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Cellular Toxic Effects and Action Mechanisms Of 2,2',4,6,6'-Pentachlorobiphenyl

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서 판 길

(포항공과대학교)

Cellular Toxic Effects and Action Mechanisms Of 2,2',4,6,6'-Pentachlorobiphenyl

Sun-Hee Kim[1], Kum-Joo Shin[2], Dohan Kim[1], Yun-Hee Kim,

Sung Ho Ryu[1] and Pann-Ghill Suh[1]

[1]Department of Life Science, Division of Molecular and Life Science, and School of Environmental Science and Engineering, Pohang University of Science and Technology, Pohang 790-784, Kyungbuk, Republic of Korea,

[2]Division of Biology, California Institute of Technology, Pasadena, CA91125

Abstract

Polychlorinated biphenyls (PCBs), one a group of persistent and widespread environmental pollutants, have been considered to be involved in immunotoxicity, carcinogenesis, and apoptosis. However, the toxic effects and physical properties of a PCB congener are dependent on the structure. In the present study, we investigate the toxic effects and action mechanisms of PCBs in cells. Among the various congeners tested, 2,2',4,6,6'-PeCB-pentachlorobiphenyl (PeCB), a highly *ortho*-substituted congener having negligible binding affinity for aryl hydrocarbon receptor (AhR), caused the most potent toxicity and specific effects in several cell types. 2,2',4,6,6'-PeCB induced apoptotic cell death of human monocytic cells, suggesting that PCB-induced apoptosis may be linked to immunotoxicity. In addition, 2,2',4,6,6'-PeCB induced mitotic arrest by interfering with mitotic spindle assembly in NIH3T3 fibroblasts, followed by genetic instability which triggers p53 activation. Which suggests that 2,2',4,6,6'-PeCB may be involved in cancer development by causing genetic instability through mitotic spindle damage. On the other hand, 2,2',4,6,6'-PeCB increased cyclooxygenase-2 (COX-2) involved in cell survival through ERK1/2 MAPK and p53 in Rat-1 fibroblasts and mouse embryonic fibroblasts, triggering compensatory mechanism for abating its toxicity.

Taken together, these results demonstrate that PCB congeners of different structure have distinct mechanism of action and 2,2',4,6,6'-PeCB causes several toxicity as well as compensatory mechanism in cells.

Introduction

Polyclorinated biphenyls (PCBs), a group of halogenated aromatic hydrocarbons (HAHs), are widely spread environmental contaminants. The high lipophilicity and chemical stability of PCBs have further resulted in widespread environmental contamination, and there is significant evidence of PCBs accumulation in biota (1). Toxic effects of PCBs range from carcinogenesis and immunotoxicity to disruption of nerve, endocrine, and reproductive system. PCBs can form 209 possible congers with respect to the number and position of chlorine atoms on the biphenyl ring (Fig. 1). The toxic effects and physical properties of a PCB congener are dependent on the structure. There are numerous PCB congeners and different congeners seem to have distinct mechanisms of action. The planar congeners without chlorine(s) substituted at *ortho*-position have relatively high affinity for aryl hydrocarbon receptor, the endogenous receptor for dioxins, and thus, considered to exhibit toxic effects through the receptor. However, the other congeners with *ortho*-substituted chlorine(s) have negligible binding affinity for the receptor; therefore, a separate mechanism may be involved in their toxic effects (2). The latter recently has been paid attention to, and studied in diverse aspects.

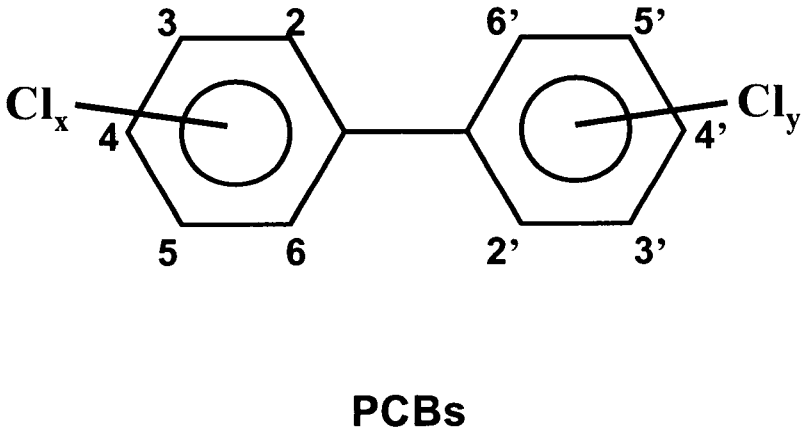

Figure 1. Structures of PCBs.

Materials and methods

Materials

PCBs (>99% pure) were purchased from AccuStandard (New Haven, CT). FuGENE^{TM} transfection reagent was from Roche Molecular Biochemicals. Luciferase activity assay reagent was purchased from Promega (Madison, WI). Propidium iodide was from Molecular Probes, Inc (Eugene, OR).

NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide) and PD98059 were from Calbiochem (La Jolla, Ca). COX-2 polyclonal antibody was from Cayman Co. (Ann Arbor, MI). Etoposide and other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture

U937 cells were maintained in RPMI supplemented with 10% fetal bovine serum and NIH3T3 and Rat-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified, 5% CO_2-controlled incubator.

Plasmids

p53-Luc- and AP-1-Luc plasmid was obtained from Stratagene (La Jolla, CA). *COX-2* promoter-luciferase wt and deletion constructs were kindly provided by Dr. Herschman (UCLA-Los Angeles Center for the Health Sciences, Los Angeles, CA). A c-Jun dominant negative expression vector was a gift of Dr. Tom Curran (St. Jude Children's Research Hospital, Memphis, TN).

Transient transfection

Cells were transfected with plasmid DNA containing luciferase reporter gene, standard plasmid DNA containing *renilla* luciferase gene using FuGENE^{TM} transfection reagent according to a procedure recommended by the manufacturer.

Luciferase reporter gene assay.

The cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.8, 1% Triton X-100, 150 mM NaCl, 2 mM DTT). The cell lysate was mixed with luciferase activity assay reagent and luminescence produced for 5 seconds was measured using luminoskan (labsystems). Data are presented as the fold of relative light unit relative to vehicle-treated control cultures.

Western blot analysis.

Whole cell lysate were prepared in lysis buffer (20 mM Tri-Hal, pH 7.4, 150 mM NaCl, 20 mM NaF, I mMEDTA, 1 mM PMSF). Proteins were separated on a denaturing 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Specific antibodies were incubated with the membranes overnight at 4 C. Secondary antibody linked to horseradish peroxidase was used at 1:10,000, and signals were visualized by the ECL technique.

RT-PCR

cDNA was reverse-transcribed from 5 μg total cellular RNA using oligo (dT) primers and murine leukemia virus reverse transcriptase. cDNA was amplified for 35cycles using the gene-specific primers. The cycling parameters were the following: 1 min at 94 °C for denaturation, 1min at 60 °C for primer annealing, and 1 min at 72 °C for polymerization. The products were visualized after electrophoresis on a 2% agarose gel containing ethidium bromide.

Cyclooxygenase Activity

Rat-1 cells were plated in 24-well plates. 1 day later, the medium was replaced with serum free DMEM for 1 day, the cells were then incubated with or without PCBs for 6 h. Media were collected from each well and analyzed for PGE_2 by enzyme-linked immunoassay as described previously.

Trypan blue exclusion and MTT assay

The cells were then harvested and resuspended in medium, and an equal volume of trypan blue was added. More than 200 cells were scored on a hemocytometer. The percentage of cell survival was calculated by taking the number of trypan blue-excluding cells following treatment and dividing it by the number of DMSO-treated control cells and multiplying by 100.

To determine cell viability, 1×10^4 cells/well was subcultured in a 96-well plate. After treatment with chemical in serum-free DMEM for the indicated times, the cells were incubated for 2 h in the presence of 0.5 mg/ml MTT reagent in a 37℃ incubator. After removal of MTT reagent and disruption of cells with DMSO, the absorbance was measured at 570 nm using a 96-well plate reader. Data are presented as the percentage of viability relative to vehicle-treated control cultures.

Immunocytochemistry and nuclear staining.

Cells treated with chemicals were washed with PBS and fixed with 4% paraformaldehyde for 30 min at RT. After incubation with 100 μg/ml RNase A and blocking with PBS containing 1% horse serum and 0.2% Triton X-100 for 30 min at RT, cells were incubated with anti-β-tubulin antibody (Sigma) for 2 hr at RT. Subsequently, cells were incubated with fluorecein isothiocyanate-labeled goat anti-mouse secondary antibody for 1 hr at RT and then, with 2.5 μg/ml propidium iodide for 10 min to visualize tubulin and nuclei, respectively.

Cell cycle analysis

Asynchronous cells were treated with vehicle, 10 ❍M 2,2',4,6,6'- or 3,3',4,4',5-PeCB for the indicated times in serum-free medium, harvested and washed with PBS/5 mM EDTA twice. Approximately 1 x 10^6 cells were resuspended with PBS and equal volume of ethanol was added with vortexing. After fixation for 30 min, followed by incubation with 40 ❍g/ml RNase for 30 min at RT, cells were stained with 50 ❍g/ml propidium iodide. DNA content was determined using a FACScan flow cytometer.

Cdc2 kinase assay

Cells treated with PeCBs for the indicated times were harvested and sonicated in lysis buffer (40 mM Tris, pH 7.5, 120 mM NaCl, 0.1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 10 mM NaF). Cell lysate 200 μg was incubated with anti-cyclin B1 antibody for 2 hr at 4 °C, followed by incubation with protein G-Sepharose beads for another 2 hr. After washing three times with lysis buffer and twice with reaction buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$), the beads were incubated with 2❍g histone H1 and 2 μCi [γ-^{32}P] ATP at 37 °C for 30 min. The reaction was stopped by addition of 5x SDS sample buffer and the samples were applied to SDS-PAGE followed by autoradiography.

Results and discussion

1. 2,2′,4,6,6′-Pentachlorobiphenyl Induces Apoptosis in Human Monocytic Cells

The evidence obtained from rodents, mammals, and even exposed humans indicates that the immune system may be a very sensitive target for PCBs (3,4,5). The monocyte/macrophage are acting as first line of defense against infection and, thus, damage on the cells may result in serious immunotoxicity. Exposure to PCBs has often been associated with nonspecific immune events such as change of nuetrophil counts and impairment of monocyte/macrophage phagocytosis (6). Apoptosis ensure the homeostasis of tissues during development as well as defense against pathogens (7). However, a dysregulation of apoptosis has been observed in auto immune diseases, acquired immune deficiency syndrome, and other viral and bacterial infections, as well as in neurodegenerative disorders and cancers (8,9). Therefore, in this study we investigated the toxic effect of PCBs on human monocytic U937 cells in the aspect of apoptosis. 15 PCB congers differing in the numbers or positions of chorine substitutions were used in this study. One of them, 2,2',4,6,6'-PeCB specifically induced DNA fragmentation, the hallmark of apoptosis in the U937 cells (Fig. 2A). The change in cell viability caused by 2,2',4,6,6'-PeCB was examined by MTT assay. Viability did not change upon treatment with up to 5 μM of the PCB, but it decreased rapidly of 10-20 μM concentrations (Fig. 2B). In order to further clarify that 2,2',4,6,6'-PeCB-induced cell death was apoptosis, we examined apoptotic features. We found the characteristic morphological markers that identify apoptosis, some of them were observed upon nuclear staining. Chromatin aggregation and apoptotic bodies were observed in the nuclei of the cells treated with 2,2',4,6,6'-PeCB (Fig. 3A). In addition, treatment of 2,2',4,6,6'-PeCB activated caspase-3, pivotal effecter in apoptosis (Fig. 3B).

Overall, we are convinced that 2,2',4,6,6'-PeCB induces apoptotic cell death of human monocytic cells by a mechanism independent of the AhR receptor. This suggests that PCB-induced apoptosis may be linked to immunotoxicity. In a further study, we intend to pursue our studies aimed at the understanding of the characteristics and action mechanisms underlying the toxic effects of human exposure to PCBs.

A. 2,2',4,6,6'-Pentachlorobiphenyl Induces Mitotic Arrest and p53 Activation

PCBs have been considered to be involved in cancers (10,11), but the underlying mechanisms are not known well. Various cancers are closely related to genetic alteration (12), therefore, we investigated the effect of PCBs on genetic stability, through p53, a guardian of genome, in NIH 3T3 fibroblasts. Among several congeners examined, 2,2',4,6,6'- PeCB specifically activated p53-dependent transcription(Fig. 4A). In addition, p53 was stabilized by treatment of 2,2',4,6,6'- PeCB and then translocated to the nucleus (Fig. 4B).

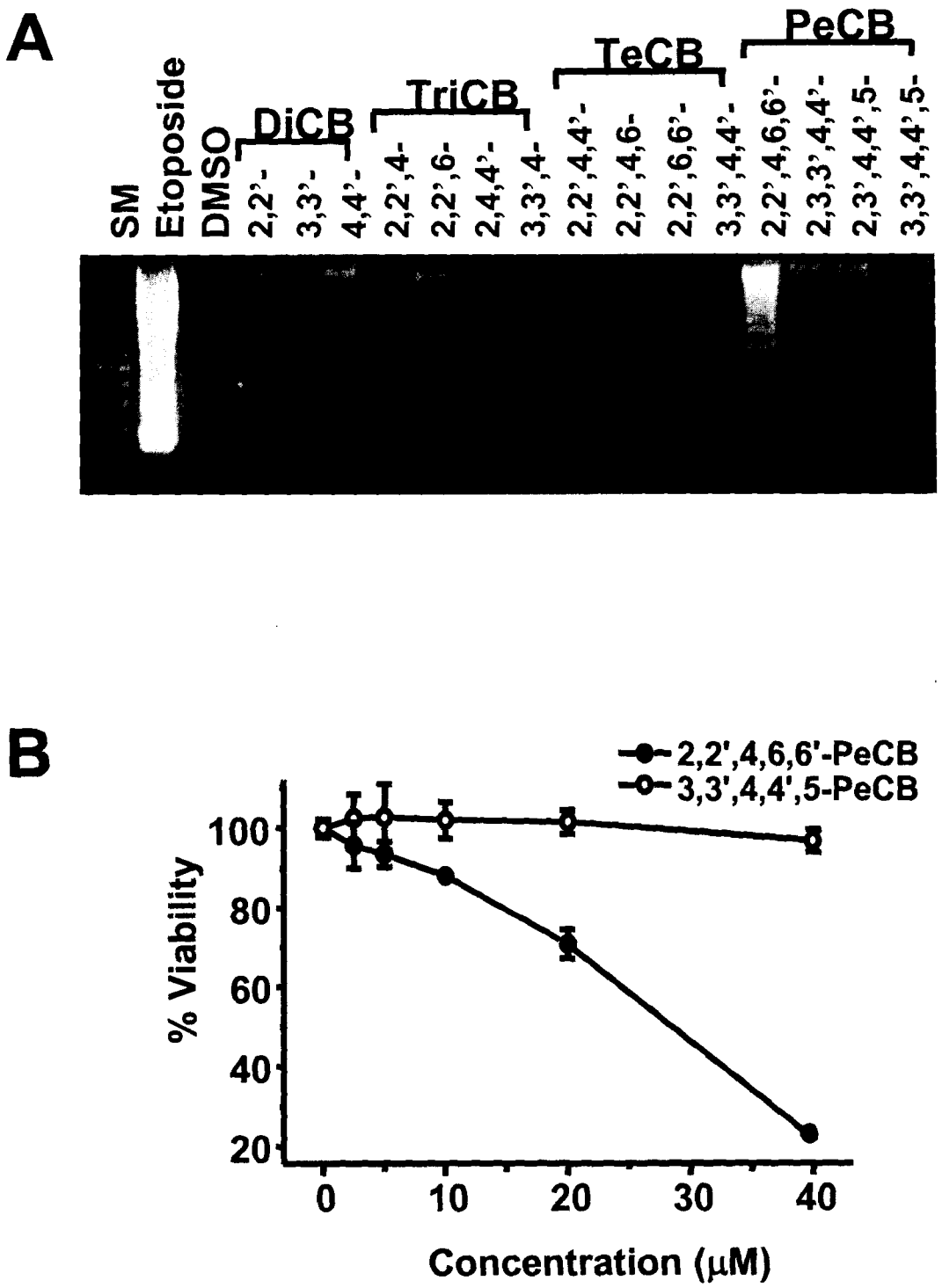

Figure 2. 2,2',4,6,6'-PeCB induces DNA fragmentation and cell death in U937 cells.
A) DNA fragmentation in cells treated with 20 μM concentration of 15 kinds of congeners. B) Dose-dependent decrease in cell viability upon 2,2',4,6,6'-PeCB treatment determined by trypan blue exclusion assay. DiCB: dichlorobiphenyl, TriCB: trichlorobiphenyl, TeCB: tetrachlorobiphenyl, PeCB: pentachlorobiphenyl.

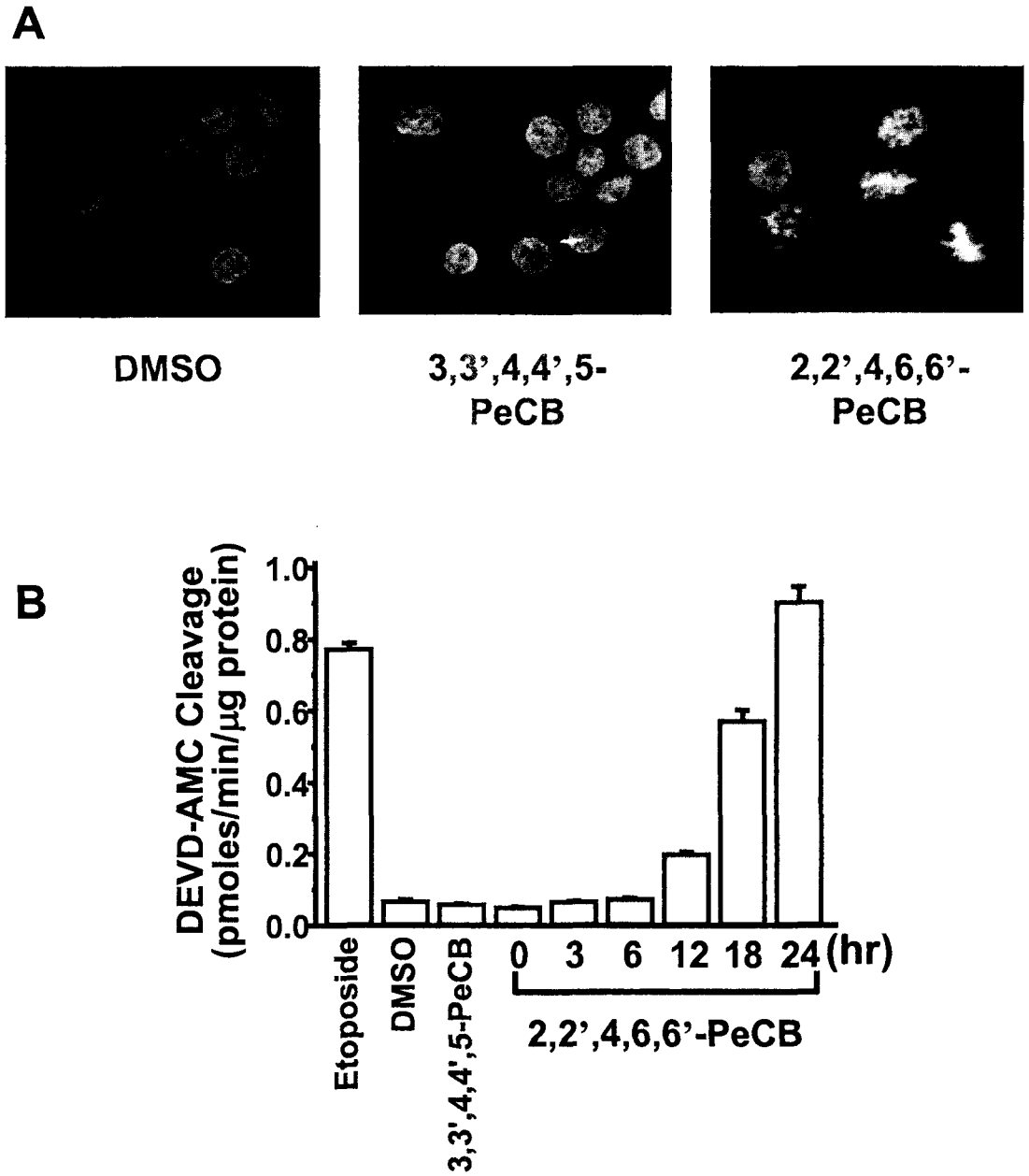

Figure 3. 2,2',4,6,6'-PeCB induces apoptotic body and activation of caspase-3.

A) The nuclei of U937 cells treated with PeCBs were stained with DAPI and observed using microscope. B) After cells were treated with PeCBs, caspase-3 activity was assayed using DEVD-AMC as a substrate.

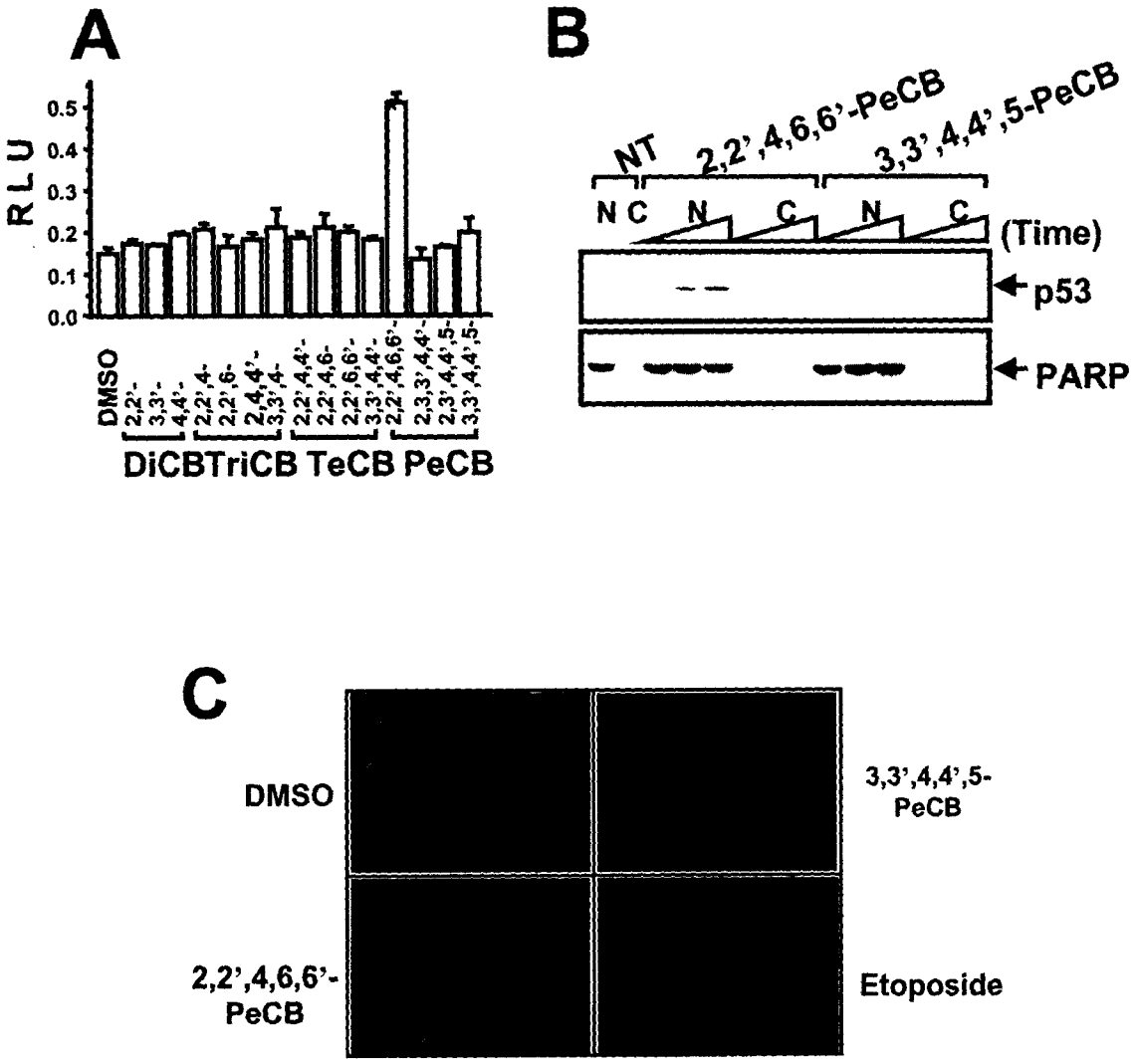

Figure 4. 2,2',4,6,6'-PeCB activates p53.

A) NIH 3T3 cells were stably transfected with luciferase reporter gene under control of p53. Transfected cells were treated with PeCBs for 12 h in serum-free medium. Luciferase activity in cell lysate was assayed as described in "Materials and Methods". RLU: relative light unit. B) NIH 3T3 cells were treated with PCBs. After preparation of cytoplasmic and nuclear extracts, p53 and PARP (a nuclear marker) were detected. N: nucleus, C: cytoplasm, NT: not treated. C) After cells were embedded in low melting point agarose, applied to gel electrophoresis, and stained with 2.5 μg/ml propidium iodide (15), cells containing DNA strand breakage were observed under confocal microscope.

DNA damage is a well-known p53-activating signal and there are several reports that PCBs might induce DNA damage (13,14), even though there is no conclusive evidence. Therefore, we addressed if 2,2',4,6,6'- PeCB can induce DNA damage using alkaline single cell gel ecectrophoresis assay, the so-called comet assay (15). Figure 4C shows that 2,2',4,6,6'- PeCB did not induce statistically significant DNA damage in comparison with DMSO, suggesting that p53 is activated by 2,2',4,6,6'-PeCB with little relevance to DNA damage.

One of the important roles of p53 is to prevent damaged cells from progressing the cell cycle (13). The result showed that the population of cells with a 4N DNA content started to increase after 3 h of treatment with 2,2',4,6,6'- PeCB (Fig. 5A). The onset of mitosis is triggered by dephospholrylation of Tyr^{15} residue of Cdc2 by phosphatase Cdc 25 and Cdc2 bound to cyclin B1 phosphorylates many proteins necessary to mitosis (16). As results, the level of phosphorylation at Tyr^{15} decreased slightly after 6 h of 2,2',4,6,6'-PeCB treatment and dramatically after 12 h (Fig. 5B). Also, Cdc2 kinase activity rose afterr 3 h and reached maximum after 12 h of 2,2',4,6,6'-PeCB treatment (Fig. 5C). proteins specifically phosphorylated at the entry into mitosis were detected using MPM2 monoclonal antibody that recognizes phosphoamino acid epitopes of M phase marker proteins (17). Figure 5D shows that phosphorylation of the proteins increase after 3 h of treatment with 2,2',4,6,6'-PeCB, suggesting 2,2',4,6,6'-PeCB induces mitotic arrest.

Mitosis is a process for equal segregation of genome to two daughter cells, and the failure of even a single chromosome to align on the mitotic spindle is sufficient to induce mitotic arrest (18). Mitotic spindle and chromosomes were visualized by staining with anti-tubulin antibody and propidium iodide, respectively. All the 2,2',4,6,6'-PeCB-treated mitotic cells had abnormal mitotic spindle and randomly distribute chromosomes (Fig. 6A).

2,2',4,6,6'-PeCB activated p53 and induced mitotic arrest producing damaged spindle. We wondered how p53 activation is related to mitotic arrest, therefore, we investigated the effect of 2,2',4,6,6'-PeCB on cell cycle progression in p53-deficient mouse embryonic fibroblasts (MEFs). As a result, 2,2',4,6,6'-PeCB increased the population of cells with a 4N DNA content in p53-/-MEF as well as wild type cells. Furthermore, cells with an 8N DNA content are the other main population in 2,2',4,6,6'-PeCB-treated p53-/-(Fig. 6B). On the other hand, cells lacking functional p53 underwent multiple rounds of DNA synthesis at S phase without undergoing cytokinesis, forming polyploidy. In wild MEFs treated with 2,2',4,6,6'-PeCB, a small subpopulation of cells having a 8N DNA content was observed (Fig. 6B), which would be predicted as cycling tetraploid cells.

In summary, 2,2',4,6,6'-PeCB, an *ortho*-substituted PCB congener, activated p53 through mitotic spindle damage during mitosis and caused polyploidy in cells deficient in functional p53; therefore, it might be related to cancer development in tumor cells that lack functional p53, through genetic instability caused by mitotic spindle damage.

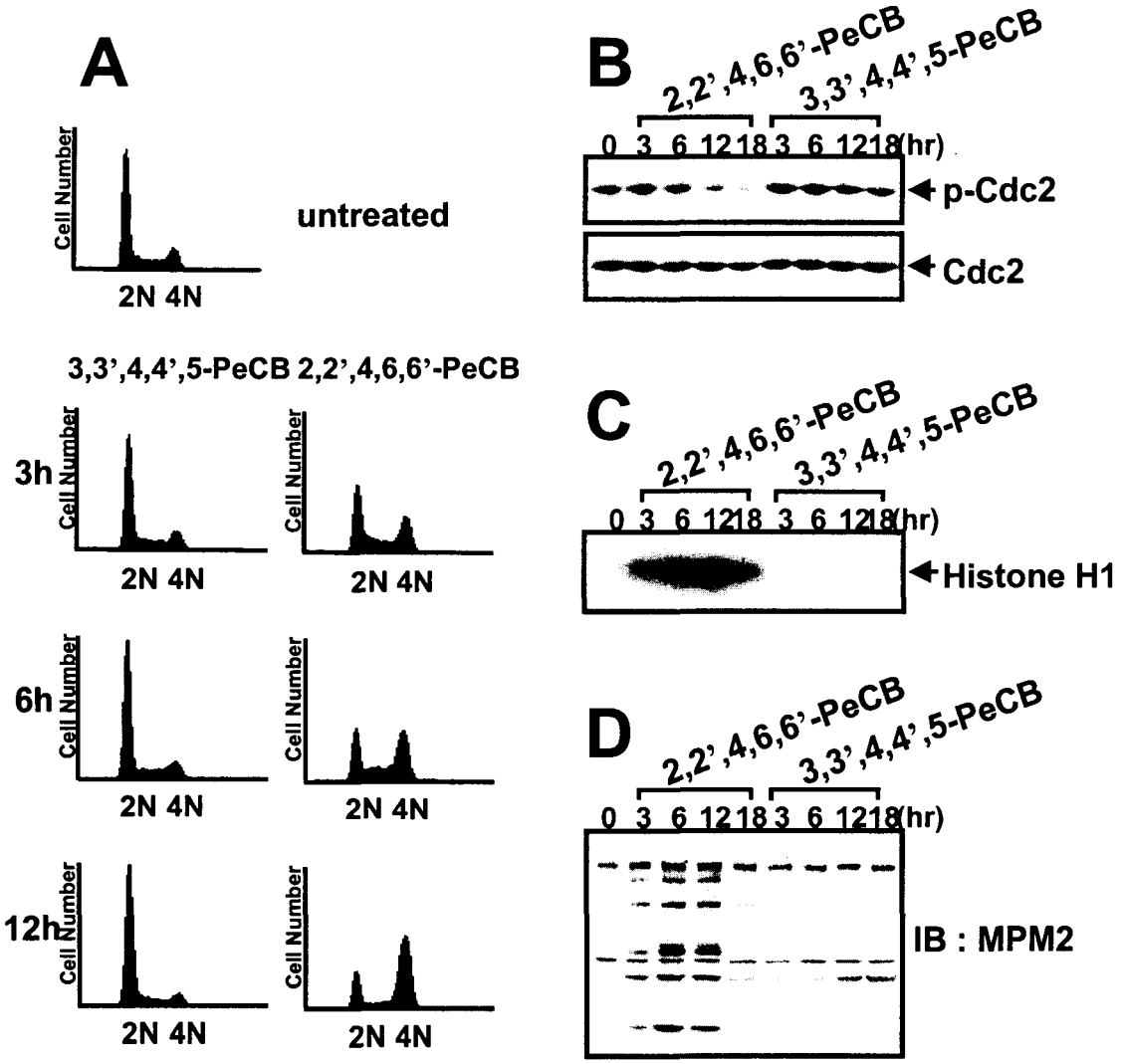

Figure 5. 2,2',4,6,6'-PeCB induces mitotic arrest.

A) Asynchronous NIH 3T3 cells were treated with 10 μM PeCBs for the indicated times. After cells were harvested, fixed and stained with 50 μg/ml propidium iodide, DNA content was analyzed. B) phospho-Tyr15Cdc2, Cdc2 and D) M phase specific phospho-proteins were detected using respective antibodies, and C) Cdc2 kinase activity was assayed using histone H1 as a substrate *in vitro*.

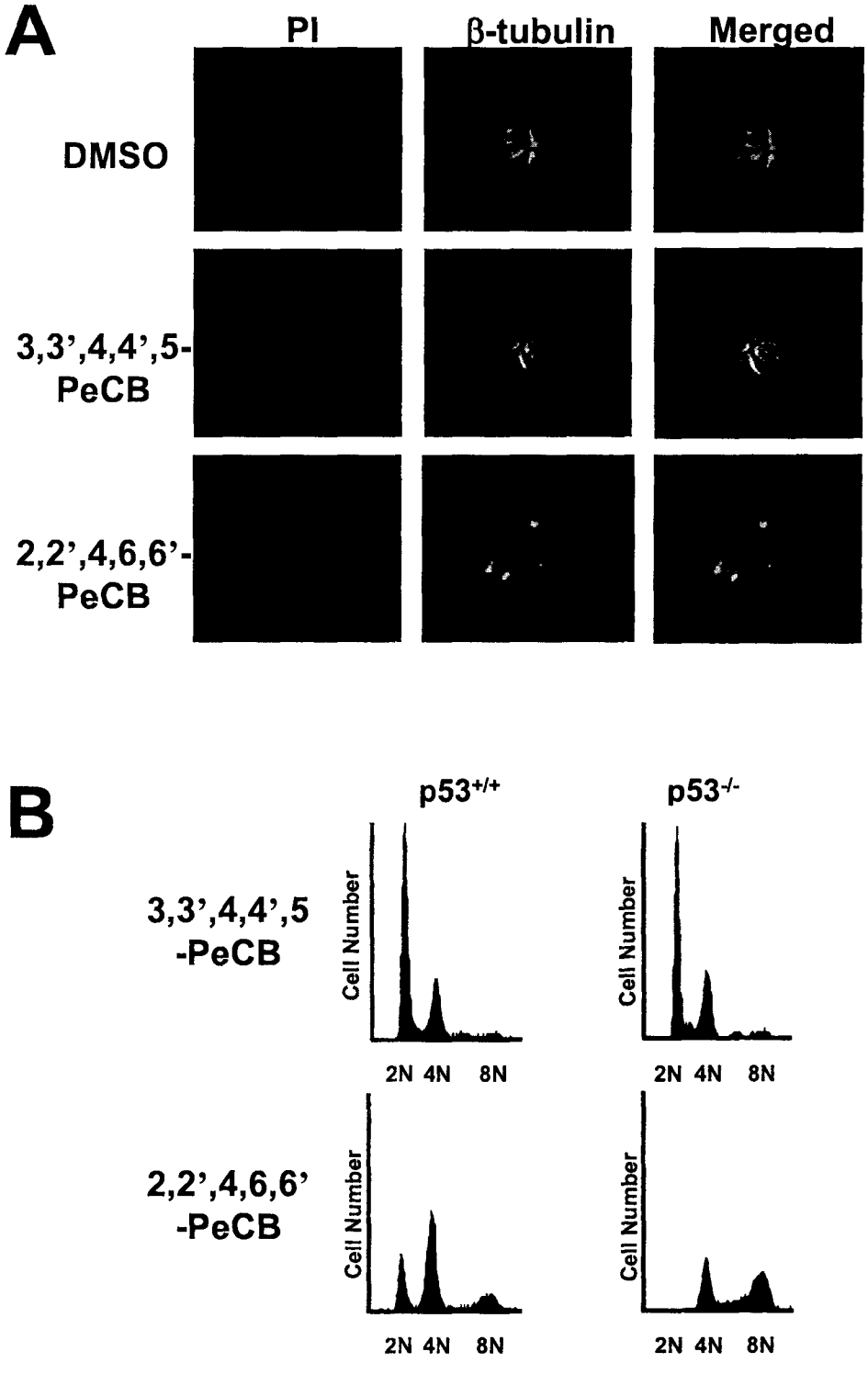

Figure 6. 2,2',4,6,6'-PeCB causes mitotic spindle damage and tetraploidy in p53-/- cells..

A) NIH 3T3 cells were treated with PeCBs for 12 h. Nuclei were stained with PI and mitotic spindles were detected by immunofluorescence using an anti-β-tubulin antibody. B) Wild type and p53-/- MEFs were treated with PCB for 12 h. After cells were harvested, fixed and stained with 50 μg/ml propidium iodide, DNA content was analyzed.

A. Apoptosis by 2,2',4,6,6'-Pentachlorobiphenyl Is Limited by Induction of Cyclooxygenase-2

Cycloogygenase-2 (COX-2), the product of a related inducible gene, is absent in most normal tissues but is expressed rapidly in response to proliferative and inflammatory stimuli such as growth factors, cytokines, and tumor promoters (19,20). It is also well documented that COX-2 is linked to cell survival (21,22). In this present study, we investigated the effect of PCBs on COX-2 expression using COX-2 promoter/luciferase assay. Among the congeners examined, 2,2',4,6,6'-PeCB specifically caused COX-2 promoter activity (Fig. 7A). Also, treatment of Rat-1 cells with 2,2',4,6,6'-PeCB increased the level of mRNA and protein of COX-2 and thereby enhanced prostaglandin E_2 (PGE_2) (Fig. 7B, C and D).

Mitogen-activated protein kinase pathways mediate the regulation of COX-2 expression to a variety of extracellular stimuli (23,24). Fig. 8A and B demonstrates that 2,2',4,6,6'-PeCB phosphorylated ERK1/2 MAPK in a time-dependent manner and treatment with PD98059, a specific inhibitor of MAPK kinase, decreased the induction of COX-2 . Recent reports have suggested that p53 can induce sustained activation of the Ras/Raf/ERK cascade. Such activation induces COX-2 expression, which counteracts p53- or genotoxic stress-induced apoptosis 25,26). Also, 2,2',4,6,6'-PeCB activates p53 through the disruption of microtubule in NIH 3T3 (Fig. 4 and 6). Therefore, we investigated whether COX-2 is induced in response to p53 in Rat-1 cells. As shown in Fig. 8C, 2,2',4,6,6'-PeCB caused the increase of p53-depentent transcription. Moreover, treatment of p53+/+mouse embryonic fibroblasts (MEFs) with 2,2',4,6,6'-PeCB increased the level of COX-2 protein. In contrast, in p53-/- MEFs, no detectable upregulation of COX-2 expression was observed in response to 2,2',4,6,6'-PeCB (Fig. 8D). These findings indicate that 2,2',4,6,6'-PeCB induced COX-2 expression through ERK1/2 MAPK and p53 activation.

2,2',4,6,6'-PeCB induces apoptosis in human monocytic cells (Fig. 2) and COX-2 has been shown to be linked to cell survival by many groups (27,28,29). Therefore, we investigated the effect of COX-2 induction on 2,2',4,6,6'-PeCB-caused apoptosis by using a selective COX-2 inhibitor, NS-398. As a result, co-treatment with 2,2',4,6,6'-PeCB and NS-398 further increased cell death and caspase 3 activity, as compared with treatment with 2,2',4,6,6'-PeCB alone (Fig. 9A and B). To examine how COX-2 reduces apoptosis by 22466-PeCB, we analyzed key steps in the mitochondrial pathway of apoptosis. NS-398 potentiated 2,2',4,6,6'-PeCB-stimulated decrease of Bcl-xL mRNA, an anti-apoptotic gene, and increase of mitochondrial cytochrome c release into the cytosol (Fig. 9C and D), suggesting that COX-2 induction restricts cell death by blocking the mitochondrial pathway of apoptosis. Furthermore, treatment of PGE_2 resulted in the inhibition of cell death caused by 2,2',4,6,6'-PeCB (Fig. 9E). These data imply that apoptosis by PCB is limited by an increase in prostaglandin resulting from induction of COX-2.

In conclusion, our study show that COX-2 is induced by 2,2',4,6,6'-PeCB via ERK1/2 MAPK and p53, and inhibition of COX-2 enhances 2,2',4,6,6'-PeCB-induced apoptosis through mitochondrial pathway. These results suggest that COX-2 expression by 2,2',4,6,6'-PeCB may be a compensatory mechanism to abate of its toxicity by reducing apoptotic susceptibility.

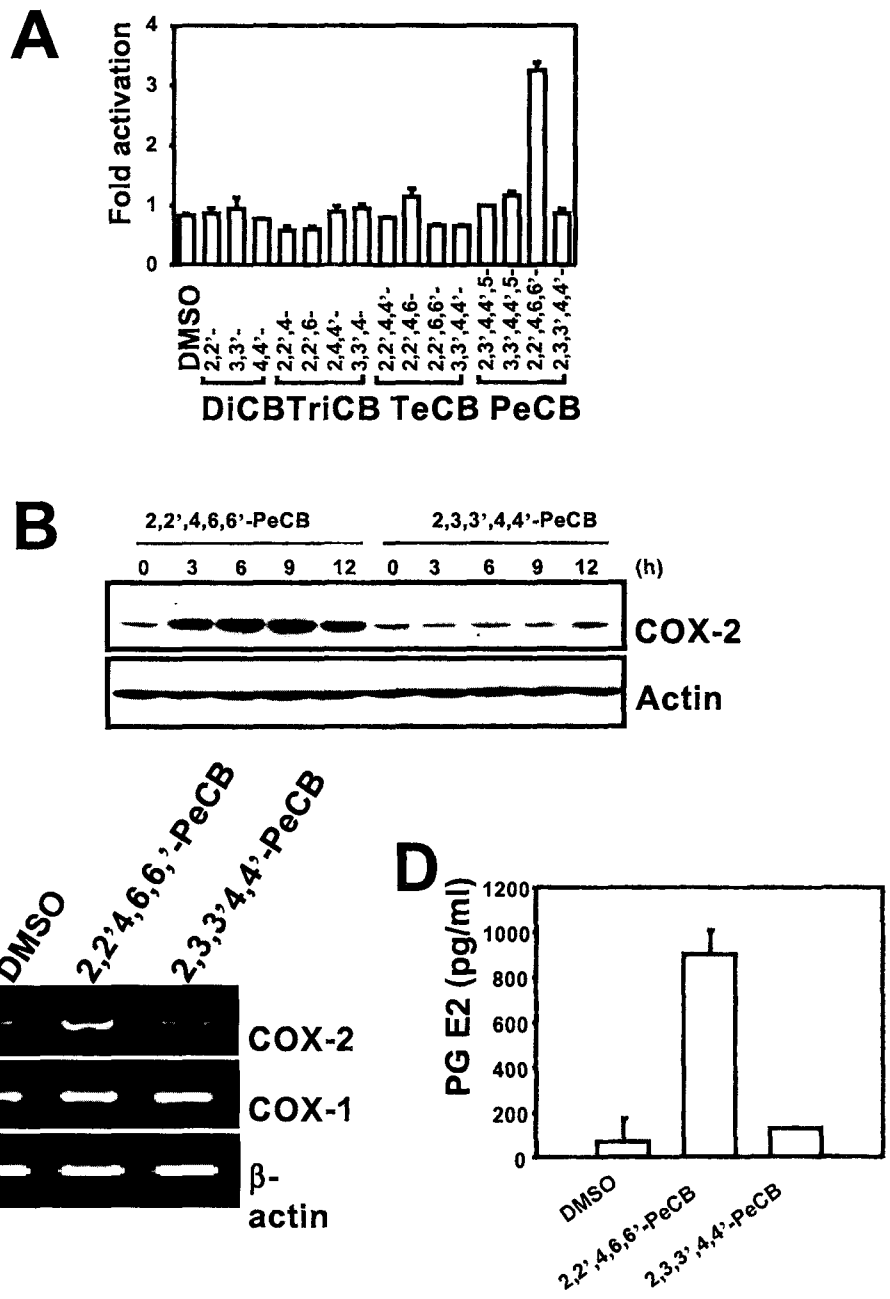

Figure 7. 2,2',4,6,6'-PeCB increases COX-2 induction.

A) Rat-1 cells transiently transfected with COX-2 promoter construct (11432/+59) ligated to luciferase. Transfected cells were treated with PeCBs for 6 h. Luciferase activity represents data that have been normalized into cotransfected renilla luciferase activity. B) 10 μM PeCBs were incubated with Rat-1 cells for the indicated times. Western blot analysis with specific antibodies against COX-2 or actin was performed. C) Total RNA was isolated from cells that were treated with PeCBs for 1.5RT-PCR with specific rat COX-1, COX-2 or actin primers was performed . D) Cells were treated with 10M PeCB for 6 h. The medium was collected to determine the synthesis of PGE2. Production of PGE2 was determined by specific enzyme immunoassay.

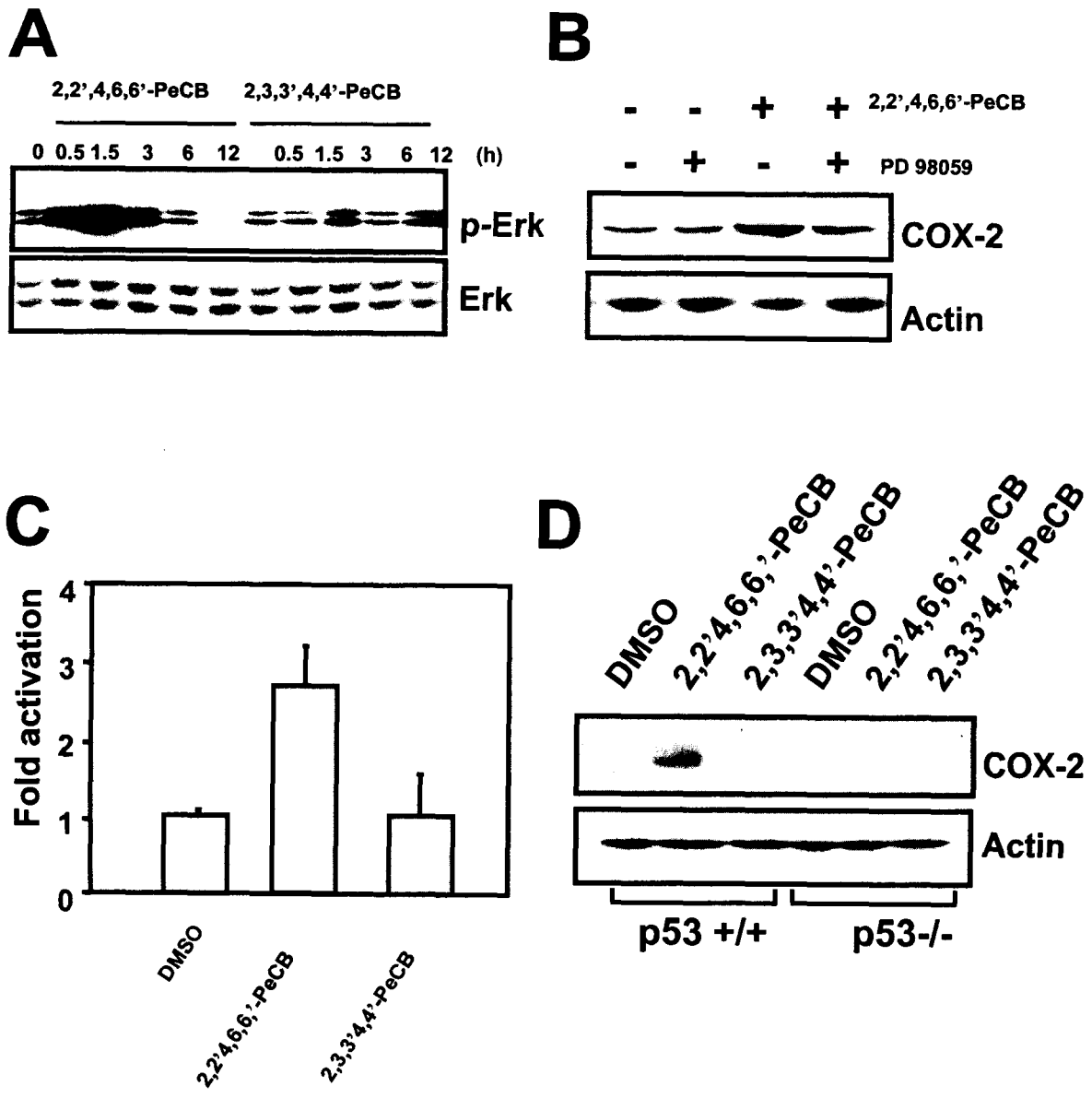

Figure 8. ERK1/2 MPAK and p53 is important for 2,2',4,6,6'-PeCB -mediated induction of COX-2. A) Rat-1 cells stimulated with PeCBs for the indicated times. Western blots were probed with antibodies to phosphorylated forms of ERK1/2. B) Rat-1 cells were pretreated with 20 μM PD98059 for 1and then stimulated with 2,2',4,6,6'-PeCB for 6h. Western blots were probed with antibody specific for COX-2 or actin. C) Rat-1 cells were transiently transfected with luciferase reporter gene under control of p53. Transfected cells were treated with PeCBs for 6 h. D) p53+/+ or p53-/-MEFs were treated with 10M 2,2',4,6,6'-PeCB for 6 h.Western blot analysis with specific antibodies against COX-2 or actin was performed.

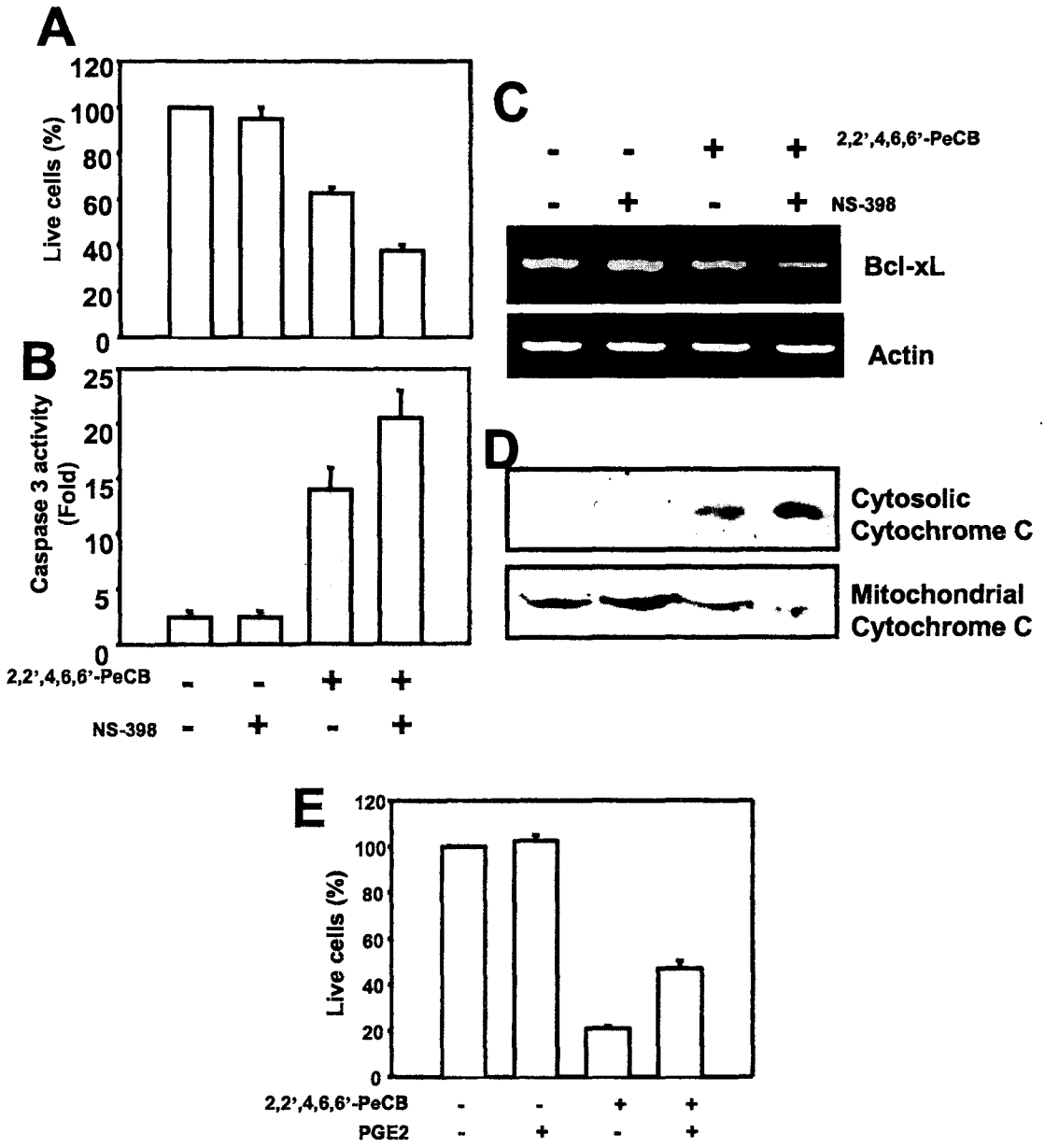

Figure 9. COX-2 selective inhibitor enhances 2,2',4,6,6'-PeCB-induced apoptosis.
A) Rat-1 cells were pretreated with 50 μM NS-398 for 1and then stimulated with 2,2',4,6,6'-PeCB for 12Cell viability was determined by trypan blue staining. B) After cells were treated with for 12 h, caspase-3 activity was assayed using DEVD-AMC as a substrate. C) Total RNA was isolated from cells that pretreated with NS-398 for 1and then stimulated with 2,2',4,6,6'-PeCB for 6h. RT-PCR with specific rat Bcl-xL or actin primers was performed. D) Rat-1 cells were pretreated with NS-398 for 1and then stimulated with 2,2',4,6,6'-PeCB for 12To determine cytochome c release into the cytosol, western blotting was performed using anticytochrome c antibody. E) Rat-1 cells were pretreated with 10 μM PGE2 for 1and then stimulated with 2,2',4,6,6'-PeCB for 24Cell viability was determined by MTT assay.

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