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Advances in Brief

Inhibition of Protein Kinase C Prevents Asbestos-induced c-*fos* and c-*jun* Proto-Oncogene Expression in Mesothelial Cells[1]

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Abstract

Asbestos and the phorbol ester tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), increase c-*fos* and c-*jun* mRNA levels and AP-1 DNA binding activity in rat pleural mesothelial (RPM) cells, a target cell of asbestos-induced mesotheliomas (N. H. Heintz *et al.*, Proc. Natl. Acad. Sci. USA, *90:* 3299–3303, 1993). Because protein kinase C (PKC) is the intracellular receptor of phorbol ester tumor promoters and asbestos is a putative tumor promoter in the respiratory tract, we hypothesized that PKC might play a critical role in asbestos-induced cell signaling pathways associated with regulation of proto-oncogenes. Using a panel of PKC antibodies, we identified PKCα as the major PKC isozyme in RPM cells. We then pretreated cells with phorbol ester dibutyrate to down-modulate PKC or with calphostin C, a specific PKC inhibitor, to determine if depletion of PKCα could block asbestos-induced c-*fos*/c-*jun* expression. Quantitation of Northern blots showed that fiber-associated c-*fos*/c-*jun* mRNA levels were significantly lower either after PKCα down-modulation or pretreatment with calphostin C. In addition, to determine whether tyrosine kinases also were involved in proto-oncogene activation by asbestos, tyrphostin AG82 or herbimycin A was added to RPM cells before exposure to asbestos. These inhibitors decreased crocidolite-induced c-*fos* but not c-*jun* levels, suggesting that tyrosine kinases have different regulatory roles in asbestos-induced c-*fos versus* c-*jun* signaling pathways. The ability to block induction of asbestos-induced proto-oncogene expression using pharmacological intervention may be important in prevention and treatment of asbestos-induced proliferative diseases including lung cancers, mesothelioma, and pulmonary fibrosis.

Introduction

Asbestos is a group of chemically and physically different types of minerals including chrysotile, crocidolite, amosite, tremolite, anthophyllite, and actinolite fibers (1, 2). Occupational exposure to asbestos, particularly crocidolite, is associated with the development of lung cancers and malignant mesothelioma (1, 2). Asbestos may have multiple effects in carcinogenesis, but the molecular mechanisms of asbestos-induced tumorigenesis are unclear. For example, asbestos can cause both chromosomal aberrations and morphological transformation, which are more striking in rodents in comparison to human cell types *in vitro* (reviewed in Ref. 2). Genotoxic events *in vitro* may occur after direct interaction of longer asbestos fibers with DNA and the mitotic spindle and/or after generation of active oxygen species from fibers either extracellularly or intracellularly (reviewed in Ref. 3). Moreover, asbestos fibers may act as classical tumor promoters in causing inflammation and cell proliferation in human lungs and rodent models of inhalation (2–5).

A likely scenario for alterations in gene expression by asbestos fibers is initiation of signaling pathways at the plasma membrane and induction of early response genes critical to inflammation, mitogenesis, and mutagenesis (6–8). Using cultures of RPM[3] cells, we have shown that exposure to crocidolite asbestos fibers leads to persistent increases in steady-state mRNA levels of the proto-oncogenes, c-*fos* and c-*jun*, and enhancement of AP-1 to DNA binding activity (7, 8). The protracted time frame (*i.e.*, ≥8 h) of these asbestos-induced events is different from the transient activation of proto-oncogenes observed with the phorbol ester tumor promoter, TPA. However, they suggest possibly common pathways of cell signaling by asbestos and TPA.

The mechanisms by which phorbol ester tumor promoters activate gene transcription from TPA response elements are complex, but accumulating data show that members of the PKC family, the largest known family of TPA-binding proteins, are critical to intracellular signaling pathways in various cell types (reviewed in Ref. 9). For example, TPA-induced activation of the ERK1 and ERK2 *in vitro* requires PKC, c-*raf*, and the mitogen-activated protein kinase activator MEK-1 (10). Unlike asbestos fibers, which activate ERKs after phosphorylation of the epidermal growth factor receptor (6), phorbol esters such as TPA have a DAG-like structure allowing them to directly activate PKC (9). This, then, may trigger down-stream signaling cascades, transcription of early-response genes such as c-*fos* and c-*jun*, and related outcomes such as induction of cell proliferation and inflammation. We hypothesized here that PKC may be a critical component in cell signaling pathways leading to proto-oncogene induction by asbestos in mesothelial cells, progenitor cells of asbestos-induced mesotheliomas (1, 2). This theory was further supported by our studies published previously showing increases in DAG in hamster tracheal epithelial cells (11). To test our hypothesis, we identified the predominant PKC isoform in mesothelial cells as PKCα. We then down-modulated PKCα in RPM cells using PDBu or pretreated cells with the specific PKC inhibitor, calphostin C, before exposure of cells to asbestos and measurement of c-*fos* and c-*jun* message levels. Because tyrosine kinases have also been implicated in the mitogen-activated protein kinase and other signaling pathways leading to alterations in cell proliferation and differentiation in many cell types, we also evaluated whether the tyrosine kinase inhibitors, tyrphostin AG82 and herbimycin A, affected c-*fos* and c-*jun* mRNA levels induced by asbestos. Whereas both down-modulation and inhibition of PKC decreased the induction of c-*fos* and c-*jun* proto-oncogenes by asbestos, tyrosine kinase inhibitors reduced asbestos-induced c-*fos*, but not c-*jun*, mRNA levels. Our studies are unique in that they show, for the first time, modification of asbestos-induced gene expression using selective inhibitors of intracellular signaling cascades.

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[3] The abbreviations used are: RPM, rat pleural mesothelial; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; DAG, diacylglycerol; PDBu, phorbol ester dibutyrate.

Materials and Methods

Asbestos and Chemicals. Processed crocidolite asbestos fibers (National Institute of Environmental Health Sciences) were obtained from the Thermal Insulation Manufacturers Association Fiber Repository (Littleton, CO) and have been described previously (8). Calphostin C (from LC Lab, Woburn, MA), herbimycin A, and tyrphostin AG82 (both from Life Technologies, Inc., Gaithersburg, MD) were dissolved in sterile DMSO (final concentration, 0.1%) before addition to culture medium. Untreated controls in these experiments were exposed to 0.1% DMSO alone in medium. cDNA probes for human c-*fos* and c-*jun* were obtained from Dr. Richard Gaynor (University of California, Los Angeles, CA). The cDNA probe for glyceraldehyde-3-phosphate dehydrogenase, used as a housekeeping gene, was a gift from Ph. Jeanteur (Laboratoire de Biochimie, Center Paul Lamarque, France). PKCα M6 monoclonal antibody was obtained from Upstate Biomedical Institute (Lake Placid, NY), and other PKC isozyme antibodies were obtained from Dr. Susan Jaken (W. Alton Jones Cell Science Center).

Cell Culture and Exposure to Agents. RPM cells were isolated from the parietal pleura of Fischer 344 rats by methods described previously (7, 8). Cells were propagated for four passages in F12/DMEM (1:1) medium (Life Technologies, Inc.) containing 10% fetal bovine serum and 100 ng/ml hydrocortisone, 2.5 μg/ml insulin, and 2.5 μg/ml transferrin (Sigma Chemical Co.). Twenty-four h prior to exposure of confluent cultures to test agents, the growth medium was replaced with medium containing 0.5% fetal bovine serum. Asbestos fibers then were suspended in HBSS (Life Technologies, Inc.) at 1 mg/ml and added directly to medium at final concentrations of 2.5–10 μg/cm^2 area of dish (11, 12). TPA (Consolidated Midland, Brewster, NY) was added to medium at 100 ng/ml from a stock solution of 1 mg/ml in ethanol. PDBu (Sigma) was added to medium at 200 nM from a stock solution of 10 mM DMSO. Inhibitors (calphostin C, herbimycin A and tyrophostin AG82) were added at predicted effective concentrations as reported by others (14) to medium 1 h prior to addition of asbestos or TPA. Cultures were exposed to calphostin C under fluorescent lighting. Untreated cultures were removed from the incubator and subjected to mock manipulations.

Western Blot Procedure. Cytosolic and membrane fractions were prepared after RPM cells at confluency had been scraped into a lysis buffer containing 250 mM sucrose, 2.5 mM EGTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. After sonication, homogenates were centrifuged at 10,000 × *g* for 30 min to separate particulate (membrane) from soluble (cytosol) fractions. Membrane pellets were resuspended in 30 μl of the above buffer containing 1% (v/v) Triton X-100 and solubilized for 30 min at 4°C and centrifuged 10,000 × *g* for 30 min to yield supernatants, *i.e.*, the membrane fraction. Protein concentrations were determined according to the method of Bradford (15). Cytosolic and membrane fractions were diluted in Laemmli buffer, applied to a 7.5% SDS-polyacrylamide gel, then transferred to nitrocellulose paper. Blots were blocked with 5% nonfat dry milk in CMF-PBS before they were incubated with PKCα primary antibody (1 μg/ml) and peroxidase-labeled IgG (Vector, Burlingame, CA) as a secondary antibody. Antibodies were diluted in CMF-PBS containing 1% BSA and washed with CMF-PBS 3 times between each step; PKCα protein was visualized using the enhanced chemiluminescence (ECL) technique (Amersham, Buckinghamshire, United Kingdom).

RNA Isolation and Northern Blot Procedures. Total RNA was prepared, and Northern blot hybridization was performed as reported in detail by Shull *et al.* (16). cDNA probes were labeled with [α-^{32}P]dCTP by random hexamer priming. Hybridization signals were quantified by measuring the radioactivity on blots with a phosphoimaging system (GS-250; Bio-Rad, Hercules, CA). To ascertain equal loading of RNA on individual lanes, gels were examined after staining with ethidium bromide. After probing for c-*fos* and c-*jun*, all Northern blots also were rehybridized with a GAPDH cDNA "housekeeping gene" probe.

Statistics. Data from Northern blots and phosphoimaging were analyzed by an ANOVA program with Duncan's procedure to adjust for multiple comparisons.

Results

Localization and Modulation of PKCα by PDBu in RPM Cells. PKC isozymes and their subcellular distribution differ in various cell types. Therefore, we first screened confluent RPM cells by immunofluorescence Western blot analysis using seven specific antibodies to PKC isozyme, α, β1, β2, γ, ε, ζ, and θ, to identify which isoforms were expressed. In comparison to other PKC isozymes that were undetectable in RPM cells (data not shown), PKCα was the predominant isoform detected. To determine whether translocation of PKCα into the membrane fraction of RPM cells occurred after exposure to PDBu and whether prolonged exposure resulted in down-modulation of PKCα, we performed Western blot analysis after exposure of cells to PDBu (200 nM) for various time periods. In untreated confluent RPM cells, PKCα was distributed in both the cytosolic and membrane fraction (Fig. 1, *Lanes 1* and *2*). However, exposure to PDBu for 15 min resulted in association of PKCα predominantly with the membrane fraction (Fig. 1, *Lanes 3* and *4*). Protracted exposure of RPM cells to PDBu showed decreases in PKCα in both membrane and cytosolic fractions at 8 h (Fig. 1, *Lanes 5* and *6*) and virtual absence of protein at 24 h (Fig. 1, *Lanes 7* and *8*).

Down-Modulation and Inhibition of PKC Inhibits Asbestos-induced c-*fos* and c-*jun* mRNA Levels. To then determine whether PKC played a role in asbestos-induced proto-oncogene induction, we first pretreated RPM cells with PDBu (200 nM) for 24 h before adding crocidolite fibers (2.5 and 5 μg/cm^2) to cells for 8 h, the time period at which proto-oncogene mRNA levels peak (7, 8). Exposure to asbestos for 8 h increased mRNA levels of c-*fos* and c-*jun* in a dose-related pattern (Fig. 2). At highest concentrations of asbestos, increases in proto-oncogene mRNA levels were approximately 3- to 5-fold higher ($P < 0.05$) than those observed in untreated control cultures ($P < 0.05$). TPA also caused striking and significant elevations in proto-oncogene expression after addition for a 1-h period (Fig. 2). Although the addition of PDBu (200 nM for 24 h) alone did not

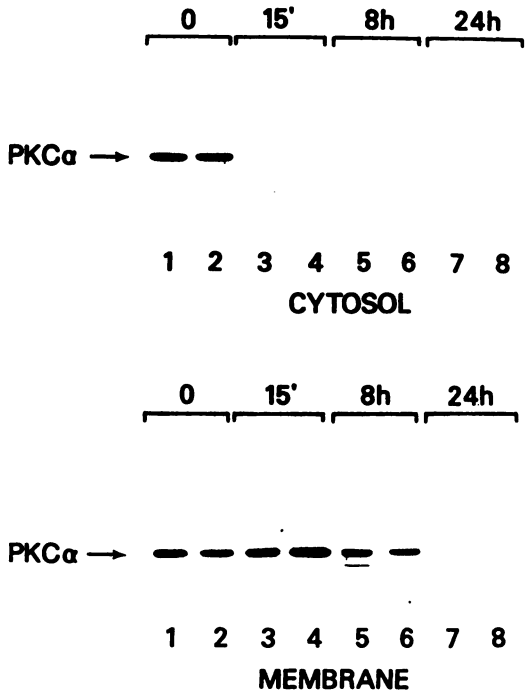

Fig. 1. Western blot showing effects of PDBu on PKCα. *Upper panel,* cytosolic fractions of RPM cells; *lower panel,* membrane fractions of RPM cells. *Lanes 1* and *2,* individual preparations from untreated controls. *Lanes 3* and *4, Lanes 5* and *6,* and *Lanes 7* and *8,* respectively, show preparations from RPM cells exposed to PDBu (200 nM) for 15 min, 8 h, and 24 h, respectively.

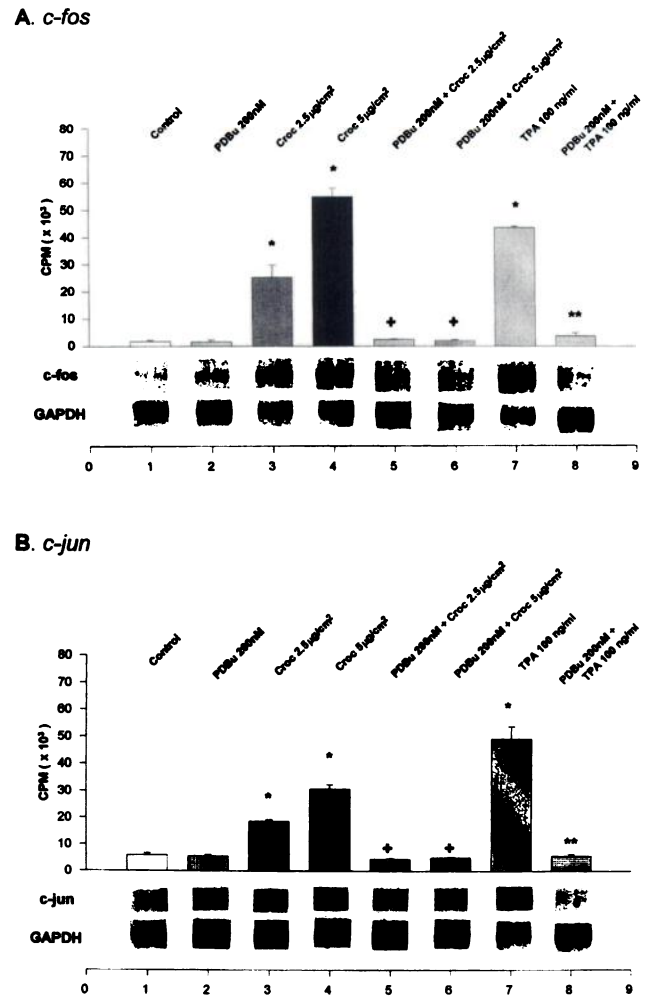

Fig. 2. Northern blot showing that down-modulation of PKCα PDBu inhibits c-*fos*/c-*jun* expression induced by asbestos. *A*, c-*fos* mRNA levels; *B*, c-*jun* mRNA levels. All results in graphs in Figs. 2–4 show quantitation of c-*fos* and c-*jun* Northern blots by phosphorimaging and are expressed as cpm (means) of $n = 2$ lanes per group; *bars*, SE. GAPDH blots are provided to indicate loading proficiency. *, $P < 0.05$ in comparison to untreated controls. +, $P < 0.05$ in comparison to asbestos alone. **, $P < 0.05$ in comparison to TPA alone. All experiments were performed in duplicate. *Croc*, crocidolite

affect levels of c-*fos* or c-*jun* mRNA, down-modulation of PKC by PDBu inhibited both asbestos- and TPA-induced c-*fos* and c-*jun* expression ($P < 0.05$).

Calphostin C, a specific inhibitor of PKC that interacts with the regulatory domain of PKC, was also used to determine whether pretreatment of RPM with this compound could inhibit asbestos-induced c-*fos* and c-*jun*. As shown in Fig. 3*A*, neither 10 nor 100 nM of calphostin C alone affected c-*fos* levels when compared to untreated cells. However, calphostin C at both concentrations inhibited increases in c-*fos* mRNA by asbestos ($P < 0.05$). Calphostin C at 10 nM was also effective in decreasing c-*jun* expression by asbestos ($P < 0.05$; Fig. 3*B*). However, a higher concentration of calphostin C (1 μM) was required to inhibit elevated c-*fos* and c-*jun* mRNA levels by TPA ($P < 0.05$).

Inhibition of Tyrosine Kinases Decreases Asbestos-induced c-*fos* but not c-*jun* Gene Expression. Tyrosine kinases may be involved at several stages in the signaling pathways governing regulation of proto-oncogenes. To determine whether tyrosine kinases were integral to c-*fos* and c-*jun* induction by asbestos, two specific tyrosine kinase inhibitors, tyrphostin AG82 and herbimycin A (17, 18) were added to RPM cells. As can be seen in Fig. 4 (groups 3 and 4), neither the addition of tyrphostin AG82 (10 and 50 μM) or herbimycin (0.1 and 1 μM) alone affected basal c-*fos* and c-*jun* expression in RPM cells. However, when added for 1 h prior to asbestos, both compounds diminished asbestos-induced c-*fos* levels at highest concentrations ($P < 0.05$; Fig. 4, *A* and *C*, *group 5*). In contrast, neither compound affected c-*jun* mRNA levels induced by asbestos (Fig. 4, *B* and *D*, *groups 5* and *6*). Pretreatment of RPM cells with either Tyrphostin AG82 (50 μM; Fig. 4, *A* and *B*, *group 8*) or herbimycin A (Fig. 4, *C* and *D*, *group 8*) did not affect c-*fos* or c-*jun* elevations by TPA.

Discussion

Mesotheliomas are tumors often associated with exposure to asbestos fibers and having an average latency period of 40 to 45 years from time of first exposure to asbestos (1, 2). The prognosis for malignant mesothelioma is extremely grim, and patient survival from diagnosis to death is generally less than 2 years, reflecting inadequate strategies for therapy (3). Mesothelioma and other asbestos-associated diseases such as lung cancer and asbestosis are characterized by chronic inflammation and cell proliferation. Thus, we focused here, using concentrations of asbestos causing increased expression of c-*fos* and

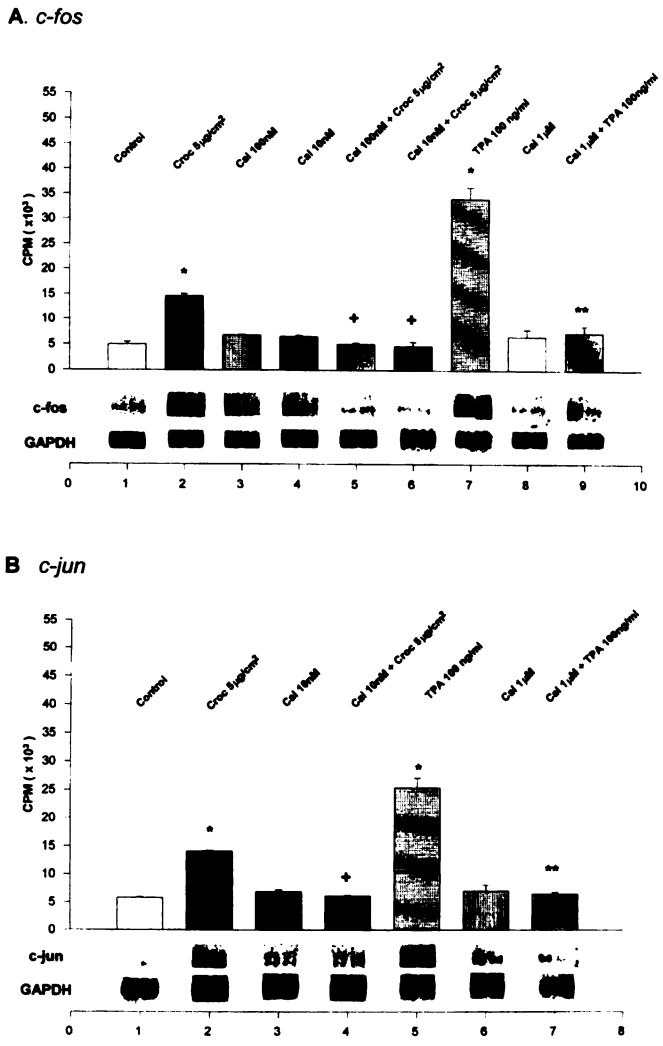

Fig. 3. Northern blot showing pretreatment with the PKC inhibitor, calphostin C (*Cal*), inhibits c-*fos*/c-*jun* mRNA levels induced by asbestos. *A*, c-*fos* mRNA levels; *B*, c-*jun* mRNA levels. Results are presented as described in the legend of Fig. 2. *, $P < 0.05$ in comparison to untreated controls. +, $P < 0.05$ in comparison to asbestos alone. **, $P < 0.05$ in comparison to TPA alone. All experiments were performed in duplicate. *Croc*, crocidolite. *Bars*, SE.

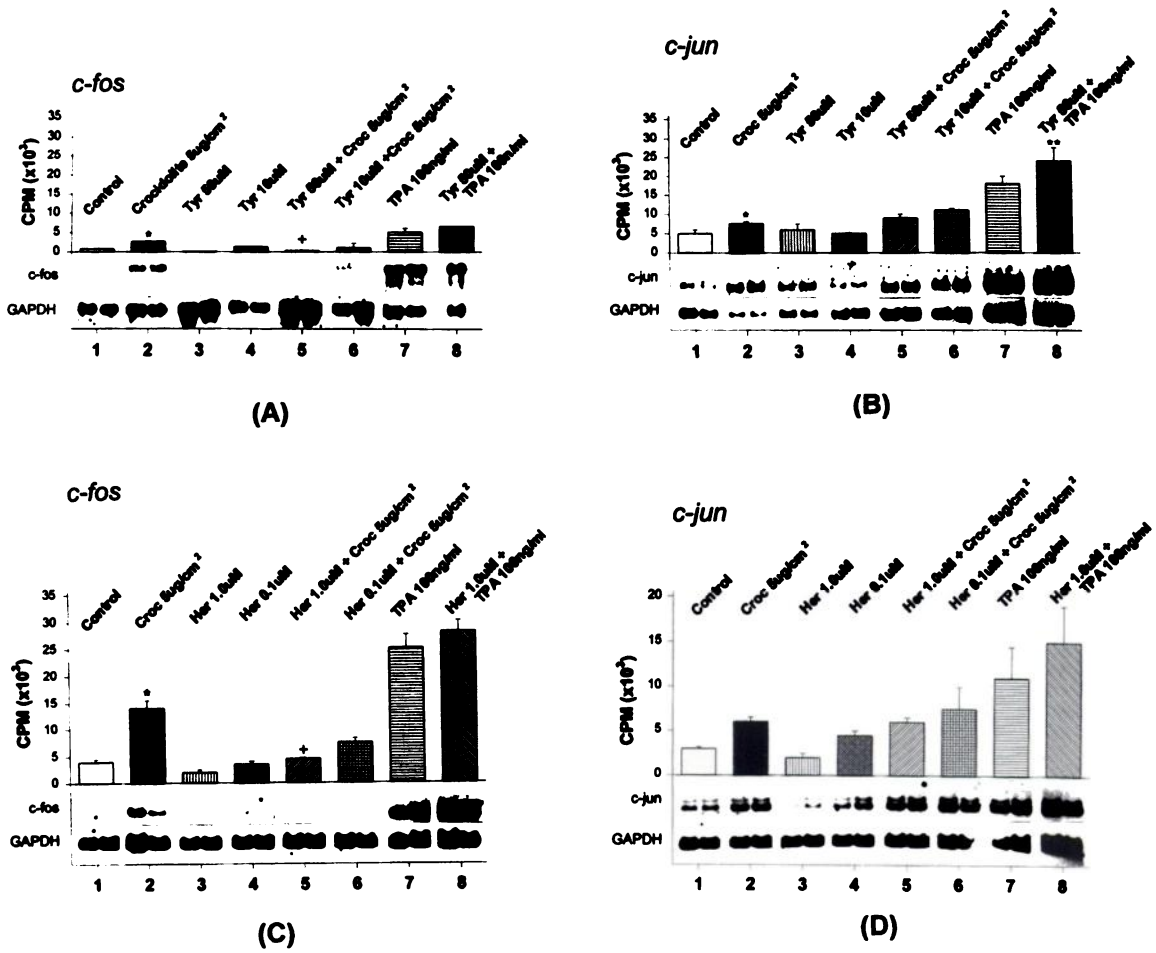

Fig. 4. Northern blot showing that the tyrosine kinase inhibitors, tyrphostin AG82 (*Tyr*; *A* and *B*) and herbimycin A (*Her*; *C* and *D*) inhibit c-*fos*, but not c-*jun*, expression by asbestos. Results and statistical significance are presented as described in the legends of Figs. 2 and 3.

c-*jun* proto-oncogenes (7, 8), on whether asbestos-induced early-response gene expression, which might be key to mitogenesis by asbestos, could be inhibited pharmacologically. Our data, based on both down-modulation experiments with PDBu and inhibition of PKC using calphostin C, suggest that activation of PKCα, the primary PKC isozyme in normal mesothelial cells, is integral to cell signaling pathways governing proto-oncogene induction by asbestos. These data are consistent with our prior experiments showing activation of PKC by asbestos in hamster tracheal epithelial cells (12), which is accompanied by rapid accumulation of DAG and hydrolysis of phosphoinositides (11).

In line with our findings here, PKC modulates diverse cellular functions including both proliferation and normal differentiation of many cell types (reviewed in Ref. 9). Various isoforms of PKC have been identified that exhibit differences in substrate specificity, kinetic properties, and phenotypic effects on cells (13). Other reports indicate that PKCα may play a facilitative role in cell regeneration, morphological transformation, and clonal expansion of tumor cells (19–22), and a recent study shows decreased growth of human tumor cell lines in nude mice by an antisense oligonucleotide inhibitor of PKCα expression (23).

In our studies, both tyrphostin AG82 and herbimycin A blocked asbestos-induced c-*fos* expression, but levels of c-*jun* mRNA by asbestos and TPA-induced proto-oncogene expression were not affected. The differential effects of both tyrosine kinase inhibitors on c-*fos* *versus* c-*jun* expression by asbestos and TPA supports a compendium of reports indicating that different signaling cascades regulate transactivation of these proto-oncogenes (reviewed in Ref. 24). Moreover, specific agents such as asbestos and TPA may activate multiple signaling cascades at different steps in these pathways in a cell type-specific manner.

In conclusion, work here shows that inhibition of PKC and tyrosine kinases differentially modify c-*fos* and c-*jun* expression by asbestos fibers, an important human carcinogen. The ability to modify cell signaling cascades leading to transactivation of proto-oncogenes by environmental agents may be important in prevention and control of asbestos-induced tumors. Because both increased cell proliferation[4] and apoptosis (25), phenotypic consequences of early-response gene transactivation in other cell types, are observed in RPM cells at concentrations of asbestos used here, further studies are planned to determine whether these physiological outcomes are affected by pharmacological approaches used here. Moreover, these strategies will be useful in rodent inhalation models of disease to show if early-response gene expression, mitogenesis, and tumor development can be modified.

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[4] J. L. Goldberg, C. I. Zanella, Y. M. W. Janssen, C. R. Timblin, L. A. Jimenez, P. Vacek, D. L. Taatjes, and B. T. Mossman. Novel cell imaging techniques show induction of apoptosis and proliferation in mesothelial cells by asbestos. Am. J. Respir. Cell Mol. Biol., in press.

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