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DISTINCT REGULATION OF EARLY GENE TRANSACTIVATION AND IL-2 GENE EXPRESSION BY SUBLETHAL LEVELS OF OXIDATIVE STRESS IN ACTIVATED HUMAN T LYMPHOCYTES: INVOLVEMENT OF INTRACELLULAR SIGNALING PATHWAYS AND TRANSCRIPTION FACTORS

DISSERTATION

Presented in Partially Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of the Ohio State University

by

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* * * * * *

The Ohio State University
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ABSTRACT

Oxidative stress is known to alter T cell functional responses, but the underlying mechanisms are not understood. The current study examined the effects of sublethal levels of oxidative stress on the expression of early activated genes and IL-2 gene, and determined the involvement of signal transduction components and transcriptional factors. The present results show that Jurkat cells acutely exposed to micromolar concentrations of H$_2$O$_2$ exhibited the rapid induction of c-jun and c-fos mRNA in a dose-dependent manner. c-jun mRNA expression induced by H$_2$O$_2$ was markedly longer than c-fos mRNA and comparable to the PHA/PMA-stimulated expression. A brief exposure of Jurkat cells to H$_2$O$_2$ was sufficient to stimulate a significant c-jun mRNA response. The selective inhibitors of PTKs and PKC blocked c-jun mRNA expression induced by H$_2$O$_2$. Exposure of Jurkat cells to H$_2$O$_2$ activated JNKs and ERK2, which are responsible for c-jun and c-fos induction, respectively. H$_2$O$_2$ also triggered the increased [Ca$^{2+}$], with a 80% contribution from extracellular influx. PTK inhibitors and Ca$^{2+}$ channel blockers suppressed the H$_2$O$_2$-induced Ca$^{2+}$ mobilization although the inhibitory patterns differed from those observed in T cell mitogen- or TCR/CD3-mediated Ca$^{2+}$ mobilization. The deletion of intracellular Ca$^{2+}$ by Ca$^{2+}$ chelator BAPTA markedly inhibited c-jun mRNA induction by H$_2$O$_2$. The H$_2$O$_2$ induction of c-jun represented the transactivation of c-jun
promoter but not stabilization of c-jun transcripts. The analyses of the transfected cells with distinct c-jun promoter constructs showed that H$_2$O$_2$ resembled PHA/PMA stimulation and initiated transactivation of full length c-jun promoter preferentially through a proximal AP-1 TRE-like element jun1. H$_2$O$_2$ facilitated the in vitro DNA-binding of AP-1 TRE and Jun1 complexes, but it could only stimulate jun1 transactivation in vivo, which was different from the induction of AP-1 TRE transactivation by mitogen or TCR/CD3 stimulation. Furthermore, the current results show that sublethal levels of H$_2$O$_2$ inhibited IL-2 production at transcriptional level. H$_2$O$_2$ suppressed the IL-2 mRNA expression and full length IL-2 promoter transactivation induced by PHA/PMA in a dose-dependent manner. H$_2$O$_2$ inhibition of IL-2 transcriptional activation was due to the transactivation suppression of Jun- and Fos-dependent transcriptional complex NFAT. Gel shift analysis revealed that H$_2$O$_2$ reduced the in vitro DNA binding activity of NFAT. The downregulation effect of H$_2$O$_2$ on NFAT binding could not be seen in the cells pretreated with PHA/PMA, suggesting that H$_2$O$_2$ inhibited the early generation of NFAT complexes rather than the binding of preformed NFAT complexes. These results suggest that oxidative signals can positively and negatively regulate the transcriptional events of T cells through influencing signal transduction components and distinct transcriptional factors.
Dedicated to my parents, Chengxun and Ying,

my lovely wife and son, Ming and Xin
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I offer special thanks to the group of my friends who gave me enjoyable moments during these years.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>Chapters</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Effects of Oxidative Stress on T lymphocytes</td>
<td>2</td>
</tr>
<tr>
<td>Intracellular Signal Transduction and Oxidative Stress</td>
<td>5</td>
</tr>
<tr>
<td>Calcium Signal and Oxidative Stress</td>
<td>9</td>
</tr>
<tr>
<td>Regulation of c-Jun and c-Fos and Oxidative Stress</td>
<td>10</td>
</tr>
<tr>
<td>Regulation of IL-2 Gene and NFA</td>
<td>17</td>
</tr>
<tr>
<td>Aims of Current Study</td>
<td>19</td>
</tr>
<tr>
<td>2. Methods</td>
<td>22</td>
</tr>
<tr>
<td>Reagents and Antibodies</td>
<td>22</td>
</tr>
<tr>
<td>Measurement of Cytosolic Free Calcium</td>
<td>23</td>
</tr>
<tr>
<td>RNase Protection Assay</td>
<td>25</td>
</tr>
<tr>
<td>EMSA</td>
<td>29</td>
</tr>
<tr>
<td>Transfection Plasmids, Transfection, and Analyses of Reporter Constructs</td>
<td>32</td>
</tr>
<tr>
<td>Immunoprecipitation and Immune Complex Kinase Assay</td>
<td>37</td>
</tr>
<tr>
<td>Measurement of IL-2 Production</td>
<td>38</td>
</tr>
</tbody>
</table>

viii
3. Results

Induction of c-jun and c-fos Transcription Expression by Sublethal Levels of Oxidative Stress and Involvement of Protein Kinase Activation in Jurkat T Cells

Oxidative Stress Induces Increase of [Ca^{2+}]_i level in Jurkat T Cells through the Mechanisms Involving Calcium Channels and PTKs

Sublethal Levels of Oxidative Stress Stimulate Transcriptional Activation of c-jun Gene in Jurkat T Cells

Sublethal Levels of Oxidative Stress Suppress IL-2 Promoter activation in Jurkat T Cells

4. Discussion

Requirements of the PTK and PKC Activities as well as Ca^{2+} Signals for Transcriptional Expression of c-jun in Jurkat T Cells Exposed to Sublethal Concentrations of H_2O_2

Ca^{2+} Channels and PTK Activity are Required for H_2O_2-Induced [Ca^{2+}]_i Increases in Jurkat T Cells

Sublethal Levels of Oxidative Stress Stimulate Transcriptional activation of c-jun Promoter Preferentially through Proximal AP-1-like Element

Sublethal Levels of Oxidative Stress Suppress Activation of NFAT-Dependent IL-2 Promoter in Jurkat T Cells

Summary

References

Appendix: Figures and Tables
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Influence of different kinase inhibitors on $[\text{Ca}^{2+}]_i$ responses of Jurkat T cells to H$_2$O$_2$</td>
<td>131</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sublethal concentrations of H$_2$O$_2$ stimulate NFkB but not NFAT activation in Jurkat cells</td>
<td>159</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Effect of ( \text{H}_2\text{O}_2 ) on cell viability</td>
</tr>
<tr>
<td>2</td>
<td>Induction of c-jun and c-fos mRNA by ( \text{H}_2\text{O}_2 )</td>
</tr>
<tr>
<td>3</td>
<td>Time-dependent expression of c-jun and c-fos mRNA by ( \text{H}_2\text{O}_2 ) or mitogen</td>
</tr>
<tr>
<td>4</td>
<td>Kinetics of c-jun mRNA induction by ( \text{H}_2\text{O}_2 )</td>
</tr>
<tr>
<td>5</td>
<td>Influence of protein kinase inhibitors on ( \text{H}_2\text{O}_2 ) induced c-jun expression</td>
</tr>
<tr>
<td>6</td>
<td>( \text{H}_2\text{O}_2 ) activates JNK and ERK kinase activities</td>
</tr>
<tr>
<td>7</td>
<td>Intracellular ( \text{Ca}^{2+} ) are required for ( \text{H}_2\text{O}_2 )-induced c-jun expression</td>
</tr>
<tr>
<td>8</td>
<td>Modulation of ([\text{Ca}^{2+}]_i ) by varying concentrations of ( \text{H}_2\text{O}_2 ) or mitogen</td>
</tr>
<tr>
<td>9</td>
<td>( \text{H}_2\text{O}_2 ) elevations of ([\text{Ca}^{2+}]_i ) require the influx of external calcium</td>
</tr>
<tr>
<td>10</td>
<td>Effects of ( \text{Ca}^{2+} ) channel blockers on ([\text{Ca}^{2+}]_i ) elevations by ( \text{H}_2\text{O}_2 ) or mitogen</td>
</tr>
<tr>
<td>11</td>
<td>Effect of protein kinase inhibitor genistein on ([\text{Ca}^{2+}]_i ) responses to ( \text{H}_2\text{O}_2 ), mitogen, or anti-\text{CD3}</td>
</tr>
<tr>
<td>12</td>
<td>AIF(^{4-}) bypasses the inhibition by PTK inhibitor of ([\text{Ca}^{2+}]_i ) elevations in Jurkat cells exposed to ( \text{H}_2\text{O}_2 ) or anti-\text{CD3}</td>
</tr>
<tr>
<td>13</td>
<td>Acute exposure of Jurkat cells to ( \text{H}_2\text{O}_2 ) does not block ([\text{Ca}^{2+}]_i ) responses to anti-\text{CD3}</td>
</tr>
<tr>
<td>14</td>
<td>Stability of c-jun mRNA in Jurkat cells exposed to ( \text{H}_2\text{O}_2 )</td>
</tr>
<tr>
<td>15</td>
<td>Schematic representation of the c-jun promoter CAT constructs</td>
</tr>
</tbody>
</table>
16 Transcription activation of c-jun promoter by H$_2$O$_2$ ........................................ 141

17 Effects of H$_2$O$_2$ or other reagents on DNA binding activities of AP-1 TRE, jun1, and jun2 ................................................................. 143

18 Identification of composition of transcription complexes recognizing AP-1 TRE, jun1, and jun2 DNA motifs .................................................... 145

19 Competition analysis of DNA binding complexes recognizing AP-1 TRE, jun1, and jun2 ........................................................................... 147

20 Differential induction of jun1 and AP-1-dependent TRE by distinct stimuli ........................................................................................................... 149

21 H$_2$O$_2$ inhibition of IL-2 production in Jurkat cells exposed to PHA/PMA .......... 151

22 H$_2$O$_2$ inhibition of IL-2 mRNA expression induced by PHA/PMA ............... 153

23 Inhibition by H$_2$O$_2$ of IL-2 promoter activation by induced by PHA/PMA .... 155

24 Inhibition of NFAT transactivation by H$_2$O$_2$ ................................................... 157

25 Correlative inhibition of IL-2 production and transcription as well as IL-2 promoter and NFAT transactivation by H$_2$O$_2$ ............................... 161

26 H$_2$O$_2$ inhibition of NFAT DNA binding activity stimulated by PHA/PMA .... 163
CHAPTER 1

INTRODUCTION

Oxidative stress is a disturbance in the balance of prooxidants and antioxidants in favor of the former (reviewed by Sies, 1991). Living cells generate reactive oxygen species (ROS) and expose themselves to oxidative stress during normal metabolic processes and in response to externally biological, chemical, and physical stimuli (reviewed by Sies, 1991; Miller and Britigant, 1995; Barnett et al, 1995). Superoxide anion (\( \cdot O_2^- \)), hydroxyl radical (\( \cdot OH \)), hydrogen peroxide (\( H_2O_2 \)), and nitric oxide (NO) are thought to be major forms of ROS, of which \( H_2O_2 \) readily diffuses across cell membrane and forms more potent oxygen radicals such as \( \cdot OH \). ROS are known to influence a variety of biologic processes and have been implicated in the pathophysiology of a number of diseases and aging (reviewed by Halliwell and Gutteridge, 1990; Ames et al, 1993). ROS can directly lead to damage of biological macromolecules, including nuclear acid, proteins, and lipid components of biologic membrane. The injury of these macromolecules results in changes in cellular structures and functions, and finally irreversible changes and cell death. In addition to direct damage, recent studies addressing the molecular and genetic effects of
ROS on living cells have revealed that sublethal levels of oxidative stress actively initiate and control the cell growth and death processes through the complicated regulation of intracellular signal transduction cascades and transcriptional factors that function to control gene expression (Shibanuma et al, 1990; Schreck and Baueuerle, 1991; Burdon and Gill, 1993; Nakamura et al, 1993; Meyer et al, 1993; Schieven et al, 1993b; Winrow et al, 1993; Pahl and Baueuerle, 1994; Wiese et al, 1995; Rao, 1996). Thus, redox state of living cells as well as changes in cellular redox potential play important roles in determining the biological behaviors of the cells.

Effects of Oxidative Stress on T Lymphocytes

Human lymphocytes are readily exposed to oxidative stress generated by "respiratory burst" of phagocytosis during inflammatory processes, and are quite susceptible to oxidative stress (reviewed by El-Hag et al, 1986; Miller and Britigant, 1995). Exposure of lymphocytes to oxidative stress can lead to the inhibition or the activation of the lymphocyte function. Exposure of T cells to micromolar levels of oxidants, such as H$_2$O$_2$, induces a state of cellular unresponsiveness to mitogenic stimuli manifested by a failure of the cells to exit G0 phase and proliferate (Duncan and Lawrence, 1990). When exposed to oxidative stress, T cells loss many important responsive functions upon stimulation by mitogen and antibody against T cell receptor complex (TCR/CD3), including production of IL-2 and expression of IL-2 receptor (Roth and Droge, 1987; Staite et al, 1987;
In addition, the deletion of or reduction in the intracellular glutathione (GSH) levels has been shown to be capable of inhibiting the proliferative response of T cells to mitogen or TCR/CD3 signaling (Messina and Lawrence, 1989; Suthanthiran et al, 1990; Kavanagh et al, 1990). Recently, Flescher et al. reported that the chronic exposure of human T lymphocytes to weak oxidative stress inhibits IL-2 production induced by TCR/CD3 signaling (Flescher et al, 1994). This inhibition of IL-2 production was related to the suppression of transmembrane and nuclear signal transduction components, including cytosol-free calcium and transcriptional factors NFAT and NFkB, but not AP-1. Furthermore, oxidative stress is capable of inducing programmed cell death (apoptosis) in lymphocytes (Reviewed by Thomas et al, 1994). Lowering the levels of intracellular GSH in Fas/CD95-resistant T cell variants reversed the cell resistance to Fas/CD95-mediated apoptosis while the reducing agents such as N-acetylcysteinine (NAC) block apoptosis induction of Fas/CD95-sensitive T cells (Chiba et al, 1996). Thiol antioxidants GSH and NAC also block antigen- or mitogen-driven activation-induced death of T-cell hybredomas (Sandstrom, 1994). In fact, the stimulation of lymphocytes by cross-linking TCR/CD3 complex, mitogen, and tumor-promoting agents leads to the excessive generation of intracellular ROS (reviewed by Buttke and Sandstrom, 1995). Activation-induced apoptosis of lymphocytes may represent the inability of cells to maintain an appropriate prooxidant-antioxidant balance. Recently, HIV-infected T cells have been demonstrated to undergo apoptotic-like death, which is closely related to the excessive generation of oxidative products triggered by HIV infection in the cells (Greenspan and Aruoma, 1994). It is believed that the excessive
prooxidants stimulate HIV replication through activating transcriptional factor NFkB and also initiate the signal transduction pathways for apoptosis.

On the other hand, oxidants have been reported to function as costimulatory signals for lymphocytes. The proliferative response of T cells in mixed lymphocyte cultures is augmented by lower concentrations of H$_2$O$_2$ and suppressed by catalase (Roth and Droge, 1987). Lower levels of oxidative stress stimulate mitogen-induced proliferation and activation in T cells (Gualde and Goodwin, 1984; Fidelus, 1988). Furthermore, subpopulations of T cells exhibit different sensitivity to oxidative stress. Exposing T cells to lower concentrations of H$_2$O$_2$ selectively depletes suppressor T cell subsets and maintains oxidant-resistant helper/inducer T cell subset (Zoschke and Staite, 1987; Staite et al, 1987). The altered CD4/CD8 ratio may lead to the imbalance of immune regulatory network. Taken together, these previous observations suggest that oxidative stress exert divergent effects on lymphocyte functions. However, the intracellular mechanisms by which oxidative stress influences the biologic behaviors of lymphocytes are not understood. Recently, oxidative stress has been shown to regulate the components of intracellular signal transduction cascades, transcriptional proteins, and gene expression in a variety of cell types including lymphocytes. At present, it is unclear how the intracellular events triggered by oxidative stress might be related to the biological effects of oxidative stress on T lymphocyte function.
The stimulation of T cells by the cross-linking of TCR/CD3 complex or mitogens leads to T cell activation and cytokine production. An immediate consequence of stimulation of TCR/CD3 complex is the activation of non-receptor-linked PTKs, including p56^{ck}, p59^{fyn}, and ZAP-70, followed by the initiation of signal transduction cascades (reviewed by Rao, 1991; Perlmutter et al, 1993; Rudd et al, 1994). The activated PTKs phosphorylate and activate phospholipases (PLCs) and small GTP-binding proteins Ras/Rac. The activation of PLC\gamma has been known to lead to the breakdown of membrane phosphotidylinosotides and subsequent PKC activation and calcium mobilization (Reviewed by Liscovitch, 1992; Majerus, 1992). The active GTP-binding form initiates the sequential phosphorylation reactions consisting of a series of protein kinases and resulting in the activation of the mitogen-activated protein kinases (MAPKs) ERKs (extracellular regulatory kinases) and JNKs (c-Jun N terminal Kinases) (reviewed by Karin, 1995). There are two pathways of the sequential kinase phosphorylation activation of MAPKs, which are identified to be activated by the active form of small GTP-binding proteins: (i) Ras-Raf1-Mek-ERKs (Minden et al, 1994); (ii) Ras/Rac-MEKK1-JNKK-JNKs (Minden et al, 1995; Reviewed by Karin, 1995). In addition to the activation by PTKs, the activity of small GTP-binding proteins may be partially regulated by PKC (Downward et al, 1990). PKC can also directly phosphorylate and activate Raf-1 (Kolch et al, 1993). ERKs and JNKs are known to be the final active kinase forms of the MAPK chain reactions. They can directly modify the biological activity of transcriptional factors and regulate the transactivation of their
target genes. For example, ERKs phosphorylate and potentiate the activity of transcription factor TCF/Elk-1 and thereby induce the expression of c-fos gene (Gille et al, 1992). JNKs can phosphorylate and potentiate the amino terminal c-Jun activation domain and thereby activate the activity of transcription factors which contain c-Jun, such as activated protein-1 (AP-1) (Hibi et al, 1993; Derijard et al, 1994). In addition to the involvement of signaling kinases in T cell activation, protein phosphatases also regulate the signal transduction. CD45-linked tyrosine phosphatase activity regulates the activation of PTKs and PLCs triggered by stimulation of TCR/CD3 complex (Kiener and Mittler, 1989; Marvel et al, 1991). A mitogen-induced tyrosine/threonine protein phosphatase has been reported to dephosphorylate and inactivate the activity of MAPKs (Zheng and Guan, 1993).

It has been known that the components of signal transduction pathways for T cell activation also participate in the apoptotic induction of T cells. Different intracellular events seem to be involved in Fas/CD95-dependent and Fas/CD95-independent induction of apoptosis in T cells. TCR/CD3 triggering through immobilized anti-CD3 antibodies induces expression of Fas/CD95 ligand and thereby results in autocrine T-cell suicide mediated by Fas/CD95 (Dhein et al, 1995). Fas/CD95-mediated apoptosis in T cells requires phosphatidylinositol turnover and increased \([\text{Ca}^{2+}]_i\) but is independent from the activity of PTKs or PKC (Izquierdo, et al, 1996; Mollereau et al, 1996; Latinis and Koretzky, 1996). Apoptosis induced by stimulation of CD2, CD4, and CD45 in T cells is mediated through Fas/CD95-independent pathway. Both CD2- and CD4-mediated
apoptosis are associated with PTK p56^{lck} activity (Di Somma et al, 1995; Mollereau et al, 1996) while CD45-induced apoptosis requires the activity of both PTKs and protein tyrosine phosphatases (PTPs) (Klaus et al, 1996). In addition, an enhanced p59^{b swallow tyrosine kinase activity in T cells undergoing apoptosis induced by in vivo staphylococcal enterotoxin B (SEB) administration was reported (Migita et al, 1995). The pretreatment of the cells with PTK inhibitors blocks SEB-induced apoptosis. Furthermore, Ras and JNKs have been implicated in apoptosis induction in T cells. The constitutive expression of v-Hras in Jurkat T cells renders the cells susceptible to apoptosis when the cellular PKC activity is inhibited (Chen and Faller, 1996). This Ras-induced and PKC-dependent cell death can be blocked by the expression of Bcl-2 protein which functions as an anti-oxidant and potent intracellular inhibitor of apoptosis (Hockenberg, et al, 1993; Steinman, 1995). The blocking of Ras-induced apoptosis by Bcl-2 is related to the formation of Bcl-2/Ras association and the subsequent phosphorylation of Bcl-2 (Chen and Faller, 1996). The persistent expression of JNK in Jurkat T cells induces apoptosis. In contrast, expression of dominant-negative mutants of the JNK kinase cascade blocks radiation- and UV-induced cell death (Chen et al, 1996). Overexpression of Bcl-2 blocks JNK activation and promotes cell survival (Park et al, 1996). Fas/CD95 ligation strongly activates JNK but not ERKs, which suggests that a signaling ratio between TCR/CD3 activation of ERKs versus JNK activation by Fas/CD95 regulates the balance between proliferation and apoptosis in T cells (Wilson et al, 1996). In addition, protein phosphatases appear to be involved in the induction of apoptosis. For example, the inhibitors of protein phosphatase 1 and 2A, okadaic acid and calyculin A, suppress IL-2-driven T cell proliferation and
induce apoptosis after IL-2 deprivation (Weller et al, 1995). Thus, the activation and apoptotic induction of T cells actually share common components of signal transduction pathways. A carefully tuned homeostatic extracellular and intracellular system modulates the proliferation-associated and death-induced events, and thereby determine T-cell fate (reviewed by Thompson, 1996).

Oxidative stress has been shown to be capable of influencing the activity of components of intracellular signal transduction cascades. Treatment of T cells or B cells with H$_2$O$_2$ or UV stimulates the activity of PTKs known to be associated with the activation of antigen receptors (Schieven et al, 1993a, 1993b, and 1994; Secrist et al; 1993; Nakamura et al, 1993). H$_2$O$_2$ and UV treatment of Jurkat T cells activate tyrosine kinase ZAP-70 and Ca$^{2+}$ signals similar to those observed following biological stimulation (Schieven et al, 1994). H$_2$O$_2$ treatment of B cells stimulates tyrosine kinase p72$^{56}$ and Ca$^{2+}$ signals which can be blocked by a inhibitor of PTKs, herbimycin A (Schieven et al, 1993a). H$_2$O$_2$ alone can not influence Src family kinases p56$^{kk}$ and p59$^{6y}$, but H$_2$O$_2$ synergizes with vanadate, an inhibitor of protein phosphatases, to induce tyrosine phosphorylation activity of both kinases in association with the activation of PLC$\gamma$ (Schieven et al, 1993b). Sublethal concentrations of H$_2$O$_2$ can also stimulate PKC activity and reduce the activity of tyrosine phosphatase and protein phosphatase 1 in Jurkat T cells (Whisler et al, 1995). Recently, Lander et al. demonstrated that several oxidative agents, including H$_2$O$_2$, nitric oxide, hemin, and Hg$^{2+}$, activated both p21$^{ras}$ and MAPK in Jurkat T cells (Lander et al, 1995). Blocking GSH synthesis made the cells more sensitive to these
agents. These findings suggest that the direct activation of Ras may be a central mechanism by which a variety of oxidative stress stimuli transmit their signals to the nucleus. Additionally, \( \text{H}_2\text{O}_2 \) can stimulate potent activation of ERK2 and moderate activation of JNK (Guyton et al, 1996). \( \text{H}_2\text{O}_2 \)-induced ERK2 activation can be blocked by NAC. This implies that ERK plays a critical role in cell survival following oxidant injury.

**Ca\(^{2+}\) Signals and Oxidative Stress**

Ionized calcium (Ca\(^{2+}\)) plays a crucial role in the activation of T lymphocytes (Gardner, 1989). Stimulation of T cells by mitogenic lectins or cross-linking of TCR/CD3 triggers the activation of protein tyrosine kinases and subsequent PLCs (reviewed by Ullman et al, 1990; Harnett and Rigley, 1992). PLC\(\gamma\) leads to the breakdown of the membrane-associated phosphatidylinositol phosphate to generate inositol-1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG), the two critical components that are tightly linked to the intracellular Ca\(^{2+}\) mobilization and the activation of PKC (Reviewed by Majerus 1992; Liscovitch, 1992). IP\(_3\) initiates Ca\(^{2+}\) internal release from the store in endoplasmic reticulum through IP\(_3\) receptors on the membrane of the organelle. Stimulation of T cells also triggers Ca\(^{2+}\) influx from the external medium through voltage-dependent (Dupuis et al, 1989; Densmore et al, 1992) or voltage-independent (Lewis et al, 1989) Ca\(^{2+}\) channels on the plasma membrane. The channel-mediated Ca\(^{2+}\) influx is directly associated with the IP\(_3\)-induced Ca\(^{2+}\) internal release and the subsequent depletion of
intracellular Ca\textsuperscript{2+} store (Dolmetsch and Lewis, 1994; Zweifach and Lewis, 1993). Both Ca\textsuperscript{2+} internal release and external influx contribute to the elevation of cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and provide the essential signals for the later intracellular events of T cell activation.

Transient increases of early [Ca\textsuperscript{2+}]\textsubscript{i} appear to be necessary for the initiation of intracellular signal transduction cascades during the activation of T cells and the induction of programmed cell death in T cells. The [Ca\textsuperscript{2+}]\textsubscript{i} elevation along with DAG may stimulate the activation of PKC (Gardner, 1989). The increase in [Ca\textsuperscript{2+}]\textsubscript{i} can also initiate the activity of Ca\textsuperscript{2+}-dependent enzymes, including Ca\textsuperscript{2+}/calmodulin kinases and calcineurin (reviewed by Perlmutter et al, 1993). These protein kinases and phosphatases play a key role in regulating the activity of other components of signal transduction pathways and the transcriptional factors (reviewed by Ullman et al, 1990). The early [Ca\textsuperscript{2+}]\textsubscript{i} increases correlate with the transcriptional activation of the so-called immediate genes including c-jun, c-fos, and c-myc, as well as the subsequent activation of genes such as c-myb, IL-2, and IL-2 receptor (reviewed by Crabtree, 1989). In addition to activating T cells, cytosolic Ca\textsuperscript{2+} can function as an early signal to control the activation-induced apoptosis in T cells (Reviewed by Golstein et al, 1991). The calcium ionophore-mediated entry of extracellular Ca\textsuperscript{2+} into the cytosol induced the apoptotic death of thymocytes (Wyllie et al, 1984; McConkey et al, 1989a) and human T cell line (Takahashi et al, 1989). Both Ca\textsuperscript{2+} increase and DNA fragmentation are prevented by the depletion of medium calcium or the treatment of cells with the Ca\textsuperscript{2+} chelator (McConkey et al, 1989b; Cantoni et al, 1989).
Furthermore, the activation of endonucleases responsible for DNA fragmentation is a calcium dependent process (Cohen and Coke, 1984).

Oxidant-induced changes of $[\text{Ca}^{2+}]_i$ have been observed in several types of cells, including endothelial cells, smooth muscle cells, and T lymphocytes. Exposure of endothelial cells and smooth muscle cells to oxidative stress such as $\text{H}_2\text{O}_2$ elevated $[\text{Ca}^{2+}]_i$ levels (Kimura et al, 1992; Roveri et al, 1992; Doan et al, 1994). Both Ca$^{2+}$ mobilization from internal stores and external influx contribute to the increase in $[\text{Ca}^{2+}]_i$. $\text{H}_2\text{O}_2$ has been shown to be capable of inducing the significant increase in $[\text{Ca}^{2+}]_i$ in Jurkat T cells, which is associated with substantial activation of PTK ZAP-70 (Schieven et al, 1993a and 1994). However, the $\text{H}_2\text{O}_2$-induced signal transduction seems to differ from those pathways induced by ultraviolet (UV) and T cell biological stimulation. Long term exposure of human T cells to oxidant polyamines, which are oxidized by polyamine oxidase to produce aldehyde, ammonia, and $\text{H}_2\text{O}_2$, stimulates persistent increase in $[\text{Ca}^{2+}]_i$, but pretreatment of T cells with the oxidation of polyamines prevents the subsequent $[\text{Ca}^{2+}]_i$ elevation and protein phosphorylation triggered by mitogen or TCR/CD3 signaling (Flescher et al, 1994). At present, biological role of the oxidant-induced regulation of $[\text{Ca}^{2+}]_i$ in T cells remain unclear.
The proto-oncogene products c-Jun and c-Fos are two major members of transcriptional protein family Jun and Fos. They have been identified as critical components in several transcriptional complexes which exert pleiotropic regulatory effects on expression of a wide variety of cellular genes (reviewed by Angel and Karin, 1991; Kovary and Bravo, 1991). Activator protein-1 (AP-1) is one of the well-studied transcriptional protein complexes which contain c-Jun and c-Fos components. The major forms of AP-1 are heterodimers and homodimers, consisting of c-Jun/c-Fos and c-Jun/c-Jun respectively (Angel et al., 1988). AP-1 recognizes the TPA (12-O-tetradecanoylphorbol 13-acetate) response element (TRE: 5'-TGAG/CTCA-3') within the promoter region of many genes and transactivates their expression. These AP-1-dependent genes include human metallothionein, collagenase, tissue plasminogen activator, glutathione S-transferase, transforming growth factor (TGF), as well as other genes (reviewed by Angel and Karin, 1991). In addition to forming AP-1 complex, c-Jun and c-Fos can interact with other transcriptional proteins to form various transcriptional complexes which include NFATp/c-Jun/c-Fos, CREB/c-Jun, c-Jun/ATF-2, and NFkBp65/c-Jun (Hai and Curran, 1991; van Dam et al., 1993; Stein et al., 1993; Northrop et al., 1994). Thus, c-Jun and c-Fos participate in several regulatory events of cell biological behaviors including cell proliferation, differentiation, and transformation. Furthermore, both c-Jun and c-Fos have been implicated in induction of programmed cell death. The increased expression of c-jun and c-fos genes correlates with the induction of

The expression of c-Jun and c-Fos can be induced by many biological factors, including growth factors, cytokines, mitogens, DNA-damaging agents, tumor promoters, and oxidative stress (reviewed by Angel and Karin, 1991). The stimulation of cells with these factors leads to the activation of intracellular regulatory elements which control the activity of c-Jun and c-Fos at transcriptional and posttranslational levels. Both c-Jun and c-Fos are nuclear phosphorylated proteins regulated by signal-dependent phosphorylation and dephosphorylation events (reviewed by Hill and Treisman, 1995; Hunter and Karin, 1992). The activity of c-Jun protein is regulated by phosphorylation and dephosphorylation at several sites. Phosphorylation of c-Jun by glycogen synthase kinase 3 (GSK-3) and casein kinase II (CKII) at three sites, Thr-231, Ser-234, and Ser-249, located immediately to the N-terminal side of DNA-binding domain, is inhibitory for c-Jun DNA-binding activity. This inhibition is reversed by phosphatase treatment and PKC-related dephosphorylation (Boyle et al, 1991; Lin et al, 1992; Papavassiliou et al, 1992). The well-identified JNKs phosphorylate Ser-63 and Ser-73 within N-terminal transactivation domain of c-Jun and potentiate c-Jun transactivation function (Hibi et al, 1993; Derijard et al, 1994; Su et al, 1994), while the dephosphorylation by phosphatase 2A may suppress the transactivation activity of c-Jun (Black et al, 1991). Relatively less is known about the modulation of c-Fos protein activity. Cyclic AMP (cAMP)-dependent protein kinase K (PKA) can phosphorylates c-Fos protein and controls its transrepression.

In addition to the posttranslational regulation, c-Jun and c-Fos are effectively controlled at transcriptional level. c-jun gene transcription is positively autoregulated by c-Jun protein itself (Angel et al, 1988b). Several transcriptional elements have been identified within c-jun promoter region (Hattori et al, 1988; Unlap et al, 1992), of which two structurally distinct AP-1 TRE-like motifs, jun1 and jun2, play a central role in regulating c-jun transactivation (Angel et al, 1988b; Stein et al, 1992; Muegge et al, 1993). Jun1 is located at position 71 to 64 from upstream of the transcriptional initiation site with a sequence 5'-TGACAATCA-3' and jun2 at 190 to 183 with a motif 5'-TTACCTCA-3'. The transactivation activity of jun1 and jun2 is independently induced by TPA and UV, although UV is a more inducer than TPA which itself is a potent inducer of consensus AP-1 TRE transactivation. The transcriptional factors responsible for c-jun induction are c-Jun/c-Jun homodimers and c-Jun/ATF2 heterodimers (Angel et al, 1988b; van Dam et al, 1993). A wide range of stimuli have been implicated in activation of c-jun promoter. The stimulation of cells is believed to enhance the activity of JNKs. The activated JNKs in turn phosphorylate c-Jun and ATF2 and potentiate their transactivation activity, leading to the subsequent activation of c-jun promoter (Hibi et al; 1993; Gupta et al, 1995).

The serum response element (SRE) within c-fos promoter region play a major role in regulating c-fos mRNA transcription. The SRE and its transcriptional factors are targets
for PKC-dependent and PKC-independent signals (Gilman, 1988). Two transcriptional factors have been identified to be associated with SRE-mediated c-fos transcriptional activation: (1) serum response factor (SRF) and (2) the ternary complex factor (TCF) of which transcriptional factor Elk-1 is a major component (Prywes et al, 1988; Hipskind et al, 1991). Both transcriptional factors can bind SRE, but TCF/Elk-1 seems only to be capable of interacting with SRF-bound SRE. SRF interacts with SRE to form binary complex which is less transactive for c-fos transcription. The subsequent interaction between TCF/Elk-1 and SRF leads to the binding of TCF/Elk-1 to SRF-bound SRE and the formation of ternary complex TCF/Elk-1-SRF-SRE. The ternary complex activates the transcription of c-fos promoter (Shaw et al, 1989; Hill et al, 1993; Schroter et al, 1990). The activity of both SRF and TCF/Elk-1 is regulated by phosphorylation. SRF constitutively binds SRE, but the serum stimulation induces the rapid and transient phosphorylation of SRF at position Ser-103 in N-terminal DNA-binding domain and increases the affinity of SRF for c-fos SRE in vitro (Janknecht et al, 1992; Rivera et al, 1993). The phosphorylation of TCF/Elk-1 seems to play a central role in regulating transactivation of SRE-mediated c-fos promoter. The phosphorylation of TCF/Elk-1 stimulates its transactivation potential in vivo and promotes its association with SRF (Gille et al, 1992; Marais et al, 1993). It has been known that MAP kinase ERK-2 mediates the phosphorylation of TCF/Elk-1 (Gille et al, 1992; Hipskind et al, 1994). Thus, many of extracellular signals inducing c-fos activate PTKs and/or PKC followed by the activation of ras-dependent kinase cascades, transcriptional factors TCF/Elk-1, and finally c-fos transcription (Hipskind, et al, 1994). In addition, a pathway independent of ras- and Elk-1
has been implicated in SRF-mediated transcription, in which increased [Ca^{2+}], stimulates SRF-dependent transcription by a Ca^{2+}/calmodulin-dependent kinase without the involvement of Elk-1 (Miranti et al, 1995).

Oxidative stress has been implicated in regulating DNA-binding activity of AP-1 and inducing expression of c-jun and c-fos genes. Treatment of Hela cells with micromolar concentrations of H_{2}O_{2} stimulates significant increases in DNA-binding activity of AP-1 in a dose-dependent manner (Devary et al, 1991). However, only weak transactivation of the AP-1-driven reporter construct by H_{2}O_{2} was observed compared with phorbol diester, although H_{2}O_{2} was a strong inducer of NFkB DNA-binding in Hela cells (Meyer et al, 1993). H_{2}O_{2} also displayed an inhibitory effect on DNA-binding and transactivation of AP-1 induced by phorbol diester. In contrast, the antioxidant pyrrolidine dithiocarbamate induced a strong activity of AP-1 DNA-binding and transactivation but a weak response of NFkB activity in Hela cells. In contrast to the relatively weak induction of AP-1, oxidative stress stimulates the strong expression of c-jun and c-fos genes in a variety of cell types (Crawford et al, 1988; Shibanuma et al, 1988; Devary et al, 1991; Amstad et al, 1992). The biological significance of this dichotomy between the induction of the AP-1 transactivation and the c-jun and c-fos gene expression by oxidative stress remains unclear. In addition, little is known about oxidative stress regulation of c-jun and c-fos gene expression and AP-1 activity in human T cells.
Regulation of IL-2 Gene and NFAT

T cell activation via the IL-2 autocrine system involves several distinct steps: first, induction of immediate early genes, including c-jun and c-fos, and IL-2 receptor alpha chain; second, induction of IL-2 gene and protein expression; and third, binding of IL-2 to its receptor leading to a mitogenic response (reviewed by Mueller et al, 1989; Crabtree, 1989). Thus, IL-2 induction is a key event in T cell activation and proliferation. T cell activation and IL-2 induction require at least two signals, generated by phorbol ester (TPA or PMA) and Ca^{2+} ionophore or TCR/CD3 stimulation and signals delivered by antigen-presenting cells or CD28 stimulation. As a result, several transcriptional factors are activated, followed by the transactivation of early activation genes and IL-2 promoters as well as their expression (reviewed by Crabtree, 1989; Rao, 1991). These transcriptional factors include AP-1, NFAT, NFkB, and NF-IL-2 (Jain et al, 1992a and 1992b; Crabtree, 1989). One of the most important actions of AP-1 components c-Jun and c-Fos in IL-2 induction is to participate in regulation of IL-2 promoter transactivation in combination with pre-existing NFAT (NFATp) (reviewed by Rao, 1994). It has been known that the activation of both JNKs and NFAT requires PKC activity and Ca^{2+} (Su et al, 1994; Hivroz-Burgaud et al, 1991), consistent with dual signal model of T cell activation and IL-2 induction.

NFAT has been shown to be an essential transcriptional factor for upregulation of the IL-2 gene promoter. It has been also implicated in control of other several cytokine gene
promoters, including IL-3, IL-4, TNF-alpha (tumor necrosis factor), and GM-CSF (granulocyte-macrophage colony-stimulating factor) (reviewed by Rao, 1994). NFAT is a multicomponent complex consisting of NFATp and members of each Jun and Fos families, such as c-Jun/c-Jun, and c-Fos/Jun (Jain et al., 1993a; Petrak et al., 1994; Yaseen et al., 1994). NFATp is present in the cytosol in its phosphorylated form prior to T cell activation although it has the ability to bind specific DNA motif (5'-GGAAAA-3'). Following T cell activation and increased intracellular Ca^{2+}, the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin is activated. The dephosphorylation of NFATp by calcineurin is associated with its subcellular translocalization from the cytosol to the nucleus (McCaffrey et al., 1993; Northrop et al., 1993; Jain et al., 1993b). NFATp contacts its DNA motif in IL-2 promoter region, but this binding is not believed to be stable nor sufficient for IL-2 induction. For stable binding of NFATp to its DNA motif and subsequent activation of IL-2 transcription, the NFATp-DNA complex must interact with Jun and Fos components in the AP-1-DNA complex which is localized several base pairs away from the NFATp-DNA complex (reviewed by Rao, 1994; Jain et al., 1993a). Thus, the formation of NFAT complex by cooperative interactions between NFATp and Jun/Fos family members is required for the effective induction of IL-2 gene expression.

The maximal expression of NFAT activity in IL-2 induction requires at least two signals: increases in [Ca^{2+}], and PKC activation (Hivroz-Burgaud et al., 1991). The requirement for Ca^{2+} signals reflects the dependence of NFATp translocalization from cytosol to nucleus on Ca^{2+}-dependent activation of calcineurin, while the PKC signal may
activate other transcriptional factors required for the induction of intact NFAT transactivation activity (Rao, 1994). Ras function has been implicated in regulating NFAT activity and controlling IL-2 gene promoter expression in T cell activation (Woodrow et al, 1993; Rayter et al, 1992; reviewed by Downward et al, 1992). The constitutively active Ras mutant can partially replace the requirement for PKC in synergizing with calcium ionophore to induce NFAT. Direct activation of PKC with phorbol ester causes a dramatic increase in GTP bound ras in T cells (Izquierdo et al, 1992). Therefore, PKC activation in T cells appears to facilitate Ras activity and initiate downstream MAPK cascades. The activated MAPKs, including ERKs and JNKs, regulate the activity of transcriptional factors required for intact NFAT activity. Recently, the requirement of JNKs activation for dual signals, PKC and Ca$^{2+}$, was reported in T cells (Su et al, 1994). Thus, the dual signals may represent the requirement for Ca$^{2+}$-dependent nuclear translocation of NFATp and PKC-dependent activation or expression of c-Jun and c-Fos, both of which are essential for IL-2 transcription activation.

Aims of current study

The observation that exposure of T lymphocytes to oxidative stress leads to either functional inhibition and apoptosis or activation enhancement suggests the importance of oxidative stress in regulating the biological behaviors of T cells. Some information is available about the intracellular mechanisms contributory to the biological effects of
oxidative stress on T cells, but many questions remain to be answered. For example, how
does the stimulation of T cells with sublethal levels of oxidative stress result in expression
of early activated genes, such as c-jun and c-fos? Are the general intracellular second
messengers, protein kinases and Ca^{2+}, involved in the linkage between the oxidative
signals and the gene expression? How does the oxidative stress regulate [Ca^{2+}]_i
mobilization in T cells and what are the biological consequences? Does the oxidative
stress promote the expression of c-jun genes through promoter regulation in T cells and
what are the mechanisms? Considering the importance of c-jun in IL-2 induction, why
does the oxidative stress induce c-jun but inhibit IL-2? What kinds of molecular
mechanisms are involved in oxidant-mediated inhibition of IL-2 production? In current
study, we employ H_2O_2 as a prototype oxidant which readily diffuses across cell
membrane, and the human T cell line, Jurkat cells which mimic the basic features of human
T cells, to attempt to elucidate these questions. Our aims are

1. To determine whether sublethal levels of H_2O_2 may induce the expression of the early
activation genes, c-jun and c-fos, and whether protein kinases and Ca^{2+} signals may be
involved in H_2O_2-induced c-jun expression.

2. To examine the influence of micromolar concentrations of H_2O_2 on intracellular
calcium mobilization and the possible cellular mechanisms for H_2O_2-mediated [Ca^{2+}]_i
mobilization compared with TCR/CD3 or T cell mitogen signals.
3. To determine how $\text{H}_2\text{O}_2$ induces the expression of c-jun in Jurkat T cells: posttranscriptional regulation or transcriptional activation; and to analyze the c-jun promoter elements and transcriptional factors which are responsible for $\text{H}_2\text{O}_2$-induced transcriptional activation of c-jun gene.

4. To investigate the possible mechanisms of oxidative stress-mediated inhibition of IL-2 production, including the influences of sublethal levels of $\text{H}_2\text{O}_2$ on IL-2 transcriptional activation, NFAT transactivation, and in vitro DNA-binding activity of NFAT.
CHAPTER 2

METHODS

Reagents and Antibodies

Hydrogen peroxide (H$_2$O$_2$, 30%) was obtained from J.T. Baker, Inc. (Phillipsburg, NJ) and diluted in phosphate-buffered saline. Phytohemagglutinin (PHA) and tyrphostin (No. A25) were purchased from GIBCO-BRL (Grand Island, NY). Phorbol myristate acetate (PMA), proteinase and phosphatase inhibitors (aprotinin, leupeptin, and sodium orthovanadate were obtained from Sigma Chemical Co. (St. Louis, MO). The specific DNA restriction enzymes were from Boehringer Mannheim Biochemical (Indianapolis, IN). Herbimycin A, l-(5-isoquinolinesulfonamide)-2-methylpiperazine (H7), N-(2-guanidinoethyl-5-isoquinioline- sulfonamide) (HA1004), nifedipine, and genistein were purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Staurosporine was from Kyowa Hakko USA, Inc. (New York, NY). The acetoxylmethylester (AM) forms of indo-1 and the Ca$^{2+}$ chelating agent BAPTA (1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'- tetraacetate) were obtained from Molecular Probes (Junction City, OR) and dissolved in desiccated dimethylsulfoxide (DMSO). Stock solutions of these reagents
were made up such that the final concentration of vehicle DMSO present in cell culture was less than 1%. Monoclonal antibody (mAb) anti-CD3 was obtained from Ortho Pharmaceuticals (Rahway, NJ). Anti-ERK2 AC and anti-JNK AC from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal rabbit anti-mouse immunoglobulin antisera from Southern Biotechnology (Birmingham, AL).

**Cell Line Maintenance**

The E6-1 subclone of the human leukemic Jurkat T cell line was obtained from American Type Culture Collection (ATCC: Rockville, MD). The cells were maintained at 5-10 x 10^5/ml in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37° C in 5% CO₂ and split every three days. In some experiments, the cells were starved in RPMI 1640 medium with 0.5% FCS for 24 hours before treatment. The cells were stimulated with different reagents in RPMI 1640 medium either with 0.5% FCS or with 10% FCS as indicated in the text.

**Measurement of Cytosolic Free Calcium \([Ca^{2+}]_i\)**

Fluorescein Indo-1 AM, the noncharged and membrane-permeable ester form of indo-
1, can chelate free calcium with a concomitant shift of its absorbent wave length, and therefore, is usually used as a reagent to monitor the level of intracellular calcium (Gryniewicz et al, 1985). In current investigation, the indo-1 AM was made in 1 mM stock solution, and aliquots were stored at -20°C in the dark.

**Loading of the Cells with Indo-1 AM**

Jurkat cells (5 x 10^6 cells) were washed twice at 200 x g for 10 minutes in phosphate-buffered saline, PBS, containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 147 μM KH_2PO_4, pH 7.3. The cells were then resuspended in 1 ml RPMI 1640 medium with 5% FCS. For cell loading, 1 μM of indo-1 AM was added to the cell suspension. The loading reaction was incubated at 37°C for 45 minutes. At the end of the loading, the cells were washed twice in saline buffer consisting of 125 mM NaCl (pH 7.4), 5 mM KCl, 1 mM Na_2HPO_4, 0.1% glucose, 25 mM HEPES, 0.1% bovine serum albumin (BSA), and 0.5 mM MgCl_2, with or without 1 mM CaCl_2. The cell pellet was resuspended at 1 x 10^6 cells/ml in the saline buffer with or without CaCl_2 and kept in room temperature. The measurement of [Ca^{2+}]_i was performed within 2 hours.

**Measurements of [Ca^{2+}]_i**

The measurements of [Ca^{2+}]_i were performed with a Perkin-Elmer Model LS5B spectrofluorimeter (Analytical Instruments, Norwalk, CT). The cells at 5 x 10^5/0.5 ml in a cuvette were maintained at 37°C in a thermostatically controlled cuvette holder. The fluorescence F was recorded with excitation at 331nm and emission at 410nm. Baseline
measurements were monitored to allow stabilization of $[\text{Ca}^{2+}]_i$ for at least 3 minutes before any stimulation. The different reagents described in the text were added to the cuvette, mixed well, and monitored immediately for the changes in fluorescence. The levels of $[\text{Ca}^{2+}]_i$ were calculated using the equation:

$$[\text{Ca}^{2+}]_i = K_d \left( \frac{F - F_{\text{min}}}{F_{\text{max}} - F} \right)$$

in which a dissociation constant $K_d$ of 250 nm for the indo-1-$\text{Ca}^{2+}$ complex was used to calculate $[\text{Ca}^{2+}]_i$ (Gryniewicz et al, 1985). $F_{\text{max}}$ was determined for each set of samples by measuring the fluorescence of the loaded cells after permeabilization with 50μM digitonin. $F_{\text{min}}$ was obtained in the presence of 4 mM EGTA (pH 8.3). The background autofluorescence of the cells in absence of indo-1 AM was subtracted from the experimental values.

**RNase Protection Assays**

RNase protection assay was employed to measure the levels of c-jun, c-fos, and IL-2 mRNAs with specific RNA probes. The procedures included (1) extraction of total cytosolic RNA, (2) preparation of plasmid templates, the syntheses of RNA probes, (3) hybridization reaction, (4) resolution of the protected double strand RNA fragments by the denaturing polyacrylamide gel, and (5) autoradiography.

**Isolation of Cytosolic RNA**

Cytosolic RNA was isolated by a one-step procedure of acid guanidium thiocyanate
/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). 1-2 × 10^6 cells were incubated in medium with 0.5 % FCS for 24 hours and then subjected to the stimulation described in the text. The cells were washed twice with PBS and lysed on ice for 20 minutes in the buffer containing 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, and 100 mM beta-mercaptoethanol (pH 7), with equal volume of water-saturated phenol, one-fifth volume of chloroform, and 0.2 M sodium acetate (pH 5.2). After centrifugation at 10,000 × g for 15 minutes, the upper phase was removed to a fresh microcentrifuge tube and precipitated by adding an equal volume of isopropyl ethanol and standing at -20° C for 1 hour. The extracts were pelleted and washed twice at 12000 × g and 4° C for 5 minutes. The RNA pellet was resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6), and quantified by absorption spectroscopy.

Preparation of Plasmid templates for Riboprobes

A plasmid template for a specific riboprobe was linearized by the digestion with an appropriate restriction enzyme such that the linearized plasmid allowed the run-off synthesis of an antisense riboprobe by using T7, T3, or SP6 polymerases. After the digestion, the linearized template was purified by phenol/chloroform extraction and precipitated by ethanol. The template pellet was resuspended at 1 mg/ml in TE buffer containing 10 mM TrisCl and 1 mM EDTA, pH 7.6, and stored at 4° C.

Synthesis of Riboprobes

The ^32P-labeled antisense riboprobes were synthesized according to the method
(Melton, 1984) with some modifications. The synthesis was performed in the total 20 ul of following reaction mixture: transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, and 10 mM NaCl), 10 mM DTT, 20 unit RNasin ribonuclease inhibitor, 2.5 mM each of ATP, GTP, and CTP, 10 μM UTP, 1μg enzyme-linearized template DNA, 50 μCi (10 mCi/ml) [α-³²P]-UTP and 20 unit T7 or T3 RNA polymerase. To the reaction 1 unit of DNase I was added to digest template DNA at the extermination of the reaction. The synthesized riboprobes were purified by phenol/chloroform and ethanol precipitation. The specific activity of each riboprobe was determined by a Scintillation Counter (Beckman, Irvine, CA). The DNA templates used for the syntheses of riboprobes include:

- **Human c-jun probe:** An AccI/PstI fragment of human c-jun cDNA from plasmid phcj-1 (a gift of M. Karin, University of California at San Diego, La Jolla, CA) (Angel, et al., 1988b) was inserted into plasmid pBluescript KS I, and then used as the template DNA for synthesis of a 228 bp riboprobe by T7 RNA polymerase, which protects 162 bp human c-jun mRNA following RNases digestion.

- **Human c-fos probe:** The c-fos cDNA was obtained from ATCC (No. 41042) and subcloned into plasmid pBluescript KS II at the sites PstI/EcoRV(PvuII). The probe synthesized from this template by T3 polymerase is 347 bp in length and protects a 287 bp fragment of human c-fos mRNA.

- **Human junB probe:** The junB riboprobe was transcribed in vitro from the plasmid template pGEM4 containing a HincII/HincII fragment of human junB cDNA (a gift from J. Minna, Southwestern Medical School, Dallas, TX). This probe protects a 447 bp
humanjunB mRNA.

- **Human IL-2 probe:** A PstI/XbaI fragment of Human IL-2 cDNA from clone pTCGF-11 of ATCC (No. 39673) was subcloned into the vector pBluescript KS II and used as the template DNA for synthesis by T3 polymerase of a 607 bp riboprobe that protects a 512 bp human IL-2 mRNA from digestion of RNases.

- **Human GAPDH probe:** The riboprobe of GAPDH was synthesized from the plasmid template pGEM4Z containing a 100 bp fragment of human GAPDH cDNA and was a gift from Dr. Susan Garfinkel, American Red Cross (Rockville, MD).

**RNA protection assay**

5 µg of cytosolic RNA was mixed with $5 \times 10^5$ cpm of specific riboprobe in 10 µl hybridization buffer containing 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA and 80% deionized formamide. An equal amount of yeast tRNA was used as a negative control in every set of experiments for each specific probe. The level of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined by the specific probe as an internal control for all sets of experiments. The hybridization reactions were denatured at 90°C for 5 minutes and renatured at 60°C for 16 hours. The hybridization products were subjected to digestion of 100 µl RNase cocktail, which containing 10mM Tris (pH 7.5), 300 mM NaCl, 5 mM EDTA (pH 8.0), 200 ng/ml RNase A and 50 units/ml RNase T1, at 30°C for 45 minutes. The digestion reaction was stopped by adding 20 µl proteinase K solution (3.5% SDS, 500 µg/ml proteinase K, and 100 µg/ml yeast tRNA) and incubated at 37°C for 30 minutes. The protected products were extracted with
water-saturated phenol and chloroform/isoamyl alcohol, and precipitated by ethanol with 0.4 M ammonium acetate. To the pellet was added 5 µl of formamide loading buffer containing 80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol FF, and 1 mg/ml bromophenol blue. The protected fragments were resolved on 6% polyacrylamide gel with 7M urea after denaturation by heating 90° C for 5 minutes. The specific protected bands were visualized by autoradiography. The densities of the bands was quantified by computerized lasser scanner and normalized by GAPDH levels.

Electrophoretic Mobility Shift Assays (EMSA)

EMSA was used to study the in vitro DNA binding characteristics of nuclear transcriptional factors interested in this investigation, including Ap-1, AP-1-like, NF-AT, NFkB, etc. The steps involved in EMSA were (1) extraction of nuclear proteins, (2) labeling of specific DNA oligonucleotides, (3) in vitro binding reaction, (4) resolution of protein-DNA complexes by polyacrylamide gel, and (5) autoradiography.

Nuclear Extracts

The nuclear extracts used in EMSA were obtained from the following procedure. After the treatment with different reagents as indicated in the text, the Jurkat cells (5 × 10^6 cells/each) were washed twice in TBS buffer (25mM Tris, 137mM NaCl, and 27mM KCl, pH 7.4). The cell pellet was resuspended in hypotonic lysis buffer containing 20mM HEPES, 10 mM KCl, 0.2mM EDTA, 0.5mM PMSF, leupeptin, aprotinin and 1mM DTT,
and incubated on ice for 15 minutes. A final concentration of 0.5% Nonidet P-40 was added, followed by vigorously vortexing and centrifugation. The pellet was then resuspended in the buffer containing 20 mM HEPES, 400 mM NaCl, 2 mM EDTA, 1 mM PMSF, leupeptin, aprotinin and 20% glycerol, and placed at 4°C for 30 minutes with constant agitation. The extractable nuclear proteins were recovered after centrifugation and the protein concentration determined with a Coomassie Plus protein assay reagent as described by the manufacturer (Pierce, Rockford, IL).

Preparation of Radiolabeled Oligonucleotide Probes

The specific oligonucleotides for EMSA were \(^{32}\text{P}\text{-end-labeled}\) by using \([\gamma^{32}\text{P}]-\text{ATP}\) and T4 polynucleotide kinase. The labeling reaction was performed at 37 °C for 30 minutes in 20 ul of the kinase buffer (50 mM Tris-HCl, 10 mM MgCl\(_2\), 100 \(\mu\)M EDTA, 5 mM DTT, 100 \(\mu\)M spermidine, pH 8.2) containing 10 ng of a specific oligonucleotide, 10 \(\mu\)Ci \([\gamma^{32}\text{P}]-\text{ATP}\) (3000 Ci/mmole), and 1 unit of T4 polynucleotide kinase. The synthesized oligonucleotide probe was purified by Sephadex G-25 spin columns and counted for the specific radioactivity by a scintillation counter.

EMSA

The protein-DNA binding reaction was performed in 20 \(\mu\)l of reaction buffer (20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, and 20% glycerol) with 5 \(\mu\)g nuclear extracts, the specific radiolabeled probe (1-2 \(\times\) \(10^4\) cpm/50 pg/\(\mu\)l), and 2 \(\mu\)g poly[d(I).C]), at 25°C for 20 minutes. The protein-DNA complexes were separated by running on a 5%
polyacrylamide gel under low ionic strength conditions, visualized by autoradiography, and quantitated by a computerized laser scanner. The sequences of the oligonucleotides used in the experiments are as follows:

**Consensus AP-1**: 5'-GGCTAGTGATGAGTCAGCCGGATC-3' (Angel et al., 1988a)

**NFKB**: 5'-AGTTGAGGGGACTTTCCCAGGC-3' (Baldwin et al., 1991)

**Jun1**: 5'-77TTGGGTTGACATCATGGGCTA56-3' (Muegge et al., 1993)

**Jun2**: 5'-197CGGAGCATTACCTCATCCCGT177-3' (Muegge et al., 1993)

**NFAT**: 5'-285GGAGAAAAACTGTTTCATACAGAAGG254-3' (Shaw et al., 1988)

**Identification of Specific Protein-DNA Binding Complexes**

The components and binding specificities of protein-DNA binding complexes in the EMSA were identified by competition assays with specific antibodies or unlabeled corresponding oligonucleotides ("cold probes"). To identify the components of a specific DNA binding protein, the cell extracts were incubated with 1 µg of the corresponding antibodies in 20 µl of the EMSA reaction buffer at 4 °C for 60 minutes, followed by the binding reaction and gel resolution described above. The existence of a specific component within the DNA binding protein was demonstrated by either a reduction in the density of the protein-DNA band or a "supershift" (retarded migration) of the band. A negative control with the same class of antibody or serum was always performed with the experiment. For the detection of the protein-DNA binding specificity, the cell extracts were first allowed to interact with an excessive amount of the corresponding unlabeled oligonucleotide probe at 25 °C for 20 minutes. The binding reaction was then performed
as described above. The binding of the nuclear protein to the radiolabeled oligonucleotide probe was competitively blocked by the pre-binding cold probe, which would result in a decrease or disappearance in the density of the band on the gel.

Transfection Plasmids, Transfection, and Analyses of Reporter Constructs

Transfection Plasmids

The different wild-type or point-mutated c-jun-CAT (chloramphenicol acetyltransferase) reporter gene constructs containing the sequence between 1600 bp upstream and 170 bp downstream of the transcriptional start site of the human c-jun promoter were used in this investigation. These constructs were kindly provided by Dr. Peter Herrlich, Institut fuer Genetik and Toxikologie, Karlsruhe, Federal Republic of Germany. Figure 11 illustrates these constructs including wild-type -1600/+170jun-CAT, point-mutated -1600/+170mjun1CAT, -1600/+170- mjun2CAT, and -1600/+170mjun1,2CAT. In the -1600/+170mjun1CAT, the wild-type jun1 sequence -71TGACATCAT-63 was replaced by the sequence -71ATCCACCAT-63, while in the -1600/+170mjun2CAT, the wild-type jun2 sequence -191ATTACCTCATC-181 replaced by -191TGGACCTCGAG-181 (Stein et al., 1992). The -79/+170 human c-jun-CAT and -79/+170 human c-jun-mut-CAT constructs as well as CAT reporter gene constructs containing a single copy or five copies of the collagenase gene promoter TRE recognizing AP-1 (1 × or 5 × TRE-CAT) (Angel et al., 1988b) were the generous gifts from Dr. Michael Karin (University of California, San Diego, CA) and Dr. Tiliang Deng (University
of Florida, Gainesville, FL). In the point mutation of -79/+170 c-jun-mut-CAT, the wild-type sequence 5'-TGACATCAT-3' in -79/+170 c-jun-CAT was replaced by the sequence 5'-ATCCACCAT-3'. The TRE AP-1 sequence in 1 x or 5 x TRE-CAT construct is 5'-TGACTCA-3', linked to the upstream of CAT reporter plasmid. The 3 x NFkB-CAT reporter plasmid was obtained from Dr. Al Baldwin (University of North Carolina, Chapel Hill, NC), containing three copies of human NFkB sequence 5'-GAGGGGACTTTCCC-3' (Baldwin, et al., 1991). Construct plasmid 5B3.1 (Matilla et al., 1990) consisting of four human NF-AT sequences upstream of a minimal gamma-fibrinogen promoter in a CAT expression vector was provided by Dr. Gerald R. Crabtree (Stanford University School of Medicine, Palo Alto, CA). Human IL-2 reporter gene construct, pIL-2-548 Luc, was obtained from Dr. Tom Williams (University of New Mexico, Albuquerque, NM). This construct contains the full length human IL-2 promoter region linked to the upstream of luciferase (Luc) gene in a Luc reporter plasmid (Williams et al., 1992)

Transfection

Jurkat cells were transfected with reporter gene constructs by DEAE-dextran procedure. The 1 x 10^7 cells were harvested from 24-48 hour culture in RPMI 1640 medium with 10% FCS and washed twice with PBS. The cell pellet was resuspended in 1 ml TBS buffer containing 10 μg reporter plasmid DNA and 500 μg DEAE-dextran, and placed at room temperature for 45 minutes. To the cell suspension was added 10 ml of RPMI 1640 medium with 50 μM chloroquine. Having been placed at 37°C for 20 minutes, the transfected cells were washed twice with PBS and incubated in RPMI 1640
medium with 10% FCS at 5% CO₂ and 37°C for 16 hours.

**Chloramphenicol Acetyltransferase (CAT) Assays**

The CAT, encoded by a bacterial drug-resistance gene, inactivates chloramphenicol by acetylation at one or both of its two hydroxyl groups. Linkage of putative regulatory sequences from an eukaryotic gene to the appropriate pCAT-vector forms a reporter gene construct. Transfection of the eukaryotic cells with a CAT reporter gene construct allows for the efficient assay of CAT activity and the studies of eukaryotic gene expression in the transfected cells. The CAT assay included the protein extract of the transfected cells, the CAT reaction, the separation of the acetylating chloramphenicol, and autoradiography.

The protein extract of the transfected cells was performed as follows. After stimulation with the reagents indicated in the text, the transfected cells were washed once with PBS and once with TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 150 mM NaCl). The cell pellet was resuspended in 50 μl of 0.25 M Tris-HCl (pH 7.8) and then subjected to 3 freeze-thaw cycles by alternatively placing the tubes in dry-ice and 37°C water bath with vigorously stirring following each thaw. The extracts were incubated in 60°C water bath for 10 minutes to reduce nonspecific reaction and precipitated at 24,000 x g for 3 minutes. The supernatant proteins were removed to fresh tubes and quantified by Coomassie Plus Protein Assay Reagent as described above.
The CAT reaction was established by adding 30 μl cell extracts prepared as above to 100 μl 0.25 M Tris-HCl (pH 7.8) containing 5 μg acetyl Coenzyme A and 50 μCi/ml 14C-chloramphenicol (50 mCi/m mole). The reaction mixture was incubated at 37 °C for 2 to 3 hours. During the reaction, CAT transfers the acetyl moiety of the cofactor to chloramphenicol. At the end of the reaction, the reaction products were extracted with 0.5 ml ethyl acetate to separate chloramphenicol from acetyl coenzyme A and stop the reaction. The acetylated and unacetylated forms of 14C-chloramphenicol partition into the organic phase, while acetyl coenzyme A remains in the aqueous phase. After centrifugation at 24,000 x g for 3 minutes, the top, organic phase was then transferred to a fresh tube and evaporated to dryness. The chloramphenicol residue was resuspended in 20 μl of ethyl acetate and spotted onto a silica gel thin layer chromatography (TLC) plate. To separate acetylated and unacetylated forms of 14C-chloramphenicol, the chromatography of the spotted TLC plate was performed for 20 to 30 minutes in a closed tank pre-equilibrated with chloroform:methanol (97:3) solution. The plate was dried and autoradiographed. The densities of acetylated and unacetylated spots of 14C-chloramphenicol on the film were measured by a computerized laser scanner. The CAT activity or conversion percentage was calculated by following formula:

\[ \text{CAT Activity or conversion percentage (\%)} = \frac{\text{Acetylated forms of chloramphenicol}}{\text{Acetylated forms} + \text{Unacetylated forms}} \times 100 \]
Luciferase Assays

The luciferase assay was used to detect the luciferase activity produced by pIL-2-548 Luc gene reporter and analyze the effects of oxidative stress or other stimulants on the transactivation of the human IL-2 gene promoter. The protocol was involved in the protein extract from the transfected cells and the analysis of the luciferase activity in the cell extract.

Following the stimulation for 18 hours and washing twice in PBS, the transfected cells (2 × 10⁶ cells/each) were extracted by resuspending the cell pellet in 100 µl of lysis buffer consisting of 25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, and 1% Triton X-100. After incubation at room temperature for 15 minutes, the cell lysate was briefly microcentrifuged to remove the cell debris. The cell proteins in the supernatant were quantitated as described above.

For the analysis of the luciferase activity, 20 µl of the cell extract was mixed with 100 µl of the luciferase assay buffer containing 20 mM Tricine, 1.07 mM (MgCO₃)₂Mg(OH)₂5H₂O, 2.67 mM MgSO₄, 100 µM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 470 µM luciferin, and 530 µM ATP, pH 7.8, immediately followed by detection for 20 seconds in a luminometer. In the reaction, the substrate luciferin is oxidized by the catalysis of the firefly luciferase existing in the cell extract, with concomitant production of a photon.
This assay was used to analyze JNK and MAP kinase activities. The Jurkat cells (5 × 10⁶ cells/each) were stimulated with stimuli in RPMI 1640 medium with 0.5% FCS for 30 minutes and washed twice in PBS buffer. The cells were lysed in 500 µl of lysis buffer consisting of 20 mM HEPES (pH 7.9), 150 mM NaCl, 2 mM EGTA, 50 mM beta-glycerophosphate, 1 mM DTT, 1 mM sodium orthovanadate, 0.1% Triton X-100, 20 µg/ml leupeptin, 18 µg/ml aprotinin, and 400 µM PMSF. After incubation on ice for 15 minutes with vortexes in intervals, the lysates were centrifuged at 24,000 × g for 10 minutes to remove cellular debris. The supernatants were removed to fresh tubes and immunoprecipitated with 2-5 µg of agarose-conjugated antibodies specifically against human JNK or ERK-2 (from Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for 1 hour to overnight with rotation. The immunoprecipitates were rinsed three times in ice-cold kinase buffer (20 mM HEPES (pH 7.9), 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, 100 µM sodium orthovanadate, and 25 mM beta-glycerophosphate). The immunoprecipitates were resuspended in 25 µl of kinase buffer containing 50 µM ATP, 50 mM MgCl₂, 3 µCi [γ-³²P]-ATP (3000 Ci/mmol), and a substrate for the specific kinase type (5 µg MBP for ERK-2 and 1.5 µg c-Jun(79) for JNK). The kinase reaction was carried out at 30°C for 20 to 30 minutes and terminated by adding an equal volume of 2 × loading buffer. After boiling for 5 minutes, the reaction products were resolved by SDS-PAGE (12% for c-Jun(79) and 15% for MBP). The gel was washed extensively in a solution containing 1% sodium pyrophosphate and 5% trichloroacetic acid. After the gel
was dried, the phosphorylated substrate proteins were visualized by autoradiography. The specific protein bands were quantified by laser scanning densitometer (LKB Ultroscan XL, Bromma, Sweden) with an absorbance at 633 nm within the linear range of exposures.

**Measurement of IL-2 Production**

IL-2 production was induced by stimulating Jurkat cells (2 × 10⁶ cells/ml) with 10 μg/ml PHA plus 50 ng/ml PMA or 5 μg/ml anti-CD3 plus 50 ng/ml PMA for 36 hours at 37 °C in RPMI 1640 medium supplemented with 10% FCS. After the stimulation, the supernatants were harvested for the analyses of IL-2 production. 100 μl out of 1 ml supernatant from one stimulation was analyzed for IL-2 production by enzyme-linked immunosorbent assay (ELISA) using Quantikine Human IL-2 Immunoassay kit. The protocol was performed as recommended by the manufacturer.
CHAPTER 3

RESULTS

Induction of c-jun and c-fos Transcription Expression by Sublethal Levels of Oxidative Stress and Involvement of Protein Kinase Activation in Jurkat T Cells

Induction of c-jun and c-fos mRNA in Jurkat T cells by sublethal concentrations of H$_2$O$_2$ and T cell mitogens

Mammalian cells can respond to oxidative stress with proliferation or apoptotic-like death which is dependent on the concentrations of oxidative products (Wieses et al, 1995). Initial experiments were performed to determine an optimal concentration range of H$_2$O$_2$ for current investigation. The viability of Jurkat T cells was evaluated by trypan blue exclusion test after the cells were exposed to different micromolar concentrations of H$_2$O$_2$ for varying times. The results in Figure 1 show that the viability of the cells tended to decrease with the increases in the concentrations of H$_2$O$_2$ and the exposure duration. When exposed to 500 µM H$_2$O$_2$ for less than one hour, there was a less than 5% reduction in viability in the Jurkat cells compared to the cells incubated in medium alone. Exposure
of cells to 500 μM H₂O₂ for 6 hours caused the reduction to 10%. The results suggest that several hour exposure of Jurkat cells to H₂O₂ at the concentrations below 500 μM did not lead to significant cell damage. The concentrations of H₂O₂ ranged from 50 to 500 μM and less than 4-hour exposure were employed in most of our experiments to avoid substantial cellular death.

Several reports have shown that oxidative stress can stimulate mRNA expression of Jun and Fos family members in different cell types (Devary et al, 1991; Schreck, et al, 1991; Amstad et al, 1992; Meyer et al, 1993). To determine whether c-jun and c-fos mRNA were induced in Jurkat T cells by H₂O₂, the cells were treated with different concentrations of H₂O₂ ranging from 25 to 500 μM. Aliquots of cells were also exposed to monoclonal antibody against TCR/CD3 (anti-CD3) and cumene hydroperoxide (CHP), a more stable form of hydroperoxide. Control cells were incubated in medium alone. After treatment for 45 minutes predetermined to be optimal, the mRNA levels of c-jun, c-fos, and JunB were measured with specific anti-sense RNA probes in RNase protection assays. The mRNA levels of GAPDH were simultaneously detected as internal controls. The results shown in Figure 2 show that as low as 25 μM H₂O₂ induced a significant expression of c-jun mRNA compared to the untreated control cells, while a slightly higher concentration of H₂O₂ (50 μM) was needed to induce c-fos mRNA expression. In both c-jun and c-fos expressions, the higher concentrations of H₂O₂, more than 300 μM, could not stimulate further increases. In fact, low levels of c-jun or c-fos mRNA were observed in the cells treated with more than 300 μM H₂O₂ compared to the cells exposed to lower
concentrations of H$_2$O$_2$. In addition, Jurkat cells expressed a higher background level of JunB mRNA. Exposure to H$_2$O$_2$ did not influence the expression level of JunB mRNA although cross-linked anti-CD3 resulted in its increase.

Both c-jun and c-fos are early activation genes that are expressed in mammalian cells in response to various stimuli (reviewed by Angel and Karin, 1991). In the next series of experiments, time-dependent induction of c-jun and c-fos mRNA was determined in Jurkat cells exposed to H$_2$O$_2$ and PHA/PMA. The cells were incubated with 200 μM H$_2$O$_2$ and the indicated concentrations of PHA plus PMA for varying times ranging from 15 minutes to 6 hours. The mRNA levels of c-jun and c-fos were measured by RNase protection assays at the different time points (Figure 3). Both H$_2$O$_2$ and PHA/PMA caused the rapid accumulation of c-jun and c-fos mRNA compared to control cells incubated in medium alone. For c-jun mRNA, H$_2$O$_2$ and PHA/PMA induced a similar time kinetic course with greater than twofold increases at 15 minutes to the first one hour and persistent expression throughout the 6-hour time course. However, the stimulated increases of c-fos mRNA levels caused by H$_2$O$_2$ and PHA/PMA exhibited different time courses. PHA/PMA stimulation resulted in 1.3 to 2.0 fold increases throughout 15 minutes to 6 hours with a peak rise at 30 to 60 minutes. By contrast, H$_2$O$_2$ treatment only induced early increases of c-fos mRNA with a peak rise (about 1.5 fold over control cells) at 30 minutes, followed by returning to the control level after one hour.

The above results indicated that the transcriptional expression of c-jun and c-fos genes
in Jurkat T cells by H$_2$O$_2$ was time- and dose-dependent. In the next experiment, we examined whether a brief exposure to oxidative stress was sufficient to trigger the activation of the transcriptional process. Jurkat cells were incubated with 100 µM H$_2$O$_2$. After 1, 5, or 15 minutes, the cells were either immediately subjected to measurement of c-jun mRNA or washed free of H$_2$O$_2$ and incubated for a further 45 minutes in medium alone, followed by c-jun mRNA measurement. The results were presented in Figure 4. A fifteen minute exposure of the cells to H$_2$O$_2$ was required for the immediate detection of c-jun mRNA (lane 4), which might reflect the time required for transcription process itself. However, after a further 45-minute incubation, even one-minute exposure to H$_2$O$_2$ was sufficient to stimulate c-Jun mRNA expression (lane 5), although longer exposure induced higher level expression of c-jun mRNA (lane 6 and 7). The results suggested that the intracellular mechanisms for c-jun gene transcription were triggered within minutes after exposure of Jurkat cells to sublethal levels of oxidative stress.

These previous results revealed that sublethal levels of oxidative stress stimulated the transcriptional expression of c-jun and c-fos genes in Jurkat T cells. Furthermore, the current data showing the persistent induction of the c-jun gene underscored the need to elucidate the possible molecular events involved in targeting c-jun during oxidative stress in human T cells.
Both serine/threonine and tyrosine protein kinases are required for the induction of c-jun mRNA expression by H$_2$O$_2$ in Jurkat T cells.

The results presented above demonstrated that micromolar concentrations of H$_2$O$_2$ induced significant expression of c-jun and c-fos mRNAs in Jurkat T cells and that a short exposure of the cells to H$_2$O$_2$ triggered the intracellular mechanisms required for c-jun expression. In the following studies, we attempted to define the intracellular molecular events that might be involved in the signal transduction pathways between oxidative stress stimulation and c-jun mRNA expression in Jurkat cells. Prior studies suggest that the transcription activation of c-jun gene is autoregulated by the expression product of c-jun gene, c-Jun protein, whose DNA binding and transactivation activity was modulated by protein kinases (Angel et al, 1988b; Boyle et al, 1991; Hibi et al, 1993; reviewed by Hunter and Karin, 1992). These observations raised the possibility that the activities of distinct protein kinases might contribute to the induction of c-jun mRNA in Jurkat cells in response to sublethal levels of oxidative stress.

To examine this possibility, different types of protein kinase inhibitors were employed to determine the possible involvement of protein kinases in H$_2$O$_2$ induction of c-jun mRNA in Jurkat cells. The cells were pretreated with the protein kinase inhibitors that suppressed the activities of PKC, PTK, or both, followed by the treatment with 100 μM H$_2$O$_2$ or PHA, and the measurement of c-jun mRNA. Both herbimycin A and tyrphostin, inhibitors of PTKs (June et al, 1990), blocked c-jun mRNA expression induced by H$_2$O$_2$ compared with the cells that were preincubated in medium alone (Figure 5A). More than 50%
inhibition of H₂O₂ induction of c-jun mRNA was observed in the cells pretreated with herbimycin A, although no dose-dependent relationship was observed in the indicated micromolar concentrations of herbimycin A. Tyrphostin pretreatment exhibited dose-dependent inhibition of c-jun mRNA with more than 80% at 200 μM and only 20% at 100 μM. Herbimycin A also suppressed PHA induction of c-jun mRNA in a dose-dependent pattern (Figure 5C). Similarly, the isoquinolinesulfonamide H7, which can inhibit PKC and to some extent cAMP- and cGMP-dependent kinases (Hidaka et al. 1984), suppressed H₂O₂ induction of c-jun mRNA expression by 45% in response to 50 μM H₂O₂ (Figure 5B). Only modest reduction (15%) was observed with 50 μM HA1004, an isoquinolinesulfonamide analog of H7 that predominantly targets cAMP- and cGMP-dependent kinases. Staurosporine, an inhibitor for the activities of both PTKs and PKC, completely abolished c-jun induction in response to oxidative stress (Figure 5A). The results indicated that either PTKs or PKC was partially involved in H₂O₂ induction of c-jun mRNA, but the optimal induction of c-jun mRNA expression was dependent on the optimal activities of both PTKs and PKC in Jurkat T cells in response to oxidative signals.

It has been known that MAP kinases are considered to be the direct upstream regulators of c-Jun and TCF/Elk-1 activities in the intracellular signal cascades. Among the MAP kinases, JNKs are believed to phosphorylate serine residues within c-Jun activation domain and thereby potentiate its transactivation function (Hibi et al. 1993), while ERKs activate TCF/Elk-1 and, in turn, induce c-fos (Gille et al. 1992). Therefore, oxidative stress induction of c-jun and c-fos expression in Jurkat cells was likely to be
linked to the activation of JNKs and ERKs. To determine whether H$_2$O$_2$ might stimulate the activities of JNKs and ERKs in Jurkat cells, immune complex kinase assays were performed with the protein extracts from the cells treated with H$_2$O$_2$ or PHA/PMA. The cells were stimulated for 30 minutes with PHA/PMA or the indicated concentrations of H$_2$O$_2$. The cell extracts were immunoprecipitated by the antibodies specific to JNK-2 and ERK-2. The in vitro immunocomplex kinase reactions were assayed using the specific substrates, c-Jun(1-93) for JNK and MBP for ERK. The results show that as low as 50 $\mu$M H$_2$O$_2$ effectively stimulated the activation of JNK (Figure 6A) and ERK-2 (Figure 6B) activities compared with the unstimulated control. The stimulation with H$_2$O$_2$ concentrations ranging from 50 to 200 $\mu$M resulted in similar levels of JNK activation equivalent to that observed in response to PHA/PMA. ERK activities induced by H$_2$O$_2$ exhibited dose-dependent relationship with H$_2$O$_2$ concentration and were lower than that triggered by PHA/PMA.

Elevations of [Ca$^{2+}$]$_i$ in Jurkat T cells in response to oxidative stress parallel the in vivo induction of c-jun mRNA

Dual signals consisting of the activation of protein kinases and the elevation of [Ca$^{2+}$]$_i$ are usually required to elicit optimal T cell activation events in response to extracellular stimulation (reviewed by Mueller et al, 1989; Gardner, 1989; Ullman et al, 1990; Rao, 1991). As oxidative stress can trigger increase of [Ca$^{2+}$]$_i$ in various cell types (Duncan and Lawrence, 1989; Kimura et al, 1992; Doan et al, 1994), experiments were conducted to see whether the exposure of Jurkat cells to micromolar concentrations of H$_2$O$_2$ might
modulate \([Ca^{2+}]_i\) and whether any alterations of \([Ca^{2+}]_i\) might be associated with c-jun mRNA expression. The results indicated that micromolar levels of \(H_2O_2\) stimulated dose-dependent increases of \([Ca^{2+}]_i\) (see next section for detail). To determine the relationship between the elevations of \([Ca^{2+}]_i\) and c-jun mRNA expression induced by oxidative stress, we employed the calcium chelator, BAPTA, to deplete the intracellular calcium of the cells and then measured \([Ca^{2+}]_i\) and c-Jun mRNA levels. Jurkat cells were first preloaded with the indicated concentrations of BAPTA in \(Ca^{2+}\)-free saline buffer containing EGTA to remove extracellular \(Ca^{2+}\). Then, the BAPTA-preloaded cells were loaded with Indo-1 AM followed by treatment of the cells with 100 \(\mu\)M \(H_2O_2\) and \([Ca^{2+}]_i\) measurements, or directly treated for 45 minutes with 100 \(\mu\)M \(H_2O_2\) followed by c-jun mRNA analyses. The results presented in Figure 7 show that the c-jun mRNA expression was inhibited by the preloading BAPTA in a dose-dependent manner (Figure 7A). There was a parallel relationship between decreases of \([Ca^{2+}]_i\) and c-jun mRNA levels (Figure 7B). In addition, preloading of the cells with 25 \(\mu\)M BAPTA blocked the constitutive expression of c-jun mRNA in untreated Jurkat cells (lane 2 in Figure 7A). Taken together, the results provide evidence that oxidative stress induction of c-jun mRNA in Jurkat T cells is dependent on intracellular calcium signal as well as protein kinase activity.
Oxidative Stress Induces Increase of \([\text{Ca}^{2+}]_i\) Level in Jurkat T Cells through the Mechanisms Involving \(\text{Ca}^{2+}\) Channels and PTKs

The \([\text{Ca}^{2+}]_i\) response is believed to be an important second signal produced during the activation of human T cells in response to extracellular stimulation (reviewed by Gardner, 1989; Rao, 1991). The current studies indicate that oxidative stress in the form of micromolar concentrations of \(\text{H}_2\text{O}_2\) elevates \([\text{Ca}^{2+}]_i\) in Jurkat T cells, which is accompanied by the induction of \(c\)-jun mRNA. In the next studies, we evaluate oxidative stress induction of \([\text{Ca}^{2+}]_i\) rise in Jurkat T cells with respect to the kinetic analysis and the possible mechanisms.

Micromolar concentrations of \(\text{H}_2\text{O}_2\) stimulate calcium influx in Jurkat T cells in a dose-dependent manner

To determine the range of \(\text{H}_2\text{O}_2\) concentrations that can modulate \([\text{Ca}^{2+}]_i\) levels, Jurkat cells were loaded with the \(\text{Ca}^{2+}\) indicator indo-1 AM and \([\text{Ca}^{2+}]_i\) monitored fluorimetrically after the exposure of cells to 50, 100, or 200 \(\mu\text{M} \text{H}_2\text{O}_2\) or to 10 \(\mu\text{g}/\text{ml}\) PHA. The results are shown in Figure 8. As previously reported (Dupuis et al, 1989), PHA was effective in stimulating the rise of \([\text{Ca}^{2+}]_i\) level in Jurkat T cells leading to an eight-fold elevation that peaked at 520 nM. The exposure of Jurkat cells to micromolar concentrations of \(\text{H}_2\text{O}_2\) also stimulated increases in \([\text{Ca}^{2+}]_i\) levels that were approximately two- to threefold greater than resting levels. The increases of \([\text{Ca}^{2+}]_i\) in response to \(\text{H}_2\text{O}_2\) were dose-dependent. Treatment with 50 \(\mu\text{M} \text{H}_2\text{O}_2\) resulted in a peak value of 280 nM and 200 \(\mu\text{M}\)
H₂O₂ induced a maximal peak value of 360 nM. Both PHA and the acute exposure of cells to H₂O₂ stimulated a similar pattern of [Ca²⁺]ᵢ kinetic changes, rapid initial elevations followed by a progressive increases in [Ca²⁺]ᵢ that reached peak values after 4 to 6 minutes.

The kinetic studies of [Ca²⁺]ᵢ changes has suggested that the elevations of [Ca²⁺]ᵢ levels in T cells stimulated by TCR/CD3 signaling or T cell mitogens consist of two phases (reviewed by Gardner, 1989; Hess et al, 1993). The increase of [Ca²⁺]ᵢ in the early phase is caused by the release from the intracellular stores leading to the rapid initial rise in [Ca²⁺]. The second phase represents the influx of extracellular Ca²⁺ that is required to maintain the late phase increase in [Ca²⁺]. To determine whether the increases in [Ca²⁺]ᵢ with H₂O₂ represent the mobilization from internal stores or the influx of extracellular Ca²⁺, the ability of H₂O₂ to induce elevations of [Ca²⁺]ᵢ levels was measured under calcium-free conditions. The results demonstrate that the initial rapid increases in [Ca²⁺]ᵢ in the cells exposed to H₂O₂ and PHA continued to be observed in the absence of external Ca²⁺, consistent with the mobilization from internal stores (Figure 9). However, the addition of 1 mM CaCl₂ to the external buffer resulted in a prompt and substantial increase of [Ca²⁺]ᵢ in the cells stimulated by H₂O₂ or PHA, which reflects the influx of extracellular Ca²⁺. Approximately 80% of the total increases in [Ca²⁺]ᵢ induced by H₂O₂ or PHA represented Ca²⁺ influx, whereas the mobilization from intracellular stores contributed about 20%.
[Ca\(^{2+}\)]_i elevations in Jurkat T cells exposed to H\(_2\)O\(_2\), PHA or anti-CD3 are differentially inhibited by calcium channel blockers and inhibitors of PTKs.

The calcium channels in the plasma membrane are responsible for the Ca\(^{2+}\) influx of T cells in response to the stimulation of T cell receptor complex, although the nature of the channels is not fully defined. The classic L-type voltage-sensitive Ca\(^{2+}\) channels do not appear to mediate Ca\(^{2+}\) influx in Jurkat T cells (Gardner, 1989), but there is evidence that Ca\(^{2+}\)-selective channels resembling T-type voltage-gated Ca\(^{2+}\) channels are involved (McDonald et al, 1993). To determine if Ca\(^{2+}\) channels might be involved in the Ca\(^{2+}\) influx of Jurkat cells exposed to H\(_2\)O\(_2\), the Ca\(^{2+}\) channel blockers, nifedipine and Ni\(^{2+}\), were employed to pretreat the cells followed by the stimulation of 100 µM H\(_2\)O\(_2\) or 10 µg/ml PHA. Nifedipine is a blocker for the L-type Ca\(^{2+}\) channel (Tsien et al, 1990), while Ni\(^{2+}\) can block other cation channels as well as the T-type Ca\(^{2+}\) channel (Silva et al, 1994).

As shown in Figure 10, the incubation of Jurkat cells with 40 µM nickel chloride effectively inhibited the H\(_2\)O\(_2\)-induced increases in [Ca\(^{2+}\)]_i, but did not suppress [Ca\(^{2+}\)]_i increases seen with PHA. By contrast, nifedipine at a dose (3 µM) that typically blocks voltage-sensitive L-type Ca\(^{2+}\) channels did not suppress [Ca\(^{2+}\)]_i increases observed with H\(_2\)O\(_2\), although there was a modest (about 30%) reduction in [Ca\(^{2+}\)]_i levels induced by PHA. The results suggest that a type of non-classic Ca\(^{2+}\) channel might mediate the Ca\(^{2+}\) influx of Jurkat T cells in response to micromolar concentrations of H\(_2\)O\(_2\). This Ca\(^{2+}\) channel may differ from the one that mediates PHA-induced Ca\(^{2+}\) influx in Jurkat cells.
[Ca\(^{2+}\)]_i elevations triggered by cross-linking the T cell receptors (TCRs) are dependent on nonreceptor PTKs (Rigley et al, 1995 and June et al, 1990). Therefore, it is conceivable that PTK activity might be needed for increased [Ca\(^{2+}\)]_i levels of Jurkat cells in response to micromolar concentrations of H\(_2\)O\(_2\). To determine if PTKs might participate in [Ca\(^{2+}\)]_i responses in Jurkat cells, indo-1 loaded Jurkat cells were pretreated with PTK inhibitor genistein (Akiyama et al, 1987), or incubated in medium alone. The cells were then divided into aliquots that were stimulated with 100 \(\mu\)M H\(_2\)O\(_2\), 10 \(\mu\)g/ml PHA, or 5 \(\mu\)g/ml cross-linked anti-CD3 MAb. The results in Figure 11A show that the pretreatment of cells with 25 or 50 \(\mu\)M genistein did not influence the [Ca\(^{2+}\)]_i responses of cells to H\(_2\)O\(_2\) compared to the control cells incubated in medium alone. By contrast, the cells pretreated with genistein exhibited reductions in the [Ca\(^{2+}\)]_i mobilization after stimulation by PHA (Figure 11B) or cross-linked anti-CD3 MAb (Figure 11C). It has been reported that genistein primarily inhibits p56\(^{ck}\) and TCR \(\zeta\) -chain phosphorylation and does not completely block the PHA- or anti-CD3-induced [Ca\(^{2+}\)]_i responses in T cells (Trevillyan et al, 1990). To characterize more fully the role of PTK activity in the [Ca\(^{2+}\)]_i responses in Jurkat T cells to oxidative stress and stimulation of membrane receptors by PHA and anti-CD3, we tested the effects of other PTK inhibitors that act by different mechanisms. Data presented in Table 1 show that Jurkat cells pretreated with staurosporine, a potent inhibitor of PTK and other classes of protein kinases, exhibited complete suppression of [Ca\(^{2+}\)]_i responses upon exposure to PHA, cross-linked anti-CD3, or 100 \(\mu\)M H\(_2\)O\(_2\) compared with the cells incubated in medium alone or in medium containing the inhibitor vehicle DMSO. Similar to staurosporine, the pretreatment of cells with selective PTK
inhibitor herbimycin A completely blocked the increases of \([Ca^{2+}]_i\) levels in responses to PHA, anti-CD3, or \(H_2O_2\). Again, cells pretreated with even higher concentration of genistein (200 \(\mu\)M) demonstrated reductions in PHA- or anti-CD3-induced \([Ca^{2+}]_i\) responses ranging from 30 to 35%, however genestein did not affect the \([Ca^{2+}]_i\) elevation induced by \(H_2O_2\).

To exclude the possibility that the suppression of \([Ca^{2+}]_i\) levels in the cells pretreated with PTK inhibitors may have simply represented nonspecific metabolic impairments rather than selective inhibition of PTKs, fluoroaluminate (AlF\(_4\)), an agent that bypasses PTK (O'shea et al, 1987), was employed to trigger the \(Ca^{2+}\) mobilization in Jurkat cells pretreated with the PTK inhibitor. The results in Figure 12 show that herbimycin A suppressed the \([Ca^{2+}]_i\) increases in Jurkat cells stimulated by anti-CD3 and \(H_2O_2\), but the subsequent treatment of the cells with AlF\(_4\) caused substantial elevations in \([Ca^{2+}]_i\) levels. Thus, the results presented here provide further evidence that different classes of PTKs might be involved in the \([Ca^{2+}]_i\) responses in Jurkat T cells induced by stimulation of T cell membrane receptors and by oxidative stress. Considering the involvement of distinct calcium channels in the \([Ca^{2+}]_i\) responses induced by these two types of stimulation as shown in our previous experiments, our data also suggest that oxidative stress may stimulate intracellular calcium mobilization in Jurkat cells through the mechanisms that differ from those triggered by biological stimulation of T cell membrane receptors.

The acute exposure of T cells to oxidative stress might impair the mechanisms of \(Ca^{2+}\).
mobilization elicited by subsequent TCR stimulation and, in turn, contribute to the possible dysfunction of T cells, as has been reported after the long-term exposure of T cells to polyamine-generated products and ROS (Flescher et al, 1994). To clarify this issue, we monitored the \([\text{Ca}^{2+}]_i\) changes in Jurkat cells pretreated with H\(_2\)O\(_2\) followed by stimulation of TCRs after a full H\(_2\)O\(_2\)-induced \(\text{Ca}^{2+}\) response reached. The results presented in Figure 13 illustrate that Jurkat cells exposed to 100 \(\mu\)M H\(_2\)O\(_2\) were still capable of further increasing \([\text{Ca}^{2+}]_i\) levels in response to anti-CD3 stimulation. In fact, the additive effects on \([\text{Ca}^{2+}]_i\) increase were observed regardless of whether the cells were exposed to H\(_2\)O\(_2\) followed by anti-CD3 stimulation or with reciprocal treatment, or treated with both at the same time.

**Sublethal Levels of Oxidative Stress Stimulate Transcriptional Activation of c-jun Gene in Jurkat T Cells**

The results presented in the previous section demonstrate that micromolar concentrations of H\(_2\)O\(_2\) induce the increases of c-jun mRNA expression in Jurkat T cells. Two distinct mechanisms may contribute to the H\(_2\)O\(_2\)-induced c-jun mRNA expression. The increased levels of c-jun mRNA induced by treatment of cells with interleukin-1 have been shown to be mediated in part by increased stability of c-jun mRNA (Muegge et al, 1993). This suggests that oxidative stress may somehow posttranscriptionally stabilize c-jun mRNA and thereby prolong c-jun mRNA half-life. Another possibility for the mechanism involved in the H\(_2\)O\(_2\)-induced c-jun mRNA expression may be the
transcriptional activation of c-jun gene by the activated transcriptional factors that can transactivate c-jun promoter region. In the following studies, we test these two possibilities.

Oxidative stress has no influence on c-jun mRNA stability in Jurkat T cells

To examine whether the sustained elevations of c-jun mRNA induced in Jurkat cells by H$_2$O$_2$ were due to stabilization of c-jun mRNA levels, the half-life of c-jun mRNA was measured. Following the accumulation of c-jun mRNA in Jurkat cells treated for 45 minutes with PHA/PMA or 200 µM H$_2$O$_2$, the transcription process was blocked by adding actinomycin D and the quantity of c-jun mRNA measured at different times by RNase protection assays. As shown in Figure 14A, no discernible differences in the degradation rate of c-jun mRNA were observed in the cells exposed to PHA/PMA or H$_2$O$_2$. The calculated half-life was 55 minutes, comparable to human T cells (Chatta et al, 1994). Protein synthesis inhibitor anisomycin has been reported to increase the half-life of c-jun mRNA in some cell lines (Edwards et al, 1992). The present experiments show that the treatment of Jurkat cells with anisomycin prolonged the half-life of c-jun mRNA to 168 minutes, but coculturing cells with H$_2$O$_2$ plus anisomycin failed to further increase the half-life of c-jun mRNA (Figure 14B). To determine if H$_2$O$_2$ might prevent the normal degradation of c-jun mRNA, Jurkat cells were pretreated with anisomycin to superinduce c-jun mRNA. After removal of anisomycin, the cells were resuspended in medium alone as control or in medium containing 200 µM H$_2$O$_2$, followed by measurement of the decline of c-jun mRNA levels. As shown in Figure 14C, the rate of decline of c-jun mRNA in the
cells exposed to $H_2O_2$ was identical with control cells. These results support the conclusion that $H_2O_2$ induction of c-jun mRNA in Jurkat cells is not caused by $H_2O_2$ influence on c-jun mRNA stability.

**Oxidative stress transactivates c-jun promoter mainly through AP-1-like site jun1 in the promoter region in Jurkat T cells**

As $H_2O_2$ does not influence c-jun mRNA stability, we considered the possibility that $H_2O_2$ might activate the transcription of c-jun gene. To determine the transcriptional regulation of c-jun gene by oxidative stress, transient transfection experiments were performed in Jurkat cells with a CAT reporter plasmid containing the complete (-1600/+170) human c-jun promoter sequence (wild-type c-jun or wtjun). The schematic structures of the c-jun CAT constructors used in the study are shown in Figure 15. After transfection, the cells were treated with varying micromolar concentrations of $H_2O_2$ and other stimuli including PMA, PHA/PMA, or okadaic acid, followed by CAT assays to analyze the transactivation of c-jun promoter. The results are shown in part of Figure 16. Figure 16A presents a representative TLC pattern of CAT assays. The results show that micromolar concentrations of $H_2O_2$ dose-dependently induced 1.3- to 2.6-fold increases in the acetylation conversion of chloramphenicol in the cells transfected with wtjun compared with the untreated control cells. Okadaic acid, a protein phosphatase inhibitor and a particularly strong inducer of c-jun mRNA in Jurkat cells (Thevenin et al, 1991), exhibited a strong induction of CAT activity (8.2-fold increase over unstimulated control cells) driven by the wild-type c-jun promoter. PMA or PHA/PMA stimulated 1.2- or 1.4-fold
elevation of CAT activity in the cells transfected with wtjun, although it was less than that observed in higher concentrations of H$_2$O$_2$ (300 or 500 µM) or okadaic acid. These results suggest that oxidative stress be able to activate the transcriptional function of c-jun gene promoter in Jurkat T cells.

There are two structurally distinct AP-1-like binding sites, jun1 and jun2, in the human c-jun promoter region (Angel et al, 1988b; Hattori et al, 1988). Both sites have been implicated in the transcriptional activation of c-jun promoter in nonlymphoid cells induced by a wide range of stimuli (Stein et al, 1992; Muegge et al, 1993). To analyze the contribution of jun1 and jun2 sites to H$_2$O$_2$-induced transactivation of c-jun promoter, the full-length -1600/+170 c-jun CAT plasmid constructs containing inactive jun1 and/or jun2 sites by site-directed mutagenesis (as shown in Figure 15) were employed to transiently transfet Jurkat cells. After recovery, the cells were incubated in medium alone or stimulated with H$_2$O$_2$, PMA, PHA/PMA, or okadaic acid for 18 hours. The activation of the c-jun constructs was determined by CAT assays. The results presented in Figure 16 indicate that the inactivation of jun1 site in the construct -1600/+170mjun1 reduced H$_2$O$_2$ induction of CAT activity to 15% or less with p < 0.05 compared with -1600/+170wtjun, while PMA or PHA/PMA inducibility was completely abolished (p < 0.05). By contrast, inactivation of jun2 site in the construct -1600/+170mjun2 produced less influence on c-jun transactivation by H$_2$O$_2$, PMA, or PHA/PMA (p > 0.05). The inactivation of both jun1 and jun2 sites in -1600/+170mjun1,2 resulted in a similar reduction of CAT activity observed in the inactivation of only jun1 site. However, okadaic acid induction of c-jun
promoter transactivation exhibits a slightly different picture. Mutation of either jun1 or jun2 site partially but not completely inhibited okadaic acid activation of full-length c-jun promoter by 40 to 50% (p < 0.05) compared with wild-type c-jun promoter. About 25% CAT activity of complete c-jun promoter remained in the cells transfected with the construct containing inactivated jun1 and jun2 sites and then treated with okadaic acid, suggesting that transactivation elements other than jun1 and jun2 in the c-jun promoter region might be also responsive to okadaic acid. Thus, the jun1 but not jun2 site was essential for the induction of c-jun promoter activity in Jurkat T cells exposed to H_2O_2, PMA, or PHA/PMA, whereas optimal enhancement of c-jun transactivation by okadaic acid was mediated through both jun1 and jun2 sites as well as other promoter elements.

Characterization of nuclear protein complexes binding to AP-1 TRE, jun1, and jun2 motifs in Jurkat T cells induced by oxidative stress and other stimuli

To induce transcriptional activity of c-jun promoter in Jurkat cells, oxidative stress and other stimuli must activate the nuclear transcriptional factors that interact with jun1 or jun2 motifs. To characterize the transcriptional proteins induced by oxidative stress, quantitative in vitro protein-DNA binding assays were performed with the nuclear extracts from the cells treated with H_2O_2, PMA, PHA/PMA, or okadaic acid. After treatment for 2 hours with the indicated stimuli, the cells were extracted for nuclear proteins, which were used for the in vitro binding with jun1, jun2, or AP-1 TRE consensus oligonucleotides. It is shown in Figure 17 that micromolar concentrations of H_2O_2, PMA, PHA/PMA, or okadaic acid all induced AP-1 DNA binding activity to the TRE recognition sequence in
Jurkat cells. More than twofold increase in AP-1 TRE binding was seen compared with the unstimulated control cells. Treatment of the cells with micromolar concentrations of H$_2$O$_2$ resulted in an 1.2- to 2.0-fold increase in complex formation with jun1 oligonucleotide, although PMA, PHA/PMA, or okadaic acid stimulated a similar elevation of the binding observed with AP-1 TRE consensus. Modest increases (1.2- to 1.5-fold) in the binding to jun2 motif were observed in the cells exposed to H$_2$O$_2$, PMA, PHA/PMA, or okadaic acid. In addition, jun2 motif tended to exhibit greater constitutive binding of nuclear proteins from the unstimulated Jurkat cells compared with the jun1 and AP-1 TRE consensus oligonucleotides.

AP-1 and AP-1-like complexes are composed of distinct components mainly from Jun and Fos families. In the following studies, we tried to characterize the components of various DNA binding complexes induced by H$_2$O$_2$, PHA/PMA, or okadaic acid in blocking experiments with Abs specific for c-Fos, c-Jun, JunB, and JunD. The results of EMSAs in Figure 18 show that the protein-DNA complexes formed between the nuclear proteins from the cells exposed to H$_2$O$_2$, PHA/PMA, or okadaic acid and the AP-1 TRE or jun1 oligonucleotide were reduced and/or supershifted by pretreatment of the nuclear extracts with Abs directed against c-Fos, c-Jun, and JunD. However, pretreatment of the nuclear extracts with Abs against JunB did not result in reduction or supershift of these complexes, suggesting that there was no JunB component in the complexes. The induced complexes formed between the jun2 oligonucleotide and the nuclear proteins from the cells stimulated with H$_2$O$_2$, PHA/PMA, or okadaic acid were decreased and/or
supershifted by the pretreatment with Abs specific for c-Jun, c-Fos, and JunD, but they were also reduced by pretreatment with anti-JunB antibody. These results indicate that the protein complexes binding jun1 DNA motif contain the same components as those interacting with AP-1 TRE motif, including c-Jun, c-fos, and JunD, when they are extracted from Jurkat cells exposed to H2O2, PHA/PMA, or okadaic acid. However, the components of the complexes binding jun2 motif are composed of JunB as well as c-Jun, c-Fos, and JunD.

Since the components of the protein complexes binding AP-1 TRE, jun1, and jun2 DNA motifs overlapped, competition experiments were used to evaluate if cross interaction might occur between the DNA motifs and the protein complexes of AP-1 TRE, jun1, and jun2. The competitive tests with unlabeled oligonucleotides were conducted in EMSAs to identify the features of DNA cross-binding among the protein complexes AP-1 TRE, Jun1, and Jun2 from the Jurkat cells exposed to H2O2, PHA/PMA, or okadaic acid. The results are presented qualitatively in Figure 19A and quantitatively in Figure 19B. A similar pattern of competitive inhibitions was observed in all nuclear proteins from the cells stimulated by H2O2, PHA/PMA, or okadaic acid. Each DNA motif was the best competitor for the binding of itself. However, jun1 motif exhibited a stronger competitive inhibition for AP-1 TRE binding than jun2 motif did, the latter only showing a slightly suppressive ability for the binding compared with an unrelated DNA motif NFkB. The jun2 motif was a better competitor for jun1 binding than AP-1 TRE motif. Reciprocally, jun1 motif strongly inhibited the binding of jun2 motif compared to a slight suppression
observed with AP-1 TRE motif. These results imply that although there are similar components among the protein complexes AP-1 TRE, jun1, and jun2, each individual protein complex may still prefer to binding its own or related DNA motifs, thereby possibly conferring functional differences among these complexes.

Differential transactivation of jun1 and AP-1 TRE reporters in Jurkat T cells induced by oxidative stress and other stimuli

The binding analyses presented above indicated that micromolar concentrations of \( \text{H}_2\text{O}_2 \), PHA/PMA, and okadaic acid all could induce the nuclear protein complexes recognizing both AP-1 TRE and jun1 motifs in Jurkat cells. however, this did not exclude that differences in the transcriptional activation functions of these complexes. To examine this possibility, Jurkat cells were transfected with the CAT reporter constructs consisting of one or five copies of AP-1 consensus TRE sites (1 x TRE and 5 x TRE) from the human collagenase gene promoter or with the -79/+170 c-jun promoter CAT construct containing the jun1 element (Angel et al, 1988b). The cells were then treated with \( \text{H}_2\text{O}_2 \), okadaic acid, or PMA or PHA/PMA. The unstimulated cells were incubated in medium alone. The fold increases of CAT activity were shown in Figure 20. As can be seen, okadaic acid was clearly a more potent inducer of -79/+170 jun1-driven promoter activity than \( \text{H}_2\text{O}_2 \), PMA, or PHA/PMA. Also, okadaic acid induced three- or five-fold increases of 1 x TRE and 5 x TRE CAT activity. Treatment of the cells with micromolar concentrations of \( \text{H}_2\text{O}_2 \) resulted in 1.5- to 2.0-fold elevations of -79/+170 jun1 CAT activity, which were internally consistent with the increases observed in the activation of
the full length (-1600/+170) c-jun promoter (Figure 16). However, H$_2$O$_2$ failed to induce any increase in CAT activity of 1 x TRE or 5 x TRE, suggesting that although H$_2$O$_2$ induced the in vitro DNA binding of AP-1 TRE (Figure 17), it could not substantially activate the in vivo transcriptional activity of AP-1 TRE in Jurkat cells. PHA/PMA stimulated threefold increases in -79/+170 jun1 CAT activity, which were significantly greater than the twofold increases observed in the full length c-jun promoter reporter (p < 0.05). In addition, 1.6- to 2.0-fold increases of CAT activity in 1 x TRE and marked increases in 5 x TRE reporter were observed. The reporters with multiple synthetic TRE sites may not accurately measure physiologic AP-1 transcriptional activity in Jurkat cells (Su et al, 1994), but they may be used as a measurement to identify the differences in the transactivation of the protein complexes induced by distinct stimuli. Thus, the results revealed that the protein complexes induced by various stimuli in Jurkat cells exhibited differential features of in vivo transactivation of jun1 and TRE CAT reporters, even though they shared similar components and recognized both jun1 and AP-1 TRE motifs in in vitro EMSA.

*Sublethal Levels of Oxidative Stress Suppress IL-2 Promoter Activation in Jurkat T Cells*

Micromolar levels of oxidative stress activate intracellular signals, induce AP-1-like transcriptional proteins, and initiate expression of c-jun and c-fos genes in Jurkat T cells. These positive effects of oxidative stress on T cells seem to be contradictory in terms of oxidative stress inhibition of IL-2 production in T cells, which has been reported by
different research groups (Roth and Droge, 1987; Staite et al, 1987; Flescher et al, 1989). NFAT has been demonstrated to be a key transcriptional protein complex consisting of two components, NFATp and either c-Jun or c-Fos protein. AP-1 has been also implicated in transactivation of IL-2 gene promoter (Jain et al, 1992a and 1992b). Given the ability of oxidative stress to induce AP-1-like activity and expression of c-jun and c-fos gene, it was important to examine the influence of oxidative stress on transactivation of IL-2 gene.

**Micromolar levels of oxidative stress inhibit IL-2 production through suppression of IL-2 transcription in Jurkat T cells induced by T cell mitogen**

To examine whether oxidative stress can inhibit IL-2 production, Jurkat T cells were treated with PHA/PMA or PHA/PMA plus varying micromolar concentrations of H$_2$O$_2$. IL-2 production of the cells was then measured by enzyme-linked immunosorbent assay (ELISA). As can be seen in Figure 21, the presence of sublethal concentrations of H$_2$O$_2$ suppressed PHA/PMA-induced IL-2 production by Jurkat cells in a dose-dependent manner, with a half inhibition of IL-2 production observed at H$_2$O$_2$ 100 μM. A similar suppression of PHA/PMA- or anti-CD3/PMA-induced IL-2 production was also observed by IL-2 production assay with IL-2-dependent cell line CTLL (the results not presented).

To determine if H$_2$O$_2$ inhibition of IL-2 production may be due to suppression of IL-2 mRNA transcription, the levels of IL-2 mRNA were measured by RNase protection assays in Jurkat T cells treated with PHA/PMA or PHA/PMA plus different micromolar
concentrations of H$_2$O$_2$. Treatment of cells with H$_2$O$_2$ resulted in a dose-dependent inhibition of PHA/PMA-induced IL-2 mRNA expression (Figure 22). The inhibition by H$_2$O$_2$ of IL-2 mRNA expression tended to parallel the suppression of IL-2 production, but it seemed that IL-2 mRNA expression was more susceptible to H$_2$O$_2$ suppression than IL-2 production.

**H$_2$O$_2$ suppresses transactivation of IL-2 promoter and NFAT-driven reporter constructs in Jurkat T cells**

The results presented above demonstrate that micromolar concentrations of H$_2$O$_2$ inhibited IL-2 production through blocking IL-2 mRNA expression but provide no information about the underlying mechanisms. In the following studies, the molecular mechanisms involved in H$_2$O$_2$-mediated suppression of IL-2 were investigated. Initial experiments examined if micromolar levels of H$_2$O$_2$ might influence transactivation of IL-2 promoter. Jurkat cells were transfected with an IL-2 luciferase reporter construct driven by a full length IL-2 promoter sequence, IL-2-luc (Williams et al, 1992). The cells were then incubated with PHA/PMA or PHA/PMA plus varying concentrations of H$_2$O$_2$. Luciferase activity was determined as a measurement for IL-2 promoter activity. As shown in Figure 23, H$_2$O$_2$ suppressed IL-2 promoter activation stimulated by PHA/PMA, again, in a dose-dependent manner. The Jurkat cells transfected with a cytomegaloviral promoter luciferase reporter (CMV-luc) were used as controls, in which H$_2$O$_2$ alone actually activated CMV promoter activity.
Because transcriptional factor NFAT, consisting of NFATp and c-Jun or c-Fos protein, plays a critical role in regulation of transactivation of IL-2 gene promoter, it is reasonable to test the influence of oxidative stress on NFAT activity. For this purpose, Jurkat cells were transfected with a NFAT CAT plasmid reporter (NFAT-CAT) containing four copies of proximal NFAT sequence of human IL-2 promoter (Matilla et al, 1990). In tandem, transfection experiments were also performed with an NFκB CAT reporter consisting of three NFκB motifs. The CAT activity was then measured in the transfected cells treated with varying micromolar concentrations of H$_2$O$_2$ to obtain information about the effects of H$_2$O$_2$ itself on the transactivation functions of NFAT and NFκB elements. Data in Table 2 shows that the ability of NFκB transactivation was gradually enhanced with the increase of H$_2$O$_2$ concentrations ranged from 50 to 500 µM, which was consistent with the reports that oxidative stress can induce NFκB activity (Schreck et al, 1991). However, no changes in NFAT reporter activity were observed in the transfected cells treated with H$_2$O$_2$ (also see Figure 24A). In the next studies, the effects of micromolar levels of H$_2$O$_2$ on PHA/PMA-induced NFAT transactivation were tested in the Jurkat cells transfected with NFAT CAT reporter. The SV40 plasmid reporter was used as a control for the transfection experiments. As can be seen in Figure 24, H$_2$O$_2$ resulted in a dose-dependent suppression of NFAT transactivation function in Jurkat cells induced by PHA/PMA. After transfection, the cells were treated with PHA/PMA or PHA/PMA plus H$_2$O$_2$, and measured for CAT activity. Collectively, H$_2$O$_2$ inhibition of (1) IL-2 production, (2) IL-2 mRNA expression, (3) IL-2 promoter activity, and (4) NFAT transactivation function correlated with one another in Jurkat T cells stimulated by
PHA/PMA, as seen in Figure 25.

Since the transactivation responses of the NFAT reporter in Jurkat cells treated with PHA/PMA were exquisitely sensitive to suppression by sublethal levels of H$_2$O$_2$, we determined whether the suppression was mediated at the level of NFAT DNA-binding. The gel shift analysis in Figure 26A shows that sublethal levels of H$_2$O$_2$ failed to induce activation of NFAT binding complexes, but suppressed the generation of NFAT binding activity induced by PHA/PMA. Moreover, the suppression occurred during the induction of NFAT complexes, as sublethal levels of H$_2$O$_2$ did not inhibit the binding of performed NFAT complexes in the cells that had been stimulated with PHA/PMA (Figure 26B).
CHAPTER 4

DISCUSSION

Acute exposure of T cells to oxidative stress is well known to trigger intracellular activation events, including the elevations of $[\text{Ca}^{2+}]_i$ and the activation of protein kinases (Nakamura et al, 1993; Schieven et al, 1993 and 1994; Whisler et al, 1995), which are major components of signal transduction pathways during T cell activation by TCR/CD3 signaling. Activation of some important transcriptional factors, such as NFkB and AP-1, in response to oxidative stress, has been reported in a variety of cell types (Schreck et al, 1991; Devary et al, 1991; Meyer et al, 1993). However, it is unclear whether the changes induced in intracellular signal pathways in T cells by oxidative stress may be linked to the activation of transcriptional proteins and the expression of some early activation genes. In present study, we examined the ability of sublethal levels of $\text{H}_2\text{O}_2$ to induce expression of c-jun and c-fos genes as well as the contribution of protein kinase activity and $[\text{Ca}^{2+}]_i$ to $\text{H}_2\text{O}_2$-induced activation of c-jun gene in Jurkat T cells. The possible mechanisms linked to $\text{H}_2\text{O}_2$-stimulated increases in $[\text{Ca}^{2+}]_i$ were investigated in terms of $\text{Ca}^{2+}$ mobilization and protein kinase activities. We also presented the experimental results concerning the
transcriptional regulation of c-jun gene promoter by sublethal concentrations of H\textsubscript{2}O\textsubscript{2}. In these studies, H\textsubscript{2}O\textsubscript{2}-induced signals and effects were evaluated along with those triggered by TCR/CD3 and/or T cell mitogens. Finally, the influence of sublethal levels of oxidative stress on the transactivation of IL-2 gene was determined in Jurkat T cells.

**Requirements of the PTK and PKC Activities as well as Ca\textsuperscript{2+} Signals for Transcriptional Expression of c-jun in Jurkat T Cells Exposed to Sublethal Concentrations of H\textsubscript{2}O\textsubscript{2}**

Hydrogen peroxide readily diffuses across the plasma membrane and generates potent free radicals such as hydroxyl radical. Mammalian cells exhibit different adaptations to H\textsubscript{2}O\textsubscript{2} exposure, which are dependent on the concentrations of H\textsubscript{2}O\textsubscript{2}. It has been reported that the exposure of mammalian cells to less than 15 μM H\textsubscript{2}O\textsubscript{2} slightly stimulated cell growth and that concentrations of H\textsubscript{2}O\textsubscript{2} ranging from 120 to 400 μM induced temporary or permanent growth-arrest with no evidence of cell death. At concentrations greater than 1 mM, H\textsubscript{2}O\textsubscript{2} resulted in apoptotic-like cell death (Wieses et al, 1995). Thus, a major determinant of effects of oxidative stress on the biological behaviors of mammalian cells is the concentration of H\textsubscript{2}O\textsubscript{2}. In several prior reports, millimolar concentrations of H\textsubscript{2}O\textsubscript{2}, which are lethal pharmacologic doses, have been employed to evaluate the stimulation of intracellular signals in lymphocytes (Schieven et al, 1993a, 1993b, and 1994). The conclusions from these studies may reflect death-induced processes rather than the physiologic and adaptive response to sublethal oxidative stress. In order to avoid the strong injurious effects of H\textsubscript{2}O\textsubscript{2} on the cells, less than 500 μM concentrations of H\textsubscript{2}O\textsubscript{2}
were employed in the majority of our experiments, in which most of the cells were alive (Figure 1).

Oxidative stress can induce the expression of c-jun and c-fos genes in mammalian cells (Crawford et al, 1988; Shibanuma et al, 1988; Amstad, et al, 1992; Devary et al, 1991 ). The current results demonstrated that sublethal concentrations of H$_2$O$_2$ strongly stimulated transcriptional expression of c-jun and c-fos genes but not junB gene in Jurkat T cells, which was comparable to TCR/CD3 signaling (Figure 2). H$_2$O$_2$ induction of c-jun and c-fos mRNA in Jurkat cells was concentration-dependent and time-dependent. A similar time-course pattern was observed with H$_2$O$_2$- and PHA/PMA-induced c-jun mRNA expressions, although c-fos mRNA expression by H$_2$O$_2$ had a short time-course compared with that by PHA/PMA (Figure 3). An acute exposure (one minute) of the cells to micromolar levels of H$_2$O$_2$ was enough to trigger the signals required for c-jun mRNA expression, although a sustained exposure (45 minutes) was required for full expression (Figure 4). This suggests that once the cells have been exposed to oxidative stress, the intracellular transduction signals required for the expression of cell genes are rapidly activated. Sustained exposure would then determine the levels or characteristics of cell responses to oxidative stress stimulation. Our results are compatible with recent studies indicating that the duration of JNK activation upon exposure of T cells to UV or $\gamma$-radiation may determine whether the cells proliferate or undergo apoptosis (Chen et al, 1996). Sustained JNK induction leads to apoptosis, whereas transient JNK activation causes cell proliferation. Because oxidative stress can activate JNK (Figure 6) and induce
cell proliferation and/or apoptosis (Guytan et al, 1996; Buttke and Sandstrom, 1995), it is plausible that the duration of exposure of T cells to oxidative stress may determine which these two opposing cell behaviors predominate.

The transactivation of c-jun gene is autoregulated by its promoter elements recognized by AP-1-like transcriptional factors consisting of c-Jun/c-Jun or c-Jun/ATF2 (Angel and Karin, 1991). Phosphorylation/dephosphorylation regulation of c-Jun protein is believed to play a major role in the activation of DNA-binding and transactivation activities of AP-1 factor (Hibi et al, 1993; Derijard et al, 1994; reviewed by Hunter and Karin, 1992; Karin, 1995). Therefore, H$_2$O$_2$ induction of c-jun mRNA in Jurkat cells might rely on the activation of protein kinases and consequently the regulation of c-Jun phosphorylation status. The current results indicated that c-jun transcriptional expression in Jurkat cells in response to H$_2$O$_2$ was totally inhibited by staurosporine, an inhibitor of both PTKs and PKC, while partial inhibition was observed with more selective PKC inhibitor H7 or PTK inhibitors herbimycin A and tyrphostin (Figure 5). These findings suggest that the activities of multiple protein kinases, including PTKs and PKC, are required for full activation of c-jun gene transcription by H$_2$O$_2$ oxidative stress. Both PTKs and PKC are known to be located upstream of the intracellular signal pathways during T cell activation by TCR/CD3 signaling. Protein tyrosine kinases ZAP-70 and p56$^{ck}$ are closely linked to TCR/CD3-mediated membrane signaling and have been reported to be activated by H$_2$O$_2$-generated ROS (Nakamura et al, 1993; Schieven et al, 1994). Activation of ZAP-70 or p56$^{ck}$ PTKs triggers the intracellular signal transduction chains consisting of ras-raf-Mek-
ERKs and ras-MEKK1-JNK1-JNKs, which, in turn, regulates the transcriptional proteins TCF/Elk1 or SRF for the transactivation of c-fos gene and c-Jun and ATF2 for c-jun gene. It has been reported that \(H_2O_2\)-induced tyrosine phosphorylation and \(Ca^{2+}\) signals in T cells bypass the requirement for T cell membrane receptors CD3 and CD45 that are needed for TCR/CD3- or UV-mediated generation of intracellular signals (Schieven et al, 1994). These reports and the current results strongly suggest that \(H_2O_2\)-generated ROS may directly activate the activity of PTKs ZAP-70 or p56\(^{lek}\) and trigger intracellular signal cascades. A PTK-independent pathway leading ras as well as raf-1 kinase activation may be mediated by PKC activation (Izquierdo et al, 1992; Kolch et al, 1993). It has been known that sublethal concentrations of \(H_2O_2\) activate PKC activity in Jurkat T cells (Whisler et al, 1994). Therefore, \(H_2O_2\) activation of PKC may trigger downstream signal cascades through a PTK-independent ras activation pathway. These results seem to be compatible with our observations showing that either PTK or PKC activity contributed partially to \(H_2O_2\) induction of c-jun mRNA. Furthermore, p21\(^{ras}\) has been demonstrated to be a common target for \(H_2O_2\)-generated ROS and other oxidative stress which can directly promote guanine nucleotide exchange on p21\(^{ras}\) and activate ras activity (Lander et al, 1995). Thus, \(H_2O_2\), unlike classical TCR/CD3-mediated signaling, can intersect intracellular transduction signal cascades at multiple levels most likely through the generated ROS in T cells.

Recent studies have identified that JNKs and ERKs are important for c-jun and c-fos gene transcriptional activation, respectively (Hibi et al, 1993; Derijard et al, 1994; Minden
et al, 1994). One target substrate of JNKs is c-Jun. JNKs phosphorylate serines 63 and 73 in the amino-terminal activation domain of c-Jun and thereby potentiate its transactivation function for c-jun gene. ERKs phosphorylate and potentiate the activity of TCF/Elk-1 and thereby induce c-fos. In our experiments, it was demonstrated that sublethal concentrations of H$_2$O$_2$ stimulated the kinase activities of both JNKs and ERKs in Jurkat T cells (Figure 6), which was internally consistent with the observation that H$_2$O$_2$ induced the expression of both c-jun and c-fos genes. H$_2$O$_2$ induction of JNK and ERK activities may be due to the activation of upstream components of signal transduction pathways as discussed above. However, our results did not formally exclude that H$_2$O$_2$ might directly regulate the activities of JNKs and ERKs.

The current results showed that the depletion of intracellular Ca$^{2+}$ by Ca$^{2+}$ chelator BAPTA in Jurkat T cells blocked c-jun expression in the cells treated with H$_2$O$_2$, and that the c-jun inhibition paralleled the reduction in [Ca$^{2+}$]$_i$ (Figure 7). This suggests that H$_2$O$_2$ induction of c-jun mRNA need Ca$^{2+}$ second signals in addition to the activities of PTKs and PKC. Because the depletion of intracellular Ca$^{2+}$ inhibited the constitutive expression of c-jun mRNA in untreated Jurkat cells, our results suggest that the level of [Ca$^{2+}$]$_i$ may be an essential factor for c-jun expression in Jurkat cells. TCR/CD3-induced activation of T cells is believed to be mediated by dual signals which can be provided by the PKC activity and Ca$^{2+}$ responses (reviewed by Mueller et al, 1989; Rao, 1991). Also, the requirement for the dual signals has been demonstrated in the stimulation of full JNK activity in T cells, consistent with TCR/CD3-mediated T cell activation (Su et al, 1994).
Similarly, H$_2$O$_2$-stimulated c-jun expression in Jurkat T cells also requires the participation of the dual signals. It is unknown whether the requirement of H$_2$O$_2$-induced c-jun expression for Ca$^{2+}$ signals may only reflect the need for Ca$^{2+}$-mediated PKC activation or other activation events. However, it seems that both Ca$^{2+}$ signals and PKC activation are required because while PKC activation is sufficient to induce AP-1 binding activity an additional [Ca$^{2+}$] increase is necessary for induction of functional AP-1 transcriptional activity in mouse T cells (Rincon and Flavell, 1994).

**Ca$^{2+}$ Channels and PTK Activity are Required for H$_2$O$_2$-Induced [Ca$^{2+}$] Increases in Jurkat T Cells**

An increase in the level of cytosolic free Ca$^{2+}$ concentration in T cells is known to function as a major signaling event for cellular biological behaviors, such as cell growth and programmed cell death (Gardner, 1989; Golstein et al, 1991). Acute changes in the redox state of T cells during oxidative stress are also known to be capable of stimulating Ca$^{2+}$ signaling pathway as well as the activation of PTKs and serine/threonine kinases (Schieven et al, 1993a and 1994). Our results indicated that c-jun transcriptional activation by sublethal levels of H$_2$O$_2$ in Jurkat cells was closely related to the Ca$^{2+}$ signaling and the activities of PTKs and PKC. However, relatively little has been known about the mechanisms involved in the oxidative stress regulation of Ca$^{2+}$ signal induction in T cells. In present study, we demonstrated that micromolar concentrations of H$_2$O$_2$ triggered marked elevations of [Ca$^{2+}$] in Jurkat T cells. Although both Ca$^{2+}$ channels and
PTK activity contributed to the Ca\(^{2+}\) mobilization in response to oxidative stress, there were differences that could be distinguished from those mediated by T cell mitogens or TCR/CD3 signaling.

Our data demonstrated that the acute exposure of Jurkat cells to micromolar levels of H\(_2\)O\(_2\) led to dose-dependent elevations of [Ca\(^{2+}\)]\(_i\) (Figure 8). The stimulation of Jurkat cells with micromolar concentrations of H\(_2\)O\(_2\) or T cell mitogen PHA resulted in similar kinetic patterns of Ca\(^{2+}\) mobilization. In absence of extracellular Ca\(^{2+}\), both H\(_2\)O\(_2\) and PHA stimulation induced the early mobilization of Ca\(^{2+}\) from internal stores, accounting for about 20% of overall [Ca\(^{2+}\)]\(_i\) increase (Figure 9). The subsequent addition of Ca\(^{2+}\) to the extracellular medium caused substantial increases (about 80% of overall increase) in [Ca\(^{2+}\)]\(_i\) in the cells that had been exposed to H\(_2\)O\(_2\) or PHA, suggesting the influx of extracellular Ca\(^{2+}\) as the major source of Ca\(^{2+}\) mobilization. These results are generally consistent with previous studies in terms of Ca\(^{2+}\) kinetic patterns in mammalian cells (Roveri et al, 1992; Hess et al, 1993; Doan et al, 1994).

Our results also showed that [Ca\(^{2+}\)]\(_i\) elevations evoked by micromolar concentrations of H\(_2\)O\(_2\) or PHA in Jurkat cells were quite sensitive to Ca\(^{2+}\) channel blockers. However, the difference in sensitivity to various Ca\(^{2+}\) channel blockers was observed between the [Ca\(^{2+}\)]\(_i\) increases induced by the two stimuli (Figure 10). The peak [Ca\(^{2+}\)]\(_i\) rise triggered by H\(_2\)O\(_2\) was inhibited more than 50% by micromolar concentrations of nickel chloride, but no suppression of PHA-mediated [Ca\(^{2+}\)]\(_i\) elevations was observed. Conversely, low
concentrations of nifedipine did not inhibit H$_2$O$_2$-stimulated [Ca$^{2+}$]$_i$ rise but suppressed [Ca$^{2+}$]$_i$ increases triggered by PHA. Ca$^{2+}$ influx mediated by either voltage-dependent and voltage-independent Ca$^{2+}$ channels have been reported in human T cells or Jurkat cells in response to TCR/CD3 or mitogen signalings (Gardner, 1989; Dupuis et al, 1989; Densmore et al, 1992; Zweifach and Lewis, 1993). These two types of T cell Ca$^{2+}$ channels seem different from either typical L-type or T-type Ca$^{2+}$ channels. Thus, at present, we do not know the exact properties of the Ca$^{2+}$ channels through which oxidative stress and T cell mitogen stimulate Ca$^{2+}$ influx in Jurkat cells. It is thought that Ni$^{2+}$ and nifedipine are effective blockers for different types of calcium channels (Tsien et al, 1990; Silva et al, 1994). However, the nickel sensitivity and nifedipine insensitivity of Ca$^{2+}$ influx in response to H$_2$O$_2$ are compatible with oxidative signals inducing Ca$^{2+}$ influx through voltage-independent cation channel (Zweifach and Lewis, 1993). The observation that H$_2$O$_2$- and PHA-mediated Ca$^{2+}$ influx in Jurkat cells were differentially interrupted by selective Ca$^{2+}$ channel blockers could represent the presence of heterogeneous Ca$^{2+}$ channels in Jurkat T cells, which differ in their sensitivities and have distinct functions in response to extracellular signalings and channel blockers.

The current observations clearly demonstrated that Ca$^{2+}$ influx in Jurkat cells induced by TCR/CD3, mitogen, and oxidative signals required the activity of PTKs. The PTK inhibitor genistein, at concentrations up to 200 μM, failed to inhibit H$_2$O$_2$-induced Ca$^{2+}$ elevations but effectively blocked anti-CD3- and PHA-stimulated Ca$^{2+}$ responses (Figure 11 and Table 1). By contrast, herbimycin A, a more potent inhibitor of PTKs, was highly
effective in blocking Ca\(^{2+}\) mobilization of Jurkat cells stimulated by either H\(_2\)O\(_2\), PHA, or TCR/CD3 signals. The complete blockade of Ca\(^{2+}\) elevations by staurosporine, a potent inhibitor of PTKs as well as several other protein kinases, was also observed in response of Jurkat cells to these stimuli, further supporting the PTK-dependent Ca\(^{2+}\) responses. In addition, herbimycin A-pretreated cells were fully capable of responding to AIF\(_i^+\) with a normal rise in \([C^{2+}]_i\), suggesting that the inhibitory effects of herbimycin A was mediated via specific suppression of PTKs rather than representing a nonspecific interference with the cell metabolism or the suppression of PLC-\(\gamma\) activity. It is, at present, unclear why \([Ca^{2+}]_i\) responses induced by H\(_2\)O\(_2\) were inhibited by herbimycin but not genistein. The failure of genistein to inhibit responses with H\(_2\)O\(_2\) could represent the differences between H\(_2\)O\(_2\) versus TCR/CD3 calcium entry channels or the types of PTKs regulating these channels. Different members of the PTK family in T cells have been proposed to respond to TCR/CD3 versus oxidative signals. At least three non-receptor PTKs, p59\(^{\text{yn}}\), p56\(^{\text{ck}}\), and ZAP-70, have been linked to T cell signal transduction following stimulation of the TCR/CD3 complex (reviewed by Perlmutter et al, 1993; Rudd et al, 1994). However, Schieven et al found that oxidative signals partially bypass the requirements of TCR/CD3- or UV-mediated activation events for CD3 and CD45 receptor expression and selectively stimulated ZAP-70 tyrosine kinase activity as well as induced Ca\(^{2+}\) elevations in Jurkat cells (Schieven et al, 1994). The activation of p59\(^{\text{yn}}\) and p56\(^{\text{ck}}\) by oxidative signals remains controversial because disparate observations have been reported (Nakamura et al, 1993; Secrist et al, 1993; Schieven et al, 1993). PTKs p59\(^{\text{yn}}\) and p56\(^{\text{ck}}\) were recently implicated in regulating Ca\(^{2+}\) influx in T cells stimulated by anti-CD3 and oxidative stress,
respectively (Rigley et al., 1995; Nakamura et al., 1993). In addition, it has been reported that neither ZAP-70 activity itself nor tyrosine phosphorylation of the TCR was required for Ca\(^{2+}\) responses in T cells (Niklinska et al., 1992). Thus, at present, it is unclear what types of PTKs may be involved in H\(_2\)O\(_2\)-induced Ca\(^{2+}\) mobilization in Jurkat cells. The fact that distinct classes of PTKs are activated by TCR/CD3 and oxidative signals along with the current results suggest that oxidative stress triggers Ca\(^{2+}\) influx in Jurkat T cells through a mechanism different from that induced by stimulation of T cell surface receptor. Another possible explanation for the failure of genistein to block H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx may be that genistein is simply a less effective inhibitor of PTK activity than herbimycin A. Although both herbimycin A and genistein can inhibit src PTKs, each has a different mechanism of action. It has been shown that herbimycin A can irreversibly inactivate PTK activity probably through interactions with protein sulphydryl groups (Vehara et al., 1989). Furthermore, the mechanisms by which the activation of PTKs by oxidative stress regulates Ca\(^{2+}\) influx remains unknown. Schieven et al reported that H\(_2\)O\(_2\) stimulated Ins-1,4,5-P3 production in a tyrosine kinase dependent manner and induced Ca\(^{2+}\) signals in B lymphocytes (Schieven et al., 1993). Ins-1,4,5-P3 is a degradation product from the breakdown of membrane phosphotidylinositide and regulates Ca\(^{2+}\) internal release from cytosolic store (reviewed by Majerus, 1992). The released Ca\(^{2+}\) from internal store is believed to activate the calcium channels on plasma membrane and subsequently Ca\(^{2+}\) influx (Dolmetsch and Lewis, 1994; Zweifach and Lewis, 1993). Whether H\(_2\)O\(_2\) induction of tyrosine kinase-dependent Ca\(^{2+}\) influx in Jurkat T cells operates in a similar way remains to be determined.
The chronic exposure of T cells to polyamine-generated oxidative products has been reported to suppress Ca$^{2+}$ responses triggered by PHA (Flescher et al, 1994). The current results demonstrated that the acute exposure of Jurkat cells to micromolar concentrations of H$_2$O$_2$ failed to inhibit Ca$^{2+}$ responses upon subsequent stimulation with cross-linked anti-CD3 antibodies. In fact, additive effects on Ca$^{2+}$ elevations were observed in the cells treated with combination of H$_2$O$_2$ and anti-CD3 antibodies (Figure 13). The contrasting effects of H$_2$O$_2$ and polyamine products on surface receptor-stimulated Ca$^{2+}$ responses might be related to the types of ROS generated, the duration of exposure to oxidative stress, or the intracellular levels of oxidative radicals generated by H$_2$O$_2$. Different concentrations of H$_2$O$_2$ can induce cell growth as well as reversible and irreversible cell growth arrest (Wiese et al, 1995). These distinct biologic effects of H$_2$O$_2$ depend on the formation of hydroxyl radicals that seem to be the active form of ROS (reviewed by Sies, 1991). By comparison, the oxidation of polyamines generates aldehydes, ammonia, and H$_2$O$_2$ (Mondovi et al, 1988; Flescher et al, 1991). Thus, these polyamine by-products in combination with ROS could conceivably mimic the action of chemical oxidants that have been reported to suppress CD3- and CD4-induced Ca$^{2+}$ responses in T cells, presumably by dissociating CD3 and CD4 receptor coupling from PTKs (Kanner et al, 1992).
Sublethal Levels of Oxidative Stress Stimulate Transcriptional Activation of c-jun Promoter Preferentially through Proximal AP-1-like Element (jun1)

Although T cell expression of c-Jun/AP-1-dependent lymphokine genes has been extensively studied, the transcriptional regulation of the c-jun gene in T cells is poorly understood. Our results demonstrate that micromolar concentrations of H$_2$O$_2$ induce substantial and sustained elevations of c-jun mRNA in Jurkat T cells (Figure 1 and 2). At least two mechanisms may contribute to oxidatively related increase of c-jun mRNA. First, oxidative signals may post-transcriptionally facilitate the stability of c-jun mRNA and thereby prolong its half-life as previously reported in okadaic acid- and IL-1-induced c-jun mRNA expression (Lee et al, 1993; Muegge et al, 1993). Second, H$_2$O$_2$ induction of c-jun transcription may represent the transactivation of c-jun promoter. Our results indicated that both micromolar concentrations of H$_2$O$_2$ and PHA/PMA did not induce c-jun mRNA stabilization (Figure 14). In addition, H$_2$O$_2$ did not influence the normal decline in c-jun mRNA induced by anisomycin. These results are consistent with prior studies of oxidative regulation of c-jun mRNA stabilization in T cells (Chatta et al, 1994). Furthermore, our results pointed toward the possible transactivation of c-jun gene promoter by H$_2$O$_2$ as a primary mechanism for the induction of c-jun mRNA.

It has been demonstrated that c-jun promoter region contains two functional AP-1-like DNA motifs, jun1 and jun2, as shown in Figure 15, which are linked to the regulation of c-jun promoter transactivation by c-Jun homodimers or c-Jun/ATF-2 heterodimer (Angel et
al, 1988b; Devary et al, 1991; Stein et al, 1992; van Dam et al, 1993). Although both AP-
1-like binding sites are functional, the proximal site jun1 seems to be a major contributor
to the responses of c-jun promoter to extracellular stimulation. The current results
showed that the jun1 promoter element was required for transcriptional activation of the
complete c-jun promoter in Jurkat cells in response to both the costimulatory signals
generated by PHA/PMA and oxidative signals produced by micromolar concentrations of
H₂O₂ (Figure 16). Inactivation of jun1 motif by site-directed mutagenesis markedly
diminished the CAT response driven by the complete c-jun promoter to PHA/PMA and
H₂O₂, whereas inactivation of distal jun2 site had no effect. The simultaneous inactivation
of both jun1 and jun2 sites resulted in similar effects on c-jun promoter activation
observed in inactivation of jun1 alone. By contrast, the full response of c-jun promoter to
okadaic acid, a potent activator of c-jun expression in Jurkat cells (Thevenin et al, 1991),
required existence of both jun1 and jun2 sites, agreeing with a previous report (Park et al,
1992). These results indicated that H₂O₂ as well as T cell mitogen activation of c-jun
promoter in Jurkat T cells preferentially needed jun1 element, although jun2 was
functional in c-jun promoter activation induced by other stimuli such as okadaic acid.

For the activation of c-jun promoter, H₂O₂ and other stimuli must have facilitated the
activity of transcriptional factors that selectively act on the required elements within c-jun
promoter region. Our results with EMSAs showed that micromolar concentrations of
H₂O₂ effectively activated the DNA-binding activity of AP-1 TRE and jun1-recognizing
factor(s) in Jurkat cells as did PMA, PHA/PMA, and okadaic acid. Relatively modest
increases in binding activities were observed for jun2-recognizing factor(s) with all stimuli (Figure 17). The DNA-binding complexes of AP-1 TRE and jun1 seemed share common components including c-Jun, c-Fos, and JunD but not JunB, which was detected in jun2-recognizing complexes. In addition, the current results also indicated that there were cross-bindings of DNA motifs among the protein complexes AP-1 TRE, jun1, and jun2. This might partially explain why we could not identify the specific complexes c-Jun homodimer for jun1 motif and c-Jun/ATF-2 for jun2 motif as reported by other groups (Angel et al, 1988; van Dam et al, 1993). However, at present, we can not exclude the possibility that the different stimulation of varying cell types might result in the formation of the protein complexes containing distinct components. For example, IL-1 induction of c-jun promoter in a human hepatoma cell line provided such example in which jun1 and jun2 protein complexes induced by IL-1 contained neither Jun nor Fos component but exhibited in vitro DNA cross-bindings between each other and with AP-1 TRE (Muegge et al, 1993).

The observation that oxidative stress and costimulatory signals provided by PHA/PMA effectively activated in vitro DNA-binding activity of AP-1 TRE and jun1 raised the possibility that the activation of DNA-binding activity might represent in vivo transactivation functions. The current results showed that H2O2 induced activation of -79/+170 c-jun reporter containing jun1 motif but no transactivation activity was observed with AP-1 TRE constructs (Figure 20). By contrast, PHA/PMA and okadaic acid activated both reporter constructs. These results further supported the observation that
jun1 element mediated H$_2$O$_2$-induced activation of c-jun promoter in Jurkat cells. However, the mechanisms underlying the absence of a direct correlation between in vitro DNA-binding and in vivo transcriptional functions of AP-1 TRE stimulated by H$_2$O$_2$ are unclear. It is possible that unlike protein-oligonucleotide interactions in EMSAs, more complicated biochemical conditions and DNA architectural structures may be involved in the in vivo protein-DNA interactions and transactivation (Pabo, 1992). In addition, we cannot exclude the possibility that the H$_2$O$_2$-induced protein complexes interacting with AP-1 TRE motif may have unrecognized differences in composition which account for the functional differences in CAT assays and thereby in functions from those stimulated by PHA/PMA. The current results also showed that the costimulatory signals provided by PHA/PMA were significantly more effective than oxidative signals in stimulating transactivation of the -79/+170 jun1 reporter ($p < 0.05$, Figure 20). However, this observation was not internally consistent with the results obtained from RNase protection assays showing that oxidative signals and PHA/PMA induced equivalent increases in the expression of c-jun mRNA (Figure 2). This apparent discrepancy can be reconciled by our demonstration that the transactivation of the complete c-jun promoter was more effectively stimulated in response to oxidative signals than to the costimulatory signals. For oxidative signals, the activation of the -79/+170 jun1 reporter paralleled the transcriptional activation of the complete c-jun promoter. By contrast, the response of the complete c-jun promoter to PHA/PMA was about 50% less than that of the -79/+170 jun1 reporter. The mutational inactivation of the jun2 motif did not improve the response of the complete c-jun promoter construct to PHA/PMA. Thus, these results suggested that
the response of the complete c-jun promoter to the T cell costimulatory signals was being positively regulated by jun1 motif as was the response to oxidative signals, but negatively regulated by c-jun promoter elements other than jun1 or jun2. Furthermore, the differences in transactivation of c-jun promoter between oxidative and T cell costimulatory signals provided further support for the concept that the distinct molecular mechanisms are involved in intracellular activation events induced by these two types of extracellular signals in Jurkat T cells, as was observed in Ca^{2+} influx regulation by the two signals.

Sublethal Levels of Oxidative Stress Suppress Activation of NFAT-Dependent IL-2 Promoter in Jurkat T Cells

TCR/CD3 and costimulatory signals stimulate T cell activation and induce the expression of IL-2. Transcriptional protein complex NFAT has been identified as a critical transcriptional regulator of IL-2 promoter activation. Except for a pre-existing cytoplasmic protein NFATp, NFAT contains Fos/Jun AP-1 components that are necessary for NFAT interaction with its DNA motifs within IL-2 promoter region and subsequent IL-2 gene transcriptional activation (Jain et al, 1993; Petrak et al, 1994; Yaseen et al, 1994). Oxidative stress inhibition of T cell production of IL-2 has been previously reported (Roth and Droge, 1987; Staite et al, 1987; Flescher et al, 1989), but the exact mechanisms remain unknown. In view of our data showing that oxidative stress induced c-jun and c-fos expression as well as AP-1 activity, the effects of sublethal levels of oxidative stress on IL-2 production and IL-2 promoter transcriptional activation were
examined.

Our results indicated that the acute exposure of Jurkat T cells to micromolar concentrations of \( \text{H}_2\text{O}_2 \) resulted in a dose-dependent inhibition of IL-2 production in response to costimulatory signals (Figure 21). \( \text{H}_2\text{O}_2 \) inhibition of IL-2 production correlated well with suppression of IL-2 mRNA expression as demonstrated by RNase protection assays (Figure 22). Experiments employing transient transfection of Jurkat cells with a CAT plasmid construct driven by a complete length human IL-2 promoter showed that micromolar levels of \( \text{H}_2\text{O}_2 \) inhibited IL-2 promoter transactivation induced by PHA/PMA-generated costimulatory signals (Figure 23). Furthermore, we demonstrated that \( \text{H}_2\text{O}_2 \) effectively suppressed in vivo transactivation ability of transcriptional complex NFAT (Figure 24), which paralleled with \( \text{H}_2\text{O}_2 \) inhibition of IL-2 production, IL-2 mRNA expression, and IL-2 promoter activation. In addition, the analyses of NFAT DNA-binding suggested that \( \text{H}_2\text{O}_2 \) could block in vitro binding of NFAT probably through interference with the formation of NFAT complexes (Figure 26). Thus, these results suggest a scenario in which micromolar concentrations of \( \text{H}_2\text{O}_2 \) inhibit the formation and transactivation potential of NFAT leading to suppression of IL-2 transcriptional activation and, in turn, IL-2 production.

The current results with EMSAs indicated that micromolar concentrations of \( \text{H}_2\text{O}_2 \) inhibited in vitro DNA-binding of NFAT. However, this inhibition might occur prior to the interaction of NFAT with its DNA motif because no \( \text{H}_2\text{O}_2 \) suppression was observed in
the Jurkat cells pre-stimulated by PHA/PMA (Figure 26B). These results also suggested that H$_2$O$_2$ could not influence preformed NFAT complexes. It has been known that several intracellular processes are involved in the generation of transcriptional activity of NFAT (reviewed by Rao, 1994). Cytosolic pre-existing component NFATp, although having specific DNA-binding activity, has to be dephosphorylated by calcineurin to acquire the ability of translocalization into nucleus and target its DNA motif. Interaction of NFATp with the members of Jun/Fos families results in formation of stable NFAT-DNA complex which activates the target gene promoter. Thus, H$_2$O$_2$ may interfere with one or more processes involved in formation of NFAT activity, including calcineurin-dependent NFATp activation, NFATp translocation, or formation of NFAT complex, although we have no evidence at present to distinguish among these possibilities. Recent reports suggest that IL-2 transcription is regulated negatively and positively by two Ca$^{2+}$/calmodulin-responsive enzymes, multifunctional Ca$^{2+}$/calmodulin kinase (CaM kinase) and calcineurin (Ngheim et al., 1994). In presence of costimulatory signals generated by phorbol diester and Ca$^{2+}$ ionophore, the Ca$^{2+}$ influx in T cells leads to activation and transcription of IL-2 via calcineurin. However, in absence of phorbol diester, Ca$^{2+}$ influx induced by Ca$^{2+}$ ionophore actually results in anergy and IL-2 transcriptional block through CaM kinase. These suggest that CaM kinase and calcineurin are differentially regulated in T cells in response to extracellular stimuli and serve to negatively and positively influence T cell responsiveness. Although our results indicated that oxidative stress induced c-jun expression in Jurkat cells via Ca$^{2+}$ and PKC dependent pathways, the possibility remains that the activation of CaM kinase and/or calcineurin may be involved. Micromolar
concentrations of H$_2$O$_2$ have been observed to activate multiple serine/threonine kinases and suppress protein phosphatases in Jurkat T cells (Whisler et al, 1995). Further work is needed to test the hypothesis that H$_2$O$_2$ may inhibit the activation of NFATp through the stimulation of CaM activity or the suppression of calcineurin activity. Our observations that there were differences in the induction of Ca$^{2+}$ signals and AP-1 transactivation activity between H$_2$O$_2$ stimulation and costimulation generated by PHA/PMA or TCR cross-linkage, may reflect the involvement of different intracellular signal mechanisms in these two types of extracellular stimuli in T cells. In fact, oxidative stress has been reported to activate the mechanisms responsible for programmed cell death in T cells (reviewed by Thomas et al, 1994). Thus, it will be important to determine whether distinct intracellular events may be triggered by oxidative and costimulatory signals in T cells.

Oxidative stress has the divergent effects on transcriptional factors, including the activation and inhibition of their DNA-binding and transactivation activities (Schreck et al, 1991; Devary et al, 1991; Meyer et al, 1993). Our results demonstrated that micromolar concentrations of H$_2$O$_2$ activated the DNA-binding of AP-1 or AP-1-like jun1 and the transactivation of NFkB or AP-1-like jun1, but not AP-1. In addition, the current data demonstrated that H$_2$O$_2$ actively inhibited NFAT DNA-binding and transactivation in Jurkat T cells. Cyclosporin A (CsA) and glucocorticoids are two well-known compounds that can inhibit IL-2 production at the transcriptional level (Matilla et al, 1990; Paliogiann et al, 1993). CsA is believed to suppress calcineurin activity and thereby the translocation
of NFATp into nucleus and the formation of activated NFAT complex. Glucocorticoid-induced inhibition of IL-2 may be due to the its interference with AP-1 action on IL-2 promoter. Interestingly, although T cells exposed to glucocorticoids undergo apoptosis, CsA can effectively protect T cells from apoptosis induced by TCR/CD3-mediated stimulation but not by glucocorticoids (Iwata et al, 1991; Zhao et al, 1995). It has been demonstrated that in Jurkat cells both CsA and dexamethasone inhibit in vitro DNA binding of AP-1 and NFAT, but not NFκB, to their corresponding sites in IL-2 promoter (Paliogianni et al, 1993). However, with the analyses of transient transfections, dexamethasone inhibited the transactivation of IL-2 promoter and AP-1, but not NFAT and NFκB, whereas CsA suppressed the transactivation of IL-2 promoter and NFAT, but not AP-1 and NFκB. Our results indicated that oxidative stress, like CsA, inhibited the transcriptional activation of both IL-2 promoter and NFAT, while stimulated the transactivation activity of NFκB and the DNA binding of AP-1 TRE. These observations are compatible with the interpretation that oxidative stress may stimulate distinct intracellular events and generate more divergent effects on transcriptional factors in T cells compared to either CsA or glucocorticoids.

In current study, the divergent effects of oxidative stress on transcriptional proteins in Jurkat T cells resulted in c-jun gene activation and IL-2 gene inhibition. The biologic significances of the oxidant-induced uncoupling of c-jun activation from AP-1 TRE and NFAT remain unclear. Exposure to low concentrations of H₂O₂ has been demonstrated to induce apoptosis in a variety of cell types (Lennon et al, 1991). Many of the biological,
chemical, and physical treatments, such as TNF, ionizing and ultraviolet radiation, are capable of evoking oxidative stress and induce apoptosis (reviewed by Thomas et al, 1994). The activation-induced apoptosis in T cells may also depend on the stimulation of intracellular oxidative stress (Sandstrom et al, 1994). In addition, some of the thiol reductants and free radical scavengers have been shown to be capable of inhibiting apoptosis, which include thioredoxin, N-acetylcysteine, and glutathione peroxidase. Recently, it has been described that Bcl-2 protein, a potent inhibitor of apoptosis caused by diverse stimuli, blocks apoptosis through an antioxidant mechanism (Hochenbery et al, 1993). All these provide strong evidence to support the concept that oxidative stress most likely plays an important role in induction of apoptosis. However, the mechanisms by which oxidative stress induces apoptosis, up to now, remain unknown. Expression of c-jun and c-fos genes prior to apoptosis has suggested that c-Jun and c-Fos may participate in apoptotic processes (Smeyne et al, 1993; Manome et al, 1993; Preston et al, 1996). Overexpression of c-Jun and persistent activation of c-Jun N-terminal kinase JNKs have provided in direct evidence that c-Jun is involved in induction of apoptosis (Chen et al, 1996). The induction of apoptosis by growth factor deprivation has been widely studied. IL-2-dependent T cells undergo apoptosis after IL-2 is removed from the medium (Collins et al, 1994). Thus, our observations that H$_2$O$_2$ activated c-jun and c-fos expression and simultaneously blocked IL-2 expression, are compatible with the possibility that oxidative stress may induce apoptosis in T cells through stimulating two different types of apoptosis-induced mechanisms, c-Jun- or c-Fos-mediated and IL-2-deprived mechanisms, although direct evidence for this possibility remains provided.
Pretreatment of lymphocytes with sublethal concentrations of \( \text{H}_2\text{O}_2 \) can elicit a response which is protective against DNA damage normally inducible in these cells by subsequent exposure to toxic doses of \( \text{H}_2\text{O}_2 \) (Barnett et al, 1995). The increase in survival is accompanied by the maintenance of the proliferative capacity of the cells and may be related to the induction of stress proteins (Marini et al, 1996). In fact, the activation of T cells by extracellular signals including the stimulation of TCR/CD3 complex also results in excessive production of intracellular ROS (Buttke and Sandstrom, 1995), suggesting that certain extracellular stimuli induce both prooxidants and antioxidants in T cells. Thus, the balance of prooxidants and antioxidants or redox status of cells in response to extracellular stimuli may determine the cell fate, proliferation or death. c-Jun/AP-1 has been implicated in the transcriptional regulation of genes for glutathione S-transferases and other enzyme systems capable of defending against ROS and genotoxins (Friling et al, 1992; Moffat et al, 1994; Pinkus et al, 1993). In addition, the depletion of intracellular GSH with buthionine-S, R-sulfoximine, or diamide stimulates AP-1-dependent transcriptional activation of the glutathione S-transferases Ya gene (Bergelson et al, 1994). These findings suggest that the highly efficient activation of c-jun by oxidative signals may serve to facilitate the expression of AP-1-dependent genes, whose products can restore cellular redox equilibrium or are otherwise protective.
Summary

Sublethal levels of H$_2$O$_2$ stimulated mRNA expression of c-jun and c-fos genes in Jurkat T cells. c-jun expression was shown to be dependent on both PTKs and PKC as specific inhibitors for PTKs and PKC partially suppressed the expression of c-jun. The phosphorylation activity of selective kinases, JNKs for c-Jun and ERK for TCF/Elk-1, were activated by H$_2$O$_2$. The elevations of [Ca$^{2+}$]$_i$ in Jurkat cells induced by H$_2$O$_2$ was a necessary signal for c-jun gene expression. Both the release from internal stores and the influx of extracellular Ca$^{2+}$ through calcium channels contributed to the H$_2$O$_2$-induced Ca$^{2+}$ mobilization. The Ca$^{2+}$ influx triggered by H$_2$O$_2$ was mediated by the Ca$^{2+}$ channels, which appeared distinct from those stimulated by TCR/CD3 antibody or T cell mitogen with respect to the sensitivity to specific channel blockers and the dependence on PTKs. H$_2$O$_2$ induction of c-jun expression occurred at transcriptional activation level. H$_2$O$_2$ effectively activated transcriptional activity of c-jun promoter preferentially through the regulation of jun1 element within c-jun promoter, which exhibited distinct responses to oxidative and costimulatory signals. Although oxidative stress activated the DNA-binding activity of several transcriptional factors, including AP-1 TRE, AP-1-like jun1, and NFkB, sublethal levels of H$_2$O$_2$ inhibited NFAT DNA-binding and transcriptional activation in Jurkat cells, which was responsible for H$_2$O$_2$-induced suppression of IL-2 promoter and IL-2 production. H$_2$O$_2$ also interfered with the formation of activated NFAT complex because the DNA-binding of the preformed NFAT complex was not affected by H$_2$O$_2$. The results suggested that sublethal levels of oxidative stress initiate the mechanisms of
intracellular signal transduction and activate or inhibit the expression of some critical genes in T cells. The intracellular events induced by oxidative stress could be distinguished from those triggered by TCR/CD3 or mitogen signals. Thus, oxidative stress is capable of regulating certain key transcriptional proteins in T cells, which are well known to play a critical role in governing the expression of many genes and the biological functions, such as cell growth and cell death.
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Biol. Chem. 268:3889-3896.


APPENDIX

FIGURES AND TABLES
Figure 1. Effects of different concentrations of $\text{H}_2\text{O}_2$ and exposure duration of cells to $\text{H}_2\text{O}_2$ on viability of Jurkat cells. The Jurkat cells ($2 \times 10^6$ cells/ml) were treated for varying times with micromolar concentrations of $\text{H}_2\text{O}_2$ as indicated in RPMI 1640 medium supplemented with 1% FCS. At the end of treatment, the cell viability was measured by trypan blue exclusion test and plotted against time points. Results are representative of three separate experiments.
Figure 1
Figure 2. Induction of c-jun and c-fos mRNA expression by micromolar concentrations of H$_2$O$_2$ in Jurkat T cells. Jurkat cells (3 x 10$^6$ cells/ml) were incubated in medium with 0.5% FCS alone (control) or exposed to varying concentrations of H$_2$O$_2$, 50 µM cumene hydroperoxide (CHP), or 5 µg/ml of cross-linked anti-CD3 MAb. After 45 minutes, the cells were harvested and washed in PBS buffer. Cytosolic RNA was extracted and RNase protection assays performed with the indicated riboprobes as described in Methods. The graphs on the top of each autoradiographic profile represent the size of each riboprobe and its protected fragment. The protected fragments specific for individual mRNAs are indicated by the horizontal arrows. The autoradiographic profiles of the different probes alone incubated in the presence (+) or absence (-) of RNase A and T1 are presented in the right two lanes. Results are representative of three separate experiments.
Figure 3. Time-dependent expression of c-jun and c-fos mRNA induced by H₂O₂ or PHA/PMA in Jurkat T cells. 3 × 10⁶ Jurkat cells in medium containing 0.5% FCS were incubated with 200 μM H₂O₂, or with 10 μg/ml PHA plus 50 ng/ml PMA. Control cells were incubated in medium alone. Total cytoplasmic RNA was isolated at the indicated incubation time. 5 μg RNA for each was analyzed by RNase protection assay as described in Methods. The size of protected fragments specific for c-jun (A) and c-fos (B) are indicated to the left. Shown are representative films from two set of repeat experiments.
Figure 3
Figure 4. Kinetics of c-jun mRNA induction in Jurkat T cells exposed to H\textsubscript{2}O\textsubscript{2} for varying time intervals. Jurkat cells were incubated in medium alone or treated with 100 \muM H\textsubscript{2}O\textsubscript{2} for different times. At the indicated time, the cells were washed free of H\textsubscript{2}O\textsubscript{2} and further incubated for 45 minutes in medium alone (lanes 5-7). Cytosolic RNA was extracted and the levels of c-jun mRNA determined by RNase protection assays and autoradiography of the protected fragment specific for c-jun.
Figure 4

<table>
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<tr>
<th>H$_2$O$_2$ Pulse</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>15</th>
<th>1</th>
<th>5</th>
<th>15</th>
<th>45</th>
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<tr>
<td>Medium</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 5. Influence of PTK and PKC inhibitors on H$_{2}$O$_{2}$ induction of c-jun mRNA expression in Jurkat T cells. 3 x 10$^{6}$ Jurkat cells in medium with 0.5% FCS were incubated in medium alone (-) as controls, or pretreated with the indicated micromolar concentrations of herbimycin A (Herb) for 18 hours or with tyrphostin (Tyrph), staurosporine (Staur), H7, or HA1004 for 2 hours. At the end of pretreatment, the cells were incubated in medium alone or exposed to 100 µM H$_{2}$O$_{2}$ or 10 µg/ml PHA for 45 minutes. The levels of c-jun mRNA were assessed by RNase protection assays and the protected fragment specific for c-jun visualized by autoradiography. Data are from a single experiment representative of two others.
Figure 6. H$_2$O$_2$ activates JNK and ERK activities in Jurkat T cells. Jurkat cells (5 x 10$^6$ cells/ml) in medium containing 0.5% FCS were incubated in medium alone (lane 1), or treated for 30 minutes with cross-linked anti-CD3 (5 μg/ml) plus PMA (20 ng/ml) (lane 2) or 50 μM (lane 3), 100 μM (lane 4), 200 μM (lane 5), and 300 μM (lane 6) H$_2$O$_2$. At the end of treatment, the cellular proteins were extracted and then immunoprecipitated by immobilized anti-JNK or anti-ERK-2 antibody. The immunocomplex kinase assays were conducted with substrate GST-c-Jun for JNK activity (A) and MBP for ERK activity (B) as described in Methods. The $^{32}$P-labeled substrates were separated by SDS-PAGE and visualized by autoradiography. Results are a representative experiment of three.
Figure 7. Inhibition of H₂O₂ induction of c-jun mRNA expression by chelation of intracellular calcium with BAPTA in Jurkat T cells. (A) Autoradiogram of c-jun mRNA levels in Jurkat cells preloaded with BAPTA and then exposed to H₂O₂. 3 × 10⁶ Jurkat cells were preloaded at 37°C with the indicated concentrations of the calcium chelator BAPTA for 30 minutes and then incubated in medium alone or exposed to 100 μM H₂O₂ for 45 minutes. RNase protection assays were performed and the protected c-jun mRNA fragments visualized by autoradiography. (B) Correlation between [Ca²⁺]ᵢ and c-jun mRNA levels after the cells were treated by BAPTA. Jurkat cells were preloaded with Indo-1 AM and different concentrations of BAPTA as described above, and then exposed to 100 μM H₂O₂, followed by RNase protection assays for c-jun mRNA levels and [Ca²⁺]ᵢ measurements as described in Methods. The levels of c-jun mRNA were quantitated by Cerenkov counting of the protected fragments. The [Ca²⁺]ᵢ in nM and c-jun mRNA levels in cpm were plotted as a function of increasing concentrations of BAPTA.
Figure 7

A

$H_2O_2 (100 \, \mu M)$ - - + + + + +

BAPTA ($\mu M$) 0 25 0 5 15 25

B

$[Ca^{2+}]$[nM] mRNA

$cjun$ mRNA (cpm)

BAPTA ($\mu M$)
Figure 8. Modulation of $[\text{Ca}^{2+}]_{i}$ in Jurkat T cells by varying concentrations of H$_2$O$_2$ compared with PHA. $5 \times 10^5$ Jurkat cells loaded with Indo-1 AM were incubated at 37°C in saline buffer until basal $[\text{Ca}^{2+}]_{i}$ stabilized. The cells were then treated with different concentrations of H$_2$O$_2$ or PHA at the time indicated by the solid arrow. $[\text{Ca}^{2+}]_{i}$ was measured and calculated by using a Perkin-Elmer spectrofluorometer as described in Methods. The curves were plotted by the values of $[\text{Ca}^{2+}]_{i}$ calculated at every 20 seconds. The results are representative of three separate experiments.
Figure 8

- 10 μg/ml PHA
- 50 μM H₂O₂
- 100 μM H₂O₂
- 200 μM H₂O₂

[Ca²⁺] (nM)

Time (Seconds)
Figure 9. H$_2$O$_2$ elevates [Ca$^{2+}$]$_i$ in Jurkat T cells by inducing the influx of extracellular calcium. Indo-1 loaded Jurkat cells were suspended in Ca$^{2+}$-free saline buffer supplemented with 0.5 mM EGTA to eliminate residual free extracellular calcium. The cells were exposed to H$_2$O$_2$ or PHA at the time indicated by the arrow and [Ca$^{2+}$]$_i$ monitored until stable. CaCl$_2$ at 2 mM was then added to the external buffer as indicated and the peak values of [Ca$^{2+}$]$_i$ determined. Each point represents the [Ca$^{2+}$]$_i$ level at 20 second interval.
Figure 9

- 10 µg/ml PHA
- 100 µM H₂O₂
- 200 µM H₂O₂

|Ca²⁺| (nM) vs Time (Seconds)

CaCl₂
Figure 10. Differential inhibition of [Ca$^{2+}$]$_i$ elevations in Jurkat T cells in response to H$_2$O$_2$ or PHA by the calcium channel blocker nickel (Ni$^{2+}$) or nifedipine. Indo-1 loaded Jurkat cells were pretreated for 10 minutes with 40 μM nickel chloride or 3 μM nifedipine, or incubated in saline buffer alone as controls. The cells were then treated with (A) 100 μM H$_2$O$_2$ or (B) 10 μg/ml PHA at the time indicated by the solid arrows. [Ca$^{2+}$]$_i$ levels recorded and plotted as described in the legend of Figure 8.
Figure 10

A. H$_2$O$_2$

B. PHA

- Control
- Ni$^{2+}$
- Nifedipine

$[Ca^{2+}]_i$ (nM) vs Time (seconds)
Figure 11. Effect of the PTK inhibitor genistein on $[\text{Ca}^{2+}]_i$ responses of Jurkat T cells to $\text{H}_2\text{O}_2$, PHA, or cross-linked anti-CD3 MAb. Jurkat cells were loaded with Indo-1 AM and incubated in saline buffer alone (none) or in buffer containing 25 or 50 $\mu$M genistein. After 15 minutes, the cells were treated at the time indicated by the solid arrows with (A) 100 $\mu$M $\text{H}_2\text{O}_2$, (B) 10 $\mu$g/ml PHA, or (C) 5 $\mu$g/ml cross-linked anti-CD3 MAb and $[\text{Ca}^{2+}]_i$ levels determined.
Genistein

- Φ None
- ● 25 μM
- ★ 50 μM

Figure 11
Table 1. Influence of different kinase inhibitors on \([Ca^{2+}]_i\); response of Jurkat T cells to
H\(_2\)O\(_2\), PHA, and anti-CD3 MAb. Jurkat cells were incubated for 18 hours in medium
alone or in medium containing 1% DMSO vehicle or 1 \(\mu\)M herbimycin A in 1% DMSO. Other cells were treated for 2 hours with 100 \(\mu\)M genistein or 0.5 \(\mu\)M staurosporine. The cells were loaded with Indo-1 AM and the basal levels of \([Ca^{2+}]_i\) established. Then, the cells were treated with 200 \(\mu\)M H\(_2\)O\(_2\), 5 \(\mu\)g/ml cross-linked anti-CD3 MAb, or 10 \(\mu\)g/ml PHA, followed immediately by recording of \([Ca^{2+}]_i\) levels. The results represent the mean ± SD of the stimulated increases in \([Ca^{2+}]_i\); over the basal \([Ca^{2+}]_i\) levels. The basal \([Ca^{2+}]_i\) levels were determined in duplicate for each pretreatment and averaged 88 ± 3 nM for 10 determinations.
Table 1. Inhibition by protein kinase inhibitors of $[\text{Ca}^{2+}]_i$ induced by $\text{H}_2\text{O}_2$, PHA, or anti-CD3

<table>
<thead>
<tr>
<th>Addition of Inhibitors</th>
<th>Stimulant</th>
<th>$\Delta [\text{Ca}^{2+}]_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-CD3</td>
<td>PHA</td>
</tr>
<tr>
<td>None</td>
<td>265 ± 21</td>
<td>233 ± 4</td>
</tr>
<tr>
<td>DMSO (vehicle)</td>
<td>261 ± 8</td>
<td>217 ± 5</td>
</tr>
<tr>
<td>Genistein</td>
<td>25 ± 3</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Herbimycin A</td>
<td>40 ± 6</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>12 ± 4</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>
Figure 12. Fluoroaluminate (AlF₄⁻) bypasses the inhibition by PTK inhibitor herbimycin A of [Ca²⁺]ᵢ elevations in Jurkat T cells exposed to H₂O₂ and anti-CD3 stimulation. Jurkat cells were cultured in medium alone or in medium containing 1 µM herbimycin A. After 18 hours, the cells were washed, loaded with Indo-1 AM, and stimulated with 5 µg/ml of cross-linked anti-CD3 MAb or 100 µM H₂O₂. Following the measurement of the peak values of [Ca²⁺]ᵢ, the indicated cells were then stimulated with AlF₄⁻ and [Ca²⁺]ᵢ recorded. Data are presented as the mean ± standard deviation (SD) from three separate experiments.
Figure 12
Figure 13. Acute exposure of Jurkat T cells to H$_2$O$_2$ does not block [Ca$^{2+}$]$_i$ responses induced by anti-CD3 MAb. Indo-1 loaded Jurkat cells were first stimulated with cross-linked anti-CD3 MAb (5 µg/ml) or H$_2$O$_2$ (100 µM), or incubated in buffer alone (-). The peak [Ca$^{2+}$]$_i$ values were recorded, followed by exposure of the cells to the second stimulus as indicated and measurement of [Ca$^{2+}$]$_i$ levels. The [Ca$^{2+}$]$_i$ values obtained with a single stimulus or sequential stimuli were compared with the [Ca$^{2+}$]$_i$ observed when the cells were simultaneously stimulated with H$_2$O$_2$ plus cross-linked anti-CD3.
Figure 13
Figure 14. Stability of c-jun mRNA in Jurkat T cells exposed to H$_2$O$_2$.  

*A*, $3 \times 10^6$ Jurkat cells in medium with 0.5% FCS were treated for 45 minutes with PHA (10 µg/ml) plus PMA (20 ng/ml) or 200 µM H$_2$O$_2$. RNA transcription was blocked at time 0 by the addition of 10 µM actinomycin D. At the specified times after addition of actinomycin D, the total cytosolic RNA of the cells was extracted. The levels of c-jun mRNA determined by RNase protection assays were measured by Cerenkov counting and plotted against the time.  

*B*, Same as for *A*, with the exception that the cells were treated with anisomycin (10 µg/ml) in the presence or absence of 200 µM H$_2$O$_2$ for 45 minutes before the addition of actinomycin D.  

*C*, Jurkat cells were preincubated for 60 minutes with 10 µg/ml anisomycin and washed twice in PBS buffer. The cells were then incubated with 10 µg/ml actinomycin D in either medium alone or in medium containing 200 µM H$_2$O$_2$. The levels of c-jun mRNA remaining after the addition of actinomycin D were quantified as illustrated for *A*.  

137
Figure 14
Figure 15. Schematic representation of the c-jun promoter CAT constructs. The sequences of the jun1 and jun2 TRE-like motifs along with their point mutations are shown.
c-jun constructs

-1600/+170 jun

mjun 2
TGACCTCGAG
ATTACCTCAT

mjun 1
TGACATCAT
ATTACCTCAT

mjun 1,2
TGACCTCGAG
ATTACCTCAT

Figure 15
Figure 16. Transcription activation of c-jun promoter by H$_2$O$_2$ and effect of point mutations in jun1 and jun2 motifs on transactivation of c-jun promoter in Jurkat T cells. 

A, Representative results of CAT assays of Jurkat cells transfected with the -1600/+170 wild-type (wt) c-jun CAT reporter construct or with the constructs carrying point mutations in jun2 (mjun2) or jun1 (mjun1) sites. The transfected cells were exposed for 18 hours to the indicated stimuli. 

B, The relative transactivation responses of different c-jun CAT constructs after exposure to various stimuli. The constructs were transiently transfected into Jurkat cells and treated for 18 hours with 50 ng/ml PMA, 10 μg/ml PHA plus PMA, 30 nM okadaic acid, or the specified concentrations of H$_2$O$_2$, followed by CAT assays. The relative CAT activity (fold induction) was calculated by dividing the CAT activity (corrected for protein content) of treated cells by the corrected control CAT activity observed for the untreated cells transfected with the same construct and incubated in medium alone. Results represent the means and SDs of four separate experiments. Actual values for the nonstimulated basal conversion of chloramphenicol to acetylated forms were wtjun: 3.2%, mjun2: 2.2%, mjun1: 2.3%, and mjun1,2: 2.1%.
Figure 17. Binding of nuclear proteins from Jurkat T cells treated with different stimuli to AP-1 TRE, jun1, and jun2 sequence motifs. Jurkat cells were either untreated or treated with 30 nM okadaic acid, 50 ng/ml PMA, 10 μg/ml PHA plus PMA, or the indicated concentrations of H₂O₂ for 1 hour before the preparation of nuclear proteins for EMSA as described in Methods. The amount of radioactivity present within each complex was quantified by Cerenkov counting in a scintillation counter. Similar results were obtained in three separate experiments.
Figure 17

jun2

jun1

AP1

Control

Okadaic

H2O2 (μM)

100

300

500

PMA

PHA/PMA
Figure 18. EMSA analyses of AP-1 TRE, jun1, and jun2 motif binding activities in Jurkat T cells treated with different stimuli using specific Abs against Fos and Jun proteins. The nuclear extracts were prepared from Jurkat cells treated for 2 hours with 300 μM H₂O₂, 10 μg/ml PHA plus 50 ng/ml PMA, or 30 nM okadaic acid. AP-1 TRE, jun1, or jun2 binding activities were assayed in the presence of 1 to 2 μg of the indicated Abs or preimmune normal rabbit serum. The protein-DNA complex formation was assessed by EMSA. Dried gels were exposed for 6 to 18 hours for autoradiography. Data shown represents one of four separate experiments. Horizontal arrows indicate Ab-induced supershifts.
Figure 18
Figure 19. Competition analysis of DNA binding complexes recognizing AP-1 TRE, Jun1, and Jun2 in Jurkat T cells treated with H$_2$O$_2$, PHA/PMA, or okadaic acid. Jurkat cells were treated for 2 hours with 200 μM H$_2$O$_2$, 10 μg/ml PHA plus 50 ng/ml PMA, or 30 nM okadaic acid followed by the extraction of nuclear proteins and EMSA. During the EMSA, the nuclear proteins were first incubated for 30 minutes with the specified molar ratios of unlabeled oligonucleotide probes, and then interacted with $^{32}$P-labeled probes as in regular EMSA. The results shown represent one of three repetitive experiments.
Figure 19

[Diagram of bar graphs and electrophoresis]
Figure 20. Differential induction of jun1 and AP-1-dependent TRE promoter activity in Jurkat T cells exposed to okadaic acid, H$_2$O$_2$, PMA, or PHA/PMA. Jurkat cells were transfected with the -79/+170 c-jun promoter construct containing the jun1 motif, or AP-1-dependent CAT reporters with one (1 × TRE) or five (5 × TRE) copies of the TRE sequence from the promoter of the human collagenase gene. After incubation for 24 hours, the cells were treated for 18 hours in the absence or presence of 30 nM okadaic acid, 50 ng/ml PMA, 10 µg/ml PHA plus 50 ng/ml PMA, or the indicated concentrations of H$_2$O$_2$ before analysis of induced CAT activity. The results represent the means ± SD of three to four separate experiments.
Figure 20

Fold Induction

Histograms showing fold induction for different conditions.

- PHA/PMA
- H3O2 (500 mM)
- H3O2 (300 mM)
- H3O2 (100 mM)
- PHA/PMA

Conditions: 79/4170 IX-TRE SX-TRE

Y-axis: Fold Induction
X-axis: Concentration
Figure 21. H$_2$O$_2$ inhibition of IL-2 production in Jurkat cells exposed to PHA/PMA. 2 ×10$^6$ Jurkat cells incubated for 36 hours in medium alone, in medium with 10 μg/ml PHA plus 50 ng/ml PMA, or in medium containing PHA/PMA plus different concentrations of H$_2$O$_2$. After incubation, the supernatants of the cell suspensions were harvested and analyzed for IL-2 production levels by IL-2 ELISA assay. The concentrations of IL-2 were plotted against H$_2$O$_2$ concentration.
Figure 21

H₂O₂ (µM)  0  50  100  300  500
PHA/PMA  +  +  +  +  +

IL-2 production (ng/ml)
Figure 22. H$_2$O$_2$ inhibition of IL-2 mRNA expression induced by PHA/PMA in Jurkat T cells. Jurkat cells ($2 \times 10^6$) were incubated for 6 hours in medium alone, or in medium containing 10 μg/ml PHA plus 50 ng/ml PMA, or PHA/PMA plus the indicated concentrations of H$_2$O$_2$. At the end of the incubation, the total cytosolic RNA was isolated, and 10 μg cytosolic RNA or yeast tRNA (control) was used for the measurement of IL-2 mRNA with a specific riboprobe in RNase protection assay. The protected fragments of IL-2 mRNA in $A$ were indicated by the horizontal arrow. The densities of bands were quantified by a scintillation counter and plotted against H$_2$O$_2$ concentrations as in $B$. The result shown represents one of three similar repetitions.
Figure 22
Figure 23. Inhibition by H$_2$O$_2$ of transactivation of IL-2 promoter in Jurkat cells
stimulated by PHA/PMA. Jurkat cells were transfected with the luciferase (luc) plasmid
constructor driven either by a full length human IL-2 promoter sequence (IL-2-luc) or
CMV promoter sequence (CMV-luc) as a control. The cells were then treated for 18
hours in medium alone, in medium with 10 μg/ml PHA plus 50 ng/ml PMA, or in medium
including PHA/PMA plus various concentrations of H$_2$O$_2$. At the end of treatment, the
cell extracts were prepared for the assay of luciferase activity described in Methods. The
levels (light units) of luciferase activity were plotted as a function against H$_2$O$_2$
concentrations. The results shown are a representative of several similar experiments.
Figure 23

Luciferase activities of IL-2-iuc (light units)

$H_2O_2$ concentrations (µM)

Luciferase activities of CMV-luc (light units x10)
Figure 24. Inhibition of NFAT transactivation by $H_2O_2$ in Jurkat T cells. Jurkat cells were transfected with $4 \times$ NFAT-CAT or SV40-CAT reporter construct, and then exposed for 18 hours to PHA/PMA alone or in the presence of the indicated concentrations of $H_2O_2$. After stimulation, the CAT assays were performed with the cell extracts and the acetylation conversion of chloramphenicol determined by TLC as described in Methods. A, A representative TLC pattern of $4 \times$ NFAT-CAT activity is presented. The cells were treated in medium alone (lane 1), with 10 $\mu$g/ml PHA plus 50 ng/ml PMA (lane 2), with PHA/PMA (P/P) plus 50, 100, 300, or 500 $\mu$M $H_2O_2$ (H) (lanes 3-6), or with the same concentrations of $H_2O_2$ alone (lanes 7-10). The SV40-CAT (lane 11) was used as a control for the transfection and CAT assay of this experiment. B, The conversion rates of chloramphenicol in CAT assays with the transfected cells performed above were calculated and presented to show the $H_2O_2$ concentration-dependent inhibition of NFAT-CAT activity induced by PHA/PMA.
Figure 24

NFAT-CAT activity (acetylation %)

+ + + + +
500 300 100 50 0

PMA/PHA

H2O2 (µM)

0 5 10 15 20 25
Table 2. Sublethal concentrations of H$_2$O$_2$ stimulate NFkB but not NFAT activation in Jurkat cells. Jurkat cells were transiently transfected with 10 μg of the indicated reporter constructs. After 24 hours, the cells were washed, divided into aliquots and incubated in medium alone or with the specified concentrations of H$_2$O$_2$ for 18 hours. CAT activity was measured in cellular lysates containing equivalent protein concentrations and expressed as the mean percent conversion of [H$^{14}$C]-chloramphenicol to acetylated forms per duplicate samples. LDH release was determined after 18 hour incubation and expressed as mean units release into the cell culture medium by 2 x 10$^6$ cells per duplicate cultures. The maximum release of LDH, 1134 units, was obtained by incubating the cells in medium containing 10 μg/ml digitonin. The numbers in parenthesis indicate the fold increase or decrease in cells incubated with H$_2$O$_2$ relative to cells incubated in medium alone. Results are representative of three separate experiments.
ND = not determined.
Table 2. Sublethal concentrations of H$_2$O$_2$ stimulate NFkB but not NFAT activation in Jurkat cells.

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (µM)</th>
<th>NFAT-CAT (% conversion)</th>
<th>NFkB-CAT (% conversion)</th>
<th>LDH release (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.22 (1.00)</td>
<td>0.87 (1.00)</td>
<td>138 (1.00)</td>
</tr>
<tr>
<td>50</td>
<td>0.20 (0.90)</td>
<td>1.45 (1.67)</td>
<td>135 (0.98)</td>
</tr>
<tr>
<td>100</td>
<td>0.21 (0.95)</td>
<td>1.89 (2.17)</td>
<td>189 (1.37)</td>
</tr>
<tr>
<td>200</td>
<td>ND</td>
<td>ND</td>
<td>297 (2.20)</td>
</tr>
<tr>
<td>300</td>
<td>0.24 (1.09)</td>
<td>2.04 (2.35)</td>
<td>561 (4.10)</td>
</tr>
<tr>
<td>500</td>
<td>0.22 (1.01)</td>
<td>2.08 (2.40)</td>
<td>552 (4.00)</td>
</tr>
</tbody>
</table>
Figure 25. Relationship among IL-2 mRNA expression, IL-2 protein production, and IL-2-luc and NFAT-CAT activities in H$_2$O$_2$ inhibition of PHA/PMA-stimulated Jurkat cells. The experimental procedures for data have been described in the legends of previous Figures. H$_2$O$_2$ inhibition of PHA/PMA-induced Jurkat cells was presented as percentages relative to PHA/PMA alone (100%). The percent values for each parameter with PHA/PMA alone and PHA/PMA plus H$_2$O$_2$ were plotted against H$_2$O$_2$ concentrations.
Figure 25

Percentage relative to PHA/PMA

$\text{H}_2\text{O}_2$ concentrations (μM)

- ○ IL-2 mRNA
- ● IL-2 protein
- □ IL-2-luc
- ■ NFAT-CAT
Figure 26. H$_2$O$_2$ inhibition of NFAT DNA-binding activity in Jurkat T cells stimulated by PHA/PMA. Jurkat cells were treated for 2 hours with 10 ug/ml PHA/50 ng/ml PMA, the indicated concentrations of H$_2$O$_2$ (H), or PHA/PMA (P/P) plus H$_2$O$_2$, or incubated in medium alone as control. EMSAs were performed with the nuclear protein extracts and the $^{32}$P-labeled oligonucleotide probe specific for NFAT as described in Methods. A. A gel pattern from one of several similar experiments is presented. The NFAT-oligonucleotide complexes were indicated by an arrow. B. NFAT binding activities in the cells treated with PHA/PMA and PHA/PMA plus H$_2$O$_2$ were quantified by scintillation counting of NFAT-oligonucleotide complex bands shown above and plotted versus H$_2$O$_2$ concentrations.
NFAT DNA-binding (% stimulated levels)

Probe alone
Untreated
H50
H100
H300
H500
P/P
P/P/H50
P/P/100
P/P/H300
P/P/H500