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# Syk Is Required for p38 Activation and G2/M Arrest in B Cells Exposed to Oxidative Stress

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## ABSTRACT

**Syk has been demonstrated to play a crucial role in oxidative stress signaling in B cells. In this study, we have investigated the role of Syk in p38 activation and the regulation of cell-cycle progression upon oxidative stress. In B cells, p38 is activated by hydrogen peroxide ($H_2O_2$) stimulation. Syk is required for p38 activation following stimulation with 10–100 $\mu M$ $H_2O_2$, but not with 1 m$M$ $H_2O_2$. $H_2O_2$-induced p38 activation is abrogated in phospholipase C-$\gamma$2 (PLC-$\gamma$2)-deficient as well as Syk-deficient cells, suggesting that Syk activates p38 through PLC-$\gamma$2 upon $H_2O_2$ stimulation. Although stimulation with 20–100 $\mu M$ $H_2O_2$ induces cellular apoptosis in B cells, pretreatment with SB203580, a p38-specific inhibitor, has no effect on $H_2O_2$-induced apoptosis. Flow cytometric analysis reveals that B cells exposed to 10–20 $\mu M$ $H_2O_2$ exhibit cell-cycle profile of G2/M arrest, and pretreatment with SB203580 inhibits only a little $H_2O_2$-induced G2/M arrest. On the other hand, Syk-deficient cells show no induction of G2/M arrest following $H_2O_2$ stimulation. These findings indicate that Syk plays a role in the regulation of cell-cycle progression in G2/M phase via p38-dependent and -independent pathways after oxidative stress.** *Antioxid. Redox Signal.* 4, 509–515.

## INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS), such as the superoxide radical, the hydroxyl radical, and hydrogen peroxide ($H_2O_2$), are continuously produced in most cells (18). It has been reported that ROS have normal roles as second messengers in signal transduction induced by cytokines (29, 30, 34, 47), growth factors (1, 25, 42), and agonists of heptahelical receptors (6, 14). On the other hand, when generation of ROS exceeds the capability of the cellular natural defense system, oxidative stress occurs (17). Oxidative stress provokes cell death, either apoptotic or necrotic, depending on the intensity of stress (12, 16, 41). In addition, many reports have shown that a sublethal level of oxidative stress causes cell proliferation (4), mitotic arrest (27), and senescent-like growth arrest (5) in various kinds of cells.

It has become clear that oxidative stress triggers the activation of multiple signaling pathways, including $Ca^{2+}$ mobilization and the activation of protein-tyrosine kinases (PTKs), mitogen-activated protein kinases (MAP kinases), phosphatidylinositol 3-kinase (PI 3-kinase)–Akt pathway, and transcription factors in various cell lines (22, 31, 33, 36, 37, 43). In B cells exposed to oxidative stress, Syk, which is one of the nonreceptor PTKs (40, 51), is rapidly tyrosine-phosphorylated and plays a crucial role in the transduction of oxidative stress signaling (31, 36, 37). Genetic studies using Syk-deficient B cells has revealed that Syk is essential for the increased tyrosine phosphorylation of cellular proteins, $Ca^{2+}$ release from intracellular stores, and c-Jun N-terminal kinase (JNK) activation after $H_2O_2$ stimulation (36, 37). Moreover, we have recently reported that Syk induces the activation of the PI 3-kinase–Akt survival pathway following $H_2O_2$ stimulation, which leads to cellular apoptosis, thereby enhancing cellular resistance to oxidative stress-induced apoptosis (8).

MAP kinase cascades are important signaling systems by which cells transduce extracellular stimuli into intracellular

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signals to control the expression of genes essential for cellular responses. MAP kinases include extracellular signal-regulated protein kinases (ERKs), JNK, and p38. In generally, ERKs are normally associated with growth factors to induce proliferation, whereas JNK and p38 are stimulated by stress responses and cytokines to mediate cell apoptosis and differentiation (38, 50). p38 has been shown to modulate apoptosis induced by cadmium (11), glutamate (24), withdrawal of trophic factors (26), and Fas activation (2, 23) in various cells. On the other hand, it has been reported that p38 plays a role in the regulation of cell-cycle progression (3, 27, 46, 49). There are some reports that p38 is required for initiation of a G2/M checkpoint after ultraviolet radiation or low-level oxidative stress (3, 27, 49).

In this article, we report that Syk is required for p38 activation in B cells following stimulation with 10–100 $\mu M$ $H_2O_2$ and plays a role in the regulation of cell-cycle progression after oxidative stress in a p38-dependent or -independent manner.

## MATERIALS AND METHODS

### *Materials*

The RPMI 1640 medium was purchased from ICN Biomedicals. Fetal bovine serum was from Sigma. Protein A–Sepharose CL-4B was from Amersham Pharmacia Biotech AB. Anti-human JNK1 antibody was purchased from Pharmingen. Anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-ERK, and anti-phospho-ERK (Thr202/Tyr204) antibodies were purchased from New England Biolabs, Inc. SB203580 was purchased from Calbiochem.

### *Cell culture*

Syk-deficient (Syk$^-$) and phospholipase C-$\gamma$2 (PLC-$\gamma$2)-deficient (PLC-$\gamma$2$^-$) DT40 chicken B cells were provided by Dr. Kurosaki (44, 45). Wild-type DT40 and its mutant cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 2 m$M$ glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml kanamycin in a humidified 95% air/5% $CO_2$ atmosphere. Cells, collected by centrifugation at 400 *g* for 5 min, were washed with phosphate-buffered saline and resuspended ($1 \times 10^7$ cells/ml) in Hanks' balanced salt solution. For all of the experiments described here, cells were stimulated with $H_2O_2$ at 37°C with gentle stirring.

### *Immunoprecipitation and immunoblotting*

Control and stimulated cells ($1 \times 10^7$ cells/ml) were lysed in ice-cold lysis buffer (50 m$M$ Tris, pH 7.4, 150 m$M$ NaCl, 5 m$M$ EDTA, 1% NP-40, 100 $\mu M$ $Na_3VO_4$, 2 m$M$ phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin). Lysates were clarified by centrifugation at 12,000 *g* for 10 min at 4°C. The supernatants were incubated sequentially (1 h for each incubation) with antibodies and protein A–Sepharose CL-4B at 4°C. Whole-cell lysates or immunoprecipitates were analyzed on sodium dodecyl sulfate–polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore). Blots were probed with the indicated antibodies, and immunoreactive proteins were revealed by the enhanced chemiluminescence detection system (Dupont NEN).

### *In vitro kinase assay*

Lysates were prepared as described above under *Immunoprecipitation and immunoblotting*. Lysates were immunoprecipitated by anti-JNK1 or anti-p38 antibodies with protein A–Sepharose. The beads were pelleted by centrifugation and washed three times with lysis buffer and twice with kinase assay buffer (20 m$M$ HEPES, pH 7.4, 10 m$M$ $MgCl_2$, 10 m$M$ $MnCl_2$, 2 m$M$ dithiothreitol, and 10 $\mu M$ sodium vanadate). Immunoprecipitates were performed in 30 $\mu$l of kinase assay buffer containing 1 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) and 5 $\mu$g of glutathione *S*-transferase (GST)–c-Jun and GST–activating transcription factor 2 (ATF2) fusion proteins as substrates for JNK and p38, respectively. After 20 min of incubation at 30°C, reactions were terminated by the addition of electrophoresis sample buffer and boiling for 5 min. Autoradiography of sodium dodecyl sulfate–polyacrylamide gels was carried out using standard procedures, and autoradiographs were quantified using a phosphoimager system (Fuji BAS 2000).

### *Cell viability*

Wild-type and mutant DT40 cells ($5 \times 10^5$ cells/ml) were stimulated using the indicated concentrations of $H_2O_2$. After the indicated times, cell viability was determined by the trypan blue dye exclusion method.

### *DNA fragmentation analysis*

Wild-type cells ($5 \times 10^5$ cells/ml) were stimulated with 50 $\mu M$ $H_2O_2$ for 8 h following pretreatment with or without 10 $\mu M$ SB203580. Collected cells ($5 \times 10^6$ cells) were lysed in 0.5 ml of lysis buffer (10 m$M$ Tris-HCl, pH 7.5, 10 m$M$ EDTA, 200 m$M$ NaCl, 0.4% Triton X-100, and 0.1 mg/ml proteinase K) for 20 min at room temperature and incubated with 0.1 mg/ml RNase A for 30 min at 50°C. DNA fragmentation was analyzed using a 2.5% agarose gel in the presence of 0.5 $\mu$g/ml ethidium bromide.

### *Flow cytometric analysis*

Either stimulated or unstimulated cells ($1 \times 10^6$ cells) were harvested and fixed with 70% ethanol and kept at 4°C overnight. Fixed cells were pelleted and resuspended in DNA staining solution (50 $\mu$g/ml propidium iodide, 100 U/ml RNase A, 1 mg/ml glucose, phosphate-buffered saline). DNA content and cell number were measured with a flow cytometer (CytoACE 300).

## RESULTS

### *p38 is activated depending on Syk following stimulation with low concentrations of $H_2O_2$*

It has been reported that oxidative stress triggers signal transduction via MAP kinases to induce activation of certain transcription factors and expression of specific genes for cel-

lular responses (22). We have demonstrated that in B cells treated with 1 m*M* $H_2O_2$, Syk is essential for activation of JNK, but not ERK (37). We examined the activation of p38 in B cells stimulated with $H_2O_2$. Wild-type or Syk-deficient cells were treated with various concentrations of $H_2O_2$, and the whole-cell lysates were subjected to immunoblot analysis using anti-phospho-p38 antibody, which recognized phosphorylated p38 on Thr180/Tyr182, an active form. In wild-type cells, p38 phosphorylation increased in a $H_2O_2$ dose-dependent manner. On the other hand, p38 phosphorylation in Syk-deficient cells stimulated with 10–100 $\mu M$ $H_2O_2$ was significantly abrogated (Fig. 1A). In wild-type cells stimulated with 100 $\mu M$ $H_2O_2$, p38 was rapidly phosphorylated and reached maximum phosphorylation at 1–5 min and attenuated at 10 min (Fig. 1B). Although phosphorylation of p38 on Thr180/Tyr182 correlates with maximal activation of p38 (39), we confirmed the enzymatic activity of p38 by *in vitro* kinase assay using ATF2 as a substrate. Consistently, the maximum activity of p38 appeared at 2–5 min after $H_2O_2$ stimulation in wild-type cells, whereas in Syk-deficient cells p38 activity was considerably inhibited (Fig. 1C). In Syk-deficient cells expressing porcine Syk, p38 phosphorylation following $H_2O_2$ stimulation is the same as that in wild-type cells (data not shown). These findings indicated that in B cells stimulated with low concentrations (10–100 $\mu M$) of $H_2O_2$, p38 was rapidly activated depending on Syk.

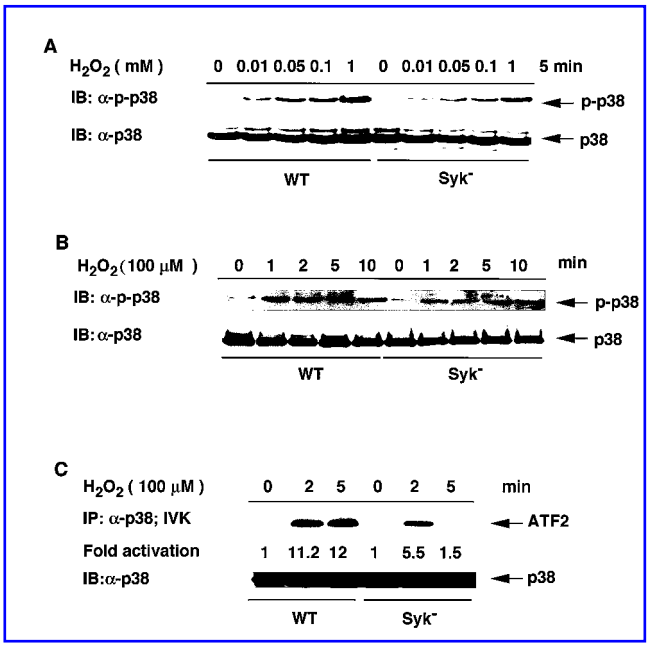


**FIG. 1. p38 is activated depending on Syk upon $H_2O_2$ stimulation.** After wild-type (WT) and Syk-deficient (Syk⁻) DT40 B cells were stimulated with the indicated concentrations of $H_2O_2$ for 5 min (**A**) or with 100 $\mu M$ $H_2O_2$ for the indicated times (**B**), cell lysates were subjected to immunoblot analysis using anti-phospho-p38 or anti-p38 antibodies. (**C**) After wild-type and Syk-deficient cells were stimulated with 100 $\mu M$ $H_2O_2$ for the indicated times, p38 activity in the immunoprecipitates was determined using a specific *in vitro* kinase assay (IVK) as described in Materials and Methods. The results shown are from one representative experiment that was repeated four times.

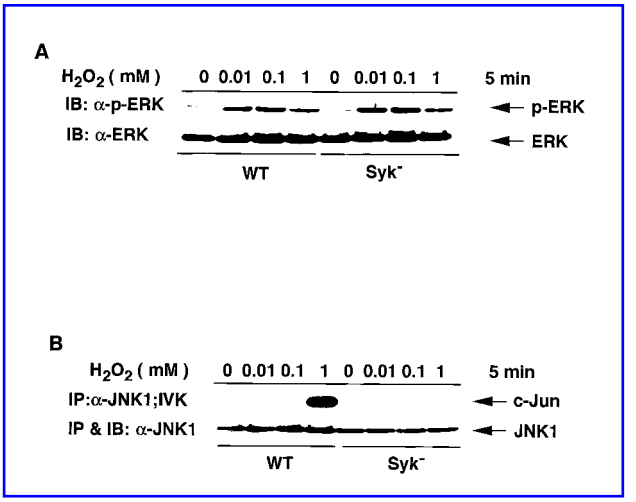


**FIG. 2. Syk is not required for ERK phosphorylation and JNK activity upon stimulation with low doses of $H_2O_2$.** (**A**) After wild-type and Syk-deficient B cells were stimulated with the indicated concentrations of $H_2O_2$ for 5 min, cell lysates were subjected to immunoblot analysis using anti-phospho-ERK or anti-ERK antibodies. (**B**) JNK activity in the immunoprecipitates was determined using a specific *in vitro* kinase assay (IVK) as described in Materials and Methods. The results from one representative experiment that was repeated four times are shown.

In the same way, we studied ERK and JNK activation upon stimulation with low concentrations of $H_2O_2$. ERK is phosphorylated independent of Syk (Fig. 2A), whereas JNK activation was not observed following stimulation with 10 or 100 $\mu M$ $H_2O_2$ (Fig. 2B). Syk was not involved in JNK activation in B cells stimulated with low concentrations of $H_2O_2$. Syk activation following $H_2O_2$ stimulation is associated with the distinct pathways for MAP kinase activation according to the intensity of $H_2O_2$ stimulation.

## *p38 activation is mediated through PLC-γ2 upon $H_2O_2$ stimulation*

It has been reported that in B cells p38 activation is essential for B-cell antigen receptor (BCR)-induced apoptosis or CD40-induced gene expression and proliferation (7, 13), and BCR-induced p38 activation was shown to require PLC-γ2-dependent signal and GTPase (19). We examined the involvement of PLC-γ2 in $H_2O_2$-induced p38 activation. As shown in Fig. 3, p38 phosphorylation was abolished in PLC-γ2-deficient as well as Syk-deficient cells upon 100 $\mu M$ $H_2O_2$ stimulation. We have demonstrated that PLC-γ2 activation after $H_2O_2$ stimulation is dependent on Syk (9). These results suggested that Syk activated p38 through PLC-γ2.

## *p38 is not involved in $H_2O_2$-induced apoptosis*

We have demonstrated that exposure of B cells to 20–100 $\mu M$ $H_2O_2$ decreases the cell viability and induces apoptosis (8). p38 has been shown to be involved in cell apoptosis induced by various stimuli (2, 11, 23, 24, 26). We examined the involvement of p38 in $H_2O_2$-induced apoptosis. Wild-type and Syk-deficient cells were pretreated with SB203580, a p38-specific inhibitor, for 1 h and then were stimulated with

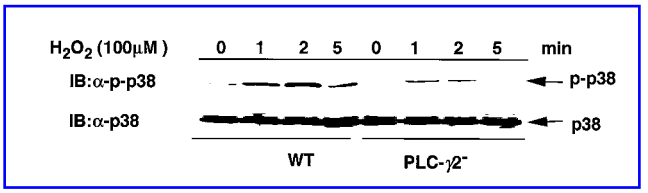


**FIG. 3. p38 activation is mediated through PLC-γ2 upon $H_2O_2$ stimulation.** After wild-type and PLC-γ2-deficient (PLC-γ2⁻) B cells were stimulated with 100 μ*M* $H_2O_2$ for the indicated times, cell lysates were subjected to immunoblot analysis using anti-phospho-p38 or anti-p38 antibodies. The results from one representative experiment that was repeated four times are shown.

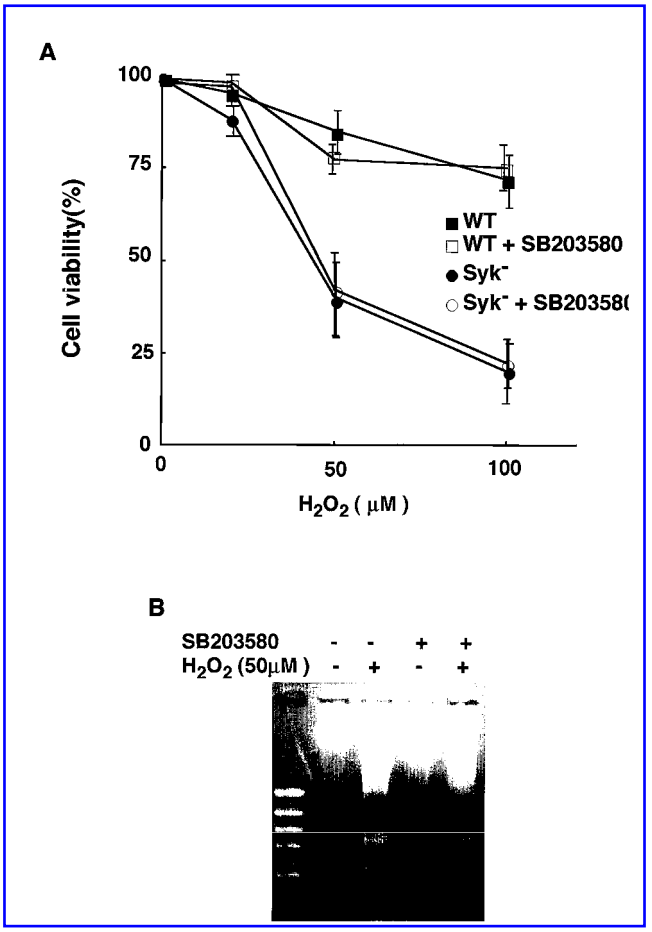


**FIG. 4. p38 is not involved in $H_2O_2$-induced apoptosis.** (**A**) Wild-type and Syk-deficient B cells were pretreated with or without 10 μ*M* SB203580 for 1 h and then treated with the indicated concentrations of $H_2O_2$ for 12 h. Cell viability was determined by the trypan blue dye exclusion method. Results shown are means ± SD from three independent experiments. (**B**) Wild-type cells were pretreated with or without 10 μ*M* SB203580 for 1 h and then treated with 50 μ*M* $H_2O_2$ for 12 h. Cell lysates were subjected to DNA fragmentation analysis as described in Materials and Methods. The results from one representative experiment that was repeated four times are shown.

20–100 μ*M* $H_2O_2$ for 16 h, and cell viability was determined by trypan blue dye exclusion method. As shown in Fig. 4A, pretreatment with SB203580 has no effect on the decrease of cell viability upon $H_2O_2$ stimulation in wild-type as well as Syk-deficient cells. In DNA ladder formation after $H_2O_2$ stimulation, there is no difference in between SB203580-pretreated and nonpretreated wild-type cells (Fig. 4B). Treatment with SB203580 for 1 h completely inhibited $H_2O_2$-induced p38 phosphorylation in B cells (data not shown). These findings suggested that p38 is not involved in $H_2O_2$-induced apoptosis in B cells.

### *Syk is required for G2/M arrest after $H_2O_2$ stimulation*

Oxidative stress has been reported to cause inhibition of cell division in the M phase through p38 activation in human promonocytic U937 cells (27). Moreover, a recent report has shown that p38 phosphorylates cdc25B and controls initiation of G2/M checkpoint after ultraviolet radiation in fibroblasts (3). These previous reports prompted us to examine whether Syk is involved in the regulation of cell-cycle progression after $H_2O_2$ stimulation through p38 activation. Wild-type and Syk-deficient cells were treated with or without 20 μ*M* $H_2O_2$ for 16 h, and cell-cycle profiles were determined by flow cytometric analysis with propidium iodide staining. As shown in Fig. 5A and D, the cell-cycle profile of unstimulated wild-type cells is identical to that of unstimulated Syk-deficient cells. In wild-type cells stimulated with $H_2O_2$, significantly more G2/M-phase than G1-phase cells were present (Fig. 5B). On the other hand, in Syk-deficient cells upon $H_2O_2$ stimulation, there is no remarkable change in the proportion of G2/M- and G1-phase cells, and a high proportion of apoptotic cells was observed (Fig. 5E). When wild-type and Syk-deficient cells were pretreated with SB203580 for 1 h and then were treated with $H_2O_2$, wild-type cells showed a somewhat reduced proportion of G2/M-phase cells, whereas SB203580 has no influence on the cell-cycle profile of Syk-deficient cells. These findings suggested the following: (a) treatment with 20 μ*M* $H_2O_2$ induced G2/M arrest in B cells; (b) p38 activation is partially involved in induction of G2/M arrest after $H_2O_2$ stimulation; (c) Syk plays an important role in $H_2O_2$-induced G2/M arrest. Further investigation is warranted to clarify how Syk regulates cell-cycle progression after oxidative stress.

## DISCUSSION

It has been reported that ROS activate p38 in many cell types (10, 15, 20, 28, 32, 35). Here, we have shown that p38 is activated through Syk following $H_2O_2$ stimulation in B cells. Syk is a central molecule in the transduction of oxidative stress signaling, such as activation of PLC-γ2, JNK, and Akt pathways in B cells (8, 9, 37). In PLC-γ2-deficient as well as Syk-deficient cells, p38 activation upon $H_2O_2$ stimulation is abolished. PLC-γ2 generates diacylglycerol and inositol 1,4,5-trisphosphate, which induce protein kinase C (PKC) activation and elevate cytoplasmic free $Ca^{2+}$, respectively. A re-

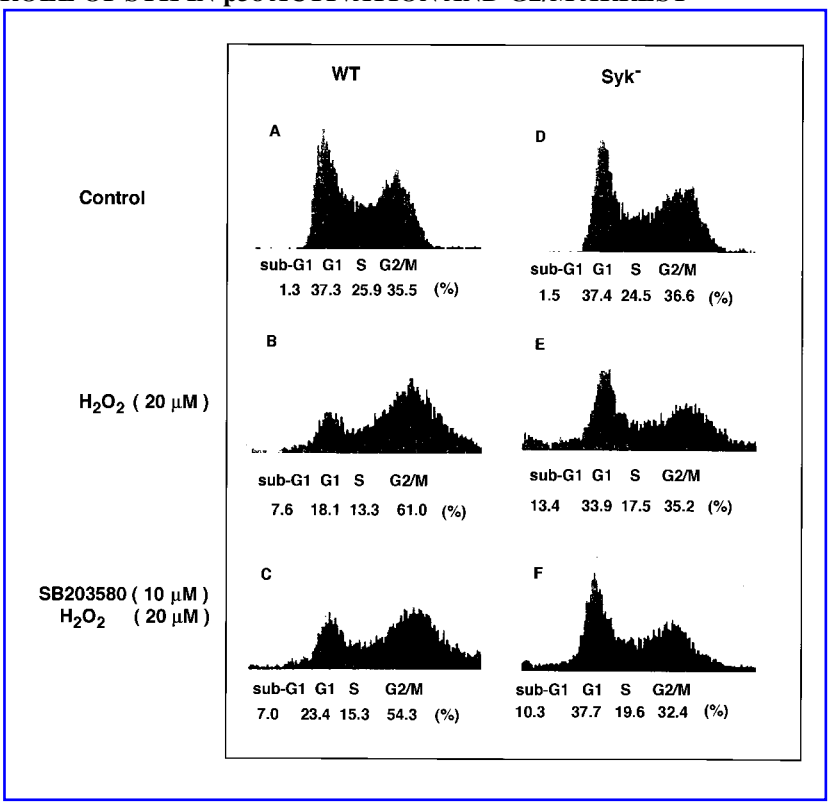


**FIG. 5. Syk is required for G2/M arrest after $H_2O_2$ stimulation.** Wild-type (**A–C**) and Syk-deficient (**D–F**) cells were unstimulated (A, D) or stimulated with 20 μ*M* $H_2O_2$ for 16 h following pretreatment with (C, F) or without (B, E) SB203580, and cell-cycle profiles were determined by flow cytometric analysis with propidium iodide staining. Results shown are from one representative experiment that was repeated four times.

cent report has shown that elevation of intracellular $Ca^{2+}$ is not essential for the $H_2O_2$-induced activation of p38 (21). We also found that an absence of both intracellular and extracellular $Ca^{2+}$ did not inhibit $H_2O_2$-induced p38 activation, and the treatment of phorbol 12-myristate 13-acetate, a PKC-specific activator, induced p38 activation independent of Syk (data not shown). We suggested that PKC was involved in Syk-mediated p38 activation upon $H_2O_2$ stimulation in B cells. It has been reported that MAP kinase kinase 6, which is a direct activator of p38, is activated by $H_2O_2$ stimulation in KB cells (32), and small GTPases are required for $H_2O_2$-stimulated p38 activation in Jurkat T cells (28). p38 activation following $H_2O_2$ stimulation probably requires a small GTPase–MAP kinase kinase kinase–MAP kinase kinase cascade. However, interaction between this small GTPase-regulated signaling pathway and PKC after oxidative stress remains unknown.

Although p38 has been shown to modulate cell apoptosis induced by ROS-producing stimuli (10, 35), there are reports that p38 is not involved in oxidative stress-induced apoptosis (48). Recently, it has been reported that treatment of U937 promonocytic cells with 20 μ*M* $H_2O_2$ causes a selective activation of p38, resulting in the induction of mitotic arrest without apoptosis (27). We also have demonstrated that a cell-cycle profile of G2/M arrest appears in B cells exposed to 10–20 μ*M* $H_2O_2$. When pretreatment with SB203580 inhibited $H_2O_2$-induced p38 phosphorylation (data not shown), there was no change in B-cell viability after $H_2O_2$ stimulation, and pretreatment of SB203580 exhibits only a little decrease of $H_2O_2$-induced G2/M arrest. These findings indicate that in B cells p38 activation is not involved in oxidative stress-induced apoptosis and has a partial influence on the induction of a G2/M arrest following oxidative stress. A recent report has shown that after ultraviolet radiation p38 phosphorylates cdc25B and regulates initiation of G2/M checkpoint (3). The role of p38 in the regulatory mechanism of oxidative stress-induced G2/M arrest remains unknown.

$H_2O_2$ stimulation induces distinct patterns of cellular responses, including necrosis, apoptosis, and mitotic arrest according to the intensity of the stimuli in B cells. We have demonstrated that in B cells the treatment with 1 m*M* $H_2O_2$ induces cell necrosis, whereas stimulation with 20–100 μ*M* $H_2O_2$ induces apoptosis characterized by DNA ladder formation (8). It is clear that Syk-deficient cells are more susceptible to $H_2O_2$-induced apoptosis than wild-type cells and p38 is not involved in this phenomenon. No differences in ERK and JNK activation between wild-type and Syk-deficient cells were observed at least at 10–100 μ*M* $H_2O_2$. We have demonstrated that Syk induces the activation of the PI 3-kinase–Akt survival pathway following oxidative stress and inhibits the activation of caspase-9 through Akt activation (8). Syk plays a role in the protection of B cells from oxidative stress-induced apoptosis through the activation of the PI 3-kinase–Akt survival pathway. In addition, this study has revealed that B cells exposed to 10–20 μ*M* $H_2O_2$ exhibit a cell-cycle profile of G2/M arrest. Interestingly, Syk-deficient cells exhibit no induction of G2/M arrest after $H_2O_2$ stimulation. These findings indicate that Syk is essential for regulation of cell-cycle progression after oxidative stress. Further investigations are required for clarifying the role of Syk in cell-cycle progression after oxidative stress.

In summary, a sublethal level of oxidative stress induces p38 activation and G2/M arrest in B cells depending on Syk. However, p38 activation has only partial influence on $H_2O_2$-induced G2/M arrest in B cells. We suggest that Syk plays a role in the

regulation of cell-cycle progression in G2/M phase via p38-dependent and -independent pathways after oxidative stress.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

ATF2, activating transcription factor 2; BCR, B-cell antigen receptor; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; $H_2O_2$, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PTKs, protein-tyrosine kinases; ROS, reactive oxygen species.

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