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Oxidative stress triggers tyrosine phosphorylation in B cells through a redox- and inflammatory cytokine-sensitive mechanism

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SUMMARY

Exposure to oxidants such as hydrogen peroxide (H_2O_2) and γ-ray irradiation has been recently shown to trigger tyrosine phosphorylation in B cells as does cross-linking surface immunoglobulin (sIg) by antigens or anti-immunoglobulins. We studied the mechanism by which H_2O_2 induced tyrosine phosphorylation in B cells and compared it with the mechanism utilized by sIg. Both anti-immunoglobulin M (anti-IgM) and H_2O_2 induced tyrosine phosphorylation through protein tyrosine kinase (PTK) activation. However, the tyrosine phosphorylation caused by H_2O_2 but not that induced by anti-IgM, was modulated by agents affecting cellular thiols and glutathione contents including dithiothreitol, 2-mercaptoethanol, and buthionine sulphoximine. Moreover, the tyrosine phosphorylation caused by the oxidant but not that induced by anti-IgM was markedly augmented by two inflammatory cytokines, tumour necrosis factor-α and interleukin-1α, although these agents by themselves did not stimulate PTK activity nor induce tyrosine phosphorylation. These findings demonstrate that oxidative stress but not surface IgM (sIgM) ligation triggers tyrosine phosphorylation through a mechanism that is sensitive to cellular thiols and these inflammatory cytokines.

INTRODUCTION

Protein tyrosine phosphorylation is an early and essential step in B-cell activation which is observed after engagement of surface immunoglobulin by antigens or anti-immunoglobulin antibodies. This event is thought to be mediated by a variety of protein tyrosine kinases (PTKs) including Src family kinases[1–4] and PTK72/p72^{syk} [5,6] that are shown to be physically associated with surface immunoglobulin or its associated molecules and to be activated after the engagement. Tyrosine phosphorylation is also shown to be independent from and responsible for an increase in phosphoinositide turnover, another early cellular event observed after the engagement. Thus, specific binding between surface immunoglobulin and its ligand has been thought to be essential to induce tyrosine phosphorylation and the resulting cellular responses such as cell activation or cell death.

Recent studies demonstrate that exposing B and T cells to oxidants such as hydrogen peroxide (H_2O_2),[7–9] diamide[9,10] and phenylarsine oxide (PAO)[11–13] or to γ-ray irradiation[14,15] results in an increase in tyrosine phosphorylation and two early biological responses, an elevation in intracellular calcium and increased phosphoinositide turnover PTK inhibitors such as herbimycin A and tyrphostin are reported to prevent tyrosine phosphorylation and the following cellular events caused by H_2O_2[7] or ionizing irradiation,[14,15] suggesting that these agents induce tyrosine phosphorylation through PTK activation and that the activation may be an early and requisite signal for inducing these cellular events. In fact, several PTKs, such as p56/53^{lyn} and p72^{syk}, which are involved in transmembrane signalling triggered via surface immunoglobulin are also activated after the exposure to H_2O_2[7] or ionizing irradiation.[14,15] Moreover, phospholipase C-γ, a physiologically relevant protein, is tyrosine-phosphorylated after surface immunoglobulin engagement as well as after exposure to γ-ray irradiation.[14] These facts suggest that similar cellular events may occur after cross-linking surface immunoglobulin and exposure to oxidative stress. Here we demonstrate that oxidative stress but not surface immunoglobulin trigger B-cell tyrosine phosphorylation through a mechanism that is sensitive to cellular thiols and inflammatory cytokines.

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MATERIALS AND METHODS

Reagents

Hydrogen peroxide and RRsrc peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) were obtained from Wako (Osaka, Japan) and Peptide Institute (Osaka, Japan), respectively. P81 phosphocellulose paper was purchased from Whatman (Maidstone, UK). Phenylmethylsulphonyl fluoride (PMSF) and [Val^5]-angiotensin II, sodium orthovanadate were obtained from Sigma (St. Louis, MO). Recombinant human

interleukin-1α (rhIL-1α) was from Oncogene Science (Uniondale, NY). Recombinant human tumour necrosis factor-α (rhTNF-α) was generously provided by Dr N. Tsuruoka (Suntory, Osaka, Japan) and [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from ICN Biochemicals (Costa Mesa, CA).

Antibodies

Monoclonal anti-phosphotyrosine antibody PY20 and rabbit polyclonal anti-phosphotyrosine antibody were obtained from ICN Biochemicals and Zymed Laboratories (San Francisco, CA), respectively. Horseradish peroxidase (HRP)-conjugated species-specific anti-mouse and anti-rabbit immunoglobulins were purchased from Amersham (Bucks, England).

Cells

The human Burkitt lymphoma lines BJAB and Ramos were generously provided by Dr George Klein (Karolinska Institute, Sweden). Cells were cultured at 37° in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Mitsubishi Kasei, Tokyo, Japan) and 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2/95% air.

Cell stimulation

Cells washed with RPMI-1640 containing 20 mM HEPES, pH 7·4 were resuspended with the same medium and incubated with agents tested at 37°. Simulation was terminated by the addition of sodium dodecyl sulphate (SDS) sample buffer. After boiling for 3 min, the resulting samples were analysed by immunoblot as described below. When PTK activity was measured, the stimulation was terminated by the addition of phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate. Then cells were pelleted down by centrifugation at 2000 *g* for 5 min and subjected to PTK assay.

Detection of tyrosine-phosphorylated proteins

Protein contents of whole cell lysates were measured using a protein assay kit (BioRad, Hercules, CA). Fifteen micrograms of the protein of the lysates were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) analysis using a 10% separation gel according to the method of Laemmli.[16] The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 0·5% gelatin in PBS at 4° overnight, the membranes were incubated with monoclonal or polyclonal anti-phosphotyrosine at a dilution of 1 : 5000 for 1 hr at room temperature. After extensive washing with 0·1% Tween-20 in PBS, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit immunoglobulin at a dilution of 1 : 5000 for 1 hr at room temperature. After washing, immunoreactive proteins on the membranes were detected using an enhanced chemiluminescence kit (ECL Western blotting kit, Amersham) according to the method recommended by the supplier.

PTK assay

Cells were suspended in a lysis buffer containing 20 mM Tris–HCl, pH 7·4, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM Na_3VO_4, 2 mM ethylenediaminetetraacetic acid (EDTA), 0·2 mM PMSF, 20 μM leupeptin, and 0·15 U/ml aprotinin and allowed to stand on ice for 20 min. The whole cell lysates were centrifuged at 16 000 *g* for 5 min and the resulting supernatants were used as postnuclear extracts. These postnuclear extracts were assayed for PTK activity according to the method of Casnellie *et al.*[17] with minor modifications. Briefly, the reaction was carried out in a 50 μl final volume containing 20 mM Tris–HCl, pH 7·4, 10 μM $ZnCl_2$, 30 μM $NaVO_3$, 0·5 mM dithiothreitol, 20 mM $MgCl_2$, 1 mM RRsrc peptide, 2 μCi [γ-^{32}P]ATP and 30 μg protein at 30° for 10 min. After the reaction was terminated by the addition of trichloroacetic acid, the reaction mixtures were added to the carrier bovine serum albumin, allowed to stand on ice for 20 min, and then centrifuged at 12 000 *g* for 5 min. The resulting supernatants were spotted onto phosphocellulose papers and the papers were washed with 0·5% phosphoric acid three times and with acetone once. The radioactivity of the papers was counted with a scintillation counter. The ^{32}P incorporation into exogenously added substrate peptide was increased in line with the amount of protein used as well as the time incubated.

RESULTS

Both anti-IgM and H_2O_2 induce tyrosine phosphorylation through PTK stimulation

We analysed protein tyrosine phosphorylation after stimulating the surface immunoglobulin M (sIgM)-positive human Burkitt lymphoma cell line, Ramos with anti-IgM antibodies or with the membrane-permeable oxidant, H_2O_2. Cells treated with these agents were lysed and cell lysates were subjected to SDS–PAGE analysis, transferred to polyvinylidene difluoride membranes, and probed for the presence of tyrosine-phosphorylated proteins using anti-phosphotyrosine antibodies. We obtained identical results with the monoclonal antibody PY20 and rabbit polyclonal antibodies. Cells stimulated with polyclonal anti-IgM antibodies showed an increase in tyrosine phosphorylation of a number of proteins in a dose-dependent manner (Fig. 1a). The increase could be observed as early as 10 seconds after the stimulation. Maximal tyrosine phosphorylation in a large number of proteins was seen after 2 min of exposure of the

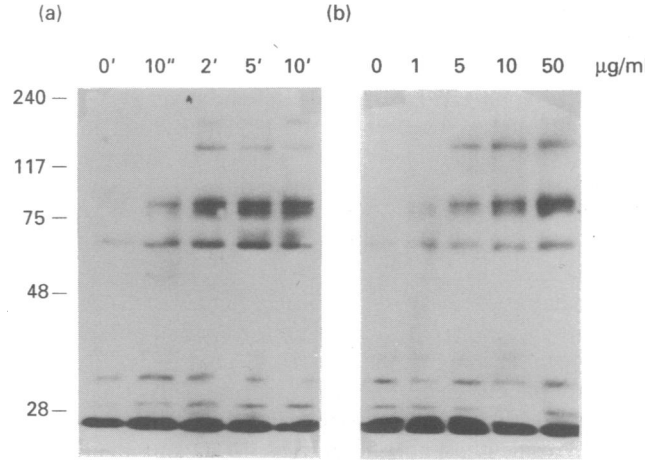

Figure 1. Anti-IgM induces tyrosine phosphorylation in B cells. Ramos cells were incubated with or without 10 μg/ml of anti-IgM for the time indicated (a) or increasing concentrations of anti-IgM for 5 min (b). Proteins of whole cell lysates were resolved by SDS–PAGE, transferred to polyvinylidene difluoride membrane and immunoblotted with anti-phosphotyrosine antibodies.

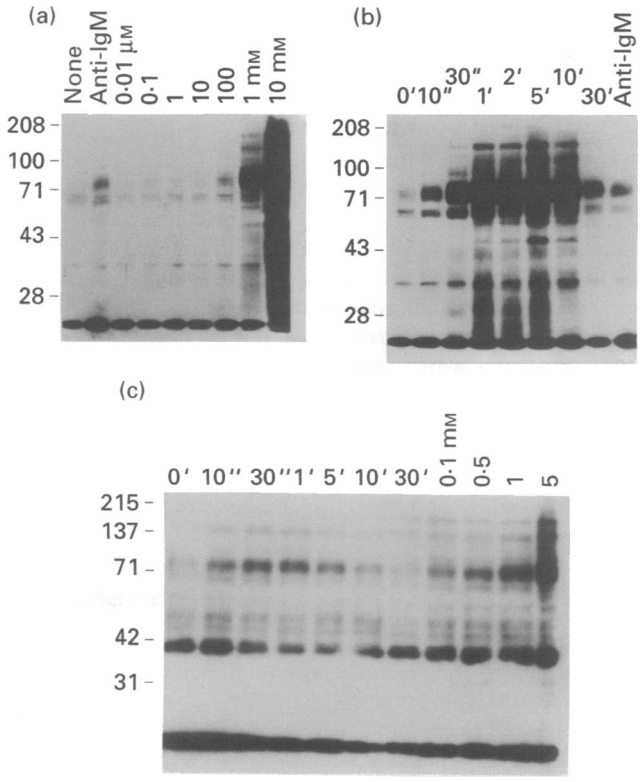

Figure 2. Hydrogen peroxide induces tyrosine phosphorylation in B cells. Ramos cells (a, b) or BJAB cells (c) were incubated with or without increasing concentrations of H_2O_2 for 5 min (a, c) or 1 mM H_2O_2 for the time indicated (b, c). As a positive control, the cells were also incubated with 10 μg/ml of anti-IgM. Tyrosine-phosphorylated proteins were detected by anti-phosphotyrosine immunoblot as described in the legend of Fig. 1.

cells to the anti-IgM antibodies (Fig. 1b). Similarly, exposure of the cells to H_2O_2 resulted in a dose-dependent increase in tyrosine phosphorylation of multiple proteins (Fig. 2a). The increase was also observed as early as 10 seconds after exposure of cells to the oxidant, reaching a maximum within 1–5 min, and declining after 30 min (Fig. 2b). It should be noticed that tyrosine phosphorylation of a similar set of proteins was stimulated upon anti-IgM stimulation and upon H_2O_2 stimulation, although at higher doses, the oxidant but not anti-IgM caused tyrosine phosphorylation of a much larger number of substrates non-specifically. Similarly a dose- and time-dependent increase in tyrosine phosphorylation after exposure to H_2O_2 was seen with another Burkitt lymphoma cell line, BJAB (Fig. 2c).

Cellular levels of tyrosine phosphorylation are balanced by the opposing actions of PTKs and protein tyrosine phosphatases (PTPases). Therefore, the observed increase in tyrosine phosphorylation could be the result of PTK activation, PTPase inactivation, or a combination of the two mechanisms. Tyrosine phosphorylation triggered by anti-immunoglobulin is shown to result from the activation of various PTKs that are physically associated with surface immunoglobulin or its associated molecules.[1–5] To determine whether H_2O_2 also causes tyrosine phosphorylation through PTK activation, we directly measured PTK activity after exposure of cells to the oxidant using specific substrates for PTK. Our preliminary

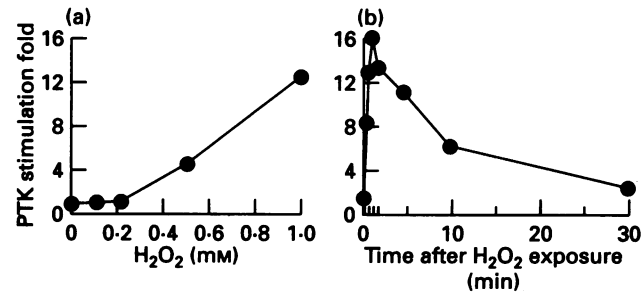

Figure 3. Hydrogen peroxide induces PTK activation in B cells. Ramos cells were incubated with or without increasing concentrations of H_2O_2 for 5 min (a) or 1 mM H_2O_2 for the indicated times (b). After the stimulation was terminated by the addition of PBS containing 1 mM sodium orthovanadate, the cells were lysed and the postnuclear fraction was assayed for phosphorylating activity towards RRsrc peptide.

experiments showed that these Burkitt lymphoma cell lines contain phosphorylating activity towards several distinct substrates, RRsrc peptide, Glu-Tyr copolymer, and [Val^5]-angiotensin II (data not shown). Since RRsrc peptide proved to serve as a good substrate, phosphorylating activity towards this peptide was measured. Consistent with the previous reports that show activation of several PTKs through surface immunoglobulin,[1–6] exposure of B cells to anti-IgM resulted in a dose-dependent elevation in the activity with a minimal effective dose of 10 μg/ml which was in good agreement with that required for inducing the tyrosine phosphorylation caused by the antibodies (data not shown). The oxidant also induced a dose-dependent elevation in the activity with a minimal effective dose of 0·5 mM (Fig. 3a). This dose was also in good agreement with that required for the tyrosine phosphorylation caused by the oxidant. The elevation could be observed as early as 10 seconds after the exposure, reached a maximum at 1 min, and declined thereafter (Fig. 3b). This time course is also quite similar to that of the increased tyrosine phosphorylation caused by the oxidant. Taken together, these results demonstrate that oxidative stress also induces tyrosine phosphorylation through PTK activation.

Involvement of a redox mechanism in H_2O_2- but not anti-IgM-induced tyrosine phosphorylation

To determine the possible involvement of oxidation of intracellular thiols in the tyrosine phosphorylation, we examined the effects of the thiol-reducing agent, dithiothreitol (DTT) on H_2O_2-induced tyrosine phosphorylation. Cells were incubated with the oxidant for 5 min and then with an excess amount (five- or 20-fold) of DTT. The lower dose of the agent partially abrogated the tyrosine phosphorylation (data not shown), while the higher dose of the agent completely abolished the tyrosine phosphorylation (Fig. 4a). The agent also abrogated tyrosine phosphorylation induced by two other thiol-reactive oxidants, diamine[9,10] and phenylarsine oxide,[11–13] but did not abrogate the tyrosine phosphorylation caused by *N*-ethylmaleimide, an agent which binds irreversibly to thiols. The results demonstrate a critical role of reversible thiol oxidation in the tyrosine phosphorylations caused by these oxidants.

Glutathione is the major intracellular thiol-containing compound that is involved in the regulation of the cellular redox state through the oxidation and reduction of its thiols.

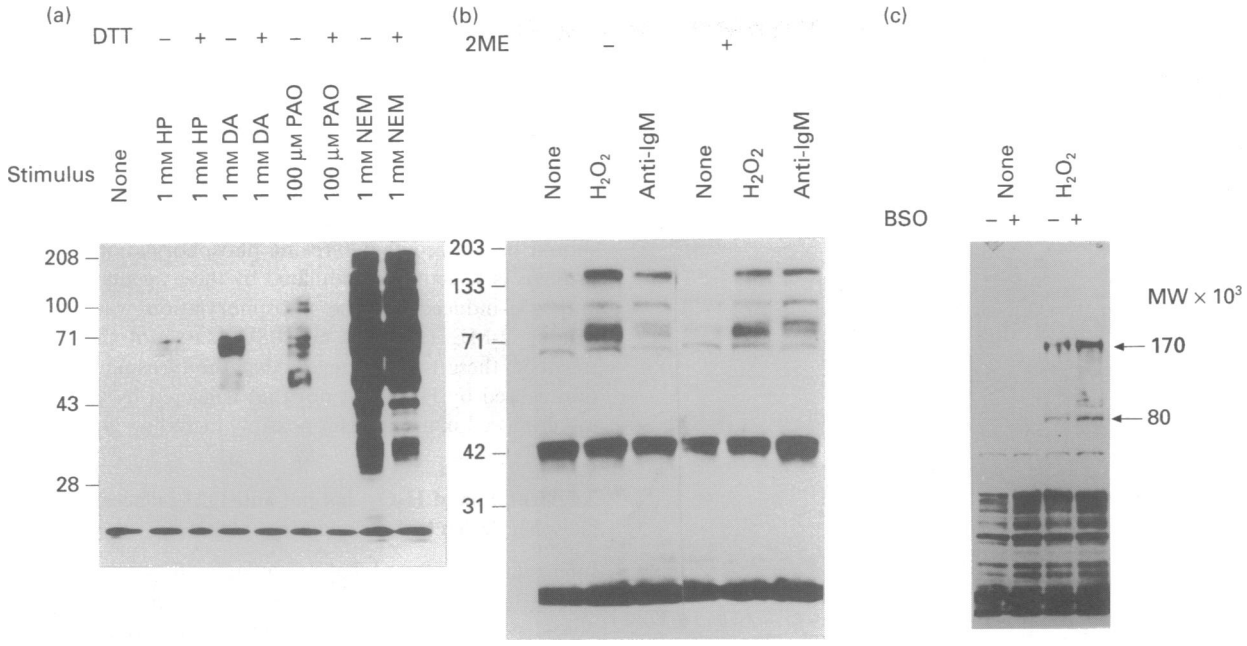

Figure 4. Agents affecting thiols and glutathione contents modulate H_2O_2- but not anti-IgM-induced tyrosine phosphorylation. BJAB cells were incubated with or without 1 mM H_2O_2 (HP), 1 mM diamide (DA), 100 μM phenylarsine oxide (PAO), or 1 mM *N*-ethylmaleimide (NEM) for 5 min and then with a 20-fold excess amount of DTT for 10 min (a). After culture for 24 hr with or without 50 μM 2-ME (b) or 100 μM BSO (c), BJAB cells were washed extensively and exposed to 10 μg/ml anti-IgM or 1 mM H_2O_2 for 5 min and then tyrosine phosphorylation was assessed.

Therefore, glutathione is a likely candidate for the H_2O_2-sensitive intracellular thiols and the oxidation of its thiols might be involved in H_2O_2-induced tyrosine phosphorylation. The involvement of glutathione in the tyrosine phosphorylation could be monitored by testing the effects of agents modulating cellular glutathione content on the tyrosine phosphorylation. Cellular glutathione content in human B cells is shown to be increased by the addition of the thiol-reducing agent, 2-mercaptoethanol (2-ME), while the content is shown to be reduced by the treatment with buthionine sulphoximide (BSO),

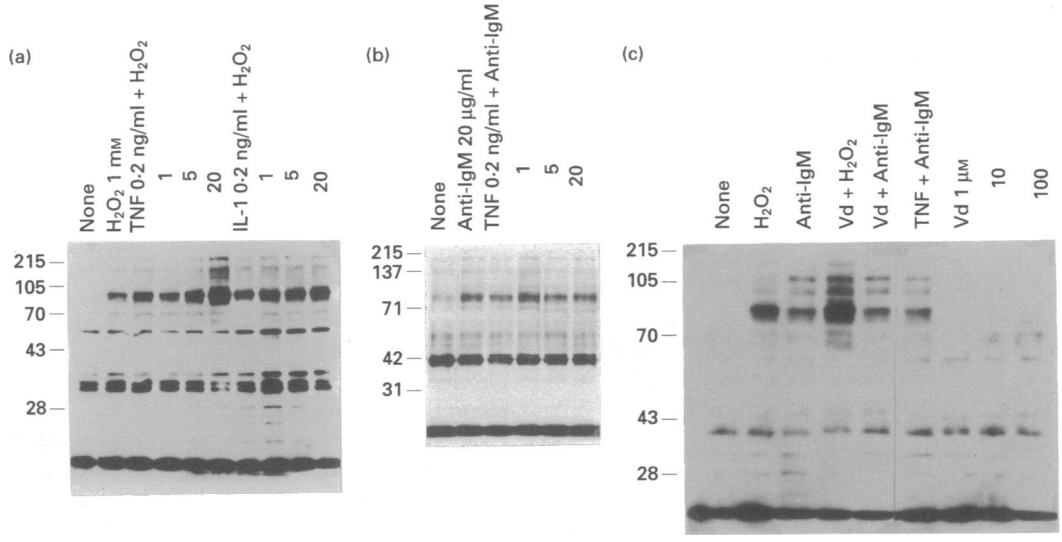

Figure 5. (a) Inflammatory cytokines augment H_2O_2-induced tyrosine phosphorylation in a dose-dependent manner. BJAB cells were incubated for 5 min with or without increasing concentrations of rhTNF-α or rhIL-1α and then incubated with 1 mM H_2O_2 for 5 min. (b) Inflammatory cytokines do not affect anti-IgM-induced tyrosine phosphorylation. The cells were incubated for 5 min with or without increasing concentrations of rhTNF-α and then incubated with 20 μg/ml anti-IgM for 5 min. (c) Orthovanadate augments H_2O_2- but not anti-IgM-induced tyrosine phosphorylation. The cells were incubated with or without 20 ng/ml rhTNF-α or 10 μM orthovanadate for 5 min and then incubated with 20 μg/ml anti-IgM or 1 mM H_2O_2 for 5 min. The cells were also incubated for 5 min with orthovanadate at the concentrations indicated and tyrosine phosphorylation was assessed.

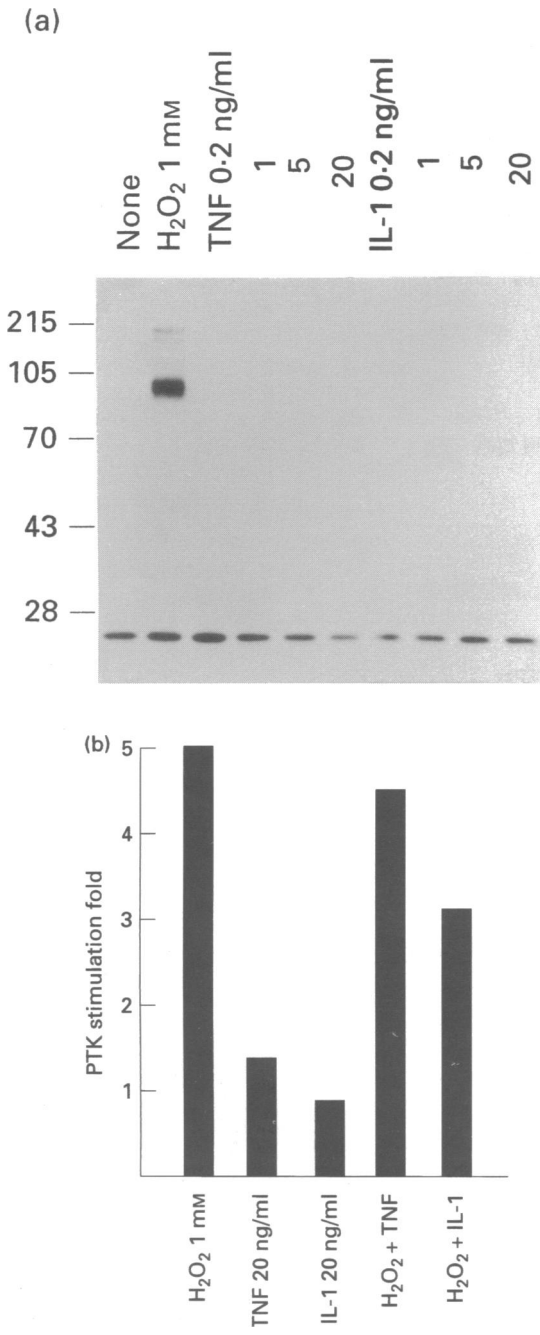

Figure 6. Inflammatory cytokine by itself does not induce tyrosine phosphorylation (a), and or stimulate PTK activity (b). (a) BJAB cells were incubated for 5 min with increasing concentrations of rhTNF-α or rhIL-1α, and tyrosine phosphorylation was assessed. H_2O_2 1 mM was used as a positive control. (b) The cells were incubated for 5 min with either H_2O_2, rhTNF-α or rhIL-1α alone, or in combination, at the concentrations indicated and PTK activity was measured.

an inactivator of γ-glutamyl-cysteine synthase,[18] a key enzyme in glutathione synthesis.[19] We therefore, tested the effects of these agents on H_2O_2-induced tyrosine phosphorylation. Cells were incubated with 2-ME or medium and then stimulated with the oxidant. Treatment with 2-ME did not affect the basal level of tyrosine phosphorylation. However, tyrosine phosphorylation of several proteins, including those of 80 000 and 170 000 molecular weights but not whole proteins, was reduced in cells incubated with 2-ME when compared with that observed in cells incubated with medium (Fig. 4b). Treatment with BSO also did not affect the basal level of tyrosine phosphorylation. However, tyrosine phosphorylation of the 80 000 and 170 000 molecular weight proteins but not whole proteins was augmented in cells incubated with BSO when compared with that observed in cells incubated with medium (Fig. 4c). It should be noticed that tyrosine phosphorylation of common proteins is selectively modulated by these agents. By contrast, anti-IgM-induced tyrosine phosphorylation was affected by neither 2-ME (Fig. 4b) nor BSO (data not shown). Taken together, these results suggest that the tyrosine phosphorylation caused by H_2O_2 but not that triggered by anti-IgM may involve a redox mechanism possibly including glutathione.

Augmentation of H_2O_2- but not anti-IgM-induced tyrosine phosphorylation by inflammatory cytokines

Interestingly, we found that two inflammatory cytokines, TNF-α and IL-1α, augment H_2O_2-induced tyrosine phosphorylation in a dose-dependent manner with a minimal effective dose of 2 ng/ml (Fig. 5a). It should be noted that tyrosine phosphorylation of several proteins including the 80 000 and 170 000 molecular weight proteins was enhanced by either cytokine. By sharp contrast, these cytokines could not affect anti-IgM-induced tyrosine phosphorylation (Fig. 5b,c). Similar results were obtained with Ramos cells (data not shown). In addition, neither cytokine by itself induced tyrosine phosphorylation (Fig. 6a) or an elevation in PTK activity (Fig. 6b). Thus, the augmentation by these cytokines is likely to involve PTPase inactivation rather than PTK activation. This idea seems to be supported by the fact that the PTPase inhibitor, orthovanadate augmented tyrosine phosphorylation caused by H_2O_2 but not anti-IgM (Fig. 5b), although the agent by itself could not induce PTK activation (data not shown) or tyrosine phosphorylation (Fig. 5b).

DISCUSSION

The data presented in this paper demonstrate both similarities and distinctions between tyrosine phosphorylation triggered by sIgM and that induced by oxidative stress in human B cells. As shown with that triggered via sIgM, the tyrosine phosphorylation induced by H_2O_2 also results from PTK stimulation. Moreover, a similar set of tyrosine-phosphorylated proteins was observed upon stimulation with anti-IgM and H_2O_2. Thus, PTK with similar substrate specificity seem to be involved in these tyrosine phosphorylations. This is consistent with the previous report that H_2O_2 induces stimulation of $p72^{syk}$, a non-Src family PTK which is shown to be physically associated with surface immunoglobulin and to be activated upon surface immunoglobulin cross-linking.[7] Indeed, also in our system, a 70 000 molecular weight protein, a likely candidate for $p72^{syk}$, became tyrosine-phosphorylated after exposing to anti-IgM or H_2O_2.

Our results also demonstrate that these tyrosine phosphorylations are distinct. First, tyrosine phosphorylation induced by H_2O_2 but not that triggered via sIgM seems to be regulated through a redox mechanism and glutathione may play an important role. Similarly a redox-sensitive mechanism is shown to be involved in tyrosine phosphorylation in B cells triggered

by the TAPA-1 antigen (CD81).[19] The authors demonstrate that tyrosine phosphorylation mediated via the antigen is sensitive to agents modulating cellular glutathione contents, such as 2-ME and BSO. Consistent with our results, the authors demonstrate that tyrosine phosphorylation mediated via sIgM is insensitive to these agents. Moreover, another laboratory has recently shown that glutathine depletion by BSO enables TNF-α to stimulate extensive tyrosine phosphorylation in T lymphocytes.[20] Interestingly, also in T cells, tyrosine phosphorylation mediated via the T-cell antigen receptor (TcR)/CD3 complex is insensitive to BSO treatment. Taken together, these facts favour the theory that both redox-sensitive and redox-insensitive mechanisms are involved in regulating lymphocyte tyrosine phosphorylation and that oxidative stress and antigen receptors trigger tyrosine phosphorylation using these two distinct mechanisms.

Several lymphocyte PTK are shown to be activated upon exposure to oxidants or thiol-reacting agents.[7–10] However, except for *Ltk*, a mouse homologue of insulin receptor kinase,[10] all these PTK are also shown to be involved in transmembrane signalling via surface immunoglobulin or the (TcR)/CD3 complex. Thus, the PTK specifically involved in such a redox-sensitive mechanism are as yet unidentified. Recent studies revealed that the TAPA-1 antigen and HLA-DR are non-covalently associated on the surface of B cells[21] and also implicated that this type of molecular complex may transduce intracellular signals through tyrosine phosphorylation using a redox-sensitive mechanism. Consistent with the idea, we have recently observed that tyrosine phosphorylation triggered by anti-major histocompatibility complex class II is also redox-sensitive (Y. Suzuki and Y. Ono, unpublished results). We are currently investigating the mechanism involved in the redox-sensitive tyrosine phosphorylation.

The tyrosine phosphorylation induced by H_2O_2 but not that caused via sIgM was augmented by two inflammatory cytokines, TNF-α and IL-1α. Since neither cytokine by itself could induce tyrosine phosphorylation or PTK stimulation, the most likely mechanism for this augmentation is PTPase inactivation. This is in good agreement with the previous studies demonstrating that an early intracellular event observed after exposing to either cytokine is the inactivation of redox-sensitive protein phosphatase possibly including PTPase.[22] Since neither cytokine could affect anti-IgM-induced tyrosine phosphorylation, our results suggest that these tyrosine phosphorylations are differently regulated by PTPase sensitive to these inflammatory cytokines.

In conclusion, we demonstrate that oxidative stress triggers B-cell tyrosine phosphorylation using a mechanism that is redox- and inflammatory cytokine-sensitive and distinct from that triggered by sIgM.

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