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Hypochlorous Acid Activates Tyrosine Phosphorylation Signal Pathways Leading to Calcium Signaling and TNFα Production

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ABSTRACT

Hypochlorous acid is an important oxidizing agent produced by neutrophils to aid in defense against pathogens. Although hypochlorous acid is known to cause tissue damage due to its cytotoxicity, the effect of this oxidizing agent on signal transduction by cells of the immune system and its effects on their responses are not well understood. In this study, hypochlorous acid was found to induce cellular tyrosine phosphorylation in both T and B lymphocytes, activate the ZAP-70 tyrosine kinase, and induce cellular calcium signaling in a tyrosine kinase-dependent manner. These signaling events also occurred in T cell lines that did not express the T-cell receptor, indicating the ability of hypochlorous acid to bypass normal receptor control. Hypochlorous acid induced tumor necrosis factor-α production in peripheral blood mononuclear cells in a tyrosine kinase-dependent manner. These results suggest that hypochlorous acid may contribute to inflammatory responses by activating signal pathways in cells of the immune system. *Antioxid. Redox Signal.* 4, 501–507.

INTRODUCTION

LYMPHOCYTES are highly sensitive to oxidative stress and a variety of agents, including hydrogen peroxide (H_2O_2), ionizing radiation, and ultraviolet (UV) radiation, are known to activate lymphocyte tyrosine phosphorylation signal pathways (20). Among the most common sources of oxidative stress normally encountered by lymphocytes are the oxidizing agents produced at sites of inflammation. Hypochlorous acid (HOCl) is one of the most important of these oxidizing agents. HOCl is formed by the reaction of H_2O_2 and chloride ion catalyzed by the neutrophil enzyme myeloperoxidase (1). HOCl is a key neutrophil product that has strong bactericidal properties. However, in addition to the role of HOCl in the innate host defense against pathogens, HOCl also has the potential to damage normal cells and tissues. HOCl can damage membranes by reaction with unsaturated lipids (17), and permeates cells to oxidize glutathione (27). HOCl can react with amines to form relatively stable chloramines such as taurine chloramine that are oxidizing agents with biological activity (13). HOCl reacts with tyrosine in proteins to generate 3-chlorotyrosine, which can serve as a biomarker for generation of HOCl *in vivo* (5). In addition to direct damage to tissues, HOCl has been reported to induce tumor necrosis factor-α (TNFα) production in a human immunodeficiency virus (HIV)-infected T-cell line through a nuclear factor-κB dependent pathway, but the mechanism by which this occurs has not been established (24).

As HOCl has the potential to be an important source of oxidative stress for lymphocytes at sites of inflammation, we examined whether HOCl can activate tyrosine phosphorylation signal pathways in T cells. We report that HOCl induces cellular tyrosine phosphorylation and activates the zeta associated protein-70 (ZAP-70) tyrosine kinase in Jurkat T cells, leading to the induction of Ca^{2+} signaling in a tyrosine kinase-dependent manner. In addition, HOCl induced TNFα production in normal human peripheral blood mononuclear cells, and this induction was tyrosine kinase-dependent.

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MATERIALS AND METHODS

Cells and reagents

The human B-cell lymphoma line Ramos, the human T-cell leukemia line Jurkat, and human peripheral blood mononuclear cells isolated from blood obtained from normal healthy volunteers were cultured in RPMI 1640 media (GibcoBRL) supplemented with 10% fetal bovine serum. BMS-3P Jurkat T cells lacking CD3 and CD4 expression have been previously described (23) and were generously provided by Robert Mittler (Emory University, Atlanta, GA, U.S.A.). Cells were put into RPMI 1640 medium without phenol red for stimulation with HOCl (Sigma, St. Louis, MO, U.S.A.). The tyrosine kinase inhibitors PP1 and herbimycin A were obtained from Calbiochem (San Diego, CA, U.S.A.). Anti-ZAP-70 rabbit polyclonal antibodies prepared against recombinant ZAP-70 protein as previously described (23) were the kind gift of Steven Kanner (Bristol–Myers Squibb, Princeton, NJ, U.S.A.). The anti-phosphotyrosine monoclonal antibody 5H1 prepared as previously described (3) was the gift of Becky Penhallow (Bristol–Myers Squibb).

Analysis of tyrosine phosphorylation and Ca^{2+} signaling

Cells were lysed on ice in Nonidet P-40 lysis buffer containing protease and phosphatase inhibitors as described previously (21). Immunoprecipitations were performed for 2 h on ice, and immune complexes were collected on protein A–Sepharose beads (Repligen, Cambridge, MA, U.S.A.). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylene difluoride membranes (Immobilon, Millipore, Bedford, MA, U.S.A.). Anti-phosphotyrosine immunoblotting was performed with the monoclonal antibody 5H1 (3), and antibody binding was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Calcium responses were measured using indo-1 (Molecular Probes, Eugene, OR, U.S.A.) and a model 50HH/2150 flow cytometer (Ortho, Westwood, MA, U.S.A.) as previously described (19). The histograms were analyzed by programs (Cicero, Cytomation, Fort Collins, CO, U.S.A.) that calculate the mean indo-1 violet/blue fluorescence ratio versus time.

Analysis of cytokine production

Human peripheral blood mononuclear cells were isolated from blood using a Ficoll–Hypaque gradient (10). T cells were isolated from human peripheral blood mononuclear cell preparations by rosetting with sheep red blood cells (9). Cells were treated in 96-well tissue culture plates (Costar, Cambridge, MA, U.S.A.) with the indicated amounts of HOCl. The cells were maintained in the treated media for 18 h, and supernatants were analyzed for TNFα production using Quantikine high-sensitivity enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, U.S.A.). Alternatively, cells were maintained in the treated media for 48 h, and viability was assessed by trypan blue staining.

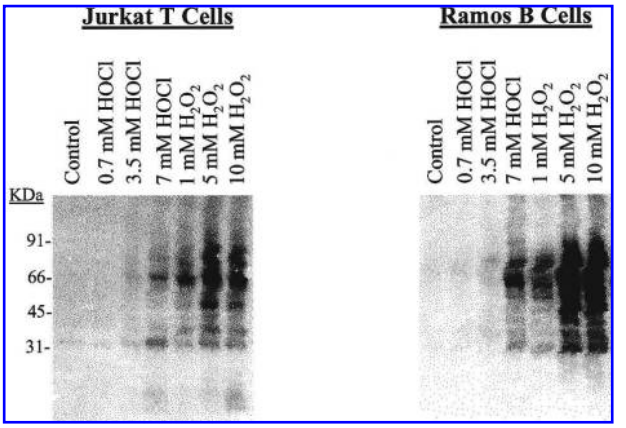

FIG. 1. Induction of cellular tyrosine phosphorylation in Jurkat T cells and Ramos B cells by HOCl. Cells were treated for 5 min with the indicated concentration of oxidizing agent and then lysed and analyzed for cellular tyrosine phosphorylation.

RESULTS

HOCl activates tyrosine phosphorylation signal pathways

We compared the ability of HOCl to induce cellular tyrosine phosphorylation with the response induced by H_2O_2, which has been extensively characterized for its ability to activate lymphocyte signal transduction (16, 22, 23). For our initial studies we utilized substantial concentrations of HOCl and H_2O_2 to obtain strong cellular tyrosine phosphorylation that could be readily detected by anti-phosphotyrosine immunoblotting. As shown in Fig. 1, HOCl was able to induce tyrosine phosphorylation in both Jurkat T cells and Ramos B cells. Although HOCl appeared less active than H_2O_2 for the induction of tyrosine phosphorylation in both cell types, a similar pattern of protein tyrosine phosphorylation was observed for HOCl and H_2O_2.

We performed additional experiments to determine the HOCl dose response for the induction of protein tyrosine phosphorylation. We compared the cellular tyrosine phosphorylation induced by anti-CD3 antibody stimulation to that obtained by treatment with HOCl in wild-type Jurkat T cells (Fig. 2). We used longer exposures of the anti-phosphotyrosine western blots than in Fig. 1, where the exposure was adjusted to show the details of the strong H_2O_2 response. Concentrations of HOCl as low as 350 μM clearly induced cellular tyrosine phosphorylation, with 700 μM HOCl inducing cellular tyrosine phosphorylation at levels similar to that observed by a maximal stimulation of the T-cell receptor by anti-CD3 antibody. The patterns of tyrosine phosphorylation induced by anti-CD3 and HOCl treatment were similar, suggesting that HOCl induces phosphorylation of many of the same proteins as T-cell receptor-induced signaling.

The similarity of the patterns of tyrosine phosphorylation observed for stimulation with HOCl and anti-CD3 antibody raised the question of whether CD3 and other T-cell receptor

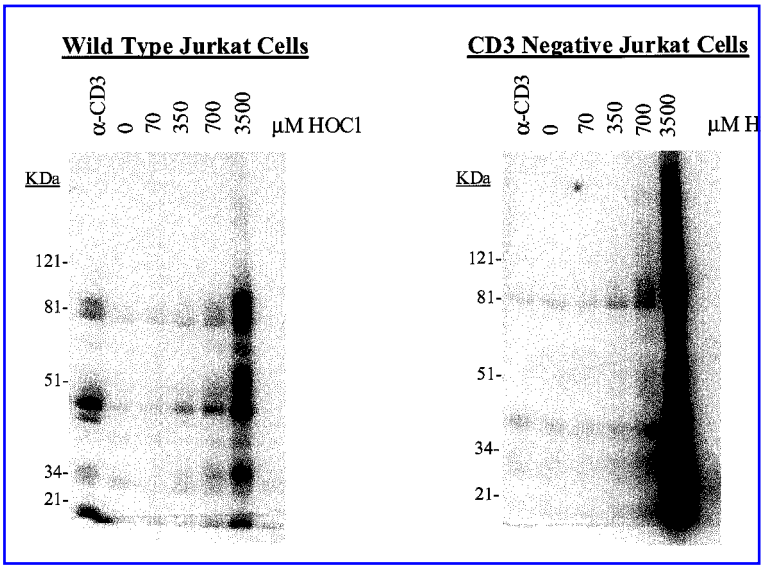

FIG. 2. Induction of cellular tyrosine phosphorylation by HOCl in wild-type and CD3-negative Jurkat T cells. Wild-type and CD3⁻ BMS-3P Jurkat T cells were treated for 5 min with the indicated concentration of HOCl and then lysed and analyzed for cellular tyrosine phosphorylation.

components are required for HOCl-induced tyrosine phosphorylation in T cells. Previous studies have demonstrated that the T-cell receptor is required for induction of tyrosine phosphorylation by UV radiation, but not for H_2O_2-induced tyrosine phosphorylation (23). We compared the patterns of HOCl-induced tyrosine phosphorylation in wild-type Jurkat T cells and BMS-3P cells deficient in CD3 and CD4 that lack expression of the T-cell receptor. The wild-type cells gave a substantial response to anti-CD3 stimulation, whereas the CD3⁻ cells did not (Fig. 2), as would be expected. However, the CD3⁻ cells and the wild-type cells both displayed very strong tyrosine phosphorylation signals when treated with 3,500 μ*M* HOCl, and lesser but substantial signals at lower concentrations of HOCl. These results indicate that HOCl does not require the T-cell receptor to induce tyrosine phosphorylation signals in T cells.

HOCl activates the ZAP-70 tyrosine kinase and induces Ca^{2+} signaling

Treatment of T cells with H_2O_2 has been previously shown to activate the key tyrosine kinase ZAP-70 (23). ZAP-70 is activated when it is tyrosine-phosphorylated by Lck, and the detection of tyrosine-phosphorylated ZAP-70 is used as a measure of its activation (6). Treatment of Jurkat cells with HOCl resulted in the dose-dependent activation of ZAP-70 (Fig. 3). At high concentrations, HOCl gave greater activation of ZAP-70 than a maximal stimulation of the T-cell receptor with anti-CD3 antibody. Furthermore, tyrosine-phosphorylated ζ chain was found to co-precipitate with the activated ZAP-70 (Fig. 3). This finding suggested that in wild-type cells, the activation of ZAP-70 was similar to T-cell receptor-induced activation, where ZAP-70 binds tyrosine-phosphorylated ζ chain via SH2 interactions, followed by direct phosphorylation of ZAP-70 by Lck (8). However, when CD3⁻ Jurkat cells were treated with HOCl, ZAP-70 was still activated, but no longer associated with the tyrosine-phosphorylated ζ chain (Fig. 3). These results indicate that HOCl is able to bypass the need for the T-cell receptor including the ζ chain to activate ZAP-70, similar to what has previously been observed for H_2O_2 (23).

Intracellular Ca^{2+} signaling is an essential step in T-cell activation, occurring downstream of ZAP-70 activation following the tyrosine phosphorylation of phospholipase Cγ (8). Intracellular Ca^{2+} signaling can be a more sensitive indi-

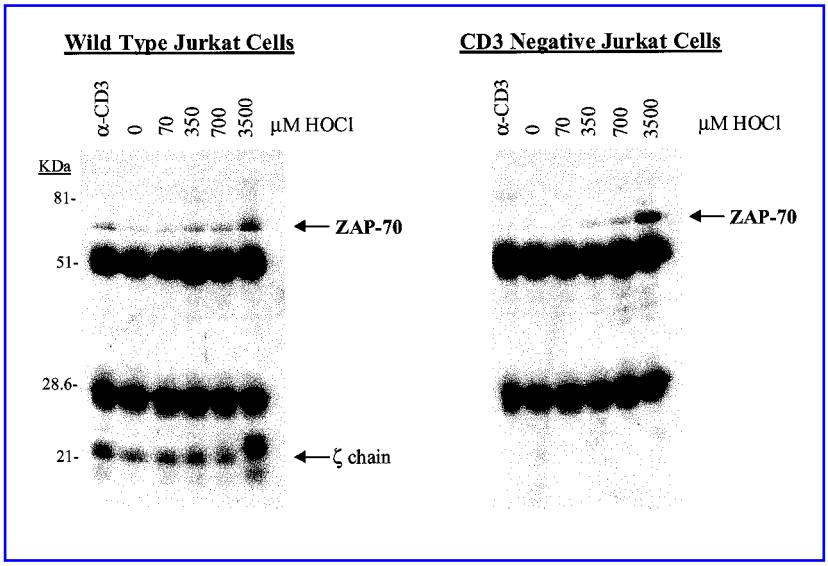

FIG. 3. Activation of the ZAP-70 tyrosine kinase by HOCl in wild-type and CD3-negative Jurkat T cells. Wild-type and CD3⁻ BMS-3P Jurkat T cells were treated for 5 min with the indicated concentration of HOCl and then lysed. ZAP-70 was immunoprecipitated, and tyrosine phosphorylation was detected by immunoblotting.

cator of cellular signaling because it is a result of a signal amplification cascade. As other oxidizing treatments such as H_2O_2 and UV irradiation have been shown to induce Ca^{2+} signaling (22, 23), we examined the ability of HOCl to induce this signal pathway. As shown in Fig. 4A, HOCl induced Ca^{2+} signals in a dose-dependent fashion, with concentrations as low as 31 μM inducing substantial signals. The HOCl-induced signal was blocked by the Src family tyrosine kinase inhibitor PP1 (4), demonstrating that the signals were tyrosine kinase-dependent (Fig. 4B). Comparison of Ca^{2+} signals induced by HOCl in wild-type and CD3$^-$ Jurkat T cells revealed that although the peak amplitude of the signal was the same in both cells (Fig. 4C), lack of T-cell receptor expression resulted in signaling for a shorter time period. These results indicate that although HOCl does not require the T-cell receptor to induce Ca^{2+} signals, the T-cell receptor is able to contribute to prolongation of the Ca^{2+} signal induced by HOCl.

HOCl induces tyrosine kinase-dependent TNFα expression

As it had been previously reported that HOCl could induce TNFα expression in an HIV-infected T-cell line, we sought to determine whether HOCl could also induce TNFα expression in normal human cells, and whether that expression was tyrosine kinase-dependent. However, as HOCl is known to be cytotoxic, we first examined the viability of human peripheral blood mononuclear cells in the presence of HOCl. As shown in Fig. 5A, >50% cell viability over a 48-h period was observed at HOCl concentrations of 120 μM or less. Similarly, human peripheral blood T-cell proliferation stimulated by anti-CD3 plus anti-CD28 antibodies was found to be inhibited by HOCl with an IC_{50} value of 155 μM in separate experiments.

In contrast to the results reported for the HIV-infected T-cell line ACH-2 (24), we did not detect induction of TNFα by HOCl in isolated normal human peripheral blood T-cells treated with HOCl for 18 h. However, monocytes and macrophages are known to be the major physiological source of TNFα. When we examined normal human peripheral blood mononuclear cells, which include monocytes and macrophages in addition to lymphocytes, HOCl was found to induce TNFα expression at 18 h, with maximal expression occurring at a concentration of 85 μM (Fig. 5B). Similar results were obtained in three independent experiments. To determine if the HOCl-induced TNFα expression was tyrosine kinase-dependent, we examined whether two unrelated tyrosine kinase inhibitors could inhibit the expression. Both PP1 and herbimycin A inhibit Src family tyrosine kinases, including Lck, and are capable of blocking T-cell receptor-induced tyrosine phosphorylation and downstream activation events (4, 7). As shown in Fig. 6, both kinase inhibitors were highly effective in blocking TNFα expression induced by 42 μM HOCl, and similar results were obtained in a second experiment with cells stimulated with 85 μM HOCl. These results obtained with two diverse tyrosine kinase inhibitors indicate that the induction of TNFα by HOCl is tyrosine kinase-dependent.

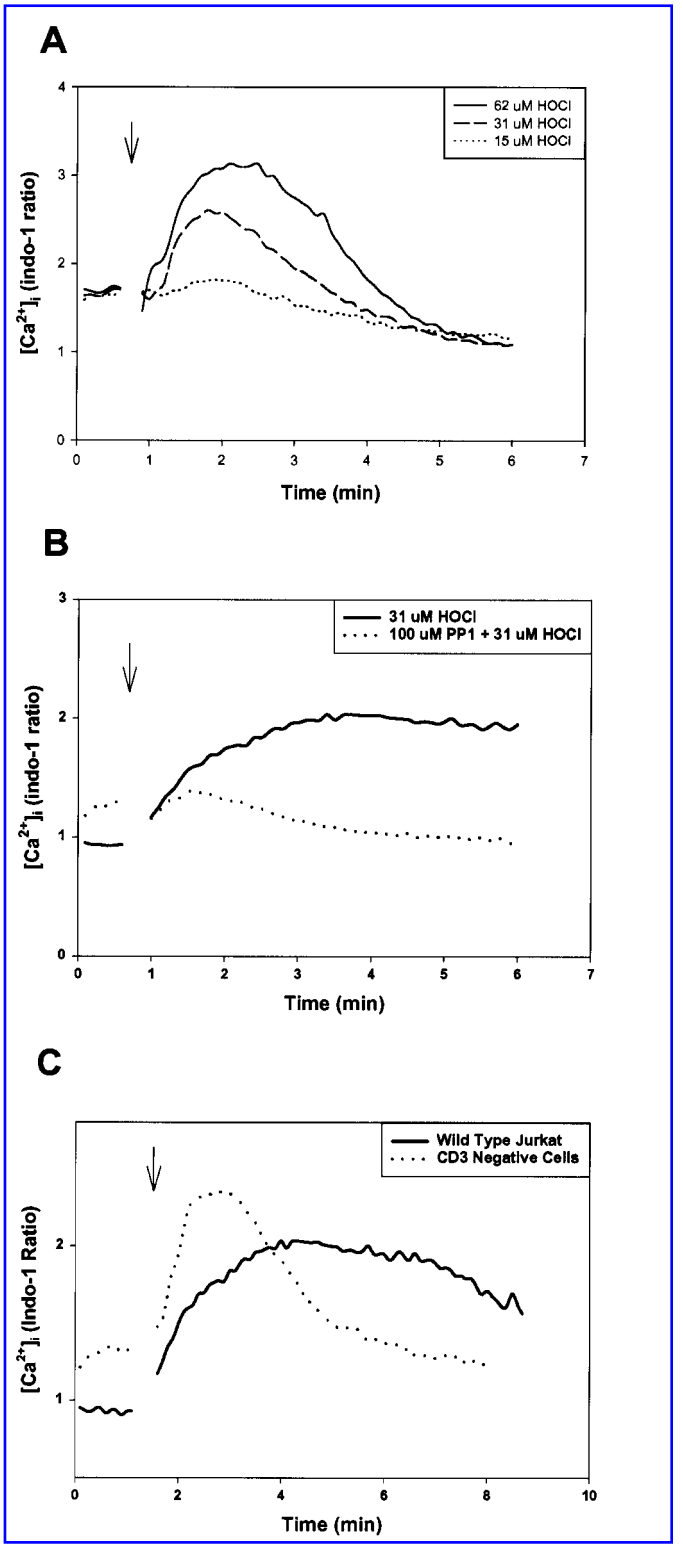

FIG. 4. Induction of Ca^{2+} signaling by HOCl. Baseline Ca^{2+} levels were recorded for 1 min, and the cells were then treated with the indicated concentration of HOCl at the time indicated by the arrow. (**A**) Response of cells to various concentrations of HOCl. (**B**) Cells were preincubated 5 min with PP1 tyrosine kinase inhibitor, the basal Ca^{2+} level was recorded for 1 min, and the cells were then treated with HOCl. (**C**) Wild-type and CD3$^-$ Jurkat T cells were stimulated with 31 μM HOCl.

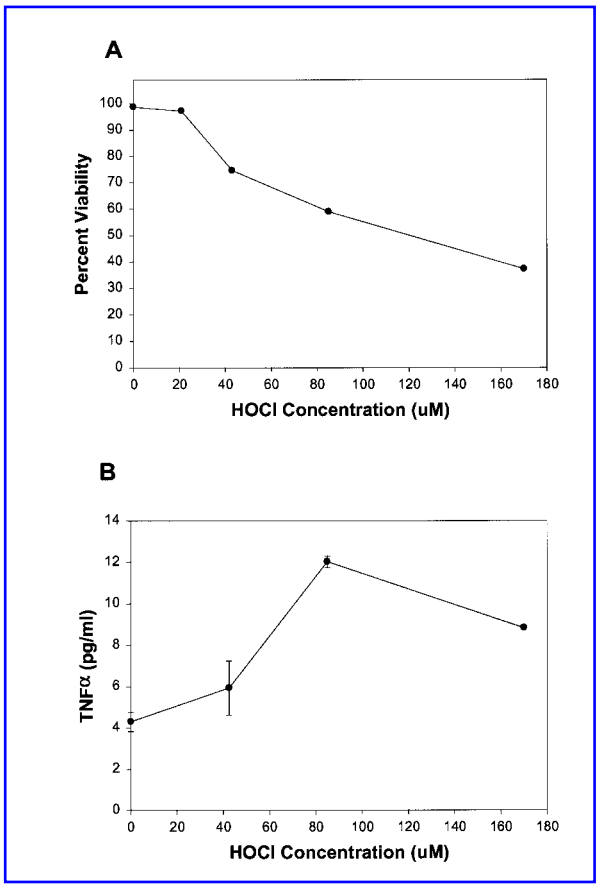

FIG. 5. Induction of TNFα production by HOCl. (**A**) Human peripheral blood mononuclear cells were treated with HOCl and viability was measured by trypan blue staining at 48 h. (**B**) Human peripheral blood mononuclear cells were treated with HOCl, and TNFα production was measured at 18 h.

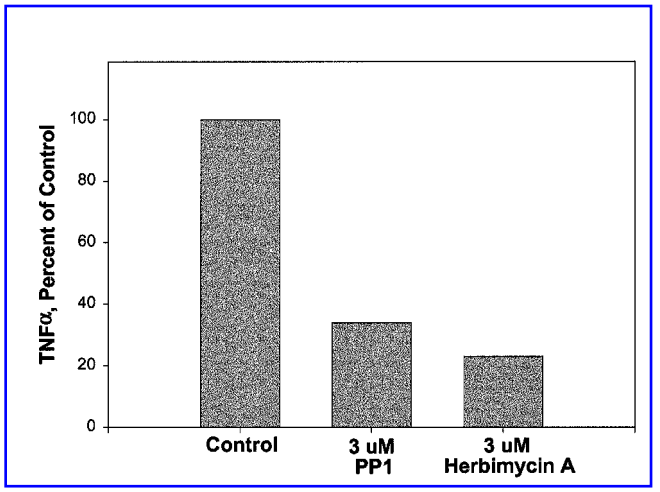

FIG. 6. Inhibition of HOCl-induced TNFα production by tyrosine kinase inhibitors. Human peripheral blood mononuclear cells were treated with HOCl in the presence of tyrosine kinase inhibitors, and TNFα production was measured at 18 h.

DISCUSSION

Neutrophils produce large quantities of superoxide and H_2O_2 upon stimulation, and much of the H_2O_2 produced by these cells is converted to HOCl in a reaction with chloride ions catalyzed by the neutrophil enzyme myeloperoxidase (1). Recent studies suggest that the action of HOCl on normal cells may contribute to immune dysfunction, including autoimmune disease. HOCl is toxic to cells and contributes to neutrophil-mediated tissue damage by this mechanism. HOCl-modified proteins have been detected in inflammatory bowel disease tissue samples (14), diseased kidney tissue (11), and atherosclerotic plaques (5, 12), suggesting a role in these diseases. However, in addition to direct tissue damage and cytotoxicity, the ability of HOCl to induce oxidative stress raises the possibility that the compound might also act to alter cellular signal transduction pathways in cells of the immune system.

HOCl is highly reactive with thiols, and glutathione can be oxidized to glutathione disulfide, glutathione thiolsulfonate, and a sulfonamide species by HOCl (28). This oxidation is irreversible, and new synthesis of glutathione is required to restore normal levels. The essential active-site cysteine residue of phosphotyrosine phosphatases must be reduced to maintain enzyme activity, and is extremely reactive (2). The active-site cysteine would not only be sensitive to the loss of glutathione, but might also react directly with HOCl, offering a potential avenue by which HOCl could affect cellular tyrosine phosphorylation signal pathways. HOCl also generates reactive chloramines on reaction with amine groups (13, 18) that could also influence signal pathways.

In the present study, we show that HOCl is able to activate cellular signal pathways normally under antigen receptor control in lymphocytes. Specifically, HOCl induced cellular tyrosine phosphorylation in T cells and activated the key signaling kinase ZAP-70 that is known to be essential for antigen receptor-induced Ca^{2+} mobilization (8). HOCl treatment of T cells induced Ca^{2+} signaling, and the signaling was blocked by a tyrosine kinase inhibitor, indicating the dependence of the response on tyrosine kinase activation. HOCl was able to bypass normal receptor control of these signal pathways, giving strong tyrosine phosphorylation and Ca^{2+} signals in cells lacking T-cell receptor expression. These results are similar to the effects that we have previously reported for H_2O_2, which is also able to bypass normal receptor control to activate tyrosine phosphorylation and Ca^{2+} signal pathways (23).

Although it had been reported that the HIV-infected T-cell line ACH-2 could be induced to produce TNFα by HOCl (24), we did not observe any induction of TNFα production by HOCl treatment of normal human T cells. This dissimilarity may reflect differences between the cell line and normal cells, or may be a consequence of the HIV infection of the cell line. By contrast, treatment of peripheral blood mononuclear cells with HOCl did induce production of TNFα. Monocytes and macrophages are major physiological sources of TNFα, and additional studies will be required to determine whether the monocytic cells are the primary source of TNFα in response to HOCl.

We demonstrated that the HOCl-induced TNFα production was tyrosine kinase-dependent by using two independent tyrosine kinase inhibitors with different mechanisms of action. PP1 has been reported to inhibit T-cell signaling by inhibiting the activity of Lck and other Src family kinases (4). Herbimycin A has been reported to block T-cell signaling by depleting cells of Lck and other Src family kinases by inducing their degradation (7). Src family kinases are also known to be essential for the lipopolysaccharide-induced TNFα *in vivo,* a response primarily mediated by monocytes, and inhibitors of Src family tyrosine kinases have been shown to block this response to lipopolysaccharide both in cells and in animals (15, 25). In the present study, concentrations of the inhibitors that have been previously established to block tyrosine phosphorylation signals by Src family kinases were found to be effective in inhibiting HOCl-induced TNFα production. These data indicate that the activation of tyrosine kinase signaling pathways in peripheral blood mononuclear cells by HOCl induces production of TNFα. It will be of interest to determine the impact of HOCl on the activation of signal pathways in monocytes, which share commonalities in signal transduction with T cells, including essential roles for Src family kinases to induce cellular responses to receptor stimulation.

Although we used concentrations of HOCl that would exceed physiological levels in some experiments in order to obtain strong tyrosine phosphorylation signals in western blot analysis of whole-cell lysates, we demonstrated that much lower concentrations of HOCl were effective at inducing tyrosine kinase-dependent Ca^{2+} signaling and TNFα production. The lower concentrations were not lethal to the majority of normal human peripheral blood mononuclear cells and fall into physiologically relevant ranges.

The activation of tyrosine phosphorylation signal transduction pathways by oxidizing agents provides a mechanism by which various inflammatory responses might be amplified outside of normal receptor control. Our findings on activation of T-cell signal pathways by HOCl can provide a basis for further study of T-cell responses to oxidative stress. In addition, HOCl induction of the tyrosine kinase-dependent production of TNFα by a cell population represented in peripheral blood mononuclear cells would be expected to induce further inflammatory responses. TNFα has been demonstrated to prime neutrophils to produce substantially increased amounts of HOCl in response to weakly stimulating agents such as unopsonized zymosan (26). The TNFα produced in a tyrosine kinase-dependent response to HOCl might thus lead to additional production of HOCl by neutrophils, increasing the inflammatory response.

ACKNOWLEDGMENTS

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ABBREVIATIONS

HIV, human immunodeficiency virus; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; TNFα, tumor necrosis factor α; UV, ultraviolet; ZAP-70, zeta associated protein-70.

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