In a previous study, we demonstrated that the length of glass fibers was a critical determinant of fiber potency in induction of tumor necrosis factor (TNF)-α and that activation of NF-κB was an important factor in this response. In the present study, we analyzed the role of mitogen-activated protein (MAP) kinases in the induction of TNF-α by glass fibers. Glass fibers induced phosphorylation of MAP kinases, p38, and ERK in primary rat alveolar macrophages, and this phosphorylation was associated with TNF-α gene expression. Long fibers were more potent than short fibers in activation of MAP kinases. Results from mechanistic analysis support that MAP kinases activate transcription factor c-Jun. The activated c-Jun acts on the TNF-α gene promoter through two binding sites, the cyclic AMP response element and the activator protein 1-binding site. These results suggest that in addition to the NF-κB pathway for TNF-α production, glass fibers are able to activate c-Jun through MAP kinase pathways that lead to induction of TNF-α expression.

Glass fibers have been popular substitutes for asbestos in the building industry. The health effects of glass fibers remain to be fully investigated. Recently, a classifier has been developed to separate fibers by length using dielectrophoresis that involves the movement of neutral particles in a gradient electric field (1, 2). The development of this classifier makes it possible to study the role of fiber length in toxicity. To this end, we have studied the biological activity of JM-100 glass fibers on macrophages and found that fiber length is a very important factor in toxicity and stimulation of macrophages (3–5). Biological effects of glass fibers of two different lengths (7 and 17 μm) have been previously studied by our laboratory (5). Long fibers were more potent than short fibers in the activation of NF-κB and the induction of TNF-α production.

Macrophages serve as sensors to external stimulation in the defense system of the body. Reaction of macrophages to glass fibers may be the first response in the lung when glass fibers are inhaled. The activated macrophages pass signals to other cells by releasing intercellular signal mediators, such as cytokines and free radicals (6, 7). In our previous study, we observed that macrophage engulfed glass fibers and released TNF-α upon exposure to glass fibers (5). TNF-α is one of the pro-inflammatory cytokines secreted by macrophages. TNF-α plays an important role in the pathogenesis of pulmonary fibrosis by stimulating proliferation of fibroblasts and production of collagen matrix (6–9).

TNF-α expression is mainly regulated at the transcriptional level. Transcription of TNF-α is controlled by multiple enhancer elements such as the κ3 site (κ NF-κB binding site), cAMP response element (CRE), and AP-1-binding site. Activities of these enhancers are regulated by several transcription factors, including NF-κB (5, 10), AP-1 (11), activated T cell factor (ATF)-2 (12, 13), c-Jun (12, 14), CRE-binding protein 1 (CREB1) (14), and nuclear factor of activated T cells (12, 15). It has been widely accepted that both the κ3 site and CRE are required for a maximal induction of TNF-α transcription, and a synergy between these two elements is necessary (12, 14). We have reported previously that the κ3 site was involved in induction of TNF-α by glass fibers (5).

The mitogen-activated protein (MAP) kinases are activated by many stress signals (16, 17). MAP kinases are serine/threonine protein kinases that participate in signal transduction of many extracellular stimuli, including UV light, bacterial derivatives, and growth factors. The major members in the MAP kinase family are extracellular signal-regulated kinase (ERK), p38 kinase, and Jun N-terminal kinase (JNK) (18–20). Activation of these kinases is marked by phosphorylation of serine/threonine amino residues in their protein molecule. ERK, p38, and JNK are cytoplasmic proteins. They act as signal transducers at the end of kinase cascades that mediate signals from the cell membrane to the nucleus. Activation of ERK, p38, and JNK leads to induction of transcription factors that in turn regulate target gene expression in the nucleus. The nuclear proteins, such as c-Jun, ATF-2, and Elk-1, are the major transcription factors that are regulated by the three MAP kinases. Reactive oxygen species (ROS) have been shown to act as MAP kinases activators (16, 17, 21).

Our previous study has demonstrated that fiber-induced ROS were required for TNF-α production in a murine macrophage cell line (5). This paper is available online at http://www.jbc.org
phage cell line (5). The present study focuses on the involvement of MAP kinase signal transduction pathway in fiber-stimulated TNF-α production by rat alveolar macrophages. MAP kinases can be activated by ROS in variety of conditions, including UV light (16, 18, 22), silica (23), and asbestos exposure (21). Thus, it is likely that MAP kinases will be activated by glass fiber-induced ROS and that they play a role in glass fiber-induced TNF-α production. This hypothesis is tested in the current study. Rat alveolar macrophages were used for analysis of MAP kinase activity after glass fiber exposure. The following questions are addressed: (a) Do glass fibers induce TNF-α production in the rat alveolar macrophages? (b) Do MAP kinases play a role in glass fiber-induced TNF-α expression? (c) In addition to NF-κB, are other nuclear factors, such as members of the AP-1 family, involved in glass fiber signaling? (d) How do AP-1 family members regulate the TNF-α gene promoter.

MATERIALS AND METHODS

Fibers—Bulk samples of JM-100 glass fibers (Manville code 100 supplied by the manufacturer) were first milled, aerosolized, and separated into three length categories using dielectrophoresis (1, 2). The dielectrophoretic classifier was operated in a differential mode so that fibers with narrow length distributions were extracted in an air suspension at the end of the classifier. These size-selected fiber samples were collected on polycarbonate (Nuclepore) filters at rates up to 1 g/day. Fibers were scraped off the filters for microscopic analysis and for biological experiments.

Samples of the length-classified fibers were prepared for size and count analysis by adding weighed portions of the dusts to freshly filtered water. These samples were then sonicated, diluted, and filtered through polycarbonate filters. Measurements of length, width, and fiber count/mass were made using a JEOL JSM-6400 scanning electron microscope (4). Measurements at each magnification were referenced to a National Institute of Standards and Technology electron microscopy standard rule.

In this study, glass fiber samples with lengths (means ± S.D.) of 7 μm (6.5 ± 2.7) and 17 μm (16.7 ± 10.6), respectively, were used to evaluate fiber effects on macrophages. Concentrations of the glass fibers used in these experiments were determined as fiber counts/ml. The glass fiber counts/ml were 3.0 × 10^3 and 2.0 × 10^4 for 7- and 17-μm fiber samples, respectively (4). The 7- and 17-μm fiber samples were heat-treated at 120 °C for 2 h and stored under sterile conditions at room temperature. Before each experiment, the glass fibers were suspended and sonicated in the complete cell culture medium and then added to cells.

The endotoxin content of the glass fiber samples was measured using the Limulus amebocyte lysate assay (24). Values ranged from 0.7 to 1.6 ng endotoxin units/mg. These values are orders magnitude lower than those found with cotton dust (1000–2000 endotoxin units/mg) or agricultural dusts (46–4000 endotoxin units/mg) where endotoxin is thought to play a role (25). The maximum dose of glass fiber used in this study was 75 μg/ml. Therefore, the maximum endotoxin concentration in this study was 0.013 ng/ml. This dose of endotoxin had no effect on TNF-α production by macrophages because the minimum effective dose of endotoxin is 1 ng/ml in this experiment system (data not shown). Therefore, the fiber results reported here cannot be attributed to endotoxin contamination.

Cells and Reagents—Pathogen free male Harlan Sprague-Dawley rats purchased from Hilltop Labs (Scottsdale, PA) were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/rat) and then sacrificed by cutting the renal artery. Following tracheal cannulation, cold sterile phosphate-buffered saline (PBS) (Ca^2+ and Mg^2+-free) was used to lavage the lungs at a volume of 6 ml for first lavage and 8 ml for subsequent lavages. Approximately 80 ml of bronchoalveolar lavage fluid was collected per rat in sterile tubes. Bronchoalveolar lavage cells were washed twice in PBS by alternate centrifugation and resuspension. Bronchoalveolar lavage cells from different rats were pooled into sterile HEPES buffer (145 mM NaCl, 2.7 mM KCl, 10 mM Na-HEPES, 1 mM CaCl_2, 5.5 mM glucose, pH 7.4). Cell counts were performed using an electronic cell counter equipped with a cell sizing attachment (Coulter model Multisizer II with a 256C channelizer, Coulter Electronics, Hialeah, FL). The number of alveolar macrophages was determined by their characteristic cell diameter (26).

A rat alveolar macrophage cell line (NR8383) was purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in complete medium containing Dulbecco’s modified essential medium supplemented with 15% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin-streptomycin. Superantigens antibodies against ERK, MAPK, and CREB (sc-44 X, sc-541 X, sc-186 X, sc-58 X, and sc-123 X), CREB1 (sc-186 X), and CREB2 (sc-200 X) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The MEK inhibitor (PD 98059; catalog number 513000), which prevents phosphorylation of ERK by MEK, and p38 inhibitor (SB203580; catalog number 559389) were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). PD 98059 and SB 203580 were dissolved in MeSO and used to treat cells with a final MeSO concentration of 0.1%. In all experiments where the inhibitors were used, MeSO was used at the same concentration in the controls.

TNF-α ELISA—Rat alveolar macrophages were plated in a 96-well plate at 1 × 10^5 cells/well in 200 μl of sterile Eagle’s modified essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM glutamine, 100 units/ml penicillin/streptomycin, and 10 μg HEPES at pH 7.2. After a 2-h incubation at 37 °C, the adherent cells were washed three times with warm sterile PBS and then cultured in 1 μl of fresh Eagle’s modified essential medium. Before exposure to glass fibers, some cells were pretreated with MAP kinase inhibitors for 1 h. The cell culture supernatant was harvested after overnight exposure to glass fibers at a cell to fiber ratio of 1:5, which resulted in an endotoxin concentration of 1 ng/ml in the supernatant of fresh macrophage cells. The supernatant was diluted 20-fold and used in the TNF-α ELISA according to the manufacturer’s instruction. An ELISA kit (catalog number KRC3010-SB) from BIOSOURCE International (Camarillo, CA) was used to determine rat TNF-α.

MAP Kinase Phosphorylation Assay—Rat alveolar macrophages were plated in a 6-well plate at 3 × 10^5 cells in 2 ml of Eagle’s modified essential medium. After a 2-h incubation at 37 °C, the adherent cells were washed three times with warm sterile PBS and then exposed to glass fibers (cell to fiber ratio of 1:5) in 2 ml of fresh Eagle’s modified essential medium. After a 2-h exposure, cells were washed once in PBS and lysed in 200 μl lysis buffer for Western blot assay. Activation of ERKs, JNKs, and p38 kinase was determined by their phosphorylation status detected with phospho-specific MAP kinase antibodies. The ERK MAP kinase assay kit (catalog number 9880) and the p38 MAP kinase assay kit (catalog number 9820) from New England Biolabs, Inc. (Beverly, MA) were utilized in this study. Phospho-specific (catalog number sc-6254) and nonphospho-specific (catalog number sc-1648) anti-JNK antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were used to detect JNK activity. The signal in Western blot was quantitated using a densitometer and normalized for protein loading.

Gel Shift Assay—A CRE binding sequence (5'-TCCAGATGAGCT-CATGGGTG TTT) in the TNF-α promoter was used to synthesize an oligonucleotide for the CRE binding. The AP-1 binding element in the collagenase gene promoter was used as an AP-1 probe to examine the AP-1 and binding activity in the cell (27). The double-stranded probe was labeled with 32P-ATP (Amersham Pharmacia Biotech) using the T4 kinase (Life Technologies, Inc.). The labeled probes were then purified using a nondenaturing polyacrylamide gel procedure (27). The harvested cells were treated with a lysis buffer. The collected nuclei were washed once in a washing buffer and then treated with an extraction buffer. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70 °C. The protein concentration was determined using a BCA protein assay reagent (Pierce). The DNA-protein binding reaction was conducted in a 24-μl reaction mixture including 1 μg of poly(dI-dC) (Sigma), 3 μg of nuclear protein extract, 3 μg of bovine serum albumin, 4 × 10^5 cpm of 32P-labeled oligonucleotide probe, and 12 μl of reaction buffer (24% glycerol, 24 mM HEPES, pH 7.9, 8 mM Tris-HCl, pH 7.9, 2 mM EDTA, and 2 mM dithiothreitol) (27). In some cases, the indicated amount of double-stranded oligomer was added as a cold competitor. The reaction mixture was incubated on ice for 10 or 20 min (with or without an antibody) in the absence of radiolabeled probe. After addition of the radiolabeled probe, the mixture was incubated for 20 min at room temperature and then resolved on a 5–8% acrylamide gel that had been prerun at 170 V for 30 min with 0.5 × TBE buffer. The loaded gel was run at 200 V for 90 min, then dried, and placed on Kodak X-Omat film (Eastman Kodak, Rochester, NY).
log number 18324-020; Life Technologies, Inc.). After transfection, the cells were washed once in PBS solution and cultured in 1 ml of the complete medium at 37 °C for 24 h. After being exposed to glass fibers for an additional 16 h, the cells were harvested for the reporter assay. The luciferase activity was determined using a luciferase assay kit (Promega, Madison, WI) and then normalized by the internal control β-galactosidase and the protein content.

Data Analysis—The data for TNF-α production and activation of the TNF-α promoter were presented as mean values ± standard deviations of three individual experiments. Results were analyzed by the Student’s t test at a confidence level of p ≤ 0.05.

RESULTS

Inhibition of TNF-α Production by MAP Kinase Inhibitors in Rat Alveolar Macrophages—In our previous study, murine peritoneal macrophages (Raw 264.7 cells) were used to study TNF-α production after exposure to size-classified glass fiber samples (5). The present study attempted to elucidate a role of MAP kinases in glass fiber-induced TNF-α production using rat alveolar macrophages. Short (7 μm) or long (17 μm) glass fibers were used to stimulate TNF-α production by rat alveolar macrophages, and TNF-α protein was determined by an ELISA after a 16-h exposure to the glass fibers. The results show that glass fibers can induce TNF-α production in rat alveolar macrophages and that short and long fibers exhibited different stimulatory potencies (Fig. 1A). The long fiber sample was twice as potent in stimulation of TNF-α production compared with the short fiber sample (7 μm length).

It is known that MAP kinases are involved in cell stress response (16). Because ROS were involved in glass fiber-induced TNF-α production (5), it is possible that the glass fibers activate MAP kinases in macrophages. The role of MAP kinases was explored by using specific MAP kinase inhibitors. The cells were pretreated with a p38 inhibitor (SB203580) or a MEK inhibitor (PD98059) for 1 h before exposure to glass fibers. Phosphorylation of p38 was induced 7-fold by long and 3.5-fold by short glass fibers (Fig. 2A). The phosphorylation signal was normalized for total p38 protein. As shown in Fig. 2A, exposure of rat alveolar macrophages to glass fibers resulted in phosphorylation of p38 MAP kinase. After being stripped, the membrane was reblotted with a regular a p38 antibody, which can specifically recognize the dual phosphorylated threonine 180 and tyrosine 182 in the p38 MAP kinase protein. The result shows that glass fibers activated p38 MAP kinase and that long fibers expressed a stronger activity than short fibers. Phosphorylation of p38 was induced 7-fold by long and 3.5-fold by short glass fibers compared with the untreated group (Fig. 2B).

ERK and JNK MAP kinases were analyzed in a similar manner. The results indicate that ERK was also activated by glass fibers (Fig. 2C). Phosphorylation of ERK was induced 14-fold by long and 7-fold by short glass fibers (Fig. 2D). In contrast, JNK activity was not activated by glass fibers (Fig. 2, E and F). These results suggest that p38 and ERK may be major components in the signal transduction pathway for TNF-α induction by glass fibers.

Association of MAP Kinase with Activation of the TNF-α Gene Promoter—The above results indicate that p38 and ERK may be involved in induction of TNF-α production by glass fibers. Because TNF-α expression is controlled at the transcriptional level, the role of p38 or ERK in activation of the gene promoter by glass fibers was examined. The promoter activity of the TNF-α gene was studied by transient transfection of rat alveolar macrophage cells (NR8383). A luciferase reporter vector that is controlled by a wild type TNF-α gene promoter was used for this purpose. Macrophages were transfected by the plasmid vector and then exposed to glass fiber samples. The
reporter assay indicates that the TNF-α gene promoter was activated by both short and long glass fibers (Fig. 3A). However, long fibers were nearly three times as potent as short fibers. Long fibers induced a 20-fold increase, whereas short fibers induced a 7-fold increase in the promoter activity.

The role of p38 or ERK in activation of the TNF-α promoter was examined by using specific p38 or MEK inhibitors. SB203580 and PD98059 were added to the cell culture at a final Me2SO concentration of 0.1%. Me2SO was used as a control. Pretreatment of the transfected cells with a p38 inhibitor (SB203580) or an MEK inhibitor (PD98059) significantly reduced the TNF-α gene promoter response to long glass fibers (Fig. 3B). Similar results were found with short fibers as well (data not shown). Each of the inhibitors resulted in approximately a 70% loss of the promoter activity induced by long fibers. These data provide evidence that p38 and ERK MAP kinases may mediate transcriptional activation of the TNF-α gene in alveolar macrophages in response to glass fibers.

Induction of AP-1 and CRE DNA-Protein Complexes—p38 and ERK MAP kinases regulate target gene transcription through activation of nuclear proteins such as c-Fos, c-Jun, and ATF-2 (18). Nuclear proteins c-Fos and c-Jun are two subunits of the transcription factor AP-1. In addition to interaction with the AP-1 site, c-Jun is able to activate a CRE element by forming homodimer or heterodimer with ATF-2. Because both AP-1 and CRE elements are enhancers in the TNF-α gene promoter, it is quite possible that p38 and ERK MAP kinases induced the TNF-α gene promoter through AP-1 and CRE elements. The protein binding activities of AP-1 and CRE elements were investigated in nuclear extracts of alveolar macrophages using an EMSA. The results show that DNA binding activity of AP-1 was significantly induced by glass fibers and that long fibers exhibited a stronger activity than short fibers (Fig. 4A, lanes 1–3). The binding specificity and protein nature of the AP-1 complex were confirmed by oligonucleotide competition and antibody supershift. The AP-1 complex was specifically removed by cold AP-1 probe (Fig. 4A, lane 5) or anti-Jun antibody (Fig. 4B, lane 2) but was not changed by a NF-κB probe (Fig. 4A, lane 6) or an ATF-2 antibody (Fig. 4B, lane 3).

The CRE probe formed three DNA-protein complexes (A, B and C) in the macrophage nuclear extracts as revealed by the EMSA (Fig. 4C and Fig. 5). Treatment with glass fibers resulted in an increase in complex A and a marginal increase in complex B (Fig. 4C, lanes 2 and 3). A change in complex C was not significant. Specificity of the three complexes was demonstrated by oligonucleotide competition. Unlabeled CRE probe effectively competed with the radiolabeled probe in the three complexes, whereas unlabeled k3 probe had no competitive effect on complex A and B (Fig. 4C, lane 4 and 5). However, the unlabeled k3 probe competed with the CRE probe in complex C. Other experimental results suggest that the k3 probe is able to bind specifically to proteins in complex C (data not shown).

Binding of c-Jun to CRE—It has been reported that c-Jun and ATF-2 are responsible for activation of the CRE element in macrophages and for induction of TNF-α transcription (14), suggesting that c-Jun and ATF-2 might be involved in formation of the CRE complexes. To examine the possibility, five antibodies to CRE binding proteins were used in supershift assays. They were CREB1, CREB2, c-Jun, c-Fos, and ATF-2 antibodies as indicated in Fig. 5. Nuclear proteins from un-
Fig. 3. Activation or inhibition of the TNF-a gene promoter in rat alveolar macrophages. The transient transfection assay was used to study the gene promoter activity of TNF-a in NR8383 cells. The cells were transfected with a luciferase reporter (0.5 µg/sample) controlled by a TNF-a gene promoter and then treated with the fibers for 16 h at a cell/fiber ratio of 1:5. The reporter activity in the cell lysate was determined using a luminometer, and the reading was normalized for the amount of protein and an internal control of β-galactosidase. Each bar represents the mean value ± S.D. of the reporter activities from three independent assays. A, induction of the promoter activity by glass fibers. * indicates a significant (p < 0.001) increase in the promoter activity stimulated by short fibers (7 µm) compared with that of the untreated cells. + indicates a significant (p < 0.001) increase in the promoter activity induced by long fibers (17 µm) compared with short fibers. B, inhibition of the promoter activity by MAP kinase inhibitors. P38 (SB203580) and MEK (PD98059) inhibitors were used at 2 and 40 µM to inhibit the long fiber (17 µm) induced promoter activity. * indicates a significant (p < 0.001) decrease in the promoter activity induced by long fibers (17 µm) in the presence versus absence of inhibitor.

Fig. 4. Activation of the DNA binding activity of AP-1 and CRE by glass fibers. The AP-1 activity was examined by EMSA after the cells were exposed to the fibers for 4 h as stated under “Materials and Methods.” The cell to fiber ratio was 1:5. A, induced AP-1 activity. Lane 1 contained the control nuclear protein; lanes 2 and 3 contained nuclear protein from the short and long fiber-treated cells, respectively. Competition assay was carried out with the nuclear protein from long fiber-treated cells (lanes 4–6) and 100 ng of cold probe. B, protein nature of the complex determined by antibody supershift. The nuclear protein of long fiber-treated cells was used. Antibodies to c-Jun (lane 2) and ATF-2 (lane 3) were used. C, induced CRE binding activity. There were three major complexes formed by incubation of the CRE probe with the nuclear protein. The short fibers (lane 2) and long fibers (lane 3) induced complex A. Binding specificity of the complexes was analyzed with oligonucleotide competition. The unlabeled CRE probe (100 µg) was used in lane 4 as a specific competitor. The same amount of unlabeled x3 probe was used in lane 5 as a nonspecific competitor.

Fig. 5. Characterization of c-Jun binding to CRE. The protein nature of CRE complexes was analyzed by antibody supershift in an EMSA. Antibodies used are listed at the top of each lane. A, c-Jun and CREB1 binding to CRE. The nuclear protein from the control cells was used. The nuclear proteins c-Jun and CREB1 were two major CRE binding proteins, because the corresponding antibodies decreased these complexes. B, increased c-Jun binding after exposure to glass fibers. The nuclear protein from long fiber-treated cells was used. c-Jun became the major binding protein in CRE after glass fiber treatment, because only c-Jun antibody removed complexes A and B.

Treated cells (Fig. 5A) and fiber-exposed cells (Fig. 5B) were analyzed in the supershift assay. The results revealed that glass fibers were able to change the components of the CRE complexes. In the control cells, complex A is formed by c-Jun because only c-Jun antibody was able to remove it completely (Fig. 5A, lane 4). Both c-Jun and CREB1 proteins are involved in formation of complex B because antibodies to either c-Jun or CREB1 partially reduced this complex (Fig. 5A, lanes 2 and 4). CREB1 is involved in complex C because CREB1 antibody partially reduced the complex (Fig. 5A, lane 2).

In the glass fiber-treated cells, DNA binding activity was increased in both complex A and complex B (Fig. 4C). It may be noted that c-Jun antibody completely removed complex A and dramatically decreased complex B. This is in contrast to the fact that c-Jun antibody only weakly reduced complex B in the control cells (Fig. 5A). This change indicates that more c-Jun protein binds to CRE element after glass fiber exposure. In the unstimulated cells, CREB1 was involved in formation of complexes B and C. Interestingly, in the glass fiber-stimulated cells, no CREB1 protein was detected in complexes B and C because CREB1 antibody did not reduce either of the two
complexes (Fig. 5B, lane 2). These results were consistent in the repeated experiments. The supershift results suggest that although the binding pattern of CRE was similar before and after fiber exposure, proteins in the complexes B and C have been changed by the fiber stimulation. c-Jun protein gained more binding activity after cell activation. Complex C was also reduced by c-Jun antibody, indicating that c-Jun was involved in complex C after fiber stimulation. Complex C was not reduced by CREB1 antibody, suggesting that CREB1 was not in the complex after fiber stimulation. These results imply that in the activated cells, c-Jun substituted for CREB1 in formation of complex C. The supershift results do not support the involvement of CREB2, c-Fos, or ATF-2 in the formation of CRE complexes.

Enhancement of TNF-α Promoter Activity by c-Jun—The above results suggest that glass fibers induce TNF-α transcription through a CRE or AP-1 element and that nuclear protein c-Jun might play a major role in this activation. To test this hypothesis, functional analysis of the TNF-α promoter was carried out by mutation and cotransfection. In the mutation analysis, the CRE element was inactivated by base pair substitution in the DNA sequence of the TNF-α promoter (14). The promoter activity was then examined in rat alveolar macrophages (NR8383) in a transient transfection assay. The results show that mutation of CRE resulted in a significant loss of the promoter response to the glass fibers (Fig. 6A). Compared with the wild type promoter, the mutated promoter lost ∼87% of its inducibility to both short and long fibers. This suggests that CRE may be required for fiber-induced TNF-α transcription.

An increase of c-Jun expression by a c-Jun expression vector resulted in activation of the TNF-α promoter. Because CRE is required for glass fiber-induced TNF-α promoter activity, and c-Jun is a main protein bound to the element, it is quite possible that c-Jun is involved in the activation of the CRE element. To verify this hypothesis, c-Jun protein level was increased in the assay system by cotransfection of a c-Jun expression vector into NR8383 cells. The TNF reporter activity was investigated under this condition. The result shows that c-Jun increased the transcriptional activity about 4-fold in the wild type promoter but only 1-fold in the CRE mutated promoter (Fig. 6B). This indicates that CRE element is required for c-Jun activity on the gene promoter. The residual promoter activity after CRE mutation might be contributed by an AP-1 binding site in the TNF-α gene promoter, which remained functional in the CRE-mutated promoter.

Inhibition of c-Jun Activity by p38 and ERK Inhibitors—MAP kinase inhibitors SB203580 and PD98059 decreased TNF-α production in response to glass fibers (Fig. 1B). The promoter response represents the end result of inhibitor effects on sequential reactions of the signal transduction pathway controlling TNF-α production. Because protein binding activities of the AP-1 and CRE elements are controlled by MAP kinases, p38 and MEK inhibitors should decrease protein binding activities of the two elements, leading to inhibition of the TNF-α promoter. To this end, effects of MAP kinase inhibitors were examined on phosphorylation of ERK, protein binding activities of AP-1 and CRE elements, and activity of the TNF-α promoter. The phosphorylation status of ERK was determined by Western blot (Fig. 7A). The results demonstrate that PD98059 specifically inhibited ERK phosphorylation (Fig. 7, A and B). Consistent with these results, protein binding activities of the AP-1 and CRE elements were decreased in the nuclear extracts from cells treated with SB203580 or PD98059 (Fig. 7, C and D). These results are in line with data that the wild type TNF-α gene promoter was inhibited by the two MAP kinase inhibitors (Fig. 3B). Together, these results strongly support that glass fibers activate MAP kinase pathways that, in turn, lead to initiation of TNF-α transcription through induction of c-Jun DNA binding.

DISCUSSION
Activation of MAP kinases by glass fibers might be due to phagocytosis by macrophages. Our previous studies have shown that macrophages could engulf glass fibers (4, 5). This phagocytosis is influenced by fiber length. Short glass fibers (7 μm) were completely engulfed, whereas long fibers were only partially engulfed by macrophages. Macrophages generate ROS upon engulfment of short fibers. It is likely that macrophage would generate more ROS during frustrated phagocytosis of long fibers. Although ROS may activate all three MAP kinases, p38, ERK, and JNK, the pattern of activation is dependent on the stimulant (16, 17). For example, we have observed that silica particles only induced activation of p38 and ERK, but not JNK (23). The present study demonstrates that...
p38 and ERK are the main MAP kinases activated by glass fibers. Our study suggests that c-Jun mediates the downstream signal of p38 and ERK. Although JNK has been proposed as a c-Jun kinase, we did not observe significant activation of JNK in rat alveolar macrophages exposed to glass fibers. In this study, activation of p38 and ERK was associated with an enhanced c-Jun DNA binding activity at both CRE and AP-1 sites, and suppression of p38 and ERK led to a decreased DNA binding of c-Jun. These results suggest that p38 and ERK MAP kinases may play a role in regulation of c-Jun activity, although it is not known how p38 or ERK phosphorylate c-Jun. Our observations support the view that p38 is required for c-Jun activity in the activation of TNF-α gene promoter (28). One possibility is that p38 induces c-Jun expression (29, 30).

The CRE element is required for induction of TNF-α transcription by several stimuli in various types of cells. In T cells, CRE is activated by the T cell receptor signal induced by phorbol 12-myristate 13-acetate or ionomycin (12, 15). In B cells, CRE is activated by CD40 ligation (13). In macrophages, CRE is activated by lipopolysaccharide (LPS) (14). In fibroblast cells, CRE can be activated by the cytokine TNF-α (28). Activity of the CRE element is mediated by different transcription factors in distinct cell types. In the T, B, and fibroblast cells, the nuclear factors c-Jun and ATF-2 have been reported as the major binding proteins of CRE (12, 13, 28). In macrophages, the binding proteins have been reported to be c-Jun and CREB1 (14). Consistent with these conclusions, CREB-binding protein is required for transcriptional induction of TNF-α by T cell receptor (31). CREB-binding protein, a homolog of p300 (32), is a transcriptional adaptor that integrates signals from many sequence-specific activators including c-Jun, ATF-2, and CREB via direct protein-protein interactions (33). It is known that CREB-binding protein/p300 is a histone acetyltransferase (34).

The present study provides evidence that CRE was required for TNF-α transcription induced by glass fibers. This evidence includes: (a) Protein binding at the CRE element was associated with fiber-induced TNF-α production (Figs. 1B and 7). Nuclear protein c-Jun was the major CRE-binding protein in rat alveolar macrophages. An increase or decrease in c-Jun binding activity led to activation or inhibition of TNF-α transcription. When c-Jun binding was induced by glass fibers (Figs. 4C and 5), TNF-α production and promoter activity were increased (Figs. 1A and 3A). When c-Jun binding was decreased by MAP kinase inhibitors (Fig. 7, C and D), glass fiber-induced TNF-α production and the promoter activity were blocked (Figs. 1B and 3B). (b) The CRE element is required for TNF-α promoter activation by glass fibers (Fig. 6). A mutation that abolished protein binding at the CRE element resulted in a loss of promoter response to glass fibers, indicating that glass fibers may regulate TNF-α transcription through the CRE element. (c) The CRE element is required for the c-Jun effect on the TNF-α gene promoter. Cotransfection of a c-Jun expression vector led to a dramatic increase in transcription activity in the wild type promoter but only weak transcription activity in the CRE mutated-promoter (Fig. 6B). This suggests that the CRE element is the major trans-acting site of nuclear factor c-Jun in the TNF-α gene promoter. The AP-1 binding site may be a weak enhancer in the promoter. The above evidence strongly supports the hypothesis that glass fibers activate the TNF-α gene promoter through induction of DNA binding activity of c-Jun at the CRE element in rat alveolar macrophages. This is consistent with c-Jun activity in human monocyte/macrophage U937 and THP-1 cells, in which c-Jun activates the TNF-α CRE element in response to stimulation by TNF-α or LPS (14, 35).

Characterization of DNA-binding proteins of the CRE element suggests that, in addition to c-Jun, nuclear factor CREB1 also binds to CRE. CREB1 was associated with the CRE element in untreated alveolar macrophages (Fig. 5A). CREB1 binding activity was reduced by glass fiber exposure, and this change was associated with an increased binding of c-Jun (Fig. 5B). This indicates that c-Jun is responsible for activation of the CRE element and that CREB1 may not be important in the glass fiber signaling pathway or might be a repressor protein. This is different from CREB1 activity in the LPS signal pathway, which induces DNA binding of CREB1 (14). Our data suggest that ATF-2 does not bind to the CRE element in rat alveolar macrophages. This result is in line with findings reported in human macrophages (14). Taken together, these results suggest that: (a) the signaling pathways of glass fibers and LPS are distinct and (b) the regulatory mechanisms of TNF-α transcription in macrophages and T cells are different. ATF-2 does not bind to the TNF-CRE element in macrophages.

The AP-1-binding site (−65/−59) in the TNF-α gene promoter is another enhancer where c-Jun can regulate transcription (11). The importance of this AP-1-binding site was verified by mutation studies on the promoter. Deletion of the AP-1 site significantly reduced both basal and phorbol 12-myristate 13-acetate-activated TNF-α promoter activity in U937 cells (11) but only marginally decreased LPS-induced TNF-α promoter activity (14). In the present study, c-Jun was able to induce a 1-fold increase in the TNF-α promoter with a mutated CRE element (Fig. 6B). Because the AP-1 binding site remains intact in the a CRE-mutated promoter, the 1-fold induction might be a result of activation of the AP-1 binding site by c-Jun. Glass fibers induce AP-1 binding activity in rat alveolar macrophages (Fig. 4A), and this is associated with a detectable response of CRE-mutated promoter to glass fibers (Fig. 6A). These data indicate that the AP-1 site is involved in the response to c-Jun
when rat macrophages were exposed to glass fibers. The CRE and AP-1 sites may have a functional synergy in activation of TNF-α transcription. Although it has been reported that in the TNF-α promoter, CRE synergizes with the NF-κB binding site (κ3 site) (10, 14), it is not clear whether CRE synergizes with the AP-1 site. AP-1 is a heterodimer formed by two subunit proteins, c-Jun and c-Fos in most cases. Dimerization of the two subunits is required for stable DNA binding activity. Expression of the c-Fos gene is controlled by the serum response element. MAP kinases, including p38 kinase, ERK, and JNK, are able to induce c-Fos expression through serum response element (18–20). Therefore, inhibition of p38, ERK or JNK would lead to an inhibition of AP-1 activity. In this study, p38 and ERK inhibitors both reduced AP-1 DNA binding activity (Fig. 7C). The inhibition may result from two effects: (a) inhibition of c-Fos expression that may explain effect of ERK inhibition and (b) inhibition of phosphorylation of c-Jun protein, which decreases c-Jun activities such as the DNA binding activity and trans-activating activity at the AP-1 binding site. If the AP-1 element synergizes with the CRE element, inhibition of either element would lead to a dramatic transcriptional inhibition. This may explain the inhibitory effect of the MAP kinase inhibitors.

In summary, we observed that glass fibers are able to induce TNF-α production in rat alveolar macrophages. In combination with our previous findings, we conclude that this induction may be dependent on at least two signaling pathways: NF-κB and MAP kinase. The present study demonstrated that glass fibers activate p38 and ERK and in turn activate TNF-α transcription. The nuclear factor, c-Jun, mediates MAP kinase signals and induces TNF-α transcription through two promoter elements, CRE and AP-1. Long fibers are more potent than short fibers in the activation of MAP kinase, c-Jun DNA binding, and TNF-α transcription.

Acknowledgments—We greatly appreciate technical assistance from Dr. Dale Porter and Mark Barger. We thank Dr. Val Vallyathan and Dr. Murali Rao for a critical review of the manuscript.

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