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Oxidative Stress Triggers STAT3 Tyrosine Phosphorylation and Nuclear Translocation in Human Lymphocytes*

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Oxidizing agents are powerful activators of factors responsible for the transcriptional activation of cytokine-encoding genes involved in tissue injury. In this study we show evidence that STAT3 is a transcription factor whose activity is modulated by H_2O_2 in human lymphocytes, in which endogenous catalase had previously been inhibited. H_2O_2-induced nuclear translocation of STAT3 to form sequence-specific DNA-bound complexes was evidenced by immunoblotting of nuclear fractions and electrophoretic mobility shift assays, and vanadate was found to strongly synergize with H_2O_2. Moreover, anti-STAT3 antibodies specifically precipitated a protein of 92 kDa that becomes phosphorylated on tyrosine upon lymphocyte treatment with H_2O_2. Phenylarsine oxide, a tyrosine phosphatase inhibitor, and genistein, a tyrosine kinase inhibitor, cooperated and cancelled, respectively, the H_2O_2-promoted STAT3 nuclear translocation. Evidence is also presented, using Fe^{2+}/Cu^{2+} ions, that $^{\cdot}OH$ generated from H_2O_2 through Fenton reactions could be a candidate oxygen reactive species to directly activate STAT3. Present data suggest that H_2O_2 and vanadate are likely to inhibit the activity of intracellular tyrosine phosphatase(s), leading to enhanced STAT3 tyrosine phosphorylation and hence its translocation to the nucleus. These results demonstrate that the DNA binding activity of STAT3 can be modulated by oxidizing agents and provide a framework to understand the effects of oxidative stress on the JAK-STAT signaling pathway.

Oxidative stress is characterized by high intracellular levels of reactive oxygen intermediates (ROI),[1] which may function as physiological mediators of a number of cellular responses by acting as second messengers for specific signaling pathways (1–3). In addition, ROI have been implicated in a variety of clinical conditions, including rheumatoid arthritis and other autoimmune diseases (4, 5), and can also play a role as tumor promoters (6). H_2O_2 as well as superoxide anions ($O_2^{\cdot-}$) have traditionally been viewed as potent microbicidal agents (7). H_2O_2 is a small, diffusible, and ubiquitous molecule that can be rapidly synthesized, as well as destroyed, in response to several stimuli. It has been proposed that H_2O_2 is converted into highly reactive $^{\cdot}OH$ radical via Fenton chemistry through its reduction by ferrous/cuprous ions (8, 9). H_2O_2 fulfills the prerequisites for intracellular second messengers. In this regard, recent studies have demonstrated that exposure of lymphocytes to oxidants, such as H_2O_2 (10–12), diamide (12, 13), or phenylarsine oxide (PAO) (14–16), results in an increased tyrosine phosphorylation of intracellular proteins.

STATs (signal transducers and activators of transcription) are a class of transcription factors bearing SH2 domains that become activated upon tyrosine phosphorylation. STATs are often activated by members of the JAK family of protein-tyrosine kinases (PTKs) in response to cytokine stimulation. This activation mechanism involves the SH2 domain-dependent recruitment of the STATs to tyrosine-phosphorylated cytokine receptors. The STATs then become phosphorylated by receptor-associated JAKs, which induces their dimerization via reciprocal SH2-phosphotyrosine interaction. STAT dimers then enter the nucleus and bind to specific DNA elements, thereby activating the transcription of a number of genes. The JAK-STAT pathway has been the subject of many recent comprehensive reviews (17–21). STAT3, a well characterized 92-kDa protein, has been shown to become activated by both epidermal growth factor and interleukin-6 in human A-431 cells (22). Because the ROI generated in response to various external stimuli can play a role both as regulators of transcription factors, including nuclear factors κB (2, 23) and AT (24), and as inhibitors of protein-tyrosine phosphatases (PTPases) (25–27), we have investigated whether H_2O_2 and other oxidizing agents could modulate STAT3 function in human lymphocytes. Enhanced phosphotyrosine accumulation could then result from the combined effects of increased phosphorylation and decreased dephosphorylation. Moreover, the DNA binding activity of STATs is known to depend primarily on tyrosine phosphorylation (19, 28–31), although serine phosphorylation is also important in modulating the binding affinity of STAT3 (32–34). Here we show for the first time that STAT3 is phosphorylated on tyrosine residue(s), translocated to the nucleus, and elicited to bind to specific DNA elements upon lymphocyte treatment with H_2O_2. An additive effect between H_2O_2 and vanadate was also evidenced suggesting that inhibition of tyrosine phosphatase(s)

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[1] The abbreviations used are: ROI, reactive oxygen intermediates; PAO, phenylarsine oxide; JAK, Janus kinase; PTK, protein-tyrosine kinases; PTPase, protein-tyrosine phosphatases; PMSF, phenylmethylsulfonyl fluoride; PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes; PAGE, polyacrylamide gel electrophoresis; SIE, *sis*-inducible element.

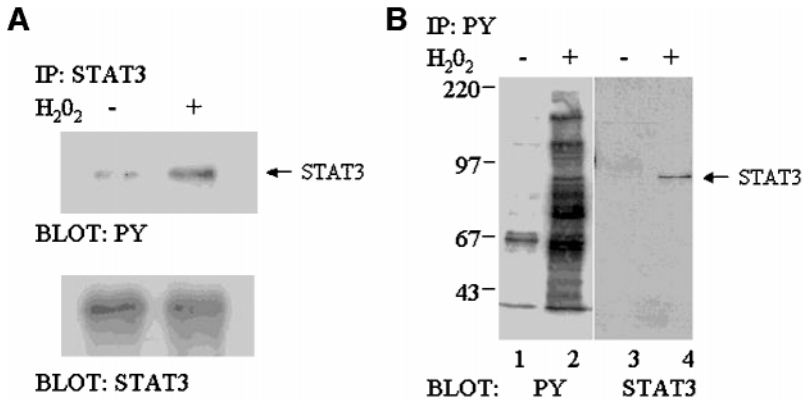

FIG. 1. **H_2O_2 induces tyrosine phosphorylation of STAT3 in human lymphocytes.** *A*, PBL were left untreated (−) or were treated (+) with 2 mM H_2O_2 for 2 min in RPMI 1640 medium at 37 °C. The cells were then lysed in buffer B, and the supernatants obtained after centrifugation were incubated with anti-STAT3 antibodies followed by precipitation of the immune complexes with protein A-Sepharose beads. The bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted sequentially with anti-phosphotyrosine antibodies (*upper panel*) or after stripping with anti-STAT3 antibodies (*lower panel*). *B*, PBL were treated and lysed as in *A*, and immunoprecipitation was carried out with anti-phosphotyrosine antibodies cross-linked to protein A-agarose. Tyrosine phosphorylation was detected by immunoblotting with anti-phosphotyrosine antibodies, and after blot stripping, STAT3 protein levels were analyzed by immunoblotting with anti-STAT3 antibodies. The sizes of molecular mass markers, expressed in kDa, are indicated at the *left* of *panel B*. *Arrows* in *A* and *B* show the position of migration of STAT3. *IP*, immunoprecipitation; PY, phosphotyrosine.

by pervanadate could take place. The effects of H_2O_2 were also enhanced by the presence of Fe^{2+} and Cu^{2+} ions, which indicates the participation of other chemical reactive species derived from oxygen. These data collectively indicate that STAT3 is a major component of the signaling pathways that become activated by oxidative stress in human lymphocytes.

MATERIALS AND METHODS

Reagents—Hydrogen peroxide (30%, v/v), 3-amino-1,2,4-triazole, Na_3VO_4, PAO, diamide, genistein, fetal bovine serum, and 1,10-phenantroline were purchased from Sigma Chemical (Madrid, Spain). Ficoll-Hypaque, phosphate-buffered saline, and RPMI 1640 were obtained from Bio-Whittaker (Verviers, Belgium). Phenylmethylsulfonyl fluoride (PMSF) and the double-stranded SIE m67 oligonucleotide (5′-GTCGACATTTCCCGTAAATC-3′) were obtained from Roche Molecular Biochemicals (Barcelona, Spain). Interferon-α was purchased from Schering-Plow (Brinny Innishannon, Ireland).

Antibodies—Polyclonal antisera directed against STAT1 and STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies (4G10) against phosphotyrosine (free or conjugated to agarose beads) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal anti-STAT5B antibody was kindly provided by Dr. J. O'Shea (Bethesda, MD). Goat anti-rabbit and anti-mouse antibodies conjugated to horseradish peroxidase were purchased from Sigma Chemical.

Cell Culture and Treatment—Human peripheral blood lymphocytes (PBL) were prepared by Ficoll-Hypaque gradient centrifugation from normal blood donors, following informed consent. Normally, PBL were used immediately after they were obtained. In other cases, PBL were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in an atmosphere of 5% CO_2 and 95% O_2 for 24 h. For stimulation treatments, the cells were washed with RPMI 1640, resuspended in fresh medium at a density of 10×10^6 cells/ml, and incubated in RPMI 1640 with the reagents indicated in each case and for the specified times at 37 °C. Stimulation was terminated by centrifugation at 5,000 rpm for 30 s and washing once with ice-cold phosphate-buffered saline supplemented with 400 μM Na_3VO_4 and 400 μM EDTA. In all the experiments in which H_2O_2 was used, the cells were previously incubated with 25 mM aminotriazole for 30 min to inhibit endogenous catalase activity, and the cells were not washed before H_2O_2 addition.

Western Blotting Analysis—Stimulated cells were pelleted and lysed in 75 μl of ice-cold lysis buffer A, containing 20 mM Tris-HCl, pH 8.0, 1% (v/v) Nonidet P-40, 137 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, and the following inhibitors of phosphatase and protease: 5 mM EDTA, 100 μM PAO, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The lysates were kept on ice for 30 min with occasional mixing and then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatants obtained constituted the cytoplasmic fraction, and the nuclei-containing pellets were washed three times with buffer A (35). Standard Laemmli sample buffer was then added to cytosolic and nuclear fractions, and after boiling for 5 min followed by centrifugation at 10,000 × *g* for 10 min, the supernatants were analyzed by SDS-PAGE on 7.5% polyacrylamide gels. The separated proteins were electrophoretically transferred to nitrocellulose membranes, and after blocking nonspecific interactions in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Tween 20) containing 3% bovine serum albumin for 1 h, incubation with anti-STAT or 4G10 antibodies (diluted 1:1000 in TBST) was carried out overnight at 4 °C. Next, the membranes were washed twice with TBST, and incubation with a 1:5000 dilution of goat anti-rabbit secondary antibody coupled to horseradish peroxidase was performed for 1 h at room temperature. After washing three times with TBST, immunoreactive bands were visualized by using the enhanced chemiluminescence assay as described previously (36).

Immunoprecipitation—Stimulated cells were pelleted and lysed in 75 μl of ice-cold lysis buffer B containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 300 mM NaCl, 100 μM PAO, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM Na_3VO_4, and 5 mM EDTA. The lysates were centrifuged at 12,000 × *g* for 5 min. For immunoprecipitation, incubation with anti-STAT3 specific antibodies (0.5 μg/70 μl) was carried out with rotation for 2 h at 4 °C, followed by addition of 40 μl of a (50%) slurry of protein A-Sepharose beads and further incubation for 2 h at 4 °C. Alternatively, anti-phosphotyrosine antibodies covalently attached to agarose (10 μg/70 μl) and a single incubation step performed for 2 h at 4 °C. The immune complexes were washed five times with ice-cold lysis buffer B, and the proteins were extracted by boiling the pellet in standard Laemmli sample buffer. After resolution by SDS-PAGE (7.5% polyacrylamide), the proteins were electrotransferred to nitrocellulose membranes and subjected to immunoblotting analysis as indicated above.

Electrophoretic Mobility Shift Assay—Stimulated cells were lysed on ice for 10 min in hypotonic buffer C, which contained 20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM Na_3VO_4, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM NaF, 1 mM dithiothreitol, and 0.2% (v/v) Nonidet P-40. After centrifugation at 13,000 rpm for 1 min at 4 °C, the supernatant obtained was collected as the cytoplasmic fraction. Nuclear extracts were prepared by resuspension of the pellet in 25 μl of high salt buffer (buffer C supplemented with 20% glycerol and 420 mM NaCl), followed by incubation on ice for 30 min with occasional mixing. After centrifugation at 13,000 rpm for 10 min at 4 °C, the supernatants obtained constituted the nuclear extracts. STAT3 DNA binding activity was assayed using the SIE m67 20-base pair oligonucleotide as a probe (see sequence above) and the digoxigenin gel shift assay kit from Roche Molecular Biochemicals. The reactions were performed in 15 μl of a binding buffer containing 20 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 6% glycerol, and 10 μg of nuclear extract in the presence of 1 pmol of digoxigenin-labeled oligonucleotide and allowed to proceed for 20 min at room temperature. For competition assays, binding reactions were performed in the presence of a 100-fold molar excess of unlabeled

oligonucleotide. The samples were finally electrophoresed on 5% polyacrylamide native gels at 4 °C in 0.25× TBE buffer.

RESULTS

Hydrogen Peroxide Promotes Tyrosine Phosphorylation and Nuclear Translocation of STAT3—To investigate the changes on the STAT3 tyrosine phosphorylation status that take place under oxidative stress conditions, PBL cells were treated with H_2O_2 for 2 min, and then STAT3 was immunoprecipitated using specific antibodies. Immunoblotting analyses revealed that, whereas the amount of STAT3 protein immunoprecipitated from H_2O_2-treated and untreated cells was comparable, the phosphotyrosine content of STAT3 was considerably higher in lymphocytes treated with H_2O_2 (Fig. 1*A*). In other experiments, cells treated with H_2O_2 and untreated control cells were lysed and subjected to immunoprecipitation with anti-phosphotyrosine antibodies. As shown in Fig. 1*B*, a high number of proteins underwent tyrosine phosphorylation upon H_2O_2 treatment, as revealed by immunoblotting using the same anti-phosphotyrosine antibodies. The presence of STAT3 as a 92-kDa protein among these phosphorylated polypeptides was further evidenced by immunobloting with anti-STAT3 antibodies. A detectable signal was only obtained in the immunoprecipitates from cells treated with H_2O_2, which was virtually absent in control untreated cells (Fig. 1*B*).

To test whether the H_2O_2-promoted increase in tyrosine phosphorylation of STAT3 was accompanied by its translocation from the cytosol to the nucleus, PBL cells treated with H_2O_2 for different times were lysed, and the cytoplasmic and nuclear fractions were subject to immunoblotting analysis using anti-STAT3 antibodies. As illustrated in Fig. 2*A,* STAT3 was clearly detectable in the nucleus after 10 min of treatment with H_2O_2, whereas in the cytosol the STAT3 signal at this time was significantly diminished. Nuclear translocation of STAT3 appeared to be completed after 30 min, because no cytosolic STAT3 was detected at this point. In some untreated cell preparations, a certain amount of STAT3 was found in the nuclei, attributable to basal levels of activation in the absence of stimulus.

The effect on STAT3 nuclear translocation of phytohemagglutinin (PHA), a well known promoter of lymphocyte proliferation, was also analyzed alone or in combination with H_2O_2. As shown, PHA by itself was able to slightly induce STAT3 nuclear translocation (Fig. 2*B*, *lane 8*). This effect was, however, considerably enhanced when PHA acted in combination with H_2O_2, conditions under which a synergistic effect was found (Fig. 2*C*). Recently, it has been documented that the treatment of T lymphocytes with interleukin-2 results in an increase of STAT3 tyrosine phosphorylation, although no such effect was observed to occur in cells treated with PHA for 3 days (37). Present results suggest that PHA enhances the transduction of signals that are required for optimal H_2O_2 effect.

Hydrogen Peroxide and Vanadate Act Synergistically to Induce Nuclear Translocation of STAT3—It has been previously proposed that the combination of H_2O_2 and orthovanadate potently inhibits PTPase activity (25, 26) and/or synergistically stimulates insulin-dependent PTK activity (38) in T lymphocytes. Also, the mixture of H_2O_2 and sodium orthovanadate promotes tyrosine phosphorylation of phospholipase Cγ1 and activates $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases (23). These facts have been explained in the light of inhibition of protein-tyrosine phosphatase(s) by pervanadate (38). Further experiments were therefore used to analyze whether a decrease in intracellular PTPase activity could be implicated in the H_2O_2-induced STAT3 nuclear translocation effect. Toward this end, human PBL were treated with orthovanadate, alone or in combination with H_2O_2, and the nuclear levels of STAT3 were

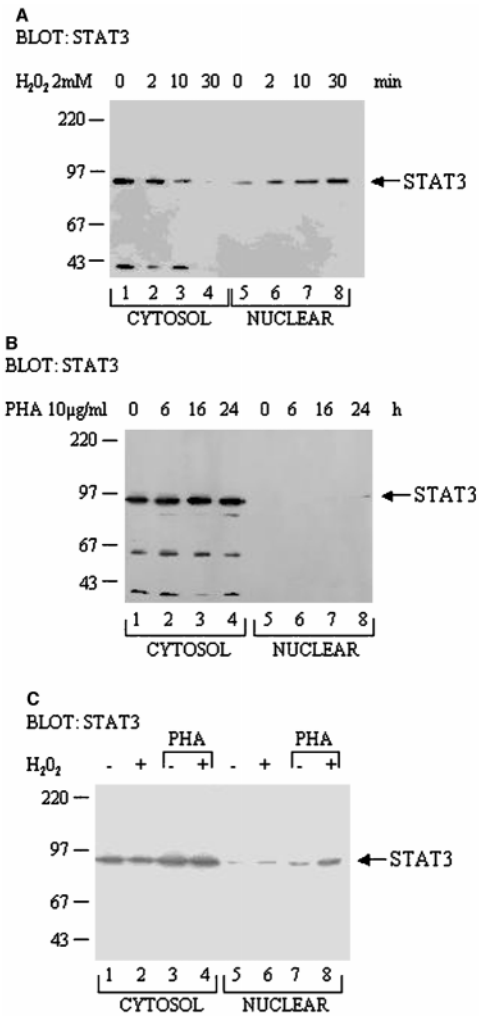

FIG. 2. **H_2O_2 and PHA induce STAT3 translocation from the cytosol to the nucleus in human lymphocytes.** PBL were treated with 2 mM H_2O_2 (*A*) or 10 μg/ml PHA (*B*) for the indicated times under culture conditions. The cells were then lysed in buffer A, and the cytoplasmic and nuclear fractions were obtained as described under "Materials and Methods." The proteins were resolved by SDS-PAGE and subject to immunoblotting analysis with anti-STAT3 antibodies. *C*, PBL were treated or not with 10 μg/ml PHA for 24 h and then were treated in the presence or absence of 2 mM H_2O_2 for 15 min, as indicated by the *minus* and *plus symbols*. The cells were then lysed, and cytoplasmic and nuclear fractions were analyzed by immunoblotting with anti-STAT3 antibodies. The sizes of molecular mass markers, expressed in kDa, are indicated at the *left* of the three panels. *Arrows* indicate the position of migration of STAT3.

analyzed. Vanadate alone at 100 μM (the maximal dose tested) promoted a slight accumulation of STAT3 in the nucleus (Fig. 3*A*, *lane 5*), and H_2O_2 alone (Fig. 3*A*, *lanes 1–4*) induced this STAT3 translocation detectable only at the highest tested concentration (2 mM). However, the combination of vanadate and H_2O_2 markedly stimulated in a synergistic fashion the nuclear translocation of STAT3, enhancing about 6-fold the effect obtained with 2 mM H_2O_2 alone. As shown in Fig. 3*A*, at 100 μM vanadate combined with a low dose of H_2O_2, a nuclear translocation of STAT3 took place (*lane 6*), whereas at higher concentrations of H_2O_2 an intense nuclear STAT3 signal was observed (*lanes 7* and *8*).

Effects of Phenylarsine Oxide, Diamide, and Genistein on H_2O_2-induced Nuclear Translocation of STAT3—Because the above effects of pervanadate could be due to the inhibition of intracellular tyrosine phosphatase(s), experiments were attempted to analyze whether other reagents known to alter the tyrosine phosphorylation status of proteins could influence the

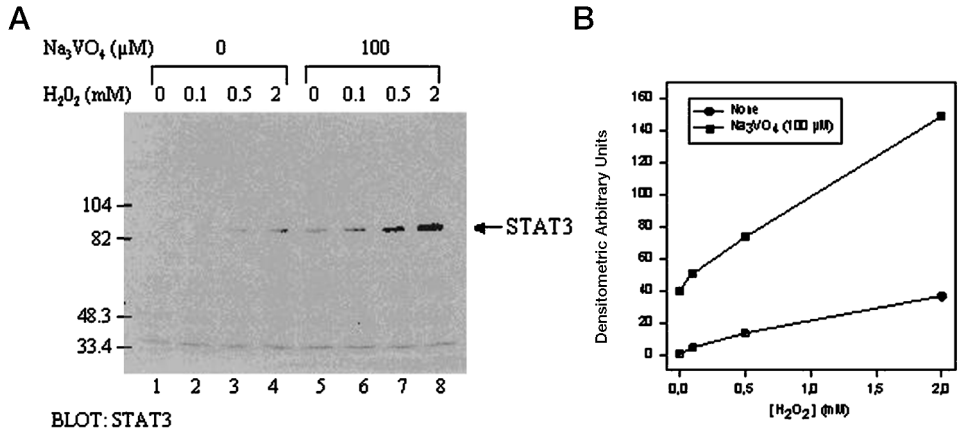

FIG. 3. **Orthovanadate and H_2O_2 cooperatively induce STAT3 nuclear translocation.** *A*, PBL were treated with several doses of H_2O_2 in the presence or absence of 100 μM sodium orthovanadate for 15 min at 37 °C. The cells were then lysed in buffer A, and nuclear fractions obtained as described under "Materials and Methods" were resolved by SDS-PAGE and analyzed by immunoblotting with anti-STAT3 antibodies. The *arrow* indicates the STAT3 band. The sizes of molecular mass markers, expressed in kDa, are indicated at the *left* of *panel A*. *B*, STAT3 nuclear levels obtained from densitometric analysis of *panel A* are plotted *versus* the concentration of H_2O_2.

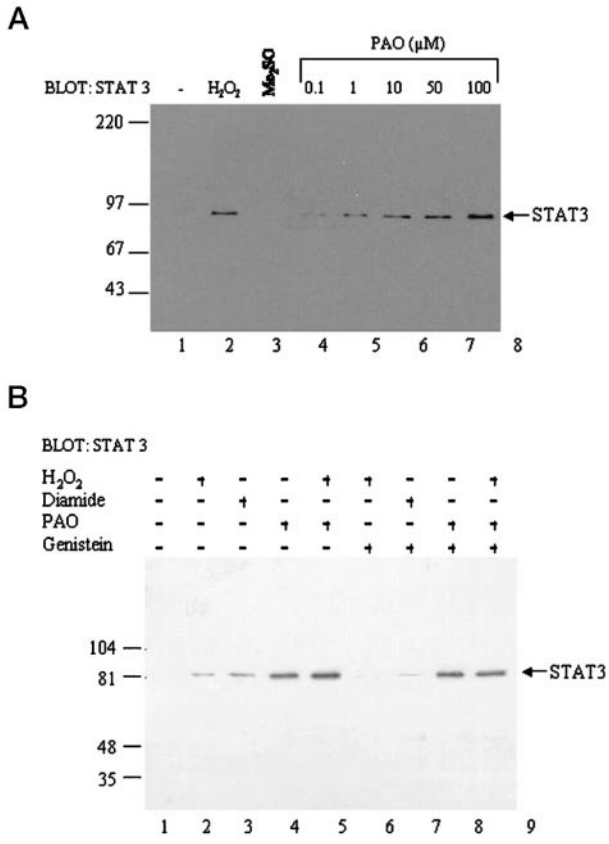

FIG. 4. **PAO induces nuclear translocation of STAT3.** *A*, PBL were left untreated (*lane 1*) or were treated for 15 min with 2 mM H_2O_2 (*lane 2*), with 1 μl of Me_2SO (*lane 3*), or with the indicated doses of PAO (*lanes 4–8*). The cells were then lysed in buffer A, and nuclear fractions were resolved by SDS-PAGE and analyzed by immunoblotting with anti-STAT3 antibodies. *B*, PBL were subjected to the indicated treatments. PBL were left untreated (*lanes 1–5*) or treated (*lanes 6–9*) with genistein (100 μM) for 30 min, followed by 2 mM H_2O_2 (*lanes 2* and *6*), 1 mM diamide (*lanes 3* and *7*), or 100 μM PAO (*lanes 4* and *8*) for 15 min. In *lanes 5* and *9*, H_2O_2 and PAO were added together at the same time. Nuclear fractions were analyzed by immunoblotting with anti-STAT3 antibodies. The *arrows* indicate the STAT3 band, and the sizes of molecular mass markers, expressed in kDa, are indicated at the *left* of both panels.

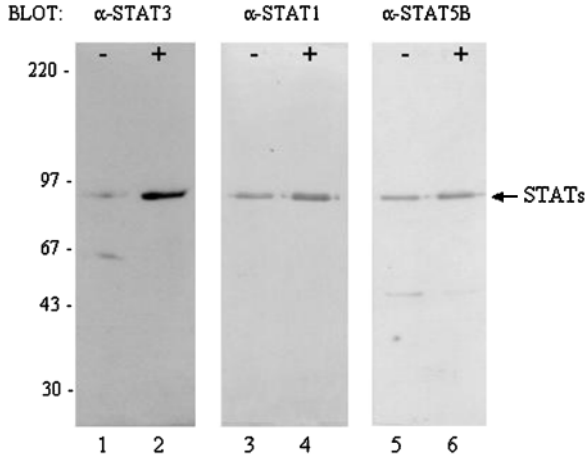

FIG. 5. **H_2O_2 induces nuclear translocation of STAT3 but not of STAT1 or STAT5B.** PBL were left untreated (−) or were treated (+) with 2 mM H_2O_2 for 15 min, and nuclear fraction samples were resolved by SDS-PAGE and analyzed by immunoblotting with anti-STAT3, anti-STAT1, or anti-STAT5B antibodies, as indicated.

nuclear translocation of STAT3. Because the effect of PAO as a PTPase inhibitor in T lymphocytes is well known (39), we decided to test its effect on STAT3 nuclear translocation in human PBL. As shown in Fig. 4*A,* PAO induced a dose-dependent enhancement of STAT3 nuclear translocation, as monitored in nuclear fractions from cells treated with different concentrations of PAO, upon immunoblotting analysis with anti-STAT3 antibodies. It could also be observed that the nuclear levels of this factor reached in the presence of 2 mM H_2O_2 could be mimicked by 50 μM PAO. Furthermore, PAO and H_2O_2 were found to act in a synergistic fashion (Fig. 4*B,* compare *lanes 2* and *4* with *lane 5*), which suggests that both molecules could be cooperatively acting as inhibitors of intracellular tyrosine phosphatases. Diamide, a compound with oxidative properties (12, 13), was also shown to stimulate the nuclear translocation of STAT3 (Fig. 4*B*, *lane 3*). This drug was reported to induce a marked enhancement of the tyrosine phosphorylation status of several cellular proteins (13, 40), possibly mediated by inhibition of tyrosine phosphatase(s) (13, 37). In an opposite fashion, genistein, an inhibitor of protein-tyrosine kinases, produced a partial inhibition of the STAT3 nuclear translocation elicited by H_2O_2 (Fig. 4*B*, *lane 6*) or diamide (*lane 7*). However, genistein was unable to counteract the stimulating action of PAO (*lane 8*). These results clearly suggest that the nuclear levels of STAT3 are balanced by the opposite actions of intracellular PTKs and PTPases.

Experiments were also conducted to study the specificity of

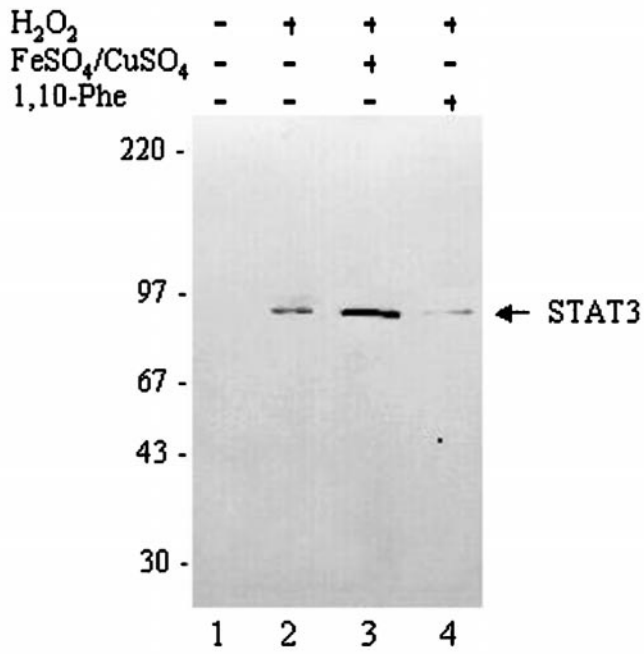

FIG. 6. **Pretreatment with $FeSO_4/CuSO_4$ or 1,10-phenantroline modulates STAT3 nuclear translocation induced by H_2O_2.** PBL were left untreated (*lane 1*), treated with 2 mM H_2O_2 (*lane 2*) for 15 min, or pretreated for 15 min with 100 μM $FeSO_4$/100 μM $CuSO_4$ (*lane 3*) or 20 μM 1,10-phenantroline (*1,10-Phe*, *lane 4*) and then exposed to 2 mM H_2O_2 for a further 15 min. Nuclear fractions were resolved by SDS-PAGE and analyzed by immunoblotting with anti-STAT3 antibodies.

oxidant-induced STAT nuclear translocation. Immunoblotting analyses of PBL nuclear extracts reveals that H_2O_2 treatment lightly induced the nuclear translocation of STAT1 and STAT5B, although to a lesser extent than that observed for STAT3 (Fig. 5). The distinct behavior displayed by these STAT proteins upon cell challenge with H_2O_2 agrees with recent observations regarding their activation by different cytokines and growth factors (41). In this regard, G-CSF induces tyrosine phosphorylation of STAT1, STAT3, and STAT5B; however, STAT5B can be activated by mutant receptors that lack tyrosine residues (41, 42). Moreover efficient STAT3 activation requires additional receptor sequences that include Tyr^{704} (42).

Effect of Fe^{2+}/Cu^{2+} Ions on H_2O_2-promoted STAT3 Nuclear Translocation—After passive diffusion through the plasma membrane, H_2O_2 can be converted into other active oxygen species, such as O_2^- and $^{\cdot}OH$ (23). The most likely mode of intracellular $^{\cdot}OH$ radical production is via Fenton chemistry, which involves the reduction of H_2O_2 by ferrous ions according to the reaction: $H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$ (8). Also, cuprous ions are elemental producers of $^{\cdot}OH$ radicals via Fenton reactions (9). We have analyzed whether the chemical species generated from H_2O_2 through Fenton reactions could affect STAT3 nuclear translocation. Fig. 6 illustrates that the prior addition of Fe^{2+} and Cu^{2+} ions notably enhanced the H_2O_2-induced nuclear translocation of STAT3 (*lane 3*). By contrast, the pretreatment of cells with 1,10-phenantroline, a chelator of copper and iron ions resulted in cancellation of the STAT3 nuclear accumulation induced by H_2O_2 (*lane 4*). These results indicate that the intracellular reduction of H_2O_2 to yield more reactive oxygen species, such as $^{\cdot}OH$, could constitute a more efficient mechanism for the positive action of H_2O_2 on STAT3.

Hydrogen Peroxide Enhances STAT3 DNA Binding Activity—The enhancement of the ability of nuclear STATs to bind to specific DNA elements in PBL cells upon cell stimulation by oxidizing agents was assessed through gel mobility shift assays using a double-stranded oligonucleotide probe containing a high affinity target sequence for STAT factors. This binding sequence, named m67, was derived from the *sis*-inducible element (SIE) of the human c-*fos* promoter (43, 44). Nuclear extracts were thus prepared from PBL challenged with orthovanadate, H_2O_2, or both and incubated with the digoxigenin-labeled SIE m67 oligonucleotide probe. Fig. 7*A* illustrates that H_2O_2 induced a rapid assembly of complexes able to bind to the SIE m67 probe within PBL nuclei (*lane 3*). The same effect was observed upon vanadate treatment (*lane 2*), and a cooperative effect was found to take place between vanadate and H_2O_2 (*lane 4*). As a positive control, we also analyzed the effect of interferon-α (18), with the same results (*lane 5*). The shifted band could be competed by an excess of unlabeled SIE m67 oligonucleotide; thus, it indicates that nuclear factor binding was specific (*lane 6*). However, quite intriguingly, we found that treatment with PAO, despite this compound enhanced STAT3 nuclear translocation, cancelled completely nuclear factor binding to the STAT specific sequence, even below basal levels of binding seen in the absence of H_2O_2 (data not shown). We cannot offer any explanation for this fact, although it could be related to the hyperphosphorylation of proteins (*e.g.* STAT3) found to occur after PAO treatment (38, 45, 46). Fig. 7*B* illustrates that the presence of Fe^{2+}/Cu^{2+} in combination with H_2O_2 greatly enhanced the capacity of nuclear factors to bind to the SIE m67 oligonucleotide (Fig. 7*B*, *lane 3*) and that the addition of 1,10-phenantroline reduced the H_2O_2-induced nuclear factor binding. These results show a clear correlation with the observations described in Fig. 6 regarding the nuclear translocation of STAT3.

DISCUSSION

The intracellular processes that become triggered upon oxidative stress constitute nowadays a focus of extensive research. Very recently, we have described that H_2O_2 and other peroxides are able to inhibit calcineurin activity and to inactivate nuclear factor κB DNA binding in human neutrophils (36). In contrast, Schreck *et al.* (2) have provided evidence that peroxides activate nuclear factor κB in lymphocytes through an undetermined mechanism involving the synthesis of ROI. Also, H_2O_2 has been implicated in the positive modulation of the activity of a number of PTKs whose function is critical for lymphocyte activation (11, 47). An involvement of STAT factors in mediating leukocyte activation elicited by interleukin-2 has been recently discovered (33, 37, 48). However, the potential role of STAT factors as mediators of peroxide-triggered lymphocyte activation, by acting as target molecules of oxidative processes, has not been previously explored. In this paper, we provide the first evidence that H_2O_2 promotes tyrosine phosphorylation of STAT3 in peripheral blood human lymphocytes, which is followed by its translocation to the nucleus and binding to a specific DNA element.

It is well established that the activation of STAT factors requires their phosphorylation on tyrosine residue(s) (34). To explain the mechanism whereby they become activated by oxidants, two possibilities can be contemplated. First, the alteration of their redox status could directly alter STAT conformation, in such a way that their interaction with cytosolic proteins responsible for nuclear targeting would be enabled. Because the structures of STAT3 and especially its presumptive redox centers are not completely understood, this hypothesis requires further investigation. The current state of knowledge thus makes more likely a second explanation based on the ability of oxidants to act as inhibitors of tyrosine phosphatases. According to this possibility, STAT3 nuclear translocation would be facilitated or promoted by its increased status of tyrosine phosphorylation, this being the consequence of inhibition by H_2O_2 of

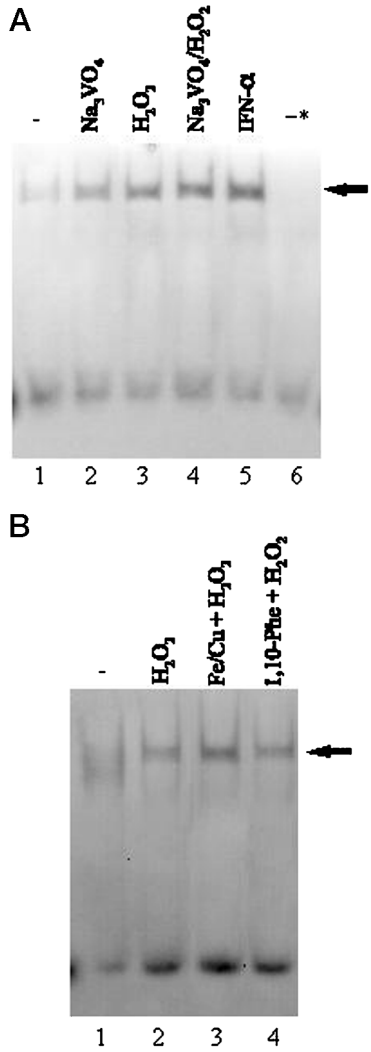

FIG. 7. **H_2O_2 enhances STAT3 DNA binding activity.** *A*, PBL were left untreated (*lane 1*) or were treated with 100 μM Na_3VO_4 (*lane 2*), 2 mM H_2O_2 (*lane 3*), both H_2O_2 and Na_3VO_4 added at the same time (*lane 4*), or 1000 units/ml interferon-α (*lane 5*) for 10 min. The cells were then lysed in buffer C, and nuclear extracts were prepared as described under "Materials and Methods." DNA binding to the digoxigenin-labeled SIE m67 oligonucleotide probe was assessed by gel mobility shift assays. The retarded band is indicated by an *arrow*. Where indicated by an *asterisk* (*lane 6*), binding reactions were performed in the presence of a 100-fold molar excess of unlabeled oligonucleotide in the reaction performed with nuclear extracts from H_2O_2-treated human lymphocytes. *B*, PBL were left untreated (*lane 1*) or treated with 2 mM H_2O_2, alone (*lane 2*) or in combination with 100 μM $FeSO_4$/100 μM $CuSO_4$ (*lane 3*) or 20 μM 1,10-phenantroline (*1,10-Phe*, *lane 4*) for 15 min. Nuclear extracts were used for gel mobility shift assays as indicated for *panel A*.

the activity of intracellular PTPases (25, 39). In this context, exogenously added H_2O_2 has been shown to increase protein-tyrosine phosphorylation in several cell types, such as lung epithelial and FAO cells (49, 50). Also, H_2O_2 can directly inhibit PTPase activity *in vitro*, and this inhibition can be completely reversed by dithiothreitol (25). All PTPases are known to contain an essential sulfhydryl group at their active site, which is susceptible to oxidation because of its unusually low pK_a (<5) (51). These observations thus suggest that PTPases may constitute targets for intracellularly generated H_2O_2 and that their inhibition by oxidants may well lead to activation of number of signaling molecules, such as STAT3, by virtue of their increased phosphotyrosine content. It can be presumed that because the specific activities of PTPases *in vitro* are 10–1000 times higher than those of PTKs (52), in most cell types the activation of a receptor tyrosine kinase upon ligand binding may not be sufficient to increase the steady-state level of protein-tyrosine phosphorylation, so that concurrent inhibition of PTPases by H_2O_2 and/or derived ROI could additionally be necessary.

The treatment of lymphocytes with vanadium derivatives induces a strong tyrosine phosphorylation of protein kinases of the Erk2 and Syk families, leading to their activation (45). Also, it has been reported that H_2O_2 treatment of blood T cells elicits tyrosine phosphorylation of Lck kinases and that a much stronger response on tyrosine phosphorylation of multiple cellular proteins is observed following treatment with the sulfhydryl oxidizing agent diamide (12). Furthermore, the Src kinases, such as $p56^{lck}$ and $p59^{fyn}$ in T cells, were transiently activated by H_2O_2 in combination with vanadate (23). It is interesting to note that vanadate by itself exerts a limited effect on the tyrosine phosphorylation of STAT3, as shown in this work. However, upon the simultaneous addition of H_2O_2 and vanadate, the resulting pervanadate enhanced severalfold the effects exerted separately by these two compounds. It is of interest that pervanadate administered as such induces the tyrosine phosphorylation and nuclear translocation of a number of STAT proteins in liver cells, including STAT3 (53). Also, pervanadate has been shown to induce T cell activation and transcription of c-*fos* (54), the activation of interferon-γ- and prolactin-dependent transcription factors, and the activation of JAK kinases in epithelia cells (55, 56). The possibility that the H_2O_2 and pervanadate effects on nuclear translocation of STAT3 could be ascribed to inhibition of tyrosine phosphatases is reinforced by experiments using PAO and diamide, two compounds able to act as PTPase inhibitors. As shown in this work, these drugs elicited an enhancement of the H_2O_2 action, whereas genistein, an inhibitor of protein-tyrosine kinases, antagonized the H_2O_2 and diamide effects. Genistein did not, however, counteract the increase in STAT3 phosphorylation promoted by PAO, possibly due to an imbalance between the phosphorylation and dephosphorylation of STAT3 at the study doses of both agents. Taken together, present results are in line with previous reports, demonstrating that H_2O_2 acts an inhibitor of tyrosine phosphatase(s) (10–12), leading in turn to enhanced STAT3 tyrosine phosphorylation. The complexity of this phenomenon is evidenced, however, by the recent demonstration that phosphorylation of STAT3 on the residue Ser^{727} is also required for competent STAT3 transcriptional activation (33, 34). It may thus be speculated that H_2O_2 could as well increase a serine kinase activity necessary for the activation of STAT3.

It is noteworthy that the enhancing effect of H_2O_2 on STAT3 nuclear translocation seems rather specific, because neither STAT1 nor STAT5B underwent migration to the nucleus under oxidative conditions, as shown in this paper. However, which is the active oxygen species directly responsible for the observed H_2O_2 effects on STAT3 activation is currently unknown. Exogenously H_2O_2 added is rapidly converted into other chemical species, and in this context we present evidence, using a combination of Fe^{2+} and Cu^{2+} ions, that $^{\cdot}OH$ radicals generated through Fenton reactions could fulfill such a role. In fact, in the presence of these metal ions, an enhanced STAT3 nuclear translocation and DNA binding was found in cells exposed to H_2O_2. Accordingly, it is clear that H_2O_2 is not the sole species implicated in the nuclear translocation of STAT3. In line with a recent paper, showing that 1,10-phenantroline, a chelator of iron and copper ions, completely prevented DNA strand breakage induced by H_2O_2 (57), we have found that this compound was able to cancel STAT3 nuclear translocation induced by H_2O_2. These results speak in favor of the possibility that in-

tracellular Fe^{2+}/Cu^{2+} ions could play a role as intermediates under H_2O_2-induced oxidative stress conditions.

In summary, present observations support a role for oxidants in the regulation of STAT3 transcription factor activity *in vivo* in human lymphocytes. In keeping with extensive work done elsewhere, our results favor the hypothesis that H_2O_2 by itself and/or derived oxidative species generated through Fenton reactions can modulate the STAT3 tyrosine phosphorylation status and hence promote its translocation to the nucleus and the formation of bound complexes at specific DNA promoter elements.

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