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Apoptotic Effect of PP2 a Src Tyrosine Kinase Inhibitor, in Murine B Cell Leukemia

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Abstract Src is a non-receptor protein tyrosine kinase that transduces signals regulating cell growth and differentiation. We report here that activation of signaling pathway after blockade of tyrosine phosphorylation by PP2 (4-amino-5-(4-chloro-phenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), a potent and selective inhibitor of the Src-family tyrosine kinase, can lead to cell death in murine B cell leukemia, 70Z/3. Death from PP2 occurred by apoptosis as indicated by the induction of caspase activation and annexin V/propidium iodide staining. Interestingly, PP2 was found to be able to enhance the DNA binding activity of nuclear factor κB (NF-κB) before induction of apoptosis without accompanying by increased phosphorylation of inhibitor of NF-κB-α (IκB-α). Additionally, immunoblotting analysis with PP2-treated cell extract demonstrated that, compared to other protein kinase C (PKC) isotypes, the translocation of novel PKC isotypes from the cytosol to membrane fraction was sustained for a longer time. These data suggest that the inhibition of Src-mediated tyrosine phosphorylation by PP2 may tilt the balance between each PKC isotypes, which in turn, activate NF-κB transcription factor, leading to apoptosis. J. Cell. Biochem. 93: 629–638, 2004.

Key words: PP2; Src; PKC; apoptosis; NF-κB

The Src family of non-receptor tyrosine kinases plays a role in the regulation of cell adhesion, growth, and differentiation through the activation of multiple intracellular signaling pathways. The Src kinase is normally maintained in an inactive state, but can be activated transiently during cellular events such as mitosis, or constitutively by abnormal events such as mutation [Bjorge et al., 2000]. Activation of Src occurs as a result of disruption of the negative regulatory processes that normally suppress Src activity [Schwartzberg, 1998]. The Src kinase is known to target PKC for dephosphorylation and activation [Zang et al., 1995].

Activation of the transcription factor, nuclear factor-κB (NF-κB), which was originally identified as a B-cell nuclear factor [Sen and Baltimore, 1986], has been shown to be a key component of immune response. At least five different genes belong to the NF-κB family: *p50*, *p52*, *Rel A* (*p65*), *Rel B*, and *c-Rel*. These transcription factors are sequestered in the cytoplasm in an inactive state by inhibitory proteins, IκB. Upon activation, IκB undergoes phosphorylation and degradation, and the NF-κB heterodimer translocates into the nucleus, where it binds to DNA and activates transcription [Rice and Ernst, 1993]. Activation of NF-κB-dependent transcription has been observed consistently following stimulation by a high molecular weight IκB kinase (IKK) complex, which consists of IKK-α, IKK-β, NF-κB-inducing kinase (NIK), and two adaptor or scaffold proteins [Woronicz et al., 1997]. However, evidence has accumulated that PKC may be associated with activation of NF-κB via IKK [Trushin et al., 1999]. Protein kinase C (PKC) has been studied extensively as a second messenger transducing diverse signals regarding cell proliferation, activation of cellular function, differentiation, and even apoptosis [Dempsey et al., 2000]. The PKC family is composed of at least 11 members which are classified into three major groups: the "conventional" isozymes

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(cPKC), including PKC-α, -β_1, -β_2, and -γ; the "novel" isozymes (nPKC), including PKC-δ, -ε, -ι, -θ, and -μ; and the "atypical" isozymes (aPKC), including PKC-ζ and -λ [Parekh et al., 2000]. It has been proposed that cPKC isozymes may play an important role in modulating 'short-term,' receptor-mediated cellular functions such as hormone secretion and neurotransmitter release in response to transient changes in cytosolic calcium [Blobe et al., 1996]. In contrast, activation of the nPKC class is proposed to rely on diacylglycerols generated by sustained phosphatidylcholine turnover, and likely is involved in 'long-term' cellular regulation, for example, cell growth, differentiation, and tumor promotion.

Recently, Hanke et al. [1996] described the tyrosine kinase inhibitor, PP2 (4-amino-5-(4-chloro-phenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) that was a potent Src family kinase inhibitor but only weakly inhibited ZAP-70 and JZK2. In the present study, we explored the intracellular signaling pathway of PP2-induced NF-κB activation and its suppressive effect on proliferation in 70Z/3 murine B-cell leukemia.

MATERIALS AND METHODS

Antibodies and Reagents

Anti-cleaved caspase-3, anti-cleaved poly ADP-ribose polymerase, and anti-poly (ADP-ribose) polymerase were obtained from Cell Signaling Technology (Beverly, MA). Anti-PKC antibody sampler kit was from GIBCO-Invitrogen (Carlsbad, CA). Anti-phosphotyrosine RC20H was from BD Biosciences Pharmingen (San Diego, CA). PD98059, SB203580, GF 109203X, and Gö 6976 were purchased from Calbiochem (La Jolla, CA). PP2 (4-amino-5-(4-chloro-phenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) was also obtained from Calbiochem and dissolved in DMSO. Drugs were freshly diluted in culture media for each experiment.

Cell Line

The murine B-cell leukemia, 70Z/3 (ATCC TIB 152), was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium supplemented with 100 U of penicillin-streptomycin/ml, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10% bovine calf serum (GIBCO-Invitrogen).

Cell Growth Assay

The cell proliferation reagent WST-1 was used for the quantitative determination of cellular proliferation and activation (Roche Molecular Biochemicals, Mannheim, Germany). The chemicals were added directly to cells, which were then cultured (5×10^3 cells/well) in 96-well microtiter plates (Costar, Cambridge, MA). The cultures were incubated at 37°C in a humidified 5% CO_2/95% air incubator. After incubation for the indicated times, the cells were incubated for additional 4 h in the presence of WST-1 labeling mixture (10 μl/well). The absorbance was measured at 450 nm using a microtiter plate reader (Molecular Devices, Sunnyvale CA).

Preparation of Cell Lysates

Whole cell lysates were prepared after chemical treatment as follows. For the experiment shown in Figure 2, cells were washed twice with ice-cold phosphate-buffered saline (PBS), suspended in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM β-glycerophosphate, and 2 mM sodium fluoride). Cell lysates were clarified by centrifugation at $15{,}000 \times g$ for 10 min at 4°C, and protein concentrations were determined with the BCA protein assay reagent kit as described by the manufacturer (Pierce, Rockford, IL). For the experiments shown in Figure 3, the cells were washed twice with ice-cold PBS, harvested by resuspension of the cell pellet in lysis buffer (50 mM Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)/NaOH, pH 6.5, 2 mM EDTA, 0.1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 mM phenylmethyl sulfonyl fluoride) and subjected to three cycles of freezing and thawing. After mixing Laemmli sample buffer and heating for 95°C for 5 min, the cell lysates were microcentrifuged and the supernatant was assayed for caspase activation by Western blot analysis.

Immunoprecipitation and Immunoblot

Immunoprecipitation was performed on the whole cell lysates using anti-Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA) with protein A-agarose beads. After incubation for 2 h at 4°C, immunoprecipitates were washed twice with

ice-cold lysis buffer. For immunoblotting, immunoprecipitates, or cell lysates were denatured in Laemmli sample buffer, and resolved by 7.5% SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose, and immunoblot analysis was performed using the antibodies indicated in figure legends. Immune complexes on nitrocellulose were detected by enzyme-linked chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ).

Apoptotic Cell Death Assay

To quantify apoptosis, 70Z/3 cells were stained with annexin V and PI using TACS^{TM} Annexin V Kits from Trevigen, Inc. (Gaithersburg, MD) following the step-by-step protocol as provided by the manufacturer and analyzed by flow cytometry. Briefly, cells were pelleted, washed with ice-cold PBS, resuspended in annexin binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$), and stained with annexin V-FITC for 10 min on ice in the dark. Apoptotic cells were counted on a flow cytometry using a dual filter set for FITC and propidium iodide.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as described by Lee and Jeon [2001]. The protein content of the nuclear extracts was determined by the Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA), and gel mobility shift assays were performed. Briefly, 5 μg of nuclear extracts were incubated with 2 μg of poly (dI-dC) (Sigma, St. Louis, MO) and ^{32}P-end-labeled DNA probe (double-stranded synthetic 26-bp oligonucleotides GATCTCAGAGGGGACTTTCCGAAGAGA containing the κB enhancer of immunoglobulin κ light chain gene). The DNA binding activity was separated from free probe using a 5% acrylamide gel in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). After electrophoresis, the gel was dried for 1 h and autoradiographed for analysis. Identity of the shifted bands was confirmed by competition with unlabeled oligomer containing NF-κB site.

Cell Fractionation

Once 70Z/3 cells reached subconfluence, the cells were incubated for additional times in the presence of PP2. The cells then were washed with ice-cold PBS, lysed in lysis buffer (20 mM Tris-HCl (pH 7.4), 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μM leupeptin), and disrupted by Dounce homogenization. The homogenates were centrifuged at 100,000 × g for 1 h. The supernatant was collected as the cytosolic fraction. The membrane pellet was suspended in the same volume of homogenization buffer with 1% Triton X-100. After incubation for 30 min at 4°C, the suspension was centrifuged at 100,000 × g for 1 h. The supernatant was collected as the membrane fraction. Equal amounts of protein from each fraction were detected by immunoblotting with the specific antibodies against PKC isotypes. The ECL (Amersham) protocol was used to visualize the immunoreactive bands.

RESULTS

Effects of PP2 on Proliferative Response

The Src-family of protein tyrosine kinases is a critical regulator of signal transduction in hematopoietic cells [Seckinger and Fougereau, 1994]. Therefore, PP2, which has been recently identified as a potent and selective inhibitor of the Src-family tyrosine kinases, was evaluated for its ability to alter proliferation of 70Z/3 cells. As shown in Figure 1, PP2 produced a dose-related inhibition of proliferation. The suppression of 70Z/3 cell proliferation was first observed at 5 μM of PP2. However, the cells treated with 5 μM of PP2, although showing growth inhibition, continued to grow. Conversely, 24 and 48 h

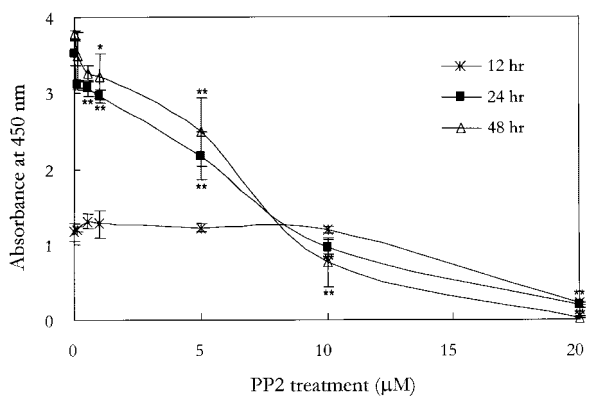

Fig. 1. Effect of PP2 on 70Z/3 cell proliferation. 70Z/3 cells were treated with PP2 and then incubated in 96-well plates for the indicated times before the addition of Cell Proliferation Reagent WST-1. After 4 h incubation periods the absorbance was determined by a microtiter plate reader. The absorbance at 450 nm is expressed as the mean ± SD of quadruplicate determinants from one of three representative experiments. $^*P < 0.05$ and $^{**}P < 0.01$ as determined by Dunnett's *t*-test as compared to the vehicle group (VH).

incubation at concentrations exceeding 10 μM PP2 resulted in more than 70% inhibition in cell proliferation, causing growth arrest.

To demonstrate that PP2 actually inhibits Src kinase in 70Z/3 cells, Raf-1 kinase, which has been known to be tyrosine-phosphorylated by Src kinase, was immunoprecipitated using anti-Raf antibody and then blotted with anti-phosphotyrosine antibody. Raf-1 kinase was tyrosine-phosphorylated after treatment with H_2O_2, as was earlier reported by Sato et al. [2001]. As shown in Figure 2, PP2 completely blocked the tyrosine phosphorylation of Raf-1 proteins.

Caspase-3-Dependent Apoptosis Was Involved in PP2-Induced Cell Death

By use of a quantitative apoptotic cell death assay, we next examined whether the observed cell growth inhibition by PP2 is accompanied by apoptotic death. As shown in Figure 3A, the apoptotic cell population (early + late apoptotic cells) increased from 12% in control to 40% after 10 μM PP2 treatment for 24 h. Paclitaxel, which has been reported to induce the apoptosis [Wang et al., 2000], was used as a positive control. In addition, caspase processing was analyzed by Western immunoblotting, because cell death from apoptosis usually results from the actions of a family of proteolytic enzymes termed caspases [Cohen, 1997]. Especially, caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key

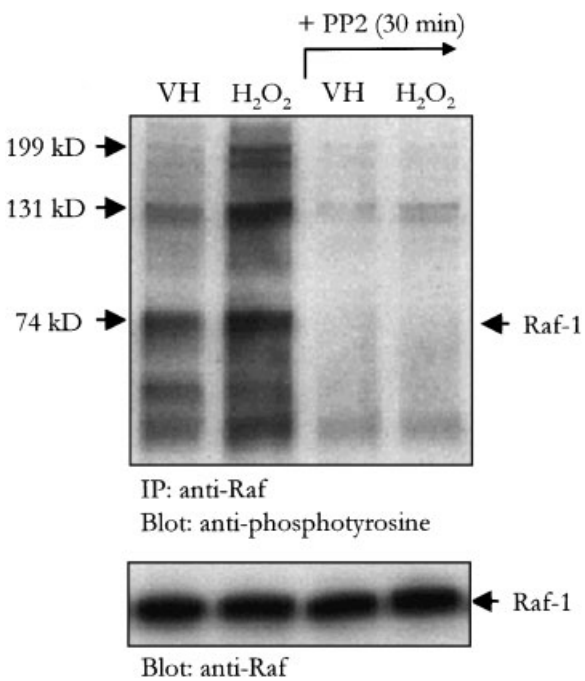

Fig. 2. The inhibitory effect of PP2 on tyrosine phosphorylation. The inhibition of tyrosine phosphorylation by PP2 was confirmed with immunoblotting using anti-phosphotyrosine antibody. 70Z/3 pre-B cells were pretreated in the presence and absence of PP2 as indicated for 30 min prior to stimulation with or without 1 mM H_2O_2 for 5 min. The cell lysates were immunoprecipitated with anti-Raf-1 antibody followed by immunoblotting with anti-phosphotyrosine antibody RC20H. The same blot was stripped and then reprobed with anti-Raf antibody. The results are representative of three independent experiments.

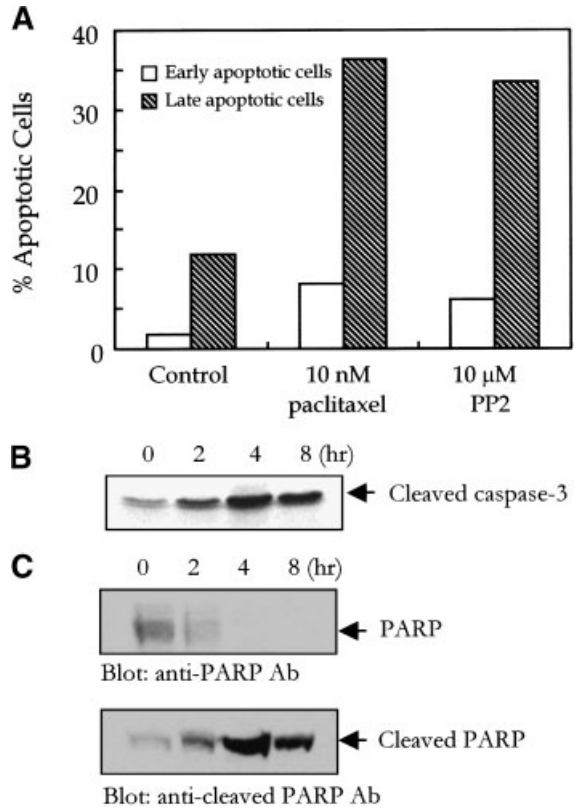

Fig. 3. PP2 induces cell death via caspase-3-dependent apoptosis. **A**: Cells were treated with either DMSO (control), 10 nM paclitaxel, or 10 μM PP2 for 24 h. Paclitaxel was used as a positive control for apoptosis. At the end of treatments, cells were harvested and stained with annexin V and propidium iodide as detailed in ''Materials and Methods.'' The data presented is representative of three independent experiments. **B**: Caspase-3 activation was assessed by Western blotting with a cleaved caspase-3 specific antibody, after cells were treated with 10 μM PP2. **C**: Poly (ADP-ribose) polymerase cleavage was assessed by Western blotting with either anti-poly (ADP-ribose) polymerase antibody that detects intact (116 kDa) products, or anti-cleaved poly (ADP-ribose) polymerase antibody that detect cleaved products (85 kDa), after cells were treated with PP2 for up to 8 h. Similar results were obtained in three separate experiments.

proteins such as the nuclear enzyme poly (ADP-ribose) polymerase. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into active p17 and p12 subunits. As shown in Figure 3B, the cleavage of caspase-3 began to increase 2 h after PP2 treatment in 70Z/3 cells and it kept increasing during the next 8 h period. To further evaluate whether processed caspase-3 were functionally active in PP2-treated 70Z/3 cells, we assessed poly (ADP-ribose) polymerase cleavage, which is an excellent marker for apoptosis, by Western blotting (Fig. 3C). Protein level of poly (ADP-ribose) polymerase was substantially reduced at 2 h after PP2 treatment. Poly (ADP-ribose) polymerase cleavage occurred at 2, 4, and 8 h after PP2 treatment, as indicated by the presence of the 85-kDa fragments at these times.

The Role of NF-κB in PP2-Induced Apoptosis

The transcription factor NF-κB has been shown to have a role in regulating the apoptotic program, either as a survival factor or an inducer of cell death in immune system [Barkett and Gilmore, 1999]. In order to ascertain whether PP2-induced apoptosis was associated with the NF-κB pathway, we determined the effects of PP2 on the nuclear expression of NF-κB in 70Z/3 cells. As shown in Figure 4A, PP2 increased NF-κB binding activity in nuclear extracts of 70Z/3 cells, suggesting that NF-κB is involved in PP2-induced apoptosis. DNA binding activity of NF-κB was sharply increased at 5–10 μM PP2 and gradually decreased at concentration exceeding 20 μM. The kinetics of NF-κB activation after PP2 exposure are also shown in Figure 4A. PP2 stimulation of NF-κB reached maximum level within 10 min and was sustained for a longer time (60 min or more) after exposure.

The event that occurs during activation of NF-κB is the phosphorylation of IκB that serves to target it for ubiquitination and degradation. Therefore, we determined IκB phosphorylation using a phospho-specific anti-IκBα antibody that detects IκBα only when activated by phosphorylation at Ser-32. An increase of phosphorylated IκBα was not detected after treatment of cells with PP2 (Fig. 4B). These observations suggest that PP2 may enhance NF-κB binding activity through the mechanism independent of the phosphorylation of IκBα without translocation of NF-κB complex to nuclear fraction.

Involvement of PKC in PP2-Induced NF-κB Activation

Many results have demonstrated a critical role for the PKC isoforms in the NF-κB pathway and in the transduction of intracellular signals initiated by Src tyrosine kinase [Zang et al., 1995; Krappmann et al., 2001; Lee and Jeon, 2001]. Therefore, we investigated the regulatory role of PKC in PP2-induced NF-κB activation, using GF 109203X (a pan-PKC inhibitor) and Gö 6976 (a conventional PKC inhibitor). As shown in Figure 5, no apparent inhibition in PP2-induced NF-κB activation was observed with a highly selective cell-permeable conventional PKC inhibitor, Gö 6976. Conversely, treatment of cells with GF 109203X, which inhibits the conventional and novel isoforms, inhibited PP2-induced NF-κB activation, suggesting a critical role for novel PKCs such as PKC-δ and PKC-ε. To assess the potential specificity of PKC isotypes mediating activation of NF-κB by PP2, we performed immunoblotting using the specific antibodies directed to PKC-α, -βI, -δ, -ε, and -ζ isotypes, which are constitutively expressed in lymphoid cells [Mischak et al., 1991]. The increase in membrane association of PKC isoforms has been widely used to demonstrate PKC isoform activation. We were able to detect a characteristic translocation noted with PKC activation when SDS-solubilized membrane and cytosolic fractions were analyzed (Fig. 6). Exposure of the cells to 10 μM PP2 for 5 min induced the membrane association of conventional PKCs such as PKC-α, -β as well as novel PKCs such as PKC-δ and -ε. However, the translocation of PKC-α and -βI from the cytosol to membrane fraction was transient and returned to cytosol between 20–30 min, while the translocation of PKC-δ and -ε was sustained for a longer time (30 min or more) after exposure of PP2. In contrast, no apparent changes in translocation were observed with atypical PKC-ζ. These observations suggest that the inhibition of Src-mediated tyrosine phosphorylation by PP2 may serve to more strongly activate novel PKC isoform compared to other PKC isoforms, allowing for a temporary alteration in PKC isoforms balance.

DISCUSSION

Src tyrosine kinases have been known to alter the regulation of the cell cycle and cell death, activating kinases that protect cells from

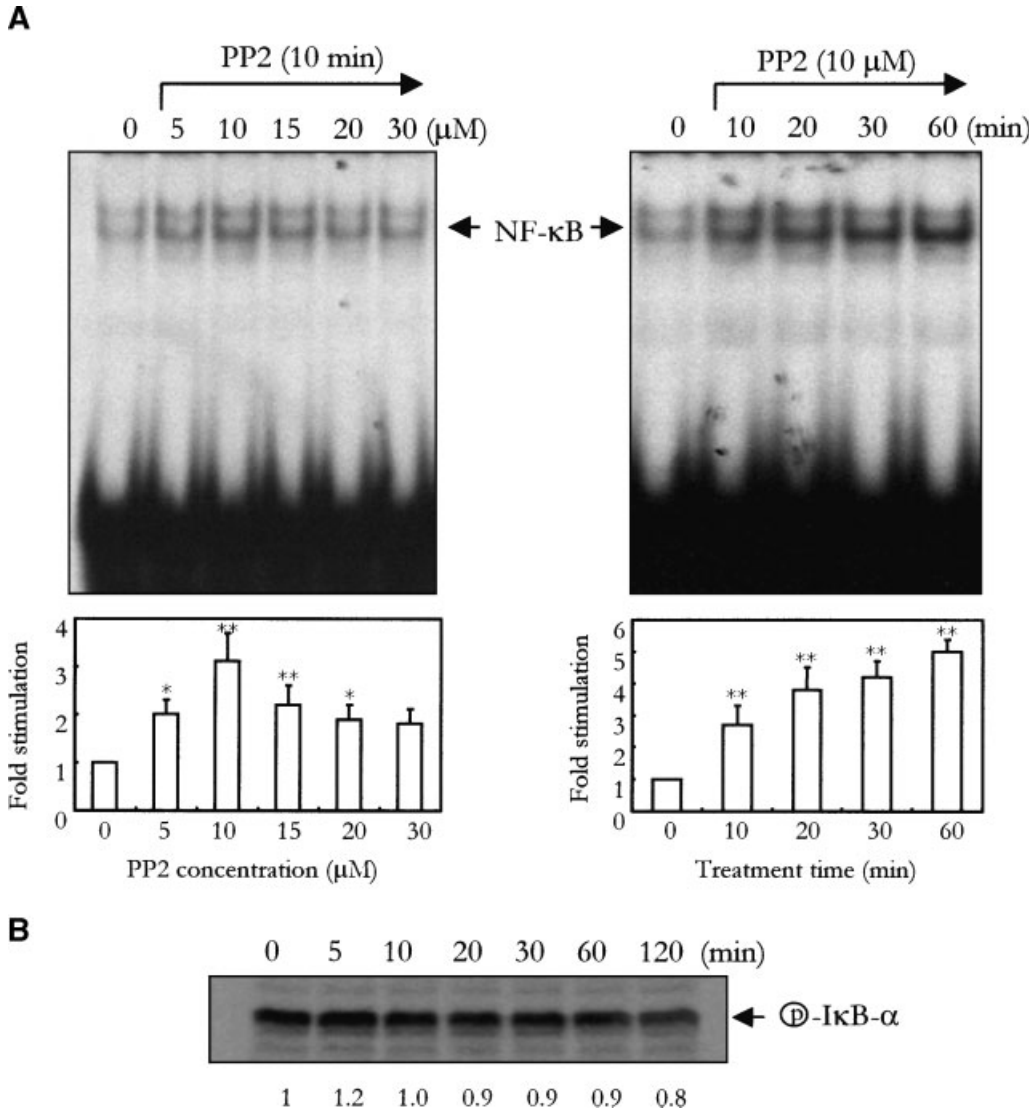

Fig. 4. NF-κB activation is involved in PP2-induced cell death. **A**: 70Z/3 cells were treated either with PP2 at the indicated concentrations for 10 min, or treated with 10 μM PP2 for the indicated time. Nuclear extracts were prepared, incubated with a ^{32}P-labeled κB probe, and analyzed by EMSA. Identity of the shifted bands was confirmed by competition with unlabeled oligomer containing NF-κB site. The **upper panel** presented is representative of three independent experiments. The **lower panel** represents data quantitated by densitometry, expressed as fold stimulation. The values are the mean of three separate experiments with error bar representing the standard deviations (*$P < 0.05$ and **$P < 0.01$). **B**: 70Z/3 pre-B cells were treated with 10 μM PP2 for the indicated time, and the whole cell lysates were analyzed by Western blot. To detect PP2-induced IκB phosphorylation, it was used a polyclonal phospho-specific anti-IκB-α antibody which detects IκB-α only when activated by phosphorylation at Ser-32. Numbers listed below each band indicate value quantified by densitometry of immunoblots, expressed as fold change in phosphorylation, in which the phosphorylation observed in unstimulated cells was defined as 1. Data presented is representative of multiple experiments with similar findings.

apoptosis [Johnson et al., 2000]. In the present studies, we demonstrated that PP2, a potent and selective inhibitor of Src-family tyrosine kinase, induced apoptosis of 70Z/3 cells through caspase cascade. After treatment of PP2, the procaspase-3, a family of cysteine proteases (caspase), which is recently reported to be mediators of apoptotic cell death pathway [Cohen, 1997], was activated through proteolytic cleavage. More interestingly, NF-κB activation seems to be attributable to PP2-induced apoptosis, contrary to the report [Mann et al., 2001] suggesting that NF-κB is a survival factor at an earlier stage in B cell development. Especially, PP2 was found to be able to enhance the DNA binding activity of NF-κB complexes without accompanying by increased phosphorylation of IκB-α.

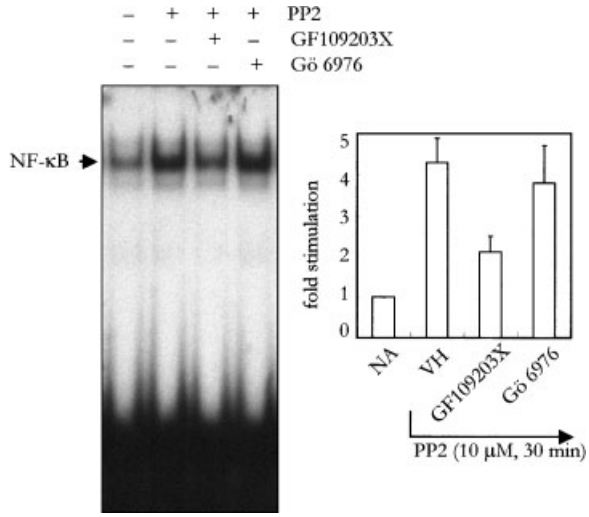

Fig. 5. Novel PKC isoforms are required for NF-κB activation. 70Z/3 cells were pretreated with either GF 109203X (1 μM), or Gö 6976 (1 μM) for 2 h before treatment with 10 μM PP2 for 30 min. EMSA was performed on nuclear extracts. The **right panel** represents data quantitated by densitometry, expressed as fold stimulation. The values are the mean of three separate experiments. The **left panel** presented is representative of three independent experiments. NA, naïve; VH, vehicle.

Treatment of cells with GF 109203X, which inhibits the conventional and novel isoforms, inhibited PP2-induced NF-κB activation, while no apparent inhibition was observed with a highly selective cell-permeable conventional PKC inhibitor, Gö 6976. This suggests that novel PKCs may contribute to activation NF-κB by PP2 in 70Z/3 cells. This finding is consistent with the reported results showing a critical role of novel PKCs for NF-κB activation in B cells [Krappmann et al., 2001]. PKC is a multimember family of serine/threonine kinases that has traditionally been associated with signal transduction regulating cell growth and differentiation. However, accumulating evidence also points to its negative regulation of cell growth and the initiation of apoptosis [Dempsey et al., 2000]. The hallmark of PKC activation in cells is its translocation to the plasma membrane. Our quantitative immunoblot analysis demonstrated the sustained translocation of novel PKCs such as PKC-δ and -ε to membrane after treatment of PP2. Conversely, the translocation of PKC-α and -βI by PP2 was transient and returned to cytosol within 20–30 min. The returning of PKC to the cytoplasm after its activation is delicately regulated [Feng and Hannun, 1998]. Failure of this regulation may result in "permanently" membrane-associated proteins with subsequent alterations in cellular signaling and functions [Dembinsky et al., 1997]. Now, it is possible that PP2 may serve to activate novel PKCs more strongly compared to other PKC isoforms, allowing for a temporary alteration in balance of each PKC isoforms. However, despite all available data on PKC translocation, our knowledge of how PP2 recruit PKC to plasma membrane is far from conclusive. Notably, Shanmugam et al. [1998] showed that tyrosine-phosphorylated PKC-δ was localized exclusively to cytosolic fractions of PMA-treated cells implying dissociation between tyrosine-phosphorylated PKC-δ (in cytosolic fraction) and catalytically active PKC-δ (in membrane fraction). Thus, PP2 is likely to localize dephosphoryleated PKC to plasma membrane by inhibiting Src tyrosine kinase. On the other hand, initial studies on PKC identified three classical activators (phosphatidylserine, Ca^{2+}, and diacylglycerol) and membrane binding modules (C1 and C2 domains) of PKC. In our study, Ca^{2+}-independent PKC isotypes, PKC-δ and -ε was translocated to plasma membrane while phosphatidylserine-dependent, diacylglycerol-independent PKC-ζ was not. Therefore, DAG is likely to be involved in recruiting PKC to plasma membrane in response to PP2. Other candidates are anchoring proteins that contribute to an elaborate level of intracellular organization and compartmentalization of signaling molecules. These include RACKs (receptors for activated C kinase), AKAP (a kinase anchoring proteins), and CG-NAP (centrosome and Golgi localized PKN-associated protein) [Newton, 2001]. Among these anchoring proteins, CG-NAP has been shown to localize unphosphorylated PKC-ε to the golgi membrane [Takahashi et al., 2000].

In particular, PKC-δ is emerging as a common intermediate in the apoptotic pathway induced by chemicals and irradiation [Dembinsky et al., 1997; Dempsey et al., 2000]. The studies from Reyland et al. [1999] indicated that PKC-δ activity is required for apoptosis at a point upstream of caspase activation. Moreover, PKC-δ is the isoform that undergoes phosphorylation by Src tyrosine kinase [Denning et al., 1996]. Isozymes other than PKC-δ showed little tendency to become phosphorylated on tyrosine. Although the role of tyrosine phosphorylation for PKC remains controversial, the tyrosine-phosphorylated PKC-δ was found to have

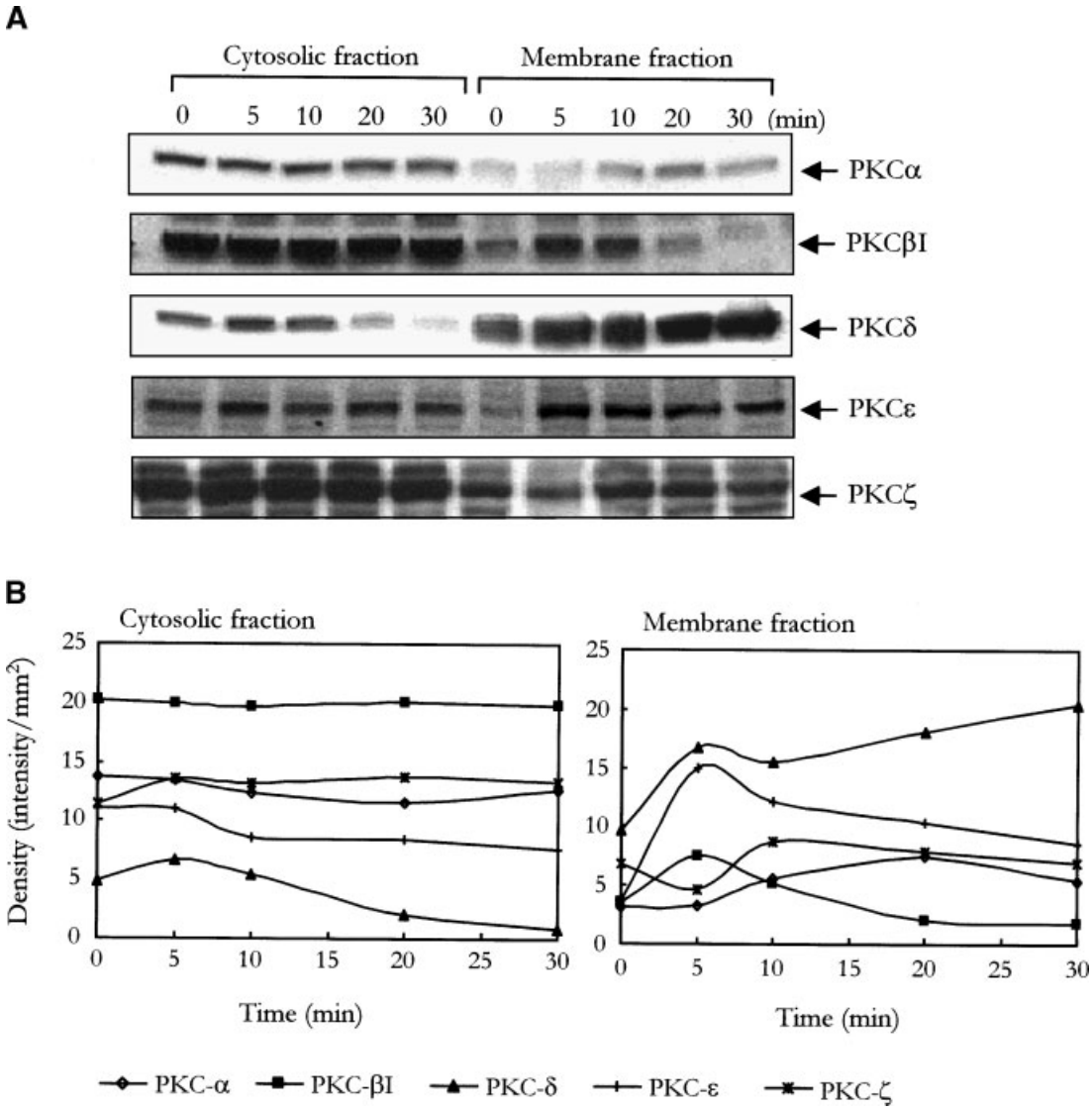

Fig. 6. Analysis of the subcellular distribution of the PKC subtypes by cell fractionation and immunoblotting. 70Z/3 cells were treated for the indicated times with 10 μM PP2. Cell lysates were fractionated by centrifugation into particulate and cytosolic fractions. 10 μg of protein/lane was applied to SDS-PAGE, and the PKC isotypes in each fraction were detected by immunoblotting. The upper panel presented (**A**) is representative of three independent experiments with similar findings. The lower panel (**B**) represents data quantitated by densitometry, expressed as intensity/mm^2.

reduced enzymatic activity relative to the non-tyrosine-phosphorylated PKC-δ [Denning et al., 1993, 1996]. In contrast to these studies, upon treatment of PKCδ-overexpressing cells with PDGF or TPA, tyrosine-phosphorylated PKCδ was found to be localized in the membrane fraction and its activity increased [Li et al., 1994]. This discrepancy can be explained by the fact that PKC activity is elevated or reduced by tyrosine phosphorylation depending on the substrate [Gschwendt et al., 1994]. Since Src tyrosine kinase correlates well with mitogenic signals, the data presented here in PP2-treated cells demonstrate that Src can down-regulate PKC-δ that has been strongly associated with inhibiting cell growth. That is, the ability to down-regulate the inhibitory PKC isoform such as PKC-δ may be important for the transforming ability of v-Src.

In addition to PKC-δ, the sustained membrane association of PKC-ε in PP2-treated cells was surprising considering previous reports that overexpression of PKC-δ inhibits cell proliferation and that overexpression of PKC-ε enhances cell growth [Mischak et al., 1993]. However, recent reports demonstrated that

the activation of PKC-ε mediates the apoptotic effects of TNF-α on intestinal epithelial cell injury [Chang and Tepperman, 2001]. On the other hand, we cannot exclude the possibility that membrane association of PKC-ε in PP2-treated cells may not correlate with an activation of its kinase activity, although it has been known that the biological activity of PKCs is closely regulated by their subcellular localization [Nishizuka, 1992].

Activation-induced cell death (AICD), a form of apoptosis, is the major mechanism by which immune cell homeostasis is maintained [Russell, 1995]. When cells were treated with PP2, we observed the activation of nuclear NF-κB DNA binding complexes similar to those seen with mitogenic stimulation. However, in the presence of PP2, cells experience growth arrest and subsequently undergo apoptosis, hence giving NF-κB a proapoptotic role in AICD. Lin et al. [1999] reported the paradoxical role of NF-κB in apoptosis, functioning as both a proapoptotic and antiapoptotic regulatory factor within a single cell type [Lin et al., 1999]. The mechanism that we suggest was summarized in Figure 7. According to this model, the inhibition of Src tyrosine kinase by PP2 results in activation of PKC-δ, which in turn, activates NF-κB transcription factor. Then, NF-κB is able to activate the pro-apoptotic pathway including caspases. In addition, cleavage and activation of PKC-δ by caspase may serve to amplify specific events in the apoptotic pathway [Dempsey et al., 2000].

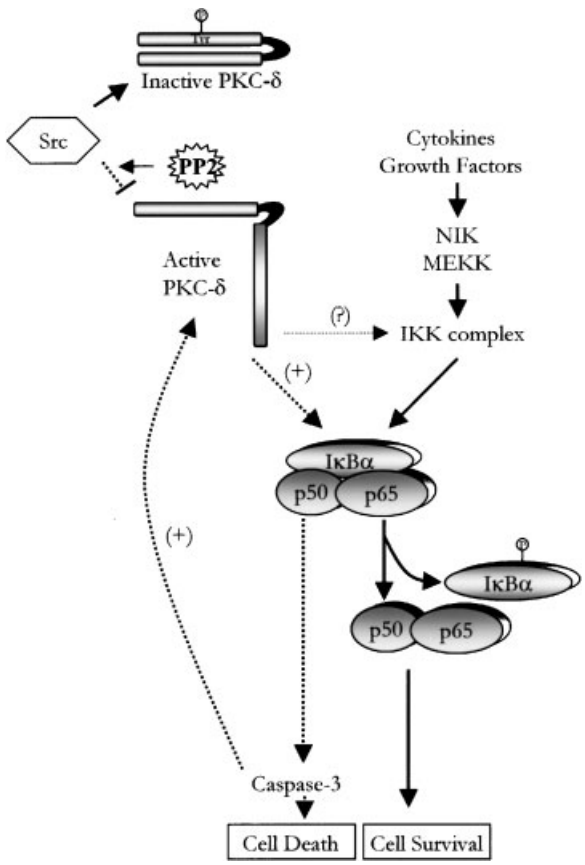

Fig. 7. Potential strategies for activating NF-κB activity as an activator for PP2-induced apoptosis. In addition to acting as a survival factor in response to growth factors, NF-κB can convert anti-apoptotic signal into pro-apoptotic signal through altering the cellular context by repressing or activating other proteins in the nucleus with which it interacts. The inhibition of Src tyrosine kinase by PP2 results in activation of PKC-δ, which in turn, activates NF-κB transcription factor. Then, NF-κB is able to activate the pro-apoptotic pathway including caspases. In addition, cleavage and activation of PKC-δ by caspase may serve to amplify specific events in the apoptotic pathway. Potential mechanisms of PP2-induced NF-κB activation are expressed as a dotted line.

In summary, our results lead to the conclusion that the inhibition of Src-mediated tyrosine phosphorylation by PP2 may tilt the balance between each PKC isotypes and activate NF-κB, leading to apoptosis. Further studies will need to address the mechanism by which PKC signaling is linking NF-κB activation to apoptotic pathway.

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