Reactive Oxygen Intermediates Activate NF-κB in a Tyrosine Kinase-Dependent Mechanism and in Combination With Vanadate Activate the p56

By Gary L. Schieven, Jean M. Kirihara, Dorothea E. Myers, Jeffrey A. Ledbetter, and Fatih M. Uckun

We have previously observed that ionizing radiation induces tyrosine phosphorylation in human B-lymphocyte precursors by stimulation of unidentified tyrosine kinases and this phosphorylation is substantially augmented by vanadate. Ionizing radiation generates reactive oxygen intermediates (ROI). Because H2O2 is a potent ROI generator that readily crosses the plasma membrane, we used H2O2 to examine the effects of ROI on signal transduction. We now provide evidence that the tyrosine kinase inhibitor herbimycin A and the free radical scavenger N-acetyl-cysteine inhibit both radiation-induced and H2O2-induced activation of NF-κB, indicating that activation triggered by ROI is dependent on tyrosine kinase activity. H2O2 was found to stimulate Ins-1,4,5-P3 production in a tyrosine kinase-dependent manner and to induce calcium signals that were greatly augmented by vanadate. The synergistic induction of tyrosine phosphorylation by H2O2 plus vanadate included physiologically relevant proteins such as PLCγ1. Although treatment of cells with H2O2 alone did not affect the activity of src family kinases, treatment with H2O2 plus vanadate led to activation of the p56

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pre-T-cell line CEM. The radiation sensitivity of these cell lines was detailed in a previous report.18

Irradiation and treatments of cells. Cells (5 x 10⁶/mL) in plastic tissue culture flasks were irradiated with 500 to 2000 cGy at a dose rate of 100 cGy/min during log phase and under aerobic conditions using a ²⁵³Cm irradiator (Model Mark I; JL Shephard and Assoc, Glendale, CA), as previously described.19 In parallel experiments, cells were treated with 3 to 9 mmol/L H₂O₂ (Sigma, St Louis MO), 100 μmol/L sodium orthovanadate (Fisher Scientific, Pittsburgh, PA), or both for 30 minutes at 37°C. For experiments involving the use of kinase inhibitors before irradiation or H₂O₂ treatment, cells were incubated for 1 hour at 37°C with (1) phosphate-buffered saline (PBS), (2) the tyrosine kinase inhibitor genestein (370 μmol/L; ICN Biomedical, Costa Mesa, CA), (3) the PKC inhibitor 1-(5-isoquinolinolysufonyl)-2-methylpiperazone (30 μmol/L; H7; GIBCO-BRL, Grand Island, NY), or for 24 hours at 37°C with (4) the potent tyrosine kinase inhibitor herbimycin A (12 μmol/L; GIBCO-BRL), using previously described protocols.²¹²² In some experiments, cells were preincubated with 20 mmol/L NAC for 1 hour according to the treatment protocol reported by Schreck et al.²³ For the measurement of [Ca²⁺], cells were used immediately after addition of stimulating agents. For IgM cross-linking, cells were treated with 10 μg/mL F(ab)², fragment goat anti-human IgM (Jackson ImmunoResearch Labs, West Grove, PA) for 1 minute. Cross-linking of CD3 alone or in conjunction with CD4 was achieved by the use of biotinylated monoclonal antibodies (MoAbs) against CD3 (G19-4) and CD4 (G17-2) followed by treatment with avidin, as previously described.²⁰

Nuclear extraction and electrophoretic mobility shift assays. Four hours after irradiation or H₂O₂ treatment, nuclear proteins were extracted according to previously published procedures.²¹²² Gel shift assays were performed as described.²³ Fifty-nanogram amounts of a double-stranded oligonucleotide containing a tandem repeat of the 11-bp consensus sequence for the NF-κB DNA binding site (GAGGGAGTTTCC; obtained in kit form from GIBCO-BRL) were end-labeled using [γ⁻³²P]ATP and T4 polynucleotide kinase according to the recommendations of the manufacturer. One nanogram of the radiolabeled oligonucleotide (400,000 cpm) was incubated with 10 μg nuclear protein for 20 minutes at room temperature in 25 mmol/L Tris-HCl, pH 7.6, 0.1 mg poly dI-dC (Boehringer Mannheim, Indianapolis, IN), 5 mmol/L MgCl₂, 0.5 mmol/L EDTA, 1 mmol/L DTT, and 10% (vol/vol) glycerol. Competition studies with unlabeled NF-κB probe were performed by preincubating the nuclear protein for 15 minutes on ice with a 500-fold excess of unlabeled oligonucleotide before the addition of the ²³²P end-labeled NF-κB probe. Controls used 500-fold excess of unlabeled AP-1 and NF-1 oligonucleotide probes for competition. DNA-protein complexes in the reaction mixture were analyzed by polyacrylamide gel electrophoresis, using a 4.5% running gel under nondenaturing conditions, in 0.25% TBE buffer (25 mmol/L Tris, pH 8.2, 2.25 mmol/L borate, 0.25 mmol/L EDTA). The gels were pre-run at 150 V for 2 hours at 4°C before the samples were loaded and electrophoresed for an additional 3 hours at 150 V. Gels were dried overnight and exposed to Kodak XAR 5 X-ray film using intensifying screens at ~70°C.

Immunoprecipitations, immunoblots, and kinase assays. Cells were lysed on ice with NP-40 lysis buffer (50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 1% NP-40, 100 μmol/L sodium orthovanadate, 100 μmol/L sodium molybdate, 8 μg/mL aprotinin, 5 μg/mL leupeptin, 500 μmol/L phenylmethylsulfonyl fluoride [PMSF]) and centrifuged at 13,000 x g to remove insoluble material. Immunoprecipitation of PLC-γ1 was performed as previously described.²⁴ Immunoprecipitation of ras GTPase-activating protein (GAP) was performed with rabbit antiserum to human GAP (Upstate Biotechnology, Lake Placid, NY) and immunoprecipitation of p34cdc2 was performed with an MoAb to human p34cdc2 (Upstate Biotechnology). Immunoprecipitation of the ζ chain of the T-cell receptor was performed using anti-ζ antibody kindly provided by J. Sancho and C. Terhorst (Beth Israel Hospital, Boston, MA).²⁵ Immune complexes were collected on protein A-Sepharose beads (Repligen, Cambridge MA), washed four times with NP-40 lysis buffer and once with PBS, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Immunoblots with anti-PLCγ1 were performed as previously described.²⁶ Antiphosphotyrosine immunoblotting was performed as previously described,²⁷ using affinity-purified rabbit polyclonal antibodies.²⁸ Immune complex tyrosine kinase assays were performed using the exogenous substrate enolase as previously described.²⁷ Samples were immunoprecipitated with antisera prepared against unique amino acid sequences of src family tyrosine kinases,²⁸ which were the kind gift of Dr Joseph Bolen (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ).

Measurement of [Ca²⁺] and inositol 1,4,5-trisphosphate (Ins-1,4,5-P₃). [Ca²⁺] responses were measured using indo-1 (Molecular Probes, Eugene, OR) and a model 50HH/2150 flow cytometer (Ortho, Westwood, MA), as previously described.²⁹ The histograms were analyzed by programs that calculate the mean indo-1 violet/ blue fluorescence ratio versus time. There are 100 data points on the X (time) axis of all flow cytometric data. Ins-1,4,5-P₃ levels were measured using a highly specific D-myo-inositol 1,4,5-[³H]-trisphosphate assay system (Amersham Corp, Arlington Heights, IL), as previously described.³⁰ This assay is based on the competition between unlabeled Ins-1,4,5-P₃ and a fixed quantity of a high specific activity tritiated Ins-1,4,5-P₃ tracer (³H-Ins-1,4,5-P₃) for a limited number of binding sites on a specific and sensitive bovine adrenal binding protein preparation.

RESULTS

The role of reactive oxygen intermediates in radiation-induced activation of NF-κB in human lymphoid cells. We used electrophoretic mobility shift assays (EMSA) to examine the effects of the ROI-scavenger NAC and tyrosine kinase inhibitors on (1) radiation-induced activation of NF-κB and (2) ROI-induced activation of NF-κB in Ramos and Daudi cells. After passive diffusion through the plasma membrane, H₂O₂ can be converted into highly reactive ROI such as the superoxide anion and the hydroxyl radical. Therefore, H₂O₂ was used as a potent ROI generator to examine the effects of ROI on NF-κB. Doses of radiation and H₂O₂ were chosen so as to show that strong signals could be clearly inhibited by NAC and tyrosine kinase inhibitors. The doses used were based on previous reports that established optimum doses of H₂O₂,³¹³² and radiation,⁶⁷ for such signals. One microgram of poly dI-dC was used to eliminate nonspecific binding. Notably, ionizing radiation with 500 to 2,000 cGy γ-rays stimulated a specific DNA binding activity, as reflected by a marked increase in intensity of a shifted band that was observed when the NF-κB probe was incubated with nuclear extracts from Ramos cells (Fig 1A). This retarded band was eliminated by competition with a 500-fold molar excess of unlabeled NF-κB oligonucleotide, confirming the specificity of the DNA-protein interactions (Fig 1A). By comparison, a 500-fold excess of unlabeled NF-1 control oligonucleotide did not compete with the binding of NF-κB probe to the retarded band (data not shown). Notably, the tyrosine kinase inhibitors genestein and herbimycin markedly inhibited NF-κB activation, in accordance
with our recent studies.\textsuperscript{16} As shown in Fig 1A, NAC substantially inhibited the formation of DNA–NF-κB protein complexes in irradiated Ramos cells. This unique observation indicates that ROI generation is a mandatory step in radiation-induced activation of NF-κB. We next compared the ability of 1,000 cGy γ-rays and 9 mmol/L \(\text{H}_2\text{O}_2\) to activate NF-κB in Ramos cells. As shown in Fig 1B, \(\text{H}_2\text{O}_2\) was as effective as ionizing radiation in inducing NF-κB DNA binding activity, and NAC prevented the activation of NF-κB in irradiated or \(\text{H}_2\text{O}_2\)-treated Ramos cells. Notably, both genistein and herbimycin were able to abrogate the \(\text{H}_2\text{O}_2\)-induced activation of NF-κB, whereas H7 was not (Fig 1B). Thus, ROI-mediated activation of NF-κB is triggered primarily by PTK activation and does not depend on PKC. Our results suggest that reactive oxygen intermediates might thus induce other kinases such that the activity of PKC is not essential for NF-κB activation. Similar results have been obtained with Daudi cells (F. Uckun, unpublished results). Activation of NF-κB was also observed at the lower concentration of \(\text{H}_2\text{O}_2\) of 300 \(\mu\text{mol/L}\) (Fig 2). However, the combination of vanadate plus \(\text{H}_2\text{O}_2\) was not significantly more effective than \(\text{H}_2\text{O}_2\) alone in inducing NF-κB activation (Fig 2), indicating that \(\text{H}_2\text{O}_2\) alone delivers a sufficiently strong signal.

\textit{Hydrogen peroxide plus vanadate strongly induce [Ca\textsuperscript{2+}]/flux and inositol 1,4,5-trisphosphate production.} We next examined the ability of the ROI generator \(\text{H}_2\text{O}_2\) alone and in combination with the phosphotyrosine phosphatase inhibitor vanadate to stimulate calcium signaling. \(\text{H}_2\text{O}_2\) gave a small [Ca\textsuperscript{2+}]\textsubscript{i} signal in Ramos cells, whereas vanadate alone gave no signal (Fig 3). However, \(\text{H}_2\text{O}_2\) plus vanadate gave a very strong signal. The signal generated by \(\text{H}_2\text{O}_2\) plus
vanadate also gave a strong signal in CEM cells (Fig 4A) and was observed in the presence of EGTA, showing that Ca²⁺ was released from internal stores as well as entered cells from the outside. The magnitude of the calcium signal was significantly greater than that observed after antibody cross-linking of CD3 with CD4 (Fig 4B), which is one of the strongest biologic stimulations for [Ca²⁺]i signals in CEM cells.¹⁰ Treatment with H₂O₂ alone gave a small but significant [Ca²⁺]i signal, whereas vanadate alone had no effect (Fig 4C). Sequential treatment with the compounds in either order was as effective as the use of both together in generating a [Ca²⁺]i signal (Fig 4D). Downmodulation of CD3 by antibody treatment had no effect on ROI-induced [Ca²⁺]i signaling (data not shown), although signaling via CD2 was inhibited, as previously reported.²⁴ The [Ca²⁺]i signal induced by ROI treatment is therefore independent of the T-cell receptor (TCR).

To investigate the basis for the ROI-induced [Ca²⁺]i signal in lymphocytes, we examined Ins-1,4,5-P₃ levels in Ramos cells after treatment with H₂O₂ and vanadate (Table 1). Treatment with H₂O₂ resulted in rapid, substantial, but transient Ins-1,4,5-P₃ production, whereas vanadate alone had little effect. The combination of H₂O₂ plus vanadate accelerated and increased Ins-1,4,5-P₃ production. The levels of Ins-1,4,5-P₃ were thus in good agreement with the [Ca²⁺]i signals observed in Ramos cells. Similar results were obtained with other B-cell lines including Daudi (early B), NALM-6 (pre-B), REH (pre-pre-B), and FL8.2 (pro-B) (data not shown), showing that the effect occurs in cells at different stages of development. These results are in accordance with previous reports that the combination of H₂O₂ and vanadate stimulated polyphosphoinositide breakdown in a variety of other cell lines.³³ As shown in Table 1, the induction of Ins-1,4,5-P₃ by ROI was blocked by the tyrosine kinase inhibitor herbimycin A, but not by the PKC inhibitor H7. Therefore, stimulation of Ins-1,4,5-P₃ production by H₂O₂ plus vanadate is dependent on tyrosine kinase activity.

Hydrogen peroxide and vanadate act synergistically to induce tyrosine phosphorylation of physiologically relevant substrates in T- and B-cell lines. We had previously reported that ionizing radiation induced tyrosine phosphorylation, which was greatly augmented by vanadate.¹⁵ The combination of H₂O₂ plus vanadate has been reported to synergistically induce tyrosine phosphorylation in insulin-responsive rat adipocytes.³¹,³² While the present study was under review, a similar effect was described for T cells,³³ but no substrates were identified. The current finding that the ROI generator H₂O₂ induced transient Ins-1,4,5-P₃ and [Ca²⁺]i signals that were augmented and stabilized by vanadate led us to examine the effects of these reagents on physio-

Fig 3. [Ca²⁺]i signals in Ramos cells in response to treatment with 1 mmol/L H₂O₂ (H) and 100 μmol/L orthovanadate (V).

Fig 4. [Ca²⁺]i signals in CEM cells in response to treatment with 9 mmol/L H₂O₂ (H) and 100 μmol/L orthovanadate (V). (A) Signal in presence of 10 mmol/L EGTA. (B) Comparison to signal generated by cross-linking CD3 and CD4. (C) Separate treatment with H₂O₂ and vanadate. (D) Sequential treatment with H₂O₂ and vanadate.
logically relevant substrates. The pattern of tyrosine phosphorylation induced by treatment with H$_2$O$_2$ plus vanadate was compared with that resulting from biologic stimulations. As shown in Fig 5A, treatment of CEM cells with H$_2$O$_2$ plus vanadate induced tyrosine phosphorylation of many proteins with molecular weights similar to those of proteins in which phosphorylation was induced by cross-linking CD3 alone or cross-linking CD3 and CD4. However, the level of tyrosine phosphorylation was approximately 10 times greater for many of the proteins after chemical stimulation. Similarly, in Ramos cells (Fig 5B), chemical stimulation induced tyrosine phosphorylation of many proteins with molecular weights comparable to those observed after sIgM cross-linking, but at much higher levels.

For both cell types, treatment with H$_2$O$_2$ plus vanadate also induced phosphorylation of proteins not readily observed after biologic stimulation.

PLC$_\gamma$1 was observed to be phosphorylated on tyrosine after treatment of cells with H$_2$O$_2$ plus vanadate for 2 minutes (Fig 6A). Under these conditions, several PLC$_\gamma$1-associated proteins, including pp35/36, which associate via the SH2 domain of PLC$_\gamma$1, were also tyrosine phosphorylated. The anti-PLC$_\gamma$1 Western blot showed that, although equal amounts of PLC$_\gamma$1 protein were recovered from cells treated with H$_2$O$_2$ or vanadate alone as from untreated cells, less was recovered from cells treated with H$_2$O$_2$ and vanadate together. Therefore, the fraction of PLC$_\gamma$1 phosphorylated on tyrosine is greater than what is initially apparent from Fig 6A. Vanadate was required to stabilize and augment the ROI signal before this phosphorylation could be detected. The tyrosine kinase-dependent induction of Ins-1,4,5-P$_3$ by ROI and the ROI-induced [Ca$^{2+}$]i signals may thus be attributed to the tyrosine phosphorylation of PLC$_\gamma$1 in these cells. As shown in Fig 6B, GAP and associated proteins, including species appearing to be the GAP-associated proteins p190$^{35}$ and p62$^{36}$, were tyrosine phosphorylated after treatment with H$_2$O$_2$ plus vanadate. The $\gamma$ subunit of the TCR was also phosphorylated (Fig 6C). Although our data indicate that ROI induction of [Ca$^{2+}$]i signals does not require surface expression of the TCR, a TCR component is acted upon under these conditions. In contrast to many other proteins, the tyrosine phosphorylation state of p34$^{52}$ remained unchanged (Fig 6B). Thus, not all proteins that can be phosphorylated on tyrosine are affected.

The combination of hydrogen peroxide plus vanadate activates p56$k$k and p59$^6k$ tyrosine kinases. The induction of tyrosine phosphorylation by H$_2$O$_2$ plus vanadate in T cells has been ascribed to inhibition of phosphatases. However, the strong phosphorylation of physiologically relevant substrates led us to examine effects on kinase activity as well. The activity of p56$k$k from Ramos cells (Fig 7) and both p56$k$k and p59$^6k$ from CEM cells (Fig 8A) was markedly stimulated by treatment of the cells with H$_2$O$_2$ plus vanadate. In addition to showing increased activity toward the exogenous substrate enolase after treatment with H$_2$O$_2$ plus vanadate, p56$k$k had a characteristic shift to a lower mobility. This shift in p56$k$k mobility has been specifically observed early in lymphocyte activation and after treatment.

### Table 1. Induction of Ins-1,4,5-P$_3$ by H$_2$O$_2$ and Vanadate

<table>
<thead>
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<th>Time</th>
<th>PBS</th>
<th>H$_2$O$_2$</th>
<th>H$_2$O$_2$ + VO$_4$</th>
<th>H$_2$O$_2$ + VO$_4$ + HERB</th>
<th>H$_2$O$_2$ + VO$_4$ + H7</th>
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<td>0</td>
<td>3.8 ± 0.3</td>
<td>3.8 ± 0.3</td>
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<td>10 min</td>
<td>1.7 ± 1.1</td>
<td>7.2 ± 0.2</td>
<td>33.0 ± 1.5</td>
<td>3.4 ± 0.1</td>
<td>26.3 ± 1.4</td>
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<td>15 min</td>
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<td>26.1 ± 2.5</td>
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Cells were treated with 3 mmol/L H$_2$O$_2$, 100 mmol/L vanadate (VO$_4$), Herbimycin A (HERB), and H7 as described in the Materials and Methods section for the times indicated and were then assayed for Ins-1,4,5-P$_3$. For both cell types, treatment with H$_2$O$_2$ plus vanadate also induced phosphorylation of proteins not readily observed after biologic stimulation.

The induction of tyrosine phosphorylation by H$_2$O$_2$ plus vanadate in T cells has been ascribed to inhibition of phosphatases. However, the strong phosphorylation of physiologically relevant substrates led us to examine effects on kinase activity as well. The activity of p56$k$k from Ramos cells (Fig 7) and both p56$k$k and p59$^6k$ from CEM cells (Fig 8A) was markedly stimulated by treatment of the cells with H$_2$O$_2$ plus vanadate. In addition to showing increased activity toward the exogenous substrate enolase after treatment with H$_2$O$_2$ plus vanadate, p56$k$k had a characteristic shift to a lower mobility. This shift in p56$k$k mobility has been specifically observed early in lymphocyte activation and after treatment.

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REACTIVE OXYGEN INTERMEDIATE INDUCED SIGNALS

Fig 6. Tyrosine phosphorylation of specific proteins after treatment of cells with 9 mmol/L H$_2$O$_2$ (H), 100 μmol/L orthovanadate (V), both (HV), or no treatment (O), as detected by immunoprecipitation followed by immunoblotting. (A) Phosphorylation of PLCγ1 and associated proteins. (B) Phosphorylation of GAP and p34$^{cd2}$. (C) Phosphorylation of ζ chain.

with phorbol myristate acetate (PMA). The amount of p56$^{Lck}$ that could be detected by immunoblot analysis was significantly reduced by treatment with H$_2$O$_2$ plus vanadate (Fig 8C). The increase in specific activity of p56$^{Lck}$ is thus greater than what is apparent in Fig 8A.

In contrast, treatment with H$_2$O$_2$ or vanadate alone did not stimulate the activity of these kinases. This finding that ROI alone did not activate p56$^{Lck}$ or p59$^{flk}$ is consistent with our previous study showing that ionizing radiation did not substantially increase the activity of these kinases. As shown in Fig 7, p56$^{Lck}$ activity did not increase after irradiation in the presence or absence of vanadate. The lack of p56$^{Lck}$ activation after irradiation in the presence of vanadate may be explained by the radiation generating lower amounts of ROI relative to treatment of cells with 9 mmol/ L H$_2$O$_2$. The activity of p62$^{yes}$ was not affected by H$_2$O$_2$ or vanadate (Fig 8A). To determine whether H$_2$O$_2$ plus vanadate acted directly on the responsive kinases, immunoprecipitates were treated directly with 9 mmol/L H$_2$O$_2$ plus 100 μmol/L vanadate for 30 minutes before the kinase reaction (Fig 8B). Chemical treatment reduced the activity of p56$^{Lck}$ and p59$^{flk}$, in terms of autophosphorylation as well as phosphorylation of enolase. Although the exact concentrations of H$_2$O$_2$ and vanadate in treated cells are unknown, these results suggest that the increase in tyrosine kinase activity observed in treated cells is not due to a direct reaction of the enzymes with H$_2$O$_2$ plus vanadate.

DISCUSSION

The biochemical mechanisms responsible for NF-κB induction have been the focus of extensive research. Brach et al have recently shown that ionizing radiation induces expression and binding activity of NF-κB in KG-1 myeloid leukemia cells. Schreck et al recently provided evidence that diverse agents activate NF-κB through a common mechanism involving the synthesis of ROI. Herbimycin A has been found to block interleukin-1-induced NF-κB activation in human lymphoid cells. Recently, we found that radiation-induced activation of NF-κB is triggered by stimulation of tyrosine-specific kinases and can be abrogated by the tyrosine kinase inhibitors herbimycin and genistein. Taken together, these observations prompted the hypotheses that the biochemical signals induced by ionizing radiation are dependent on the generation of ROI and that many of the pleiotropic effects of ROI on human lymphoid cells are triggered by stimulation of tyrosine-specific protein kinases. We tested this hypothesis by determining whether NAC and herbimycin could inhibit strong signals induced by substantial doses of H$_2$O$_2$ and radiation previously reported to be optimal. Furthermore, we examined cell responses within a short time of treatment. Although 20 Gy of gamma irradiation will kill over 90% of Ramos cells, this does not occur for 3 to 7 days and no change in trypan blue dye exclusion occurs in the first 24 to 48 hours (F. Uckun, unpublished results). The short exposures of Ramos cells to H$_2$O$_2$ reported here result in no change in viability even 48
hours after treatment. The ability of NAC and herbimycin to inhibit the signals from such substantial dosages shows the essential role of ROI and tyrosine kinase activation. Our findings that lower doses of H\textsubscript{2}O\textsubscript{2} (300 μmol/L) and radiation (5 Gy) also gave similar results shows that the effects are not unique to high doses. NAC alone does not depress tyrosine phosphorylation in unstimulated cells (F. Uckun, unpublished results), indicating that it is acting only as a free radical scavenger and not as a kinase inhibitor. The inhibitor studies thus indicate that activation of NF-κB by radiation was dependent on ROI generation and, furthermore, activation of NF-κB by ROI was dependent on tyrosine kinase activity.

The augmentation of tyrosine phosphorylation when cells were treated with H\textsubscript{2}O\textsubscript{2} plus vanadate was similar to the effect previously observed when vanadate-treated cells were irradiated,\textsuperscript{13} indicating a common mechanism. The ROI-induced tyrosine phosphorylation in lymphoid cells led to downstream events including NF-κB activation, Ins-1,4,5-P\textsubscript{3} generation, and [Ca\textsuperscript{2+}]\textsubscript{i} signals. Additional signal transduction pathways such as those involving ras and GAP may also be affected. Thus, ROI induction of tyrosine phosphorylation may account for many of the pleiotropic effects of ROI in lymphoid cells, whether generated chemically or by ionizing radiation. This hypothesis is further supported by the recent finding that the mammalian UV response in HeLa cells leading to c-Jun induction is triggered by src kinases and inhibited by elevation of intracellular glutathione levels.\textsuperscript{39} The identification of the particular ROI species involved in the kinase activation, as has been performed by spin trapping precursors of thymine damage in X-irradiated DNA,\textsuperscript{40} could provide further insights into the mechanisms involved.

The level of tyrosine phosphorylation in cells is the result of a dynamic equilibrium between the opposing activities of tyrosine kinases and phosphotyrosine phosphatases.\textsuperscript{41} H\textsubscript{2}O\textsubscript{2} and vanadate are potent phosphotyrosine phosphatase inhibitors, which, when used together, strongly induced tyrosine phosphorylation in insulin-responsive cells such as FAO cells due to activation of the insulin receptor’s tyrosine kinase activity.\textsuperscript{42,43} It has been shown that the potent phosphotyrosine phosphatase inhibitor phenylarsine oxide (PAO) induces tyrosine phosphorylation in T cells by inhibition of phosphatases without kinase activation,\textsuperscript{44} and the effects of H\textsubscript{2}O\textsubscript{2} and vanadate on lymphocytes has similarly been believed to be only due to phosphatase inhibition.\textsuperscript{33} However, we have now found that the combination of two potent phosphatase inhibitors, H\textsubscript{2}O\textsubscript{2} and vanadate, stimulates tyrosine kinases as well. This combination of kinase activation and phosphatase inhibition provides a mechanism for the hyperinduction of tyrosine phosphorylation in T- and B-cell lines. We propose that the triggering of an ROI-sensitive tyrosine kinase constitutes the initial signal, followed by stabilization of the resultant tyrosine phosphorylation due to inhibition of phosphatase activity. The signal is then further amplified by the activation of src family kinases such as p56\textsuperscript{Lck} and p59\textsuperscript{Srm}. The continued inhibition of phosphatase activity would then maintain the signal at high levels.

Notably, two kinases known to be involved in T-cell signaling, p56\textsuperscript{Lck} and p59\textsuperscript{Srm}, were activated, whereas p62\textsubscript{cbl}, which has not been reported to be involved in T-cell signaling, was not affected. Furthermore, direct reaction of H\textsubscript{2}O\textsubscript{2} plus vanadate did not activate the kinases. Taken together, these results suggest that regulatory elements of the signal transduction pathways mediate the effects of chemical treat-
ment on these kinases. The activation of the kinases by H$_2$O$_2$ plus vanadate does not appear to be a function of phosphatase inhibition alone because PAO has not been found to activate kinases. CD45 phosphotyrosine phosphatase is required for TCR- and CD2-mediated activation of protein tyrosine kinases. In contrast to these biologic receptor-mediated stimulations, the ability of a combination of two potent phosphatase inhibitors to activate tyrosine kinases shows that the requirement for phosphatase activity can be circumvented.

The combination of an ROI generator plus vanadate led to high levels of tyrosine phosphorylation of physiologically relevant proteins. This phosphorylation showed evidence of specificity in that tyrosine phosphorylation of p34$^{cdk}$ was not affected. Because phosphorylation of p34$^{cdk}$ is regulated in response to incompletely replicated DNA, the chemical treatment may not have affected that particular signal transduction pathway. The effects of H$_2$O$_2$ plus vanadate offer a method we are currently investigating to produce substantial quantities of tyrosine phosphorylated substrates for purification and identification.

The effects of ROI on lymphoid cell signal transduction may have important consequences for a variety of disease states. H$_2$O$_2$ is well suited as a messenger because it can freely diffuse across cell membranes and in healthy individuals the concentration of H$_2$O$_2$ is normally quite low. Thus, the elevation of H$_2$O$_2$ upon viral infection or inflammation could act as a significant signal. For cells already activated, this signal might be expected to amplify their response by boosting tyrosine phosphorylation, NF-$\kappa$B activation, and calcium signaling. However, for resting T cells, ROI could induce a calcium signal under nonmitogenic conditions that is potentially sufficient to induce a state of nonresponsiveness, as has been observed for calcium signals induced by modulation of CD3 or by pulsing with calcium ionophores. It has been proposed that many childhood acute lymphoblastic leukemias arise from a combination of initial mutations followed by a later infection. The ROI generated during the course of infection may play an important role in this process, because ROI are tumor promoters. Signals induced by ROI in lymphoid cells with accumulated mutations could conceivably tip the biochemical balance between oncogenic and tumor suppressor proteins in favor of leukemogenesis. The replication of human immunodeficiency virus-1 has been reported to be induced by ROI and inhibition by NAC via regulation of NF-$\kappa$B activation. Our results suggest that ROI-induced tyrosine phosphorylation is likely to play an essential role in this process. ROI-induced signals in lymphoid cells thus offer several avenues for future investigation.

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Reactive oxygen intermediates activate NF-kappa B in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the p56lck and p59fyn tyrosine kinases in human lymphocytes

GL Schieven, JM Kirihara, DE Myers, JA Ledbetter and FM Uckun