrosine residue of Src-family tyrosine kinases [30,31], suggesting that the role of CD45 in T cell signaling may depend on the types of stimuli and the accessibility of the substrates to the phosphatase. SHP-1 is a PTP which is predominantly expressed in hematopoietic cells [32]. Thymocytes from SHP-1 deficient mice (designated motheaten) showed the enhancement of constitutive as well as induced tyrosine phosphorylation of the TCR complex [33], suggesting a negative role of SHP-1 in TCR signaling. Supporting this concept, SHP-1 has been shown to dephosphorylate SLP76 [34] and Zap70 [35], which are important signaling molecules in T cell activation. HePTP is expressed exclusively in hematopoietic cells [36]. Overexpression of HePTP in T cells resulted in down-regulation of ERK activation and IL-2 promoter activation, suggesting that HePTP has a negative role in T cell activation [37]. It has been shown that HePTP dephosphorylates the activating tyrosine residue of ERK [38], and PMA- and TCR-induced ERK activation is increased in spleen cells from HePTP knockout mice [39], indicating that ERK is an authentic substrate of HePTP.

One well documented result of cell stimulation with H_2O_2 is MAPK activation [40]. The MAPK family members considered in this report include

ERK, p38, and JNK. ERK activation has been mainly implicated in proliferation in response to growth factors, whereas p38 and JNK activation are more important to stress responses, such as in inflammation [41]. It has been suggested that the combination of the magnitude and kinetics of activation of each member of MAPK family determines the appropriate response of the cell according to the specific stimulus [42]. In the case of T cells, the distinct activation profile of three members of the MAPK family has been shown to influence the specific stages of thymocyte development as well as the precise effector function of mature T cells [43].

In this report, the role of PTPs on H_2O_2-induced MAPK activation was analyzed by measurement of PTP activity immunoprecipitated from intact cells and by transfection of PTP vectors. Treatment of T cells with H_2O_2 inhibited PTP activity of CD45, SHP-1, and HePTP, suggesting that PTPs are targets of H_2O_2. Ectopic expression of wtSHP-1 inhibited H_2O_2-induced ERK and JNK phosphorylation without inhibiting p38 phosphorylation. On the other hand, ectopic expression of HePTP specifically affected H_2O_2-induced ERK and p38 phosphorylation without affecting JNK phosphorylation. The differential effect of PTP transfection suggests that each PTP controls the distinct signaling pathway leading to MAPK phosphorylation. The activity of Src-family tyrosine kinase was necessary for H_2O_2-induced ERK, p38, and JNK phosphorylation, whereas PLC and PKC activity was dispensable in the case of p38 and JNK phosphorylation induced by H_2O_2. Taken together, results in this study suggest that inhibition of

PTPs by H_2O_2 contributes to the distinct activation profile of three members of MAPK family.

MATERIALS AND METHODS

Reagents and antibodies. H_2O_2 was purchased from Sigma (St. Louis, MO). PP2, U73122 and Ro-31-8425 were obtained from Calbiochem (La Jolla, CA). Antibodies used in this study were obtained as follows: phospho-ERK (New England Biolabs, Beverly, MA); phospho-p38 and phospho-JNK (Promega, Madison, WI); ERK2, JNK, normal IgG, and CD45 for immunoprecipitation (Santa Cruz Biotechnology, Santa Cruz, CA); CD45 for western blotting and SHP-1 (Transduction Laboratories, Lexington, KY); haemagglutinin (HA) antibody, clone 16B12 (Babco, Richmond, CA); horseradish peroxidase-conjugated donkey anti-rabbit antibody and sheep anti-mouse antibodies (Amersham, Arlington Heights, IL); and horseradish peroxidase-conjugated rabbit anti-goat antibody (Calbiochem). cDNAs for wtSHP-1, C/S-SHP-1, wtHePTP and C/S-HePTP containing in the pEF/HA vector were generously provided by Dr. T. Mustelin [37,44].

Cell culture and stimulation. Jurkat T cells and Jurkat TAg cells were cultured in RPMI 1640 medium with 25 mM HEPES buffer (Biofluids: Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies: Rockville, MD), streptomycin/penicillin (100 units/ml) and 2 mM glutamine (Life Technologies). Cells were maintained in an exponential growth phase (0.2-1.0 × 10^6 cells/ml). Cells were reconstituted in the concentration of 1 × 10^6 cells/ml media described above before stimulation. Because PP2, U73122, and Ro-31-8425 were dissolved in dimethyl sulfoxide (DMSO), the same volume

of DMSO was included in the control sample whenever these reagents were used.

Western Blotting. Cells were washed twice with ice-cold phosphate-buffered saline and then lysed in lysis buffer (Cell Signaling: Beverly, MA) for 30 min on ice. Cell nuclei were pelleted by centrifugation at 8,000 × g at 4°C for 10 min. Lysates were separated on a 10% SDS-PAGE gel and proteins were transferred to nitrocellulose membrane. Immunoblotting was performed as previously described [45]. Bands were visualized by chemiluminescence (Amersham).

Transfection. Transient transfection of Jurkat TAg cells was performed using DMRIE-C (Life Technologies). Typically, 6 μl of DMRIE-C was mixed with 24 μg of SHP-1, HePTP vector or pEF/neo control vector in 250 μl of OPTI-MEM media (Life Technologies) at room temperature to form lipid-DNA complexes. After 40 min, 0.5×10^6 cells in 50 μl of OPTI-MEM were added to the mixture. After 5 hrs of incubation at 37°C, 1 ml RPMI medium containing 10% FBS was added. After two days, cells were stimulated and subjected to lysis.

Immunoprecipitation and PTP assay. Cells were stimulated by H_2O_2 and, after washing with PBS, subject to lysis with immunoprecipitation buffer (15 mM KCl, 10 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 0.1% NP40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF) for 10 min in the ice. Cell supernatants were incubated with antibody at 4°C for the indicated times followed by addition of 30 μl Gammabind-Sepharose beads (Amersham) and incubation for an additional two hours at 4°C. The beads were washed and then

mixed with 100 μl PTP buffer (25 mM imidazole (pH 7.2), 45 mM NaCl, 1 mM EDTA) containing 20 mM or 40 mM pNPP followed by incubation at 37°C for 5 min or 30 min. Absorbance of the supernatant was determined at 410 nm. For each PTP activity calculation the absorbance of pNPP hydrolyzed from beads with control IgG (or anti-HA IgG) alone was subtracted from the absorbance of lysate immunoprecipitates. The absorbance obtained from PTP immune complexes was then normalized to the absorbance obtained with control IgG immunoprecipitates from lysates to determine fold change. In the case of HePTP immunoprecipitation, the absorbance was normalized to that of anti-HA immune complexes from cells transfected with control vector. In all cases, average values with standard deviation were obtained after three independent experiments. The statistical analysis was done using student's t test and *p* value was determined. After measurement of PTP activity, the beads were recovered and subjected to SDS-PAGE gel for the analysis for western blotting to detect the presence of PTPs.

RESULTS

H_2O_2 induces activation-related phosphorylation of ERK, p38, and JNK MAPKs. The goal of this study is to understand the mechanism of H_2O_2-induced signaling leading to MAPK phosphorylation and activation. The phosphorylation level of MAPKs was measured by western blotting with antibodies that specifically recognize both phospho-Tyr and phospho-Thr residues that are regarded to be necessary and sufficient for the activation of MAPKs. As shown in Fig. 1, stimulation of Jurkat T cells with H_2O_2 induced specific phosphorylation of all three members of MAPK family, ERK, p38, and JNK. ERK phosphorylation rapidly increased by 10 min (lane 2) of H_2O_2 stimulation and decreased after 30 min. p38 phosphorylation also rapidly increased, but the phosphorylation was more sustained compared to ERK. In contrast, JNK phosphorylation was delayed when compared to ERK and p38. The peak of JNK phosphorylation was at about 30 min (lane 4), and this phosphorylation level was still detected up to 2 hrs after stimulation. Accordingly, in subsequent analysis in this report, the level of ERK and p38 phosphorylation was measured at 10 min and JNK phosphorylation was measured at 30 min after H_2O_2 stimulation.

Treatment of cells with H_2O_2 inhibits the activity of PTPs. In this report we wished to test the hypothesis that the observed specific activation of MAPKs was, at least in part, due to the inhibition of PTPs which influence both upstream signaling pathways leading to MAPK phosphorylation, and to the direct dephosphorylation of the MAPKs themselves.

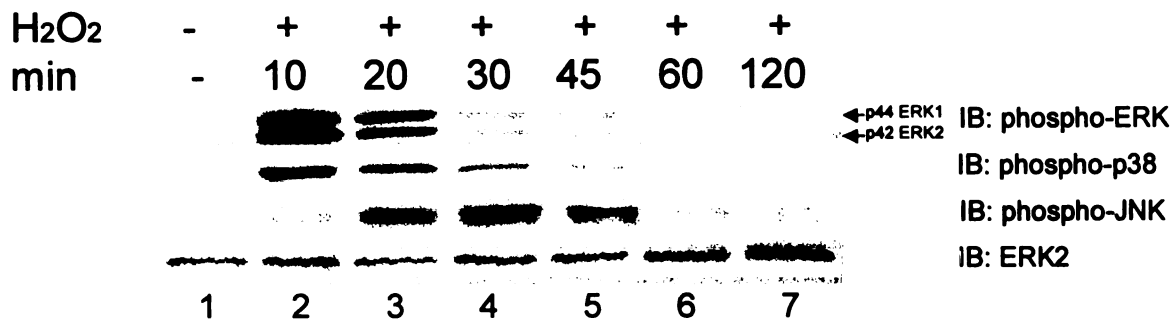


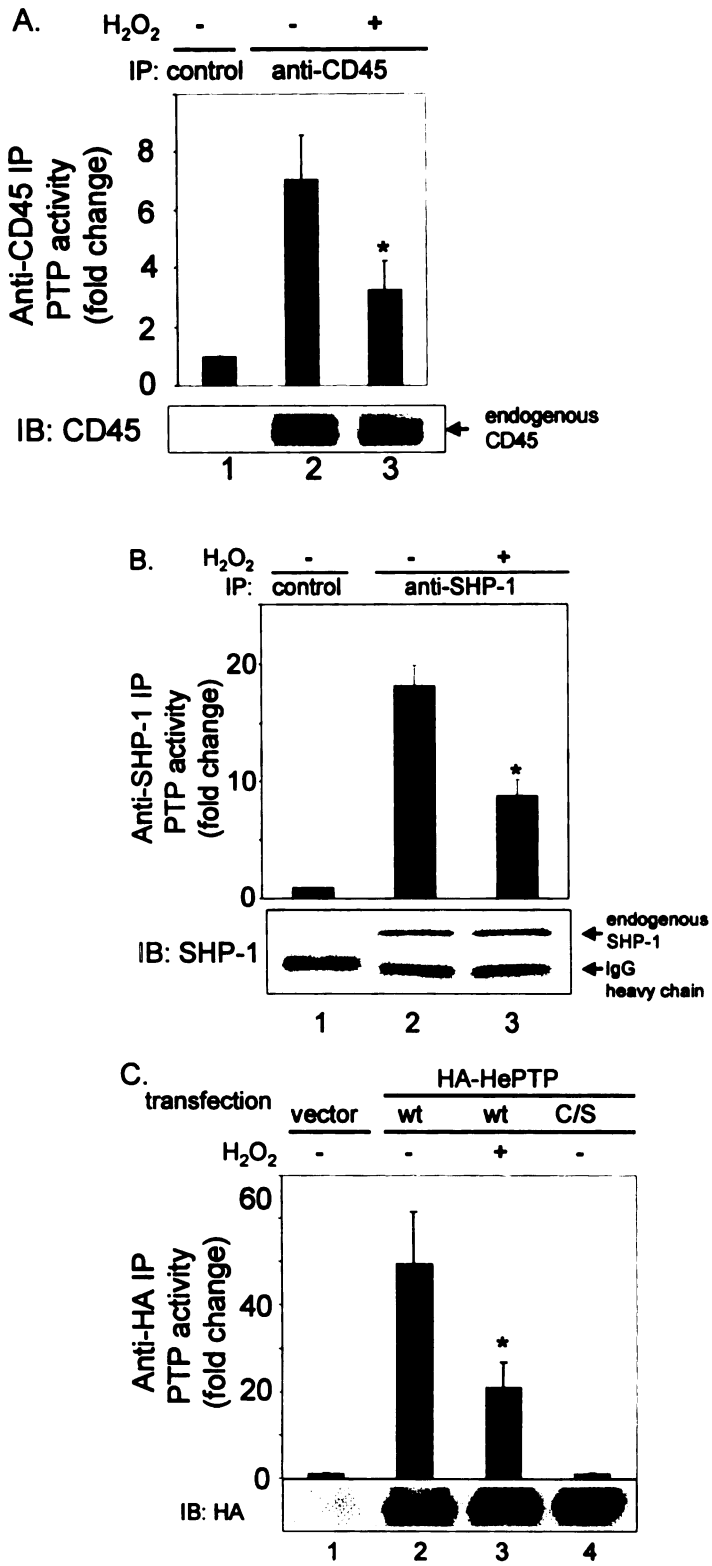
Figure 1. H_2O_2 induces MAPK phosphorylation. Jurkat T cells were stimulated by 400 μM H_2O_2 for the indicated times. Phosphorylation levels were measured by immunoblotting the whole cell lysate with antibodies specific for the phosphorylated forms of the enzymes. The blot was reprobed with ERK2 antibody to show that equivalent amounts of protein were in each lane. The representative blots of three independent experiments are shown.

CD45 is the most predominant phosphatase in T cell membrane and the enzyme is critical for T cell activation through regulation of Src-family kinase (Lck, Fyn in T cell) activity [46,47]. Treatment of Jurkat T cells with H_2O_2 for 10 min inhibited PTP activity of endogenous, immunoprecipitated CD45 by 60% (Fig. 2A, lanes 2 and 3; N = 3, P < 0.005). Western blotting with CD45 antibody confirmed the presence of CD45 in the immune complex (Fig. 2A, anti-CD45 immunoblot).

Because we hypothesized that SHP-1 is also a target of H_2O_2, we measured the PTP activity of SHP-1 after H_2O_2 treatment. Immunoprecipitates of endogenous SHP-1 showed ~18 fold higher activity compared to the activity of control IgG immunoprecipitates (Fig. 2B, lanes 1 and 2). H_2O_2 treatment of the cells for 10 min inhibited the PTP activity of SHP-1 immunoprecipitates by about 50% (Fig. 2B, lanes 2 and 3; N = 3, P = 0.027). Western blotting the immune complex with SHP-1 antibody showed that endogenous SHP-1 was successfully immunoprecipitated with the antibody (Fig. 2B, anti-SHP-1 blot).

We next wished to examine the activity of the MAPK tyrosine phosphatase, HePTP, which is expressed in T cells. Because a precipitating antibody for endogenous HePTP was not available, Jurkat TAg cells ectopically expressing haemagglutin (HA) tagged HePTP [37] were used and HA-HePTP was immunoprecipitated using anti-HA antibody. Cells transfected with vectors were treated with H_2O_2 for 10 min followed by anti-HA immunoprecipitation and measurement of PTP activity. Anti-HA immunoprecipitates of wtHePTP transfectants exhibited about a 50 fold greater PTP activity compared to

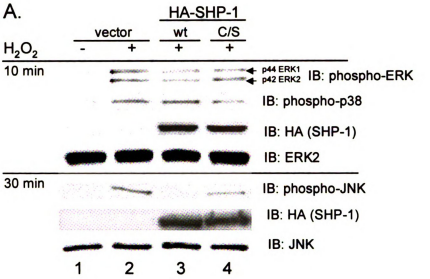
Figure 2. Treatment of cells with H_2O_2 inhibits the activity of PTPs. (A) After Jurkat T cells were stimulated with 400 μM H_2O_2 for 10 min, CD45 was immunoprecipitated with anti-CD45 Ab. The immune complex was incubated with 20 mM pNPP for 5 min and the activity was calculated as described in the Methods. *, $p < 0.005$ *versus* the activity of CD45 immune complex from cells without H_2O_2 stimulation. The bottom blot shows the presence of endogenous CD45 in the anti-CD45 immune complex. (B) After Jurkat T cells were stimulated by 400 μM H_2O_2 for 10 min, SHP-1 was immunoprecipitated with SHP-1 antibody for two hours and the washed immune complex was incubated with 40 mM pNPP for 30 min. The activity was calculated as described in the Methods. *, $p = 0.027$ *versus* the activity of SHP-1 immune complex from cells without H_2O_2 stimulation. The bottom blot shows endogenous SHP-1 immunoprecipitated by the antibody. (C) Jurkat TAg cells were transfected with 12 μg of HePTP vector or pEF/neo vector. After two days, cells were stimulated by 400 μM H_2O_2 for 10 min. Ectopically expressed HePTP with HA tag was immunoprecipitated using HA antibody for two hours and the immune complex was incubated with 20 mM pNPP for 5 min. The activity was calculated as described in the Methods. *, $p <$ 0.01 *versus* the activity of anti-HA immune complex from wtHePTP transfected cells without H_2O_2 stimulation. The bottom blot shows the presence of HA-tagged HePTP in anti-HA immune complex. In each case, the value in the first lane was set as 1 and the values in the other lanes were represented as fold change with standard deviation after three independent experiments.

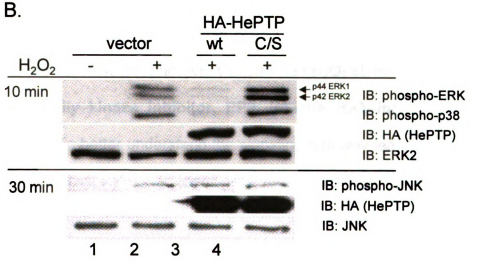

A.
H_2O_2 - - +
IP: control anti-CD45
Anti-CD45 IP PTP activity (fold change)
0 2 4 6 8
*
IB: CD45
endogenous CD45
1 2 3
B.
H_2O_2 - - +
IP: control anti-SHP-1
Anti-SHP-1 IP PTP activity (fold change)
0 10 20
*
IB: SHP-1
endogenous SHP-1
IgG heavy chain
1 2 3
C.
transfection vector HA-HePTP wt wt C/S
H_2O_2 - - + -
Anti-HA IP PTP activity (fold change)
0 20 40 60
*
IB: HA
1 2 3 4

immunoprecipitates from cells transfected with empty vector (Fig. 2C, lanes 1 and 2). The activity of HePTP immunoprecipitated from H_2O_2-treated cells was inhibited by about 60% (Fig. 2C, lanes 2 and 3; N = 3, P < 0.01). As a control, immunoprecipitates from cells expressing the catalytically inactive Cys to Ser mutant (C/S) form of HePTP had no phosphatase activity (Fig. 2C, lanes 1 and 4). The presence of similar amounts of HePTP in each immune complex was confirmed by western blotting with anti-HA antibody (Fig. 2C, anti-HA blot). These experiments demonstrate that, under the conditions of H_2O_2 stimulation which lead to MAPK activation, the PTP activity of several key PTPs was indeed inhibited.

wtSHP-1 overexpression inhibits H_2O_2-induced ERK and JNK phosphorylation without affecting p38 phosphorylation. The effect of PTP overexpression on H_2O_2-induced MAPK phosphorylation was analyzed in order to address the question of whether the inhibition of PTPs by H_2O_2 was relevant to MAPK activation. SHP-1 was chosen as a representative enzyme that regulates the upstream signaling pathways leading to MAPK phosphorylation. SHP-1 has been suggested to dephosphorylate proximal tyrosine kinases such as Lck and Zap70. Overexpression of wtSHP-1 moderately reduced H_2O_2-induced ERK phosphorylation and substantially inhibited JNK phosphorylation (Fig. 3A, lane 3). In contrast, overexpression of SHP-1 did not affect H_2O_2-induced p38 phosphorylation. The effect of SHP-1 overexpression was dependent on the catalytic activity of SHP-1, because overexpression of catalytically inactive SHP-1 containing a mutation of the catalytic Cys to Ser (C/S) did not suppress the

Figure 3. PTP overexpression affects H_2O_2-induced MAPK phosphorylation. (A) Jurkat TAg cells were transfected with pEF/neo vector, wtSHP-1, or C/S-SHP-1 vector; and (B) with pEF/neo vector, wtHePTP, or C/S-HePTP vector. After two days of transfection, cells were stimulated with 200 μM H_2O_2 and the ERK and p38 phosphorylation levels were measured after 10 min and the JNK phosphorylation level was analyzed after 30 min. After blotting with phospho-specific antibodies, the blots were reprobed with ERK2 or JNK antibody to show that equivalent amount of protein were in each lane. Anti-HA blot confirmed the expression of HA-tagged SHP-1 and HA-tagged HePTP in the transfected cells. The representative blots from three independent experiments are shown.


A.
HA-SHP-1
vector
wt
C/S
H2O2
-
+
+
+
10 min
p44 ERK1
p42 ERK2
IB: phospho-ERK
IB: phospho-p38
IB: HA (SHP-1)
IB: ERK2
30 min
IB: phospho-JNK
IB: HA (SHP-1)
IB: JNK
1
2
3
4


B.
HA-HePTP
vector
wt
C/S
H2O2
-
+
+
+
10 min
p44 ERK1
p42 ERK2
IB: phospho-ERK
IB: phospho-p38
IB: HA (HePTP)
IB: ERK2
30 min
IB: phospho-JNK
IB: HA (HePTP)
IB: JNK
1
2
3
4

H_2O_2-induced phosphorylation of the MAPKs (Fig 3A, lane 4). Because ectopically expressed SHP-1 contains HA tag in the N terminus [44], the expression of transfected SHP-1 vectors was verified by anti-HA antibody (Fig 3A, anti-HA blot).

wtHePTP overexpression suppresses H_2O_2-induced ERK and p38 phosphorylation, but not JNK phosphorylation. HePTP was chosen as an example of a PTP which directly dephosphorylates MAPK at the activation loop Tyr residue. Overexpression of wtHePTP substantially inhibited H_2O_2-induced ERK and p38 phosphorylation (Fig. 3B, lane 3). Conversely, overexpression of C/S-HePTP potentiated ERK and p38 phosphorylation induced by H_2O_2, suggesting a dominant-negative effect (Fig. 3B, lane 4). The enhancement of H_2O_2-induced p38 phosphorylation by C/S-HePTP overexpression was less dramatic than the potentiation of ERK phosphorylation. In contrast with ERK and p38, JNK phosphorylation was not affected by either wt or C/S-HePTP overexpression. These results suggest that SHP-1 and HePTP may differentially regulate each member of MAPK phosphorylation when H_2O_2 stimulates T cells.

The Src-family kinase inhibitor, PP2, inhibits H_2O_2-induced MAPK phosphorylation. To better understand the signaling pathway leading to MAPK activation by H_2O_2, reagents that specifically inhibit signaling enzymes were applied during H_2O_2 stimulation. Src family tyrosine kinases such as Lck and Fyn are associated with cytoplasmic portions of TCR components and their activation is the first step in TCR activation [48]. We treated cells with the Src family kinase-specific inhibitor, PP2, to assess the involvement of these kinases in

H_2O_2-induced MAPK phosphorylation. PP2 treatment inhibited the H_2O_2-induced phosphorylation of all three MAPKs (Fig. 4A, lane 3), suggesting that the activity of the proximal tyrosine kinases for T cell activation (such as Lck and Fyn) was required for H_2O_2-induced MAPK phosphorylation. The possibility that PP2 treatment made the cells nonfunctional was negated by the observation that PMA-induced ERK phosphorylation was not inhibited by the same condition of PP2 treatment (data not shown), as previously reported [49].

U73122 (PLC inhibitor) and Ro-31-8425 (PKC inhibitor) inhibit H_2O_2-induced ERK phosphorylation, whereas p38 and JNK phosphorylation are not affected. PLC and PKC are important enzymes that mediate the signaling response in T cell activation. PLCγ is activated by tyrosine phosphorylation [50] during TCR stimulation, and it hydrolyzes PIP_2 to form IP_3 and diacylglycerol (DAG). It is believed that PKC contributes to ERK phosphorylation by promoting Raf-1 activation when T cell receptor is triggered [51]. We used reagents which specifically inhibit PLC and PKC to assess their involvement in H_2O_2-induced MAPK phosphorylation. Treatment of cells with either U73122 or Ro-31-8425 abrogated H_2O_2-induced ERK phosphorylation (Fig. 4B, lanes 3 and 4). Surprisingly, neither inhibitor at the same concentration affected H_2O_2-induced p38 and JNK phosphorylation (Fig. 4B, lanes 3 and 4). These results suggest that the activity of PLC and PKC is especially required for H_2O_2-induced ERK phosphorylation.

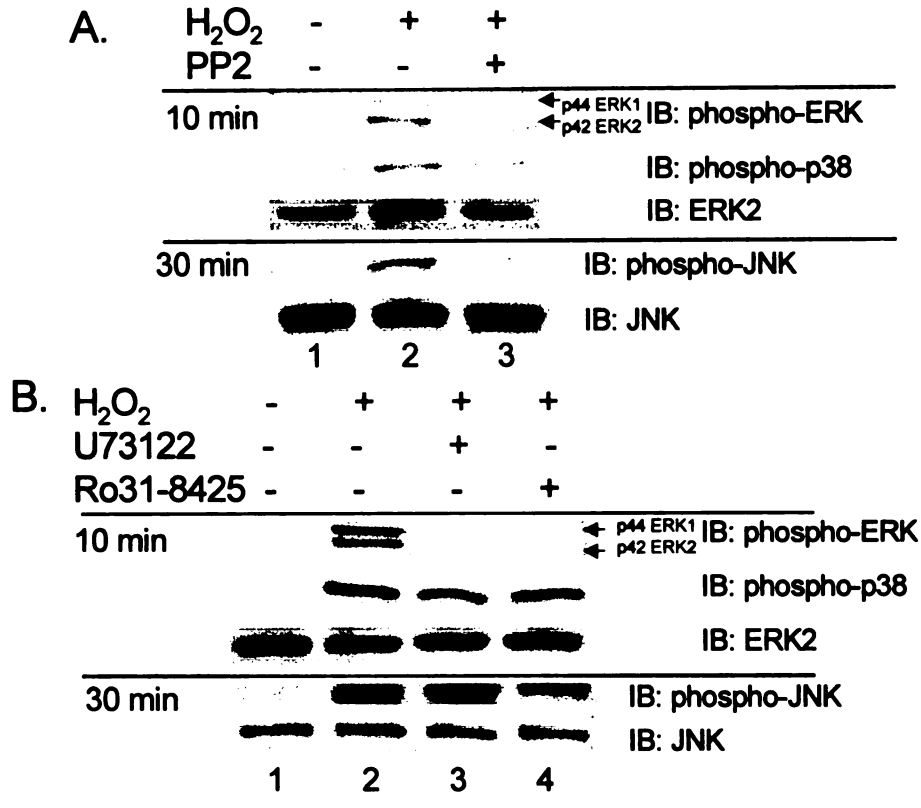


Figure 4. H_2O_2-induced MAPK phosphorylation is regulated by differential signaling pathways. (A) Cells were incubated with 20 μM PP2 for 2 hrs and stimulated by 400 μM H_2O_2. (B) Cells were incubated with 2 μM U73122 or 1 μM Ro-31-8425 for 10 min and stimulated by 400 μM H_2O_2. In both (A) and (B), ERK and p38 phosphorylation levels were measured after 10 min stimulation with H_2O_2 and the JNK phosphorylation level was analyzed after 30 min stimulation. After blotting with phospho-specific antibodies, the blots were reprobed with ERK2 or JNK antibody to show that equivalent amounts of protein were in each lane. The representative blots from three independent experiments are shown.

DISCUSSION

The level of tyrosine phosphorylation inside the cell is determined by the balance between the activity of protein tyrosine kinases (PTKs) and of protein tyrosine phosphatases [52]. Although various potential schemes for the regulation of PTKs have been proposed, rigorously verified regulatory mechanisms for PTPs are poorly understood [53]. Inhibition of PTP activity by ROS is regarded as one physiological means of regulation of PTPs. It has been documented that ROS are produced endogenously and inhibit PTP activity when receptors are stimulated, and blockade of the receptor-triggered ROS production restores PTP activity [23]. In addition, there are various situations involving inflammatory responses and associated diseases in which T cells are exposed to exogenous ROS. Because of the potential importance of ROS modulation of T cells by inhibition of PTPs, we designed experiments to use Jurkat cells treated with H_2O_2 as a model system to understand the dynamic relationship between several PTPs and signaling events leading to MAPK activation.

The current experiments were designed to evaluate the role of several interacting PTPs important for T cell activation and for MAPK activation. We chose to study CD45, SHP-1 and HePTP because of their important involvement in T cell activation, as well as for their respective roles in the stages of the signaling events leading to MAPK activation. Our strategy was to verify the effect of H_2O_2 on PTP activity in intact cells followed by verification of the role of PTPs in MAPK phosphorylation by ectopic expression of individual PTPs.

When Jurkat T cells were stimulated by H_2O_2, all three members of MAPK family, ERK, p38, and JNK, become phosphorylated at the activating Tyr and Thr residues as detected by phopho-specific antibodies (Fig. 1). The peak of H_2O_2-induced JNK phosphorylation was delayed (about 30 min) compared to that of ERK and p38 phosphorylation (about 10 min). The fact that this kinetic pattern of MAPK phosphorylation was similar to that described for TCR-induced MAPK phosphorylation [54] suggests that phosphorylation induced by H_2O_2 treatment and TCR triggering share similar signaling pathways. The lag in JNK phosphorylation could be due to mechanical events involving cytoskeletal rearrangement required to fully activate JNK. GTPases leading to the activation of JNK, such as Rac and cdc42, are also effectors of actin filament organization [55]. Recently, it has been shown that agents that disrupt the cytoskeleton inhibit both ROS production and JNK activation induced by TNFα in ECV-304 cells [56].

To test whether H_2O_2 treatment of Jurkat T cells inhibited PTP activity, CD45, SHP-1, and HePTP were immunoprecipitated from cells and the PTP activity of the immunoprecipitates were measured. Stimulation of Jurkat T cells with H_2O_2 substantially inhibited the activity of all three tested PTPs (Fig. 2), indicating that PTPs are potential targets of exogenously produced H_2O_2. All the tested PTPs seemed to have a similar sensitivity to H_2O_2. This result supports the idea that PTP inhibition by H_2O_2 is an intrinsic property of PTPs which universally contain a highly reactive, negatively charged cysteine residue in the catalytic site [57].

As a way of addressing the hypothesis that PTP inhibition by H_2O_2 is relevant to H_2O_2-induced MAPK activation, the effect of SHP-1 and HePTP overexpression on MAPK phosphorylation by H_2O_2 was analyzed. If PTPs are targets of H_2O_2, overexpression of PTP would be expected to overcome the effect of H_2O_2. An example of this approach is seen in a study in which the overexpression of SHP-1 inhibited the activation of NF-AT induced by peroxyvanadium, an inhibitor of PTPs [58]. SHP-1 overexpression also reduced the activation of UV-induced MAPKAP kinase 2, which is thought to occur by generation of ROS [59]. Our results also show that overexpression of wtSHP-1 inhibited H_2O_2-induced ERK and JNK phosphorylation (Fig. 3A). The catalytic activity of SHP-1 was necessary for the effect, because the overexpression of the catalytically inactivated C/S-SHP-1 did not reduce H_2O_2-induced ERK and JNK phosphorylation. In contrast, SHP-1 overexpression did not affect H_2O_2-induced p38 phosphorylation, suggesting that SHP-1 is primarily involved in the ERK and JNK activation pathways (summarized in Fig. 5). The participation of SHP-1 activity on ERK activation has been previously suggested by the reports showing that the Ras-ERK MAPK pathway was enhanced in motheaten thymocytes [33]; and SHP-1 overexpression reduced TCR-induced ERK activation [44]. The possibility that SHP-1 negatively regulates JNK activation comes from the report that SHP-1 associates with Vav [60], a GEF (GTP-exchanging factor) of Rac1 that has been implicated in JNK activation [61].

HePTP expression inhibited H_2O_2-induced ERK and p38 phosphorylation without affecting JNK phosphorylation (Fig. 3B). This result suggests that ERK

and p38 are substrates of HePTP. Analysis of HePTP knockout mice confirmed that ERK is the authentic substrate of HePTP, because TCR- and PMA-mediated ERK phosphorylation was enhanced in the absence of HePTP [39]. Sorbitol-induced p38 phosphorylation was not enhanced in the same study, raising doubt that p38 is a substrate of HePTP. The study of K562 cells stably expressing HePTP also provides the evidence that ERK rather than p38 is a substrate of HePTP, because C/S-HePTP bound to Tyr-phosphorylated ERK2 more efficiently than p38 [62]. HePTP expression in our study also suggests the highest affinity between ERK2 and C/S-HePTP, because the H_2O_2-induced phosphorylation level of ERK2 was enhanced by C/S-HePTP overexpression (Fig. 3B, lane 4). However, the ability of wtHePTP expression to inhibit H_2O_2-induced p38 phosphorylation was as effective as for ERK phosphorylation (Fig. 3B, lane 3), raising the possibility that the mutation of Cys to Ser in C/S-HePTP inadvertently changed the affinity of ERK and p38 for HePTP. The possibility that p38 is a substrate of HePTP should still be considered because wtHePTP overexpression inhibits TCR-induced p38 phosphorylation as well as ERK phosphorylation; and p38 MAPK is detected in the endogenous HePTP immunoprecipitation complex [38].

To better understand the signaling pathway leading to H_2O_2-induced MAPK phosphorylation, the effect of reagents inhibiting specific signaling enzymes was analyzed. When the H_2O_2-induced signaling pathway was investigated using the Src-family kinase inhibitor, PP2, it was found that Src-family tyrosine kinases (most likely Lck and Fyn in T cells) are required for

phosphorylation of all three MAPK forms (Fig. 4A). This finding correlates with reports showing that H_2O_2 treatment of Jurkat T cell leads to the increase of Lck activity [63]. This result suggests that the proximal tyrosine kinases responsible for T cell activation may be subject to ROS regulation. Because it has not been demonstrated that the direct addition of H_2O_2 to the purified Src-family kinases increases the tyrosine kinase activity *in vitro* [1], it is probable that the activation of Src-family tyrosine kinases in intact cells treated by H_2O_2 occurs by the inhibition of CD45 and/or SHP PTPs, which regulate these tyrosine kinases. It is interesting that the activity of Src-family kinases is required for H_2O_2-induced MAPK phosphorylation in a situation where H_2O_2 substantially inhibits the activity of CD45 (Fig. 2A), because CD45 is the primary phosphatase implicated in the dephosphorylation of the inhibitory Tyr residue of Src-family kinases in T cells. If CD45 activity is inhibited by H_2O_2, unchecked phosphorylation of the inhibitory Tyr residue may contribute to the inactivation of Src-family kinases. However, the inhibition of CD45 activity by H_2O_2 may also lead to the hyperphosphorylation of activating Tyr residues of Src-family kinases. It has been reported that Lck is phosphorylated at both activating Tyr394 and inhibitory Tyr505 residue in CD45 negative cell line [30]. H_2O_2 treatment of T cells also leads to the phosphorylation of both the activating Tyr394 and the inhibitory Tyr505 residues of Lck [63]. Importantly, the activity of Lck (which has phosphates on both Tyr residues after H_2O_2 treatment [64] or in the absence of CD45 [31]) was actually increased, suggesting that activation of Lck by phosphorylation of Tyr394 is dominant over inhibition induced by Tyr505 phosphorylation. Therefore, it is

probable that H_2O_2 treatment leads to the activation of Src-family kinases by inhibiting CD45 activity.

We found that wtSHP-1 overexpression did not inhibit H_2O_2-induced p38 phosphorylation (Fig. 3A) while the Src-family kinase inhibitor, PP2, effectively inhibited p38 phosphorylation (Fig. 4A). This results suggest that the activity of Src-family kinases (Lck and Fyn) was not completely controlled by SHP-1. There is controversy surrounding the idea that Lck is the direct substrate of SHP-1. While it has been shown that co-expression of Lck and SHP-1 resulted in the dephosphorylation of activating Tyr394 residue of Lck in 293 cells [65], co-expression of Lck and SHP-1 did not change the activity of Lck in COS-7 cells [66]. The discrepancy may be caused by the fact that SH2-domain deleted form of SHP-1 was used for expression in the former study. The two tandem SH2 domains in the N terminus of SHP-1 inhibit PTP activity by sterically blocking the catalytic domain [67]. Because the removal of SH2 domains increases the catalytic activity of SHP-1, the form of SHP-1 without SH2 domains has often been used for transfection to increase the effect of overexpression. However, SH2 domains of SHP-1 are important for localization of SHP-1 because the SH2 domains have been implicated in the binding of tyrosine-phosphorylated motifs in inhibitory receptors such as Kir [68] and CD5 [69]. The localization of SHP-1 may be important for the phosphatase to access specific substrates. Because the overexpression of wtSHP-1 with intact SH2 domains effectively inhibited H_2O_2-induced JNK phosphorylation (Fig. 3A) and PI3K activity in COS-7 cells [66], the overexpression of wtSHP-1 may have the enough potential to affect the

signaling pathway in which endogenous SHP-1 participates. Therefore, caution is needed for the identification of the substrates of SHP-1 by overexpression of SH2 domain-deleted SHP-1.

Exogenous treatment of H_2O_2 has been shown to induce tyrosine phosphorylation of PLCγ and to initiate PLC-mediated hydrolysis of inositol phospholipids [70,71]. We found that PLC activity was required for H_2O_2-induced ERK phosphorylation because the PLC inhibitor U73122 inhibited H_2O_2-induced ERK phosphorylation (Fig. 4B). It has been known that PKC activation by DAG is required for PKC phosphorylation and activation of Raf-1 [72,73]. PKC activity was also necessary for H_2O_2-induced ERK phosphorylation because the PKC inhibitor Ro-31-8425 inhibited H_2O_2-induced ERK phosphorylation (Fig. 4B). The sequential activation pathway (PLC→PKC→Raf-1) may explain why PLC activity is needed when H_2O_2 induces ERK phosphorylation.

In contrast to H_2O_2-induced ERK phosphorylation, the PLC inhibitor and PKC inhibitor had no effect on H_2O_2-induced p38 and JNK phosphorylation (Fig. 4B). This result suggests that PKC activation by DAG production from PLC activity may not be involved in the effect of H_2O_2 on p38 and JNK phosphorylation. ASK1 is a characterized redox-regulated MAPKKK which associates with Trx (thioredoxin). The dissociation of ASK1-Trx complex occurs after the oxidation of Trx and ASK1 becomes activated and mediates the activation of p38 and JNK MAPK pathway [74]. It will be interesting to check whether PKC activity is dispensable for the activation of ASK1 by H_2O_2 in Jurkat T cells.

Taken together, the differential effect of PTP overexpression and of enzyme inhibitors on the induction of phosphorylation among MAPK members by H_2O_2 suggests that each member of MAPK is activated by H_2O_2 through distinct signaling pathways (Fig. 5). The observation that PTP overexpression specifically affects H_2O_2-induced MAPK phosphorylation and that PTP activity is decreased by H_2O_2 treatment indicates that the inhibition of PTPs by H_2O_2 is one important mechanism for H_2O_2 to modulate the signaling pathways leading to differential MAPK pathway activation.

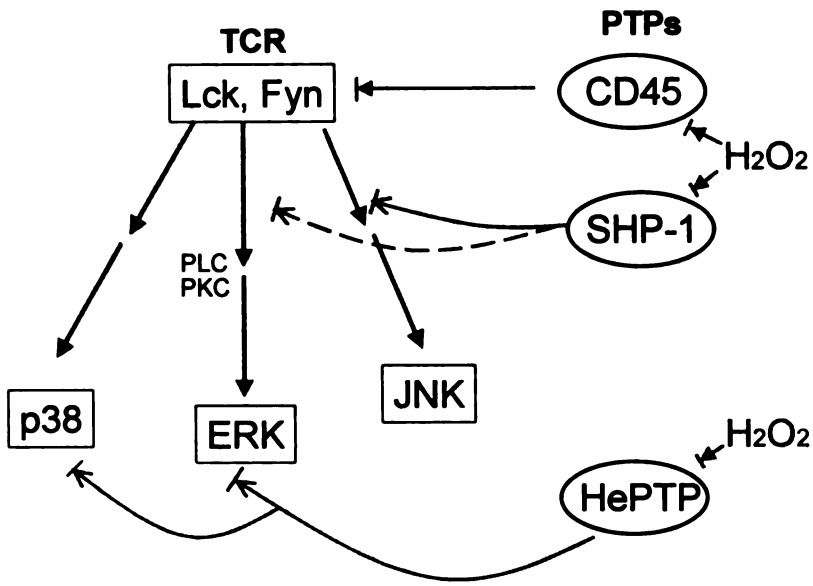


Figure 5. PTP inhibition by H_2O_2 regulates the activation of distinct MAPK pathways. The solid arrows indicate MAPK pathways of TCR activation, whereas the blocked arrows indicate predicted sites of inhibition by PTPs. The dashed line indicates a partial effect.

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CHAPTER FOUR

INHIBITION OF CD45 BY REACTIVE OXYGEN SPECIES (ROS) MEDIATES 1-CHLORO-2,4-DINITROBENZENE (DNCB)-INDUCED IMMUNOSTIMULATION OF T CELLS

FOOTNOTES

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[3] Abbreviations used in this paper: GSH, gluathione; Trx, thioredoxin; DNCB, 1-chloro-2,4-dinitrobenzene; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular-signal regulated kinase; p-JNK, phospho-JNK; p-ERK, phospho-ERK; p-p38, phospho-p38; TrxR, thioredoxin reductase; LMW, low molecular weight; CM-H_2DCF-DA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester; PLC, phospholipase C; PKC, protein kinase C; EGF, epidermal growth factor; PDGF, platelet-derived growth factor

ABSTRACT

DNCB has been utilized to modulate T cell activation in contact hypersensitivity and in the treatment of melanoma. Because It has been suggested that DNCB treatment of cells leads to the production of reactive oxygen species (ROS) by inhibiting thioredoxin reductase and by depleting glutathione, the relevance of ROS production on DNCB-induced immunostimulation of T cells was investigated. The specific immunostimulatory effect of DNCB was shown by the observation that DNCB strongly potentiated TCR-induced p38 and JNK phosphorylation, while having no effect on ERK phosphorylation. DNCB treatment of Jurkat cells increased intracellular ROS level, which was prevented by an antioxidant, N-acetylcysteine (NAC). The inhibition of Lck activity by PP2 or NAC corresponded with the decrease of DNCB-induced JNK and p38 phosphorylation. The activity of CD45, a protein tyrosine phosphatase (PTP) that regulates Lck, was inhibited when cells were stimulated by DNCB or H_2O_2. Inclusion of DTT in the lysate or pretreatment of the cells with NAC restored CD45 activity inhibited by DNCB, suggesting that ROS were involved in the process. To assess the mechanism of CD45 inhibition by ROS, the sequential modification of recombinant CD45 with differential isotypes of iodoacetic acid was applied. H_2O_2 inhibited the incorporation of the first modifying agent to the active site cysteine residue (Cys817) of CD45, indicating that the modification of the cysteine residue was responsible for the inhibition of CD45 activity by H_2O_2. Taken together, these results suggest that ROS-induced inhibition of CD45 activity is responsible for the subsequent

activation of Lck and the differential regulation of MAPKs in DNCB-induced immunostimulation of T cells.

INTRODUCTION

The topical application of DNCB to the skin has been known to provoke a delayed-type hypersensitivity (DTH) reaction (1). In addition to the experimental use of the compound to study DTH, DNCB has been tried to improve the conditions of dermatoses, HIV infection and malignant melanoma (2-4). Recently, it has been shown that chemotherapy for melanoma using DNCB is dependent on T cell-elicited immune response (5). Therefore, it is important to understand the mechanism of DNCB-induced immunostimulation of T cells. Although DNCB has been thought to induce DTH by functioning as a hapten (6), it has been reported that immunostimulatory properties of DNCB cannot be solely explained by the hapten model (7). An additional mechanism of immunostimulation by DNCB involves the inhibition of mammalian thioredoxin reductase (TrxR) activity by alkylating the catalytic selenocysteine and cysteine residues (8). With the inhibition of TrxR activity, thioredoxin (Trx) and other substrates of TrxR such as lipid hydroperoxides will remain in the oxidized state and the reducing capacity of the cell will be impaired (9). Another interesting feature is that TrxR alkylated by DNCB produces superoxide with the consumption of NADPH, like NADPH oxidase (10). In addition, DNCB depletes GSH by forming a conjugate with GSH, in a reaction catalyzed by glutathione S-transferase (GST) (11). All these effects change the intracellular milieu to a pro-oxidative state which contributes to the profound immunostimulatory effect of DNCB (12, 13). The relevance of DNCB-induced inhibition of TrxR for the immunostimulatory effect of the compound is supported by the reports that TrxR

activity is decreased in vitiliginous skin that shows impaired DTH reactions by DNCB (14, 15).

Cells maintain specific redox gradient in their intracellular compartments with various regulatory machinery including glutathione (GSH) and thioredoxin (Trx) (16-18). The change in the intracellular redox status affects diverse aspects of the cell including proliferation and apoptosis (19, 20). Recent reports have shown that the change in the redox status is used to modulate the appropriate responses of the cells to extracellular stimuli. The most well documented response in this regard is the production of endogenous ROS upon exposure to extracellular stimuli (21). Phagocytic cells contain NADPH oxidase in the plasma membrane, which produces high amount of ROS which has a role in bacterial killing (22). Nonphagocytic cells also harbor a family of NADPH oxidase-like enzymes, the homologues of which are being continuously cloned and characterized (23, 24).

One of the potential molecules directly affected by ROS are the protein tyrosine phosphatases (PTPs). Because the catalytic cysteine residue of PTP exhibits a low pKa as compared to other cysteine residues (25), it has been suggested that ROS inhibit PTP activity by modifying catalytic cysteine residue. Supporting this concept, treatment of purified PTPs with H_2O_2 inhibits the activity of the enzyme (26) and exposure of cells to H_2O_2 also leads to the downregulation of PTP activity (27, 28). Stimulation of cells with insulin or EGF inhibits PTP1B activity with endogenous production of ROS (29, 30). PDGF increases intracellular ROS and inactivates LMW PTP and SHP-2 by oxidation

(31, 32). These results suggest that PTP inhibition by ROS has physiological significance during cell stimulation. However, it has been difficult to experimentally elucidate the mechanism of PTP inhibition by ROS because few methods have been developed to examine the status of the active site cysteine residue.

In this study, the relevance of ROS production to immunostimulation induced by DNCB was investigated in Jurkat T lymphocytes. The immunostimulatory effect of DNCB was manifested by the selective enhancement of TCR-induced JNK and p38 phosphorylation. Stimulation of Jurkat cells with DNCB was found to produce endogenous ROS. The importance of ROS production for DNCB-induced immunostimulation was shown by the observations that an antioxidant, N-acetylcysteine, inhibited DNCB-induced ROS production, Lck activation, and MAPK phosphorylation. The PTP activity of CD45 was downregulated by DNCB or H_2O_2 treatment of the cell, which was reversed by the addition of DTT to the lysates. H_2O_2 inhibited the incorporation of C^{13}-iodoacetic acid to the active site cysteine residue of CD45, indicating that the modification of the cysteine residue is responsible for the inhibition of CD45. Taken together, these results suggest that ROS production by DNCB results in the reversible inhibition of CD45 activity in T cells and subsequent activation of Lck mediates the immunostimulatory effect of DNCB by promoting JNK and p38 phosphorylation.

MATERIALS AND METHODS

Reagents and Antibodies

H_2O_2 and DNCB were purchased from Sigma (St. Louis, MO). PP2, U73122, and Ro31-8425 were obtained from Calbiochem (San Diego, CA). Monoclonal anti-CD3 clone 235 (IgM type) and anti-CD28 clone NE51 (IgG type) Abs were provided by Dr. Shu Man Fu (University of Virginia, Charlottesville, VA). p-ERK Ab was purchased from New England Biolabs (Beverly, MA). p-p38 and p-JNK Abs were obtained from Promega (Madison, WI). JNK1, ERK2, normal IgG, Lck and CD45 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated rabbit Ab was purchased from Amersham (Arlington Heights, IL). Horseradish peroxidase-conjugated goat Ab was obtained from Calbiochem.

Cell culture and stimulation

Jurkat cells, clone E6-1, were grown in RPMI 1640 with 25 mM HEPES buffer (Biofluids: Rockville, MD) supplemented with 10% heat-inactivated FBS (Life Technologies: Rockville, MD), 100 units/ml streptomycin/penicillin (Life Technologies) and 2 mM glutamine (Life Technologies). Cells were reconstituted in the concentration of 10^6 cells/ml in serum-free RPMI before stimulation. Because DNCB, PP2, U73122, and Ro31-8425 were dissolved in DMSO, same volume of DMSO was included in control sample. Western blotting procedure was done as previously described (33).

Measurement of ROS

After 5 min of stimulation with DNCB, 5 μM CM-H_2DCF-DA (Molecular Probes: Eugene, OR) was added. After 1 hr incubation with the fluorochrome, cells were analyzed by FACS at an excitation wavelength of 488 nm and emission at 520 nm.

Lck kinase assay

Lck was immunoprecipitated from cells by incubation of lysates with Lck Ab for 2 hrs followed by incubation with protein A agarose for the additional 2 hrs. The beads were washed three times with immunoprecipitation buffer and two times with kinase buffer (20 mM PIPES (pH 7.2), 10 mM $MnCl_2$). The immune complexes were incubated in kinase buffer containing 5 μCi [γ-^{32}P] ATP and 20 μM ATP for 30 min at 30 °C. After the addition of Lammelie sample buffer and boiling, the supernatants were separated by SDS-PAGE gel. The gel was dried and autoradiographed using a phosphoimager.

Immunoprecipitation and PTP assay

After stimulation by DNCB, cells were washed with PBS and subject to lysis with lysis buffer (10 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 15 mM KCl, 0.1% NP40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF) for 30 min in the ice. Where indicated, 5 mM DTT was included in the lysis buffer. After centrifugation for 30 s at room temperature, supernatants were incubated with CD45 Ab at 4°C for two hours followed by addition of 30 μl Gammabind-Sepharose beads (Amersham) and incubation for the additional two hours at 4°C. The beads were mixed with 100 μl PTP buffer (25 mM imidazole (pH 7.2), 45 mM NaCl, 1 mM EDTA) containing 20 mM pNPP followed by incubation for 90

s at 37°C. Absorbance of the supernatant was determined at 410 nm. The value obtained from CD45 immunoprecipitates was subtracted by the value obtained from control IgG immunoprecipitates.

PTP assay of recombinant CD45

The bacterial expression vector pET3D-His6CD45, which expresses the cytoplasmic domain of murine CD45 with a His6 tag in the amino terminus, was kindly provided by Dr. Pauline Johnson (University of British Columbia, Vancouver, Canada). The purification of CD45 from bacteria was done as described (34). 1 μg of protein in 100 μl PTP buffer was incubated with 100 μM H_2O_2 or with 100 μM DNCB for 1 h at room temperature. PTP activity assay was initiated by adding 20 mM pNPP. After 5 min of incubation at room temperature, absorbance was determined at 410 nm.

Carboxymethylation with iodoacetic acid, in-gel digestion, and MALDI-MS analysis of CD45

10 μg of recombinant CD45 in 100 μl of 50 mM MES (pH 6.6) buffer was incubated with and without 1 mM H_2O_2 for 30 min at room temperature. Subsequently, 250 μM of C^{13}-iodoacetic acid was added and the mixture was incubated for 30 min in the darkness. After the incubation, the solution was incubated with 10 mM DTT for 30 min. After adding Lamealli sample buffer, the solution was boiled and subject to SDS-PAGE electrophoresis. After staining of the gel with Coomassie reagent (Bio-rad), CD45 band was cut out and chopped into 1 mm-size pieces. After washing the gel pieces with ammonium bicarbonate, the gel pieces were dehydrated by acetonitrile and vaccum-dry.

After reducing the gel pieces by incubation with DTT at 56°C for 30 min, the gel pieces were subject to alkylation by C^{12}-iodoacetic acid for 20 min at room temperature in the darkness. The gel pieces were washed with ammonium bicarbonate, dehydrated by acetonitrile and dried under vacuum. The gel pieces were incubated by trypsin (Promega) in the ice for 45 min and subsequently at 37°C overnight. After harvesting the supernatant, the gel pieces were suspended in the solution containing 60 % acetonitrile and 3 % trifluoroacetic acid and were sonicated for 30 min. After mixing the supernatant from sonication with the previous harvest, the solution was dried down to 20 μl under vacuum. 6 μl of 3 % trifluoroacetic acid was added to the solution and the mixture was sonicated for 10 min. The solution was analyzed by MALDI-MS.

Results

DNCB potentiates TCR- and H_2O_2-induced MAPK phosphorylation. To determine whether DNCB treatment affects T cell activation process, the effect of DNCB on the phosphorylation in the activation domain of MAPKs was analyzed. DNCB treatment induced JNK and p38 phosphorylation and potentiated TCR-induced JNK and p38 phosphorylation (Fig. 1A, p-JNK and p-p38 immunoblots). Interestingly, DNCB was a poor stimulator of ERK phosphorylation and did not enhance TCR-induced ERK phosphorylation (Fig. 1A, p-ERK blot). To compare the sensitivity of MAPK phosphorylation induced by TCR activation and by direct oxidant challenge, cells were stimulated by H_2O_2 with and without DNCB. In contrast to TCR activation, H_2O_2-induced ERK phosphorylation was potentiated by DNCB as effectively as JNK and p38 phosphorylation (Fig. 1B). This result indicated that TCR-induced JNK and p38 activation is specifically affected by cellular redox status.

DNCB induces endogenous ROS production in T cells. To determine whether DNCB treatment influences the oxidative potential of the T cell, the intracellular ROS level was measured by the cell-permeable and ROS-sensitive fluorochrome, CM-H_2DCF-DA. Stimulation of T cell with DNCB increased the fluorescence of the intracellular fluorochrome (Fig. 2, lanes 1 and 2). This result confirmed the previous report that DNCB treatment produced endogenous ROS in Jurkat T cells (35). Pretreatment of the cells with NAC inhibited basal level of fluorescence (lanes 1 and 3) and DNCB-induced increase of fluorescence (lanes 2 and 4), indicating that NAC functioned as antioxidants.

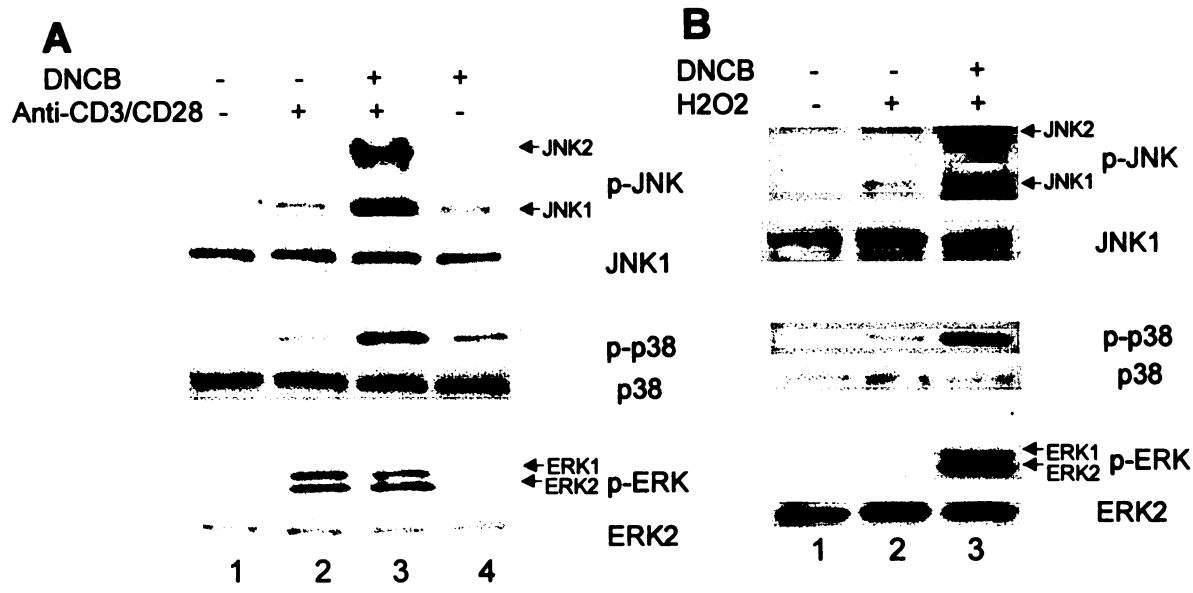


Figure 1. DNCB enhances TCR- and H_2O_2-induced MAPK phosphorylation. **(A)** Cells were incubated with 30 mM DNCB for 1 hr and were subsequently stimulated with anti-CD3 (1:100) and anti-CD28 (1:100) for 30 min. **(B)** Cells were incubated with 30 mM DNCB for 1 hr and were subsequently stimulated with 200 mM H_2O_2 for 30 min. After blotting with phospho-MAPK antibodies, the membranes were reprobed with ERK2 antibody to show that equivalent amount of protein was present in each lane. The blots were the representative from three independent experiments.

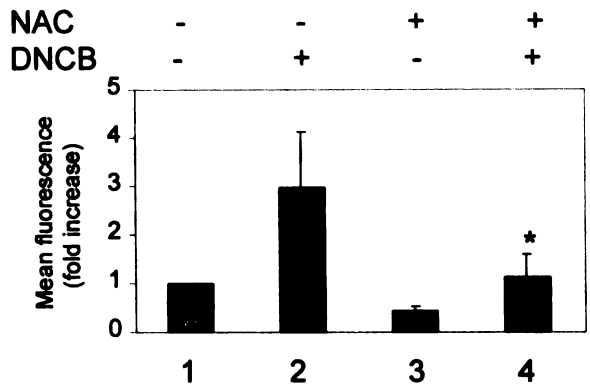


Figure 2. NAC inhibits DNCB-induced intracellular ROS production. Cells were stimulated with 30 mM DNCB and incubated with 5 mM CMH_2DCF-DA for 1 hr. The fluorescence of the unstimulated cells was set 1 and the fluorescence from DNCB-treated cells was represented as fold increase with standard deviation after four independent experiments. *, $p < 0.05$ *versus* value obtained from cells stimulated by DNCB alone.

NAC inhibits DNCB-induced JNK and p38 phosphorylation. Because we observed that NAC inhibited DNCB-induced ROS production, we checked the effect of ROS on DNCB-induced MAPK phosphorylation by pretreatment of cells with NAC. As shown in Fig. 3A, NAC inhibited DNCB-induced JNK and p38 phosphorylation. NAC pretreatment did not inhibit PMA-induced ERK phosphorylation (data not shown), indicating that application of NAC specifically affected JNK and p38. This result suggested that ROS production is required for DNCB-induced JNK and p38 phosphorylation.

Src-family kinase inhibitor, PP2, inhibits DNCB-induced JNK and p38 phosphorylation. We analyzed the signaling pathways leading to MAPK phosphorylation by DNCB with the application of the reagents which specifically inhibit signaling enzymes. Src family tyrosine kinases such as Lck and Fyn are associated with cytoplasmic portions of TCR components and their activation is the first step in TCR activation [48]. To determine whether the activity of the kinases is required for DNCB-induced JNK and p38 phosphorylation, the Src-family kinase inhibitor, PP2, was pretreated before DNCB stimulation. PP2 inhibited DNCB-induced JNK and p38 phosphorylation (Fig. 3B). PP2 treatment did not make cells nonfunctional because the same concentration of PP2 did not inhibit PMA-induced ERK phosphorylation (data not shown), as previously reported (36). This result suggested that Lck (and Fyn) activation is required for DNCB-induced JNK and p38 phosphorylation.

The PLC inhibitor (U73122) and the PKC inhibitor (Ro-31-8425) do not affect DNCB-induced JNK and p38 phosphorylation. Activation of PLCγ by

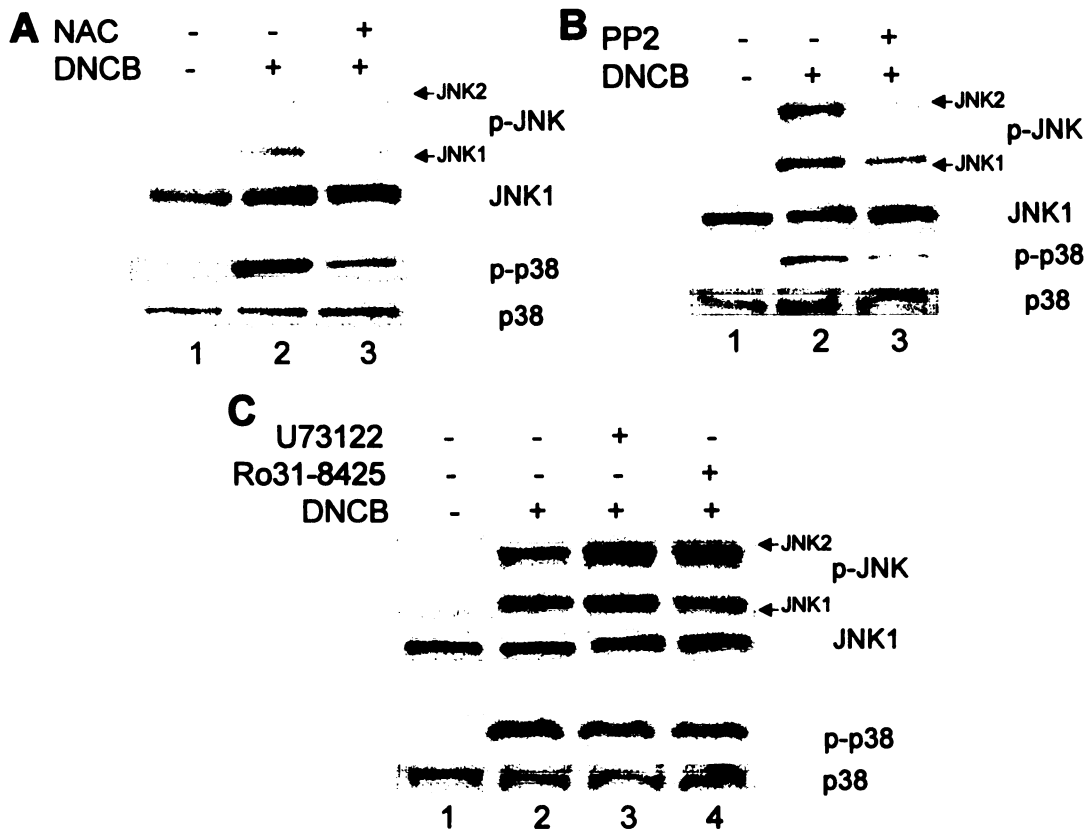


Figure 3. PP2, the Src-family kinase inhibitor, and NAC inhibit DNCB-induced JNK and p38 phosphorylation. **(A)** Cells were pretreated with 20 mM PP2 for 2 hrs and subsequently incubated with 30 mM DNCB for 1 hr. **(B)** Cells were pretreated with 20 mM NAC for 30 min and subsequently stimulated with 30 mM DNCB. **(C)** Cells were pretreated with 4 mM U73122 or with 1 mM Ro31-8425 for 10 min and stimulated with 30 mM DNCB for 1 hr. After blotting with phospho-MAPK antibodies, the membranes were reprobed with ERK2 antibody to show that equivalent amount of protein was present in each lane. The blots are the representative of three independent experiments.

tyrosine phosphorylation and subsequent activation of PKC by diacylglycerol is an important event in T cell activation. To determine the involvement of these enzymes in DNCB-induced JNK and p38 phosphorylation, cells were treated with the PLC inhibitor, U73122, or with the PKC inhibitor, Ro-31-8425, before stimulation with DNCB. Both inhibitors did not inhibit DNCB-induced JNK and p38 phosphorylation (Fig. 3C).

NAC inhibits DNCB-induced Lck kinase activity. Because we observed that PP2 inhibited DNCB-induced JNK and p38 phosphorylation, we checked whether Lck is activated when cells are stimulated with DNCB. DNCB treatment increased Lck kinase activity, judged by autophosphorylation of the enzyme (Fig. 4, lanes 1 and 2). NAC pretreatment inhibited the induction of Lck kinase activity by DNCB (Fig. 4, lanes 2 and 3), suggesting that ROS production was involved in the activation of Lck.

DNCB or H_2O_2 treatment of Jurkat T cells reversibly inhibits PTP activity of CD45. Because it has been reported that the direct addition of oxidants did not increase Lck activity (37), we measured the PTP activity of CD45 that regulated Lck activation. CD45 PTP activity, measured in specific immunoprecipitates after treatment of Jurkat cells with DNCB or H_2O_2, was found to decrease by about 40% (Fig. 5A, lanes 2 and 3). The addition of 5 mM DTT in lysis buffer did not significantly affect the basal CD45 activity (Fig. 5A, lanes 1 and 4). However, a considerable portion of CD45 activity inhibited by DNCB or by H_2O_2 was reversed by 5 mM DTT (Fig. 5A, lanes 2 and 5 and lanes 3 and 6). This result suggested that inhibition of cellular CD45 activity by DNCB was

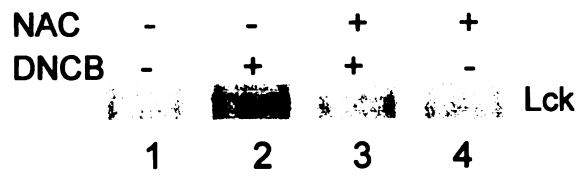
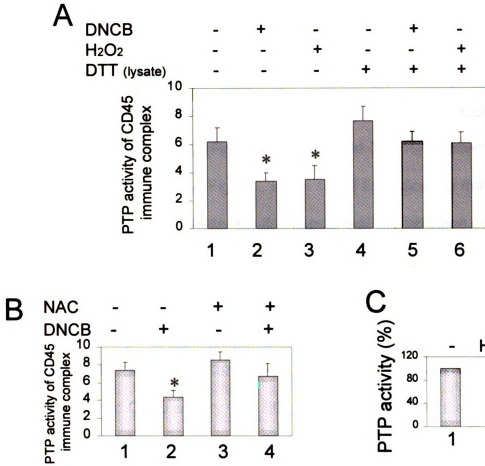


Figure 4. NAC inhibits DNCB-induced Lck activation. Cells were stimulated with 30 mM DNCB for 1 hr. Lck was immunoprecipitated and autophosphorylation activity in the immune complex was assayed by incorporation of [g-32P] ATP. The radiograph is the representative of three independent experiments.

Figure 5. DNCB reversibly inhibits CD45 activity in Jurkat cells. (A) Cells were stimulated with 30 mM DNCB or with 300 mM H_2O_2 for 1 hr. 5 mM DTT was included in lysis buffer at the indicated. PTP activity of CD45 immunoprecipitates was measured and average value was represented with standard deviation after three independent experiments. *, $p < 0.05$ *versus* CD45 activity without treatment (lane 1). **(B)** Cells were incubated with 20 mM NAC for 30 min and subsequently stimulated with 30 mM DNCB for 1hr. PTP activity of CD45 immunoprecipitates was measured and average value was represented with standard deviation after three independent experiments. *, $p < 0.05$ *versus* CD45 activity without treatment (lane 1). **(C)** Recombinant CD45 (1 mg) was incubated with 100 mM DNCB or with 100 mM H_2O_2 for 1 hr. The PTP activity without treatment was set 100% and the activity with DNCB or H_2O_2 was represented as % activity with standard deviation after four independent experiments. **, $p < 0.05$ *versus* CD45 activity without treatment (lane 1).


A
DNCB - + - - + -
H2O2 - - + - - +
DTT (lysate) - - - + + +
PTP activity of CD45 immune complex
10
8
6
4
2
0
*
*
1 2 3 4 5 6
B
NAC - - + +
DNCB - + - +
PTP activity of CD45 immune complex
10
8
6
4
2
0
*
1 2 3 4
C
PTP activity (%)
- H2O2 DNCB
120
80
40
0
1 2 3

mediated by reversible modification of a thiol group by ROS. NAC pretreatment prevented the decrease of CD45 activity by DNCB (Fig. 5B), further supporting the notion that DNCB-induced inhibition of cellular CD45 activity was mediated by ROS. The possibility that DNCB inhibited CD45 activity by direct alkylation was excluded by the observation that the addition of DNCB to recombinant CD45 did not affect PTP activity (Fig. 5C, lanes 1 and 3). H_2O_2 effectively inhibited recombinant CD45 activity (Fig. 5C, lanes 1 and 2), indicating that ROS directly inhibited CD45 activity. These results suggest that the inhibition of CD45 activity by DNCB-induced ROS production mediates the immuno-stimulatory effect of DNCB (Fig. 6).

H_2O_2 inhibits the modification of the active site cysteine residue of recombinant CD45 by C^{13}-iodoacetic acid. Because we observed that H_2O_2 directly inhibited CD45 activity (Fig. 5C), we tried to examine the mechanism how ROS inhibit CD45 activity. Since it has been suggested that the active site cysteine of PTPs is a peculiar target of ROS because of the low pKa, we determined to look at the status of the active site cysteine residue (Cys817) of CD45. Iodoacetic acid reacts with the reduced sulfhydryl group (-SH), but not with the oxidized forms such as sulfenic (-SOH), sulfinic ($-SO_2H$), or sulfonic ($-SO_3H$) acids. Therefore, we used the reactivity with iodoacetic acid as the indicator of oxidation. After incubation of CD45 with and without H_2O_2, the protein was subject to modification by C^{13}-iodoacetic acid. The solution was subsequently incubated with DTT to reverse the oxidized residues. The protein was purified by SDS-PAGE electrophoresis and the gel piece containing CD45

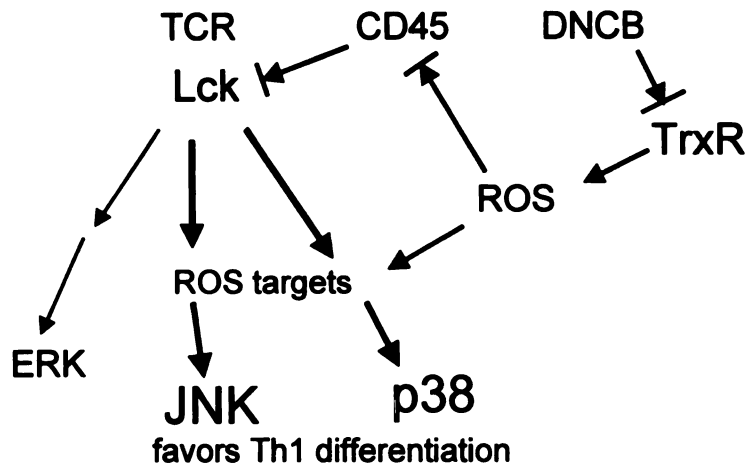
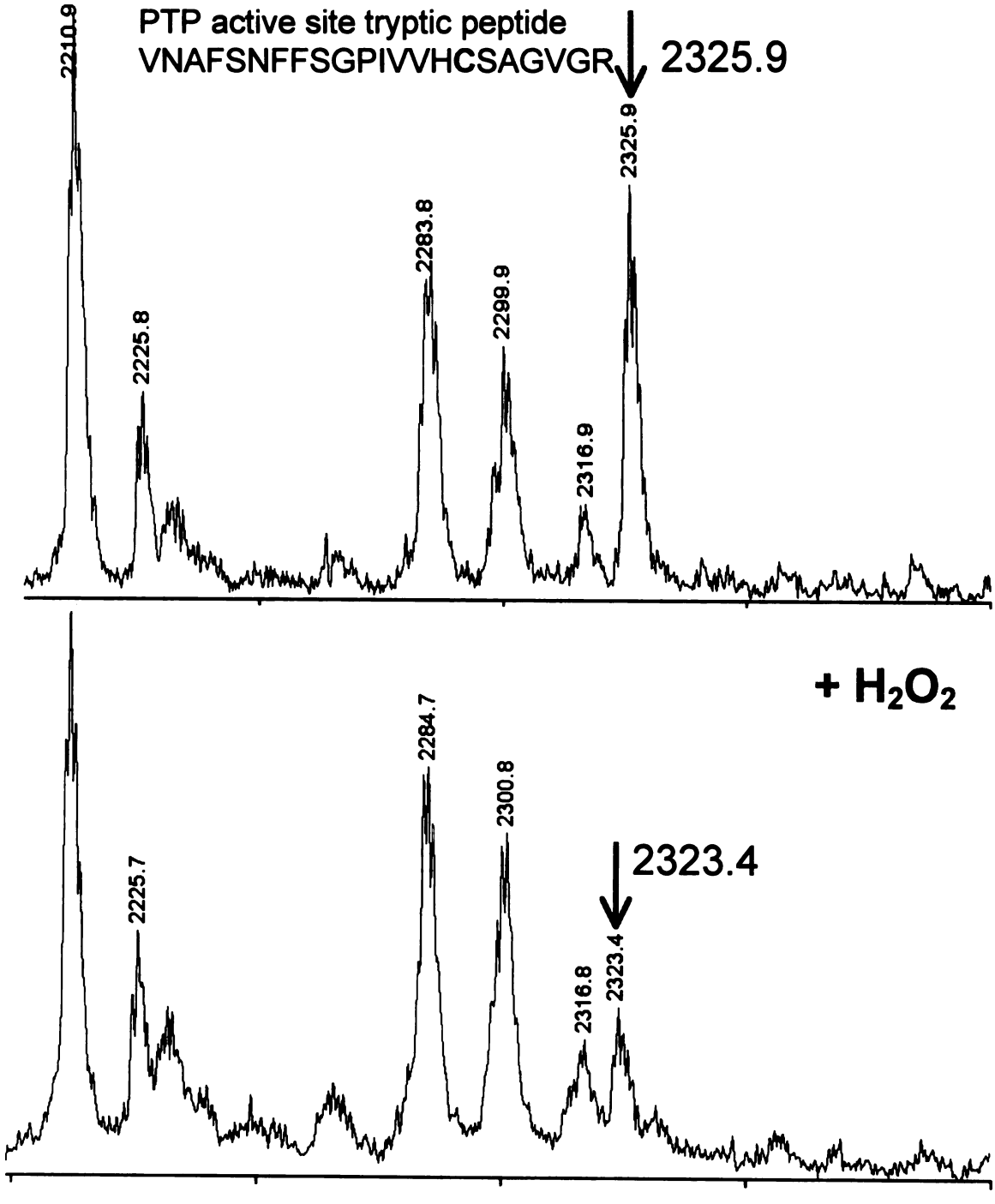


Figure 6. Inhibition of CD45 by ROS regulates DNCB-induced activation of distinct MAPK pathways. Arrows indicate activation pathways and blocked arrows indicate inhibition.

Figure 7. H_2O_2 inhibits the modification of the active site cysteine residue of recombinant CD45 by C13-iodoacetic acid. Recombinant CD45 (10 μg) was incubated with or without 1 mM H_2O_2 for 30 min. After incubation with C13-iodoacetic acid, the protein was reduced by DTT and was subsequently purified by SDS-PAGE electrophoresis. The protein in the gel was subject to alkylation with C12-iodoacetic acid and was digested by trypsin. The peptide fragment was analyzed by MALDI-MS. The results shown are representative of two independent experiments.


PTP active site tryptic peptide
VNAFSNFFSGPIVVHCSAGVGR
2325.9
2210.9
2225.8
2283.8
2299.9
2316.9
2325.9
+ H2O2
2225.7
2284.7
2300.8
2316.8
2323.4
2323.4

underwent the second modification by C^{12}-iodoacetic acid. CD45 was digested by trypsin and the masses of the resultant peptide fragments were analyzed by MALDI-MS. The mass of the peptide fragment containing Cys817 from H_2O_2-treated sample was about 2 Da lower than the mass of the peptide fragment from untreated sample (Fig. 7B). This mass difference corresponded to the mass difference between C^{13}-iodoacetic acid and C^{12}-iodoacetic acid. The result indicated that the first modification by C^{13}-iodoacetic acid was inhibited by H_2O_2. After treatment of DTT, the unmodified cysteine residues from H_2O_2-treated sample became more available to the second modification by C^{12}-iodoacetic acid. The relative intensity of the peak of the peptide containing Cys817 from H_2O_2-treated sample was lower than that from untreated sample. This result indicated that some of the cysteine residues oxidized by H_2O_2 underwent the irreversible oxidation into sulfinic or sulfonic acids, which were not reduced by DTT.

DISCUSSION

It has been reported that DNCB-induced contact sensitivity is correlated with NADPH-dependent oxygen consumption and elevated glutathione disulfide (GSSG) level in mouse skin (7). Because it has been demonstrated that DNCB inhibits TrxR and depletes GSH inside the cell, the DTH reaction invoked by DNCB may linked to its pro-oxidant properties. As T cells are important mediators of DNCB-induced hypersensitivity and chemotherapy (5), the relevance between the cellular redox alteration induced by DNCB and the immunostimulation of T cells was investigated.

DNCB treatment of Jurkat cells induced JNK and p38 phosphorylation and potentiated TCR-induced JNK and p38 phosphorylation (Fig 1A). In accordance with the report that a mild oxidative shift enhances T cell signaling events including JNK and p38 activation (38), this result suggests that the redox change induced by DNCB may be involved in the modulation of T cell activation process. The report that JNK2 and p38 activation stimulates CD4+ T cell differentiation into Th1 cells (39) is consistent with the observations that DNCB treatment promotes Th1-type cytokine expression (5, 9). Therefore, our result suggests that direct stimulation of T cells by DNCB contributes to the differentiation into Th1 type by selectively augmenting TCR-induced JNK and p38 activation. Interestingly, DNCB was a poor stimulator of ERK phosphorylation and did not enhance TCR-induced ERK phosphorylation. The enhancement of JNK and p38 phosphorylation without affecting ERK phosphorylation is a characteristic of CD28 costimulation and IL-7 treatment (40-42). It will be interesting to check

whether the alteration of redox potential is involved in the activation of MAPKs by those stimuli. It has been previously reported that CD28 activates NF-κB by ROS-dependent signaling pathway involving 5-lipoxygenase activation (43).

The Src-family kinase inhibitor, PP2, decreased DNCB-induced JNK and p38 phosphorylation (Fig. 3). This result suggests that DNCB-induced Lck (and Fyn) activation is required for the downstream MAPK activation. In contrast with PP2, the PLC inhibitor (U73122) or the PKC inhibitor (Ro-31-8425) did not affect DNCB-induced JNK and p38 phosphorylation. The functionality of these inhibitors was confirmed by the observation that the same concentration of inhibitors abrogated H_2O_2-induced ERK phosphorylation (28). H_2O_2-induced JNK and p38 phosphorylation, like DNCB, was not affected by PLC and PKC inhibitors (28). In addition, it has been reported that chelerythrine-induced JNK and p38 activation was inhibited by antioxidants but not by PKC inhibitors (44). These observations suggest that PLC and PKC activity are not required for ROS-induced JNK and p38 activation and support the specificity of ROS-induced signaling pathway.

Stimulation of Jurkat cells with DNCB resulted in the activation of Lck (Fig. 4). NAC pretreatment inhibited DNCB-induced Lck activation, suggesting that ROS production was involved. It is not likely that ROS produced by DNCB directly activate Lck because the addition of oxidants to purified Lck did not increase kinase activity (37). Therefore, ROS may indirectly regulate Lck activity by affecting PTPs that control the phosphorylation of Lck. It has been known that phosphorylation of Tyr394 in the activation loop of Lck induces kinase activity

(45), whereas phosphorylation of Tyr505 in the C-terminal domain inhibits the activity (46). It has been reported that CD45 regultes Lck activity by dephosphorylating both residues of Lck. Although the model that CD45 activates Lck by dephosphorylating Tyr505 in T cell activation has been upheld, it has been reported that Lck may be hyperphosphorylated at Tyr 394 in some CD45 deficient T cells (47, 48). H_2O_2 treatment of Jurkat cells inhibited CD45 activity and led to the hyperphosphorylation of both residues of Lck (49). Importantly, Lck kinase activity is actually increased in cells stimulated by H_2O_2 or in cells deficient in CD45 (47). Those results suggest that the activating effect of Tyr394 phosphorylation is dominant over the inhibitory effect of Tyr505 phosphorylation when both residues are phosphorylated. Therefore, the net effect of CD45 inhibition can lead to the activation of Lck (50). The importance of the inhibition of Lck activity by CD45 has been shown in the report that mice expressing constitutively active Lck by mutating Tyr505 into Phe develop thymic lymphoma on a CD45-/- background (51).

CD45 is the prototypical PTP that is most abundantly expressed in T cells. Because it has been suggested that the catalytic cysteine residues of PTPs are susceptible to oxidation, the effect of DNCB-induced ROS production on PTP activity of CD45 was investigated. As shown in Fig. 5A, the exposure of Jurkat cells to DNCB or to H_2O_2 resulted in the substantial inhibition of CD45 activity immunoprecipitated from the cells. When 5 mM DTT was included in the lysis buffer, significant portion of CD45 activity that was inhibited by DNCB and H_2O_2 was recovered. This result suggests that both the extracellular addition of

ROS and the intracellular ROS production by DNCB reversibly inhibit CD45 activity. Pretreatment of cells with NAC before DNCB stimulation also prevented the inhibition of CD45 activity, further indicating the involvement of ROS for the process. In contrast with H_2O_2, DNCB did not directly inhibit recombinant CD45 activity. The result excluded the possibility that DNCB inhibited CD45 activity by direct modification. If intracellular environment somehow induces the modification of CD45 by DNCB without involving ROS, the inhibited portion of CD45 activity as such may not be restored by DTT because TrxR inhibition by DNCB-mediated alkylation is irreversible (8).

Although it has been suggested that ROS inhibit PTP activity by oxidizing the catalytic cysteine residue of the active site, only a few PTPases that are small in size have been shown to contain redox-sensitive catalytic cysteine residues. It has not been amenable to demonstrate the principle in the case of receptor-like PTPs such as CD45 which contain the tandem PTPase domains. As a way to elucidate the mechanism of PTP inhibition by ROS, we developed the method to detect the status of the active site cysteine residue of CD45 by sequential modification with differential iodoacetic acids by mass. H_2O_2 treatment inhibited the first modification of the active site cysteine residue by C^{13}-iodoacetic acid because the oxidized cysteine residue was resistant to the alkylation. However, the active site cysteine residue from CD45 sample treated with H_2O_2 became modified by the second modification with C^{12}-iodoacetic acid because the oxidized cysteine residue was reduced by DTT before C^{12}-iodoacetic acid labeling. The lower peak intensity of the peptide fragment containing Cys817

from H_2O_2-treated sample compared to that from untreated sample suggested that not all the oxidized cysteines were reduced by DTT. This result indicated that the catalytic cysteine residue of CD45 was indeed oxidized by H_2O_2 into both reversible and irreversible state.

NAC inhibited DNCB-induced ROS production, CD45 inhibition, Lck activation, and JNK and p38 phosphorylation. The correlative sensitivity of those signaling events to NAC indicates that they are intimately connected processes. NAC is a precursor of GSH that is the most abundant intracellular thiols and has the capacity to directly scavenge ROS. It has been known that NAC and other antioxidants inhibit JNK and p38 activation induced by other alkylating agents and chemotherapeutic compounds. Therefore, JNK and p38 activation induced by the alteration of the redox status is the important process for those reagents including DNCB to induce appropriate cellular signaling.

Taken together, these results show that CD45 activity is reversibly regulated by ROS in T cells and suggests a role for ROS for the subsequent activation of Lck and differential regulation of MAPKs in DNCB-induced immunostimulation (Fig. 6). The change in redox state by DNCB affected T cell activation by specific activation of the JNK and p38 MAPK signaling pathways. Since JNK and p38 activation has been associated with induction of Th1 cells (39), our results provide the potential molecular mechanism how DNCB promotes Th1 differentiation. With the use of differential iodoacetic acid, the catalytic cysteine residue of CD45 was probed as the target of ROS. Since it has been documented that TCR triggering produces endogenous ROS (54), it will be

interesting to determine whether PTP activity is regulated by redox status during T cell activation.

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CHAPTER FIVE

CURCUMIN INHIBITS STAT1 AND STAT3 PHOSPHORYLATION AND C-FOS EXPRESSION IN JURKAT T LYMPHOCYTES

ABSTRACT

Curcumin and resveratrol are polyphenolic compounds present in dietary products that show anti-inflammatory, anti-neoplastic and antioxidant properties. However, the association of antioxidant properties of the polyphenols with their clinical effects has not been convincingly established. To understand the relationship between anti-inflammatory effects and antioxidant properties of curcumin and resveratrol, we compared the effect of the compounds on signaling events in Jurkat T cells stimulated by interferon-α (IFN-α) and Concanavalin A (Con A). The antioxidant properties of curcumin and resveratrol were manifested by the inhibition of Con A-induced Reactive Oxygen Species (ROS) production. Curcumin, but not resveratrol, inhibited phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT3 induced by IFN-α and Con A. This result suggests that the effect of curcumin on STATs is mediated by its non-antioxidant properties. Both curcumin and resveratrol inhibited Con A-induced IL-2 mRNA expression, indicating that antioxidant properties of the compounds also contribute to their anti-inflammatory effects. Although curcumin and resveratrol comparably inhibited Con A-induced c-Jun NH_2-terminal kinase (JNK) phosphorylation and *c-jun* promoter activation, curcumin displayed more profound inhibition of AP-1 activity. The differential sensitivity of AP-1 was attributed to the finding that curcumin, but not resveratrol, ablated Con A-induced c-Fos expression. Con A-induced Elk-1 activity and CREB phosphorylation were not affected by either curcumin or resveratrol, suggesting that non-antioxidant properties of curcumin inhibited c-Fos expression by blocking STAT1 and STAT3

activation. Taken together, these results demonstrate that non-antioxidant and antioxidant properties of curcumin and resveratrol contribute to their overlapping but distinct anti-inflammatory effects.

INTRODUCTION

Recently there has been a renewed level of interest in the anti-inflammatory properties of naturally occurring polyphenols such as curcumin and resveratrol [1]. The anti-inflammatory activity of this class of compounds has been known for many years and recent studies support their beneficial effects in clinical models of inflammation and cancer [2, 3]. In accordance with their clinical effects, polyphenolic compounds have been reported to modulate a broad spectrum of signaling events in a variety of types of cells [1, 4]. Many of the polyphenols, including curcumin and resveratrol, have been demonstrated to have antioxidant properties [5, 6]. Accordingly, it has been suggested that the effect of the compounds is mediated by the regulation of redox-sensitive signaling pathways [4]. However, the association of antioxidant properties of polyphenols with their clinical effects has not been convincingly established [3]. Therefore, it is important to address the question whether all the effects of the polyphenolic compounds are due to their antioxidant properties.

Curcumin is a dietary yellow pigment from the rhizome of *Curcuma longa L.* . Curcumin displays anti-tumor and anti-inflammatory properties in models of tumorigenesis, atherosclerosis, arthritis, Alzheimer's disease and multiple sclerosis [7-11]. The antioxidant properties of curcumin have been described in the reports showing that curcumin inhibits the activities of lipoxygenase, cyclooxygenase and xanthine oxidase [12-14]. With regard to immune cells, curcumin inhibited superoxide production of macrophages stimulated with phorbol myristate acetate (PMA) [15] and inhibited the response of blood

mononuclear cells to phytohemagglutinin (PHA) and mixed lymphocyte reactions [16]. Recently it has been reported that curcumin inhibits the activation of signal transducers and activators of transcription (STATs) in cells stimulated by oncostatin M and interleukin (IL)-12 [11, 17].

Resveratrol is a polyphenolic compound naturally present in high amounts in the skin of red grapes. Resveratrol has been regarded as a factor involved in the "French Paradox", in which it has been observed that moderate consumption of red wine is associated with the low incidence of coronary heart disease [18]. The antioxidant properties of resveratrol have been mechanistically related to its beneficial effect on atherosclerosis by research showing that resveratrol inhibits the oxidation of low-density lipoprotein (LDL) particles, vascular reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, lipoxygenase and cyclooxygenase [19-21]. The anti-neoplastic effect of resveratrol has also been associated with its antioxidant properties [22]. Incubation of U937 lymphoma cells with resveratrol decreased tumor necrosis factor (TNF)-α-induced ROS production and nuclear factor (NF)-κB activation [23]. Recently, it has been shown that resveratrol inhibits Con A-induced IL-2 gene transcription and NF-κB activation in splenic T cells [24].

In order to understand the relationship between anti-inflammatory effects and antioxidant properties of curcumin and resveratrol, we compared the effect of the compounds on signaling events in Jurkat T cells stimulated by IFN-α and Con A. Examination of the signaling effects of the two related antioxidant polyphenols identified the signaling pathways sensitive to both compounds as well as the

pathways that are differentially regulated. The comparison of the cellular effects between curcumin and resveratrol led us to the conclusion that curcumin acts in a non-antioxidant manner in suppressing STAT1 and STAT3 activation and subsequent c-Fos expression.

MATERIALS AND METHODS

Reagents and Antibodies Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) and resveratrol (5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol 3,4′,5-trihydroxy-trans-stilbene) were purchased from Sigma (St. Louis, MO). Curcumin and resveratrol were dissolved in dimethyl sulfoxide (DMSO) freshly prior to use. Human recombinant IFN-αA was obtained from Calbiochem (San Diego, CA). Con A was obtained from Amersham (Piscataway, NJ). Antibodies used in this study were obtained as follows: p-STAT1 (Tyr701), p-STAT3 (Tyr705), p-ERK1/2 (Thr202 and Tyr204), STAT1, STAT3 and p38 (Cell Signaling, Beverly, MA); p-p38 (Thr180 and Tyr182) and p-JNK1/2 (Thr183 and Tyr185) (Promega, Madison, WI); p-CREB (Ser133) (Upstate, Charlottesville, VA). ERK2 and JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA); horseradish peroxidase-conjugated donkey anti-rabbit antibody and sheep anti-mouse antibodies (Amersham); and horseradish peroxidase-conjugated rabbit anti-goat antibody (Calbiochem).

Plasmids Luciferase reporter plasmids containing *c-jun* and *c-fos* promoters were kindly provided by Dr. Y. Kawakami (La Jolla Institute, CA). Luciferase reporter containing five Gal4 binding sites with E1B minimal promoter and the expression vector for Gal4-Elk1 transactivation domain fusion were the generous gifts from Dr. R. A. Maurer (Oregon health science Univ., OR). Dr. M. Karin (University of California at Davis, CA) generously provided luciferase reporter containing two AP-1 binding sites from collagenase-α enhancer with rat prolactin minimal promoter.

Cell culture Jurkat cells, clone E6-1, were grown in RPMI 1640 media with 25 mM HEPES buffer (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated FBS (Life Technologies, Rockville, MD), 100 units/ml streptomycin/penicillin and 2 mM glutamine (Life Technologies). Cells were maintained in an exponential growth phase ($0.2 - 1.0 \times 10^6$ cells/ml).

Measurement of ROS 1×10^6 cells were reconstituted in 1 ml serum-free RPMI medium. After stimulation, cells were incubated with 5 μM of 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H_2DCF-DA) (Molecular Probes, Eugene, OR) was added. After 1 hr incubation with the fluorochrome, fluorescence-activated cell sorter (FACS) analysis was conducted at an excitation wavelength of 488 nm and emission at 520 nm.

Immunoblotting 1.5×10^6 cells were reconstituted in 1.5 ml serum-free RPMI medium. After stimulation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in lysis buffer (Cell Signaling) for 30 min on ice. Lysates were pelleted by centrifugation at 8,000 × g at 4°C for 10 min and supernatants were recovered. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membrane. Blotting of the membrane was performed as previously described [25].

Measurement of IL-2 mRNA 4×10^6 cells were reconstituted in 4 ml RPMI medium containing 10% FBS. After stimulation of cells, RNA was extracted using Trizol reagent (Life Technologies) and the amount of RNA was quantified by spectrophotometer. The amount of IL-2 mRNA was measured by

Quantikine mRNA kit (R&D systems, Minneapolis, MN) according to manufacturer's instruction.

Transfection and luciferase assay The luciferase reporter vectors and β-gal reporter plasmids were mixed with DMRIE-C reagent (Life Technologies) in OPTI-MEM media (Life Technologies) at room temperature. After 40 min, 0.5×10^6 cells were added to the mixture. After 5 hrs of incubation at 37°C, 1 ml RPMI medium containing 10% FBS was added. After 16 hrs, cells were stimulated and lysed with lysis buffer (Promega). The luciferase activity was determined by luciferase reagent (Promega) and was normalized by β-galactosidase activity determined by the galactosidase reagent (BD Biosciences, Palo Alto, CA).

Statistical analysis Data are expressed as means with standard deviation. Data were analyzed by Student's *t*-test to determine the level of significance ($P < 0.05$)

RESULTS

Curcumin and resveratrol inhibit Con A-induced ROS production. We compared the antioxidant properties of curcumin and resveratrol to determine whether they exhibit comparable antioxidant capacity. Intracellular ROS levels in Jurkat T cells were measured by the use of the cell-permeable and ROS-sensitive fluorochrome, CM-H_2DCF-DA. IFN-α stimulation did not induce a significant change in fluorescence (Fig. 1, lanes 1 and 2). In contrast, Con A stimulation led to about a 3 fold increase in fluorescence (Fig. 1, lanes 1 and 3). This result suggests that endogenous ROS were produced in T cells stimulated by Con A but not IFN-α. The increase of fluorescence by Con A was significantly inhibited by curcumin (lanes 3 and 4) and by resveratrol (lanes 3 and 5). This result confirmed that curcumin and resveratrol have antioxidant properties. The degree of inhibition exhibited by curcumin and resveratrol was not statistically different (lanes 4 and 5), indicating that curcumin and resveratrol manifest the comparable antioxidant capacity in the system examined in this study.

Curcumin but not resveratrol blocks IFN-α-induced STAT1 and STAT3 phosphorylation. The activation of STATs is the primary signaling event activated by interferon [26]. Binding of interferons to their cognate receptors leads to the activation of Janus kinases (JAKs) associated with the receptors. When STATs become phosphorylated at the conserved tyrosine residue (Tyr701 in STAT1 and Tyr 705 in STAT3) by JAKs, they undergo dimerization and subsequent translocation to the nucleus to bind cognate DNA sequences. As a way to examine the effect of curcumin and resveratrol on the

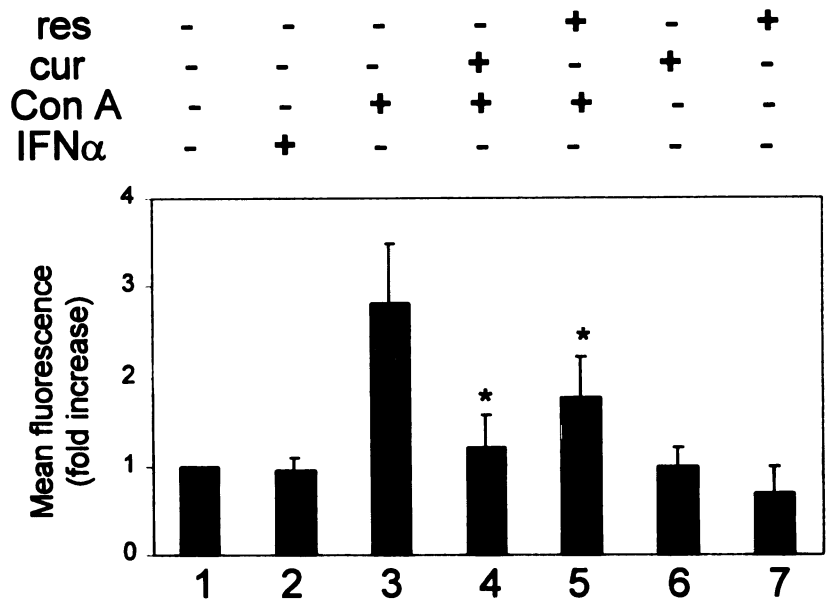
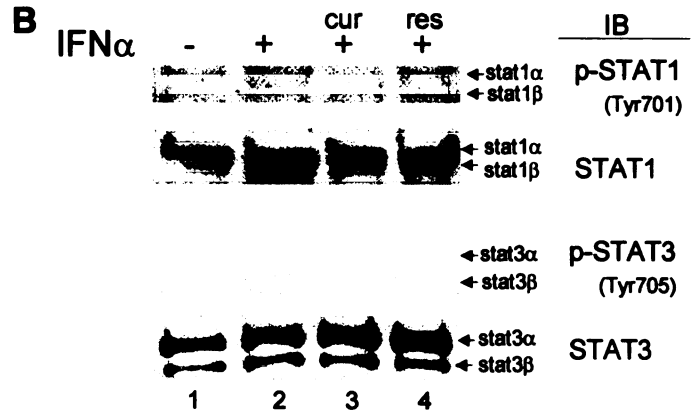


Figure 1. Curcumin and resveratrol inhibit Con A-induced ROS production. Cells were preincubated with 20 μM curcumin (cur) or with 50 μM resveratrol (res) for 30 min and were subsequently stimulated with 50 μg/ml Con A or with 10^5 units/ml IFN-α. After 5 min of stimulation, 5 μM CM-H_2DCF-DA was added. After 1 hr, cells were analyzed by FACS at an excitation wavelength of 488 nm and emission at 520 nm. The mean fluorescence of the sample in the first lane was set as 1 and the fluorescence of the other samples were presented as fold increase. The average value with standard deviation is presented (resveratrol: n = 4, curcumin: n = 3, IFN-α: n = 3). *, $p < 0.05$ *vs.* the value obtained from cells from Con A stimulation alone.

IFN-α-induced signaling pathway, we compared the effect of the compounds on IFN-α-induced STAT1 and STAT3 phosphorylation. Treatment of Jurkat cells with IFN-α resulted in the robust phosphorylation of both STAT1 and STAT3 (Fig. 2A, lanes 1 and 2). Both isoforms of STAT1 (STAT1α and STAT1β) and STAT3 (STAT3α and STAT3β) were phosphorylated in proportion to their cellular abundance. Curcumin blocked IFN-α-induced STAT1 and STAT3 phosphorylation (Fig. 2A, lanes 2 and 3). In contrast, resveratrol did not affect either STAT1 or STAT3 phosphorylation (Fig. 2A, lanes 2 and 4). This result suggests that the observed inhibitory effect of curcumin on STAT1 and STAT3 phosphorylation was not due to its antioxidant properties because a related antioxidant, resveratrol, had no effect.

Curcumin but not resveratrol blocks Con A-induced STAT1α and STAT3β phosphorylation. It has been known that STATs are activated not only by interferons but also by cytokines and growth factors [27]. To determine whether the inhibitory effect of curcumin on STAT1 and STAT3 phosphorylation is stimulus-specific, Con A-induced STAT phosphorylation was examined. Con A stimulation induced phosphorylation of STAT1α (91 kDa) without detectable STAT1β phosphorylation (84 kDa) (Fig. 2B, p-STAT1 blot, lanes 1 and 2). Because blotting with STAT1 antibody (Ab) showed that the amount of STAT1α was much higher than STAT1β, preferential phopshorylation of STAT1α may have resulted from the low signal strength from Con A stimulation. Interestingly, Con A-induced STAT3 phosphorylation involved only the STAT3β isoform (82 kDa) (Fig. 2B, p-STAT3 blot, lanes 1 and 2), even though STAT3α (93 kDa) was

Figure 2. Curcumin, but not resveratrol, inhibits STAT1 and STAT3 phosphorylation induced by IFN-α and Con A. Cells were pretreated with 20 μM curcumin (cur) or with 50 μM resveratrol (res) for 30 min and subsequently stimulated **(A)** with 5×10^4 units/ml IFN-α or **(B)** with 50 μg/ml Con A for 10 min. After blotting with p-STAT1 and p-STAT3 Abs, the membranes were reprobed with STAT1 and STAT3 Abs to show that equivalent amount of protein was present in each lane. Blots are representative of three independent experiments. (IB: immunoblot)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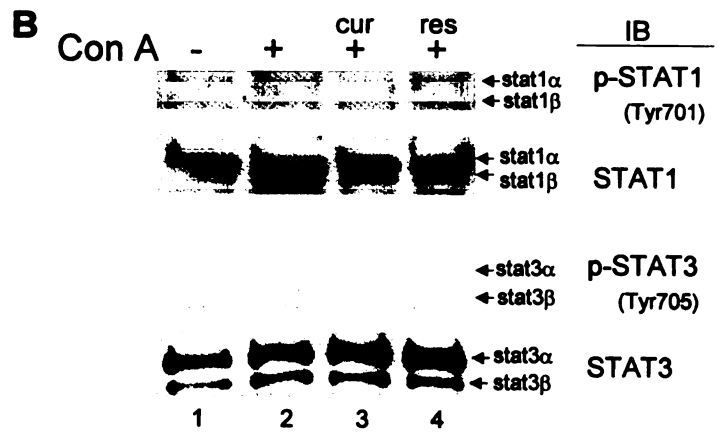
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far more abundant as determined by a STAT3 Ab blot. Curcumin completely inhibited Con A-induced STAT1α and STAT3β phosphorylation (Fig. 2B, lanes 2 and 3). However, a significant level of STAT1α and STAT3β phosphorylation was still detectable in the presence of resveratrol (Fig. 2B, lanes 2 and 4). The selective effect of curcumin on Con A-induced STAT1α and STAT3β phosphorylation further confirmed that the inhibition of STAT phosphorylation by curcumin was mediated by its unique non-antioxidant properties.

Curcumin and resveratrol inhibit Con A-induced IL-2 mRNA expression. IL-2 is the pivotal cytokine produced when T cells are activated. To determine the effect of curcumin and resveratrol on the outcome of T cell activation, the level of IL-2 mRNA was assessed. Stimulation of Jurkat T cells by Con A increased IL-2 mRNA level (Fig. 3, lanes 1 and 3). IFN-α did not increase IL-2 mRNA level (Fig. 3, lanes 1 and 2). Curcumin effectively blocked the increase of IL-2 mRNA induced by Con A (Fig. 3, lanes 3 and 4). Resveratrol also significantly decreased the amount of IL-2 mRNA induced by Con A (Fig. 3, lanes 3 and 5). The inhibition of IL-2 mRNA expression by both curcumin and resveratrol strongly supports the idea that endogenous ROS production has an important role in Con A-induced IL-2 gene expression.

Curcumin and resveratrol selectively inhibit Con A-induced JNK phosphorylation. To understand the mechanism how curcumin and resveratrol inhibit Con A-induced IL-2 mRNA expression, the effect of curcumin and resveratrol on Con A-induced signaling pathways was further compared. As mitogen-activated protein kinases (MAPKs) are important mediators of the T cell

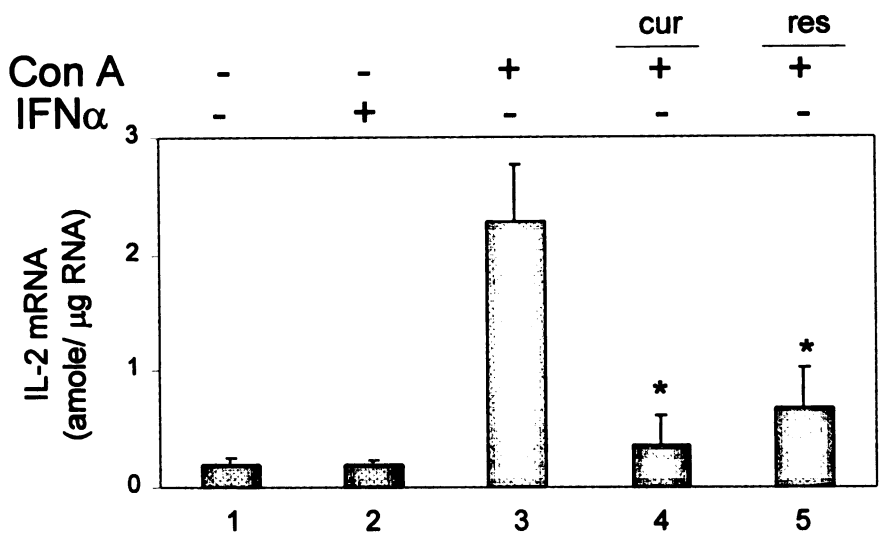


Figure 3. Curcumin and resveratrol inhibit IL-2 mRNA induction by Con A. Cells were incubated with 20 μM curcumin (cur) or with 50 μM resveratrol (res) for 30 min, followed by 25 μg/ml Con A stimulation for 4 hrs. The amount of IL-2 mRNA was measured by Quantikine mRNA kit. The value of the amount of IL-2 mRNA (attomole) divided by the amount of RNA extracted (μg) is presented with standard deviation after three independent experiments. *; $p < 0.05$ *vs.* value from cells stimulated with Con A alone.

activation process [28], the status of activation-associated phosphorylation of c-Jun NH_2-termianl kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) MAPKs was examined. Con A stimulation of Jurkat cells induced phosphorylation of all three MAPK members, judged by western blotting with phospho-specific antibodies (Fig. 4A and 4B, lanes 1 and 2). Pretreatment of cells with curcumin or resveratrol partially inhibited JNK phosphorylation (Fig. 4A and 4B, p-JNK blot, lanes 2 and 3). In contrast, the level of p38 and ERK phosphorylation was not affected by either curcumin or resveratrol (Fig. 4A and 4B, p-p38 and p-ERK blot, lanes 2 and 3). The selective effect of both antioxidants on JNK phosphorylation suggests that the signaling pathway leading to JNK phosphorylation harbors a component regulated by endogenous ROS.

Curcumin and resveratrol comparably inhibit c-jun promoter activation but differentially inhibit AP-1 activation. The activation of JNK leads to the phosphorylation and the activation of c-Jun, and one of the target genes activated by c-Jun is the *c-jun* promoter itself [29]. Accordingly, *c-jun* promoter activity was measured as the readout of JNK activation. Con A stimulation increased the activity of luciferase reporters containing *c-jun* promoter (*c-jun*-luc) (Fig. 5A, lanes 1 and 2). Pretreatment of the cells with either curcumin or resveratrol significantly inhibited Con A-induced *c-jun*-luc activation (Fig. 5A, lanes 2, 4 and 6). This result suggests that the inhibition of JNK activation by curcumin and resveratrol leads to the decrease of *c-jun* promoter activation. Since AP-1 is composed of Jun and Fos dimers, we next measured AP-1 activity as the consequence of c-Jun expression. Con A stimulation increased the

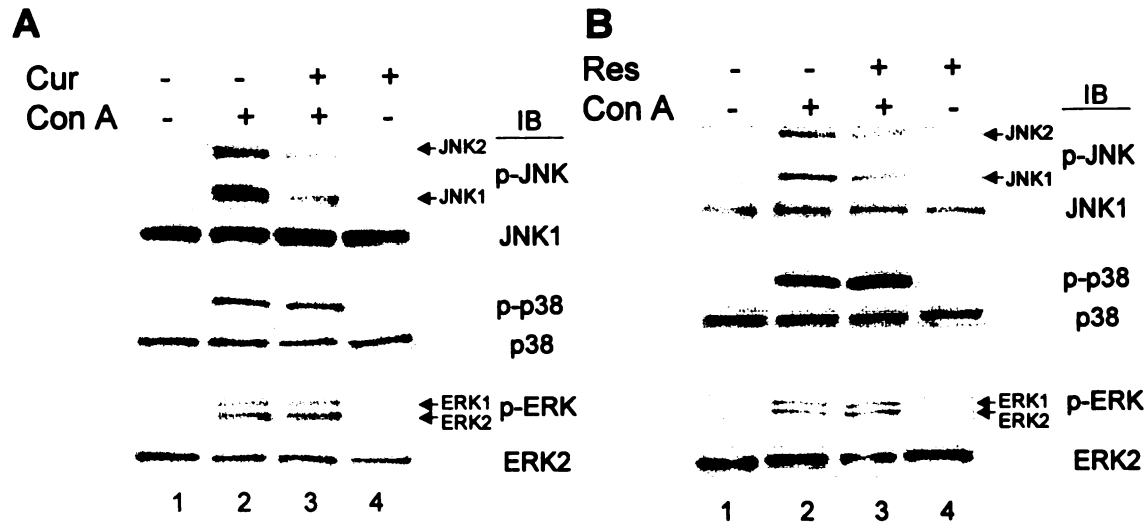


Figure 4. Curcumin and resveratrol inhibit Con A-induced JNK phosphorylation. Cells were pretreated **(A)** with 20 μM curcumin (cur) or **(B)** with 50 μM resveratrol (res) for 30 min and subsequently stimulated with 25 μg/ml Con A for 1 hr. After blotting with phospho-specific MAPK Abs, blotting with JNK1, p38, and ERK2 Abs showed that equivalent amount of protein was present in each lane. The blots are representative of three independent experiments. (IB: immunoblot)

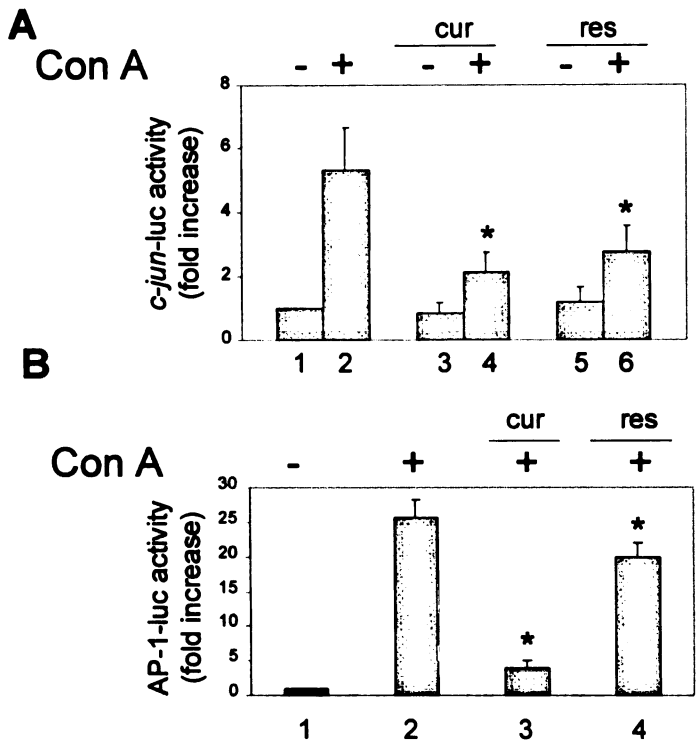


Figure 5. Curcumin and resveratrol comparably inhibit Con A-induced *c-jun* promoter activation but distinctly inhibit AP-1 activation. Cells were transfected with **(A)** 0.5 μg luciferase vector containing *c-jun* promoter (*c-jun*-luc) or **(B)** with 1 μg luciferase reporter containing two AP-1 binding sites (AP-1-luc). After 16 hrs, cells were preincubated with 20 μM curcumin (cur) or with 50 μM resveratrol (res) for 30 min and subsequently stimulated by 25 μg/ml Con A for 4 hrs. The normalized luciferase activity of the sample in lane 1 was set as 1 and the activities of the other samples were represented as fold increase. The average value with standard deviation is from three independent experiments. *; $p < 0.05$ *vs.* activity from cells stimulated with Con A alone.

activity of luciferase reporters containing AP-1 binding sites (AP-1-luc) (Fig. 5B, lanes 1 and 2). Surprisingly, there was a dramatic difference in the capacity of curcumin and resveratrol in the inhibition of AP-1-luc. Curcumin almost completely inhibited AP-1-luc (~ 80%) (Fig. 5B, lanes 2 and 3), while resveratrol exhibited only modest inhibition (~ 20%) (lanes 2 and 4).

Curcumin but not resveratrol inhibits Con A-induced c-Fos expression. To understand the differential sensitivity of AP-1-luc to curcumin and resveratrol, we examined another member of AP-1 family, c-Fos. Con A stimulation of Jurkat cells induced c-Fos protein expression (Fig. 6A, lanes 1 and 2). Of the two bands detected with anti-c-Fos Ab, the upper band most likely represents the hyperphosphorylated form of the protein [30]. Curcumin completely abrogated the Con A-induced c-Fos expression (Fig. 6A, lanes 2 and 3). In contrast, resveratrol did not affect Con A-induced c-Fos expression (Fig. 6A, lanes 2 and 4). The observation that curcumin but not resveratrol inhibited Con A-induced c-Fos expression indicates that the inhibitiory effect of curcumin on c-Fos expression is mediated by its non-antioxidant properties.

To determine whether the inhibitory effect of curcumin on c-Fos expression is exerted at the level of *c-fos* gene expression, we measured the activity of luciferase reporters containing *c-fos* promoter (*c-fos*-luc). Curcumin inhibited Con A-induced *c-fos*-luc activation by 50 % (Fig. 6B, lanes 2 and 3), while resveratrol showed no effect (lanes 2 and 4). This result indicates that c-*fos* promoter inhibition by curcumin is responsible for the decrease in c-Fos expression.

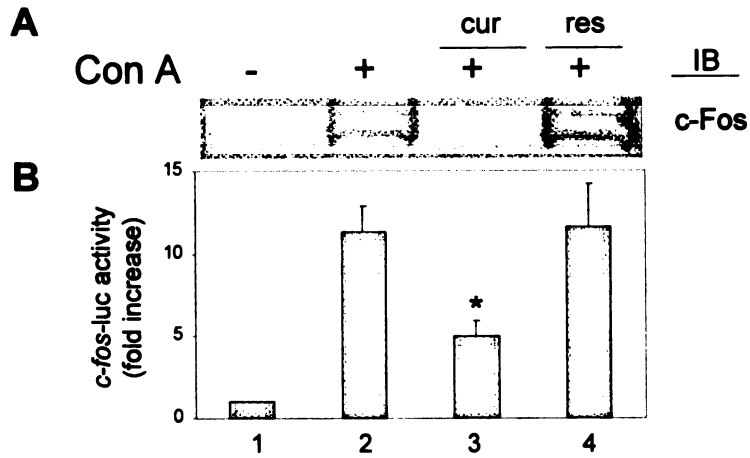
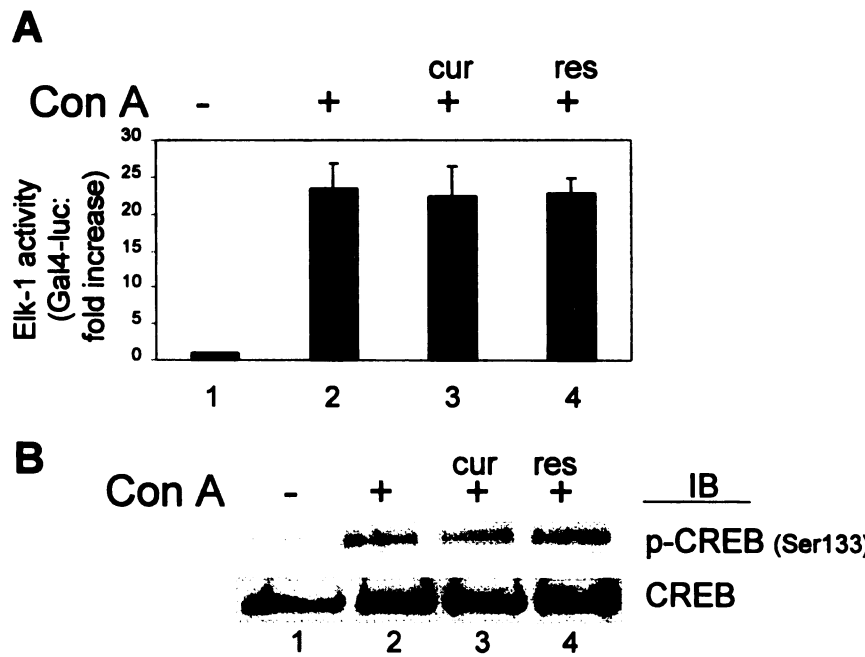


Figure 6. Curcumin but not resveratrol inhibits Con A-induced c-Fos expression. **(A)** Cells were pretreated with 20 μM curcumin (cur) or with 50 μM resveratrol (res) for 30 min and subsequently stimulated with 25 μg/ml Con A for 1 hr. The blot is the representative of three independent experiments. (IB: immunoblot) **(B)** Cells were transfected with 0.5 μg luciferase vector containing *c-fos* promoter (*c-fos*-luc). After 16 hrs, cells were preincubated with 20 μM curcumin or with 50 μM resveratrol for 30 min and subsequently stimulated by 25 μg/ml Con A for 2 hrs. The normalized luciferase activity of the sample in lane 1 was set as 1 and the activities of the other samples were represented as fold increase. The average value with standard deviation is from three independent experiments. *, $p < 0.05$ *vs.* activity from cells stimulated with Con A alone.

Curcumin and resveratrol do not inhibit Con A-induced Elk-1 activity and CREB phosphorylation. The transcriptional induction of *c-fos* is known to be regulated by the following factors [reviewed in [31]]: 1) serum response element (SRE) recognized by serum response factor (SRF) and a ternary complex factor such as Elk-1; 2) cyclic adenosine monophsophate response element (CRE) recognized by CRE-binding protein (CREB); and 3) *sis*-inducible element (SIE) recognized by STAT proteins. Accordingly, we determined whether the effect of curcumin on c-Fos expression could be attributed to the activation status of transcription factors other than STAT1 and 3. The transcriptional activity of Elk-1 is potentiated when MAPKs phosphorylate Elk-1 in the transactivation domain [32]. The level of Elk-1 phosphorylation was assessed by an assay in which a Gal4-Elk1 transactivation domain fusion protein was co-expressed with a Gal4-dependent luciferase reporter (designated as Gal4-luc). In this system, Gal4-luc activity increases when the Gal4-Elk1 fusion protein is activated by phosphorylation of the Elk-1 transactivation domain [33]. As shown in Fig. 7A, Con A stimulation led to about 20 fold increase of Gal4-luc activity (lanes 1 and 2). Pretreatment of the cells with either curcumin or resveratrol did not inhibit Con A-induced Gal4-luc activity (Fig. 7A, lanes 2, 3 and 4). This result suggests that Elk-1 phosphorylation was intact in the presence of the compounds.

It has been known that CREB phosphorylation at Ser133 residue by protein kinase A (PKA), calcium/calmodulin-dependent protein kinase (CaMK) or MAPK-dependent kinases induces transcriptional activity of the protein [34]. The

Figure 7. Curcumin and resveratrol do not inhibit Con A-induced Elk-1 activity or CREB phosphorylation. (A) Cells were transfected with 0.25 μg luciferase reporter containing Gal4 binding sites (Gal4-luc) plus 0.25 μg Gal4-Elk-1 expression vector. After 16 hrs, cells were preincubated with 20 μM curcumin (cur) or with 50 μM resveratrol (res) for 30 min and subsequently stimulated by 25 μg/ml Con A for 2 hrs. The normalized luciferase activity of the sample in lane 1 was set as 1 and the activities of the other samples were represented as fold increase. The average value with standard deviation is presented from three independent experiments. **(B)** Cells were pretreated with 20 μM curcumin or with 50 μM resveratrol for 30 min and subsequently stimulated with 25 μg/ml Con A for 1 hr. After blotting with p-CREB Ab, the membrane was reprobed with CREB Ab to show the equal amount of the protein in each lane. Blots are representative of three independent experiments. (IB: immunoblot)


A
Con A
-
+
cur
+
res
+
Elk-1 activity
(Gal4-luc:
fold increase)
30
25
20
15
10
5
0
1
2
3
4
B
Con A
-
+
cur
+
res
+
IB
p-CREB (Ser133)
CREB
1
2
3
4

level of CREB phosphorylation was determined by blotting with phospho-CREB antibodies. As shown in Fig. 7B, Con A stimulation induced CREB phosphorylation (lanes 1 and 2). Treatment of cells with either curcumin or resveratrol did not affect Con A-induced CREB phosphorylation (Fig. 7B, lanes 2, 3, and 4). This result suggests that the CRE site in the *c-fos* promoter is not the mediator of the inhibitory effect of curcumin. The observation that curcumin did not inhibit Elk-1 and CREB phosphorylation strongly suggests that curcumin inhibited Con A-induced c-Fos expression (Fig. 6) by inhibiting STAT1 and STAT3 activation (Fig. 2B).

DISCUSSION

In this report, we have studied the effect of curcumin and resveratrol on IL-2 and c-Fos promoters to understand how these agents affect complex inflammatory signal integration. It has been known that the signaling pathways leading to the activation of AP-1 and NF-κB transcription factors contain redox-sensitive components [35]. Accordingly, antioxidant polyphenols including curcumin and resveratrol have been reported to inhibit AP-1 and NF-κB activation in a wide range of stimuli [23, 36, 37]. However, the degree and the mechanism of the effect exerted by these compounds show considerable variation depending on the types of stimuli and on the cellular systems examined (reviewed in [1]). More importantly, it has not been clearly demonstrated that all the effects displayed by the polyphenols are due to their antioxidant properties. As an approach to disentangle the complexity of their effect on the inflammatory signaling, we have compared two related but distinct polyphenols (curcumin and resveratrol) in Jurkat cells stimulated by IFN-α and Con A.

Stimulation of Jurkat cells with Con A, but not IFN-α, induced endogenous ROS production (Fig. 1). This result demonstrates that ROS production is regulated by specific stimulus. Both curcumin and resveratrol inhibited Con A-induced ROS production (Fig. 1), suggesting that they have comparable antioxidant capacities in the system examined in this study. Based on inhibitor studies, NADPH oxidase and lipoxygenase have been implicated in ROS production during T cell stimulation [38, 39]. Further support comes from a report showing that 5-lipoxygenase was activated following T cell receptor (TCR)

stimulation [40] and from a report describing the abundant expression of Nox5, a homologue of gp91^{phox} NADPH oxidase component, in B and T lymphocytes [41]. Curcumin and resveratrol may inhibit the activity of these ROS-producing cellular enzymes and/or may directly scavenge ROS (a known *in vitro* effect).

Curcumin inhibited IFN-α- and Con A-induced STAT1 and STAT3 phosphorylation (Fig. 2). The related antioxidant, resveratrol, had no effect on either STAT1 or STAT3 phosphorylation. In addition, curcumin inhibited STAT1 and STAT3 phosphorylation induced both by Con A that produced ROS, and by IFN-α that did not produce ROS (Fig. 1). These observations led us to the conclusion that the inhibitory effect of curcumin on STATs is mediated by its non-antioxidant properties. The mechanism of the inhibition of STAT phosphorylation by curcumin is not clear. Curcumin may affect the activities of tyrosine kinases that phosphorylate STATs [11] or the activities of protein tyrosine phosphatases (PTPs) that dephosphorylate STATs. Alternatively, curcumin may affect protein-protein interaction that is important for STAT phosphorylation [42]. An example of a non-antioxidant effect of curcumin is the inhibition of sarco endoplasmic reticulum Ca^{2+}-ATPases (SERCA) [43]. Whether this and other non-antioxidant properties of curcumin are linked to the inhibition of STAT phosphorylation will be investigated further.

Alternative splicing of STAT1 and STAT3 gene results in the shorter isoforms (STAT1β and STAT3β) that do not contain C-terminal region of the longer isoforms (STAT1α and STAT3α) [44]. Although it has been suggested that shorter isoforms of STAT1 and STAT3 may inhibit the transcriptional

activation of their respective longer isoforms, it has been shown that STAT1β and STAT3β act as distinctive genetic regulators [45, 46]. Con A stimulation of Jurkat cells led to the phosphorylation of STAT1α without STAT1β phosphorylation (Fig. 2B). This result may be caused by the weak signal strength from Con A stimulation, because the intensity of Con A-induced STAT phosphorylation was much lower compared to that of IFN-α. In contrast, Con A stimulation induced STAT3β phosphorylation without STAT3α phosphorylation. This result is not explained by the weak signal strength from Con A stimulation, because the cellular abundance of STAT3α is much higher than STAT3β. Therefore, this result suggests that there is a mechanism that distinctively regulates STAT3α and STAT3β phosphorylation. It has been previously shown that expression and phosphorylation of STAT3α and STAT3β is differentially regulated during granulocyte differentiation [47]. Because STAT3β can be transcriptionally active under conditions where STAT3α is not [46], phosphorylation of STAT3β without STAT3α phosphorylation may lead to the activation of STAT3β. The importance of the transcriptional activity of STAT3β is supported by a report showing that specific ablation of STAT3β resulted in the impairment of c-*fos* promoter activation without affecting STAT3α expression and phosphorylation [48].

Reactive oxygen species (ROS) not only participate in killing bacteria but also play an active role in pro-inflammatory signaling [49]. The exposure of immune cells to exogenous ROS at the inflammatory sites as well as endogenous ROS produced by cellular activation contributes to the etiology of

various inflammatory conditions [50]. The inhibition of Con A-induced IL-2 mRNA expression by curcumin and resveratrol (Fig. 3) is consistent with the previous reports showing that the attenuation of the oxidative environment by antioxidants inhibits T cell proliferation as well as the expression of IL-2 [51-53]. Consequently, the results in this study and others indicate that endogenous ROS production as exemplified by Con A stimulation has an important role in cellular activation.

It has been suggested that the distinct activation profile of MAPK members contributes to the precise outcome of T cell activation [28]. While it has been demonstrated that exogenous oxidants such as H_2O_2 activated JNK, p38, and ERK MAPKs in Jurkat cells [54], it is not clear to what extent these kinases are regulated by endogenous ROS production. In the context of Con A stimulation, JNK may be the most sensitive target of endogenous ROS because both curcumin and resveratrol inhibited JNK phosphorylation without affecting p38 and ERK (Fig. 4). There are several candidate mediators for redox-dependent activation of JNK. Apoptosis signal-regulating kinase 1 (ASK1) is a MAPK kinase kinase (MAPKKK) that is activated by the dissociation of thioredoxin (Trx) from the kinase. The dissociation is stimulated when Trx is oxidized by exogenous oxidants as well as by endogenous ROS-producing stimuli such as TNF-α [55]. Glutathione S-transferase (GST) binds to JNK to inhibit its activation. The interaction of JNK with GST is disrupted by ultraviolet (UV) irradiation or H_2O_2 treatment, conferring JNK activation [56]. In addition, phosphatases which directly dephosphorylate JNK such as M3/6 [57] may be

regulated by oxidants because the phosphatases contain reactive cysteine residues in their active sites.

The correlation of the inhibition of JNK phosphorylation (Fig. 4) and the reduction of *c-jun* promoter activation and AP-1 activity (Fig. 5) by curcumin and resveratrol suggests that the inhibition of transcriptional activity may be mediated by the decrease of JNK activation. Because AP-1 is one of the critical transcriptional regulators for IL-2 promoter activation [58], the inhibition of AP-1 activity by curcumin and resveratrol may be responsible for the inhibition of IL-2 mRNA expression (Fig. 3). Interestingly, the degree of inhibition of IL-2 mRNA expression by resveratrol (~70 %) (Fig. 3) was more profound than the degree of inhibition of AP-1 activity (~20 %) (Fig. 5B). This result supports the idea that AP-1 has a cooperative effect with other transcription factors in IL-2 promoter activation [58]. In addition, resveratrol has been shown to inhibit other transcription factors important for IL-2 expression such as NF-κB [24]. The other consideration is that inhibition of JNK pathway by resveratrol may cause a negative effect on IL-2 mRNA stabilization [59].

The inhibition of c-Fos expression by curcumin (Fig. 6A) explains its more robust inhibition of AP-1 activity compared to resveratrol (Fig. 5B). Inhibition of c-Fos expression by curcumin was attributed to the reduction of *c-fos* promoter activation (Fig. 6B). It was interesting that inhibition of c-Fos expression by curcumin was almost complete (Fig. 6A), although *c-fos* promoter activation was downregulated by 50% (Fig. 6B). This result suggests that posttranscriptional and posttranslational regulation of c-Fos expression may also be affected by

curcumin [30, 60]. When we examined the activity of transcription factors regulating *c-fos* promoter, it was observed that curcumin did not inhibit Elk-1 or CREB phosphorylation (Fig. 7). Because it has been shown that the complex of STAT1 and STAT3 binds to the SIE region of *c-fos* promoter [61, 62], the inhibitory effect of curcumin on *c-fos* promoter activation may be mediated by the impairment of STAT1 and STAT3 activation at the SIE region (although effects at other promoter sites cannot be completely eliminated).

Taken together, the results in this study show that both antioxidant properties and non-antioxidant properties of curcumin and resveratrol mediate their overlapping but distinct effects in Jurkat T cells (Fig. 8). The concomitant inhibition of ROS production, JNK activation, and IL-2 mRNA expression by both curcumin and resveratrol provides the evidence that antioxidant properties of the compounds contribute to their anti-inflammatory effects. On the other hand, the observation that curcumin but not resveratrol inhibited STAT1 and STAT3 phosphorylation induced by IFN-α and Con A indicates that the inhibitory effect of curcumin on STATs is mediated by its non-antioxidant properties. The inhibition of Con A-induced STAT1 and STAT3 activation by curcumin resulted in the downregulation of c-Fos expression, illustrating that non-antioxidant properties of curcumin participate in its anti-inflammatory effect. Because constitutive activation of STATs and c-Fos potentially leads to cellular transformation [63, 64], the use of curcumin for the inhibition of STAT activation and c-Fos expression may provide a promising modality for the amelioration of inflammatory and neoplastic diseases.

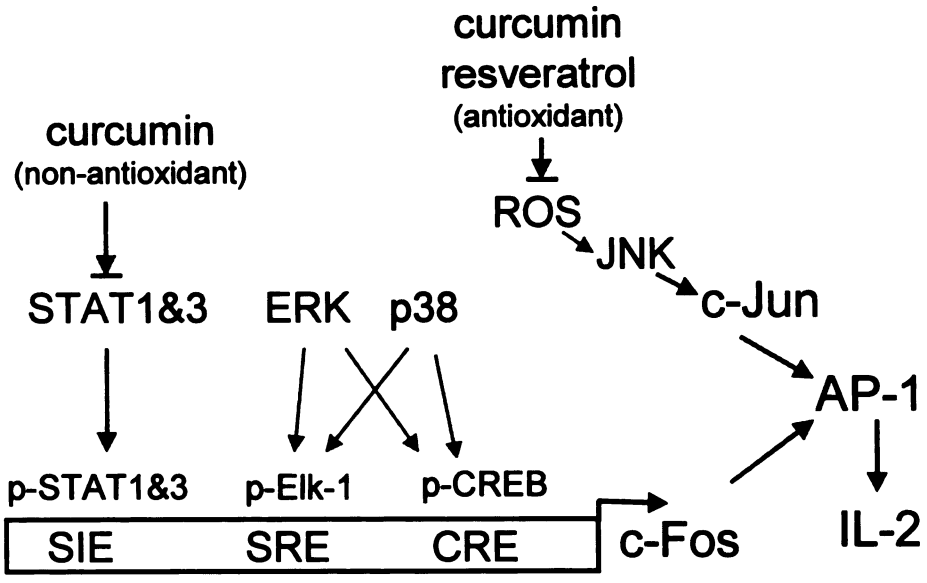


Figure 8. Anti-inflammatory effects mediated by antioxidant and non-antioxidant properties of curcumin and resveratrol. Arrows indicate activation pathways, whereas blunted arrows indicate inhibition.

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