Molecular and Cellular Biochemistry **234/235:** 161–168, 2002.

Involvement of hydrogen peroxide in asbestos-induced NFAT activation

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Abstract

The present study investigated the role of reactive oxygen species (ROS) in activation of nuclear factor of activated T cells (NFAT), a pivotal transcription factor responsible for regulation of cytokines, by asbestos in mouse embryo fibroblast PW cells. Exposure of cells to asbestos led to the transactivation of NFAT in a time- and dose-dependent manner. Scavenging of asbestos-induced H_2O_2 with N-acety-L-cyteine (NAC, a general antioxidant) or catalase (a specific H_2O_2 inhibitor) resulted in inhibition of NFAT activation. In contrast, an increase in H_2O_2 generation by the addition of superoxide dismutase (SOD) slightly enhanced asbestos-induced NFAT activation. In addition, pretreatment of cells with sodium formate did not exhibit any inhibition of NFAT activity induced by asbestos. These results demonstrated that H_2O_2 appeared to play an important role in asbestos-induced NFAT transactivation. Furthermore, it was observed that incubation of cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) not only resulted in NFAT activation by itself, but also enhanced asbestos-induced NFAT induction. Pretreatment of cells with cyclosporin A (CSA), a pharmacological inhibitor of the phosphatase calcineurin, blocked both asbestos- and TPA plus asbestos-induced NFAT activation. These data suggest that asbestos is able to induce NFAT activation through H_2O_2-dependent and CSA-sensitive pathways, which may be involved in asbestos-induced carcinogenesis. (Mol Cell Biochem **234/235**: 161–168, 2002)

Key words: reactive oxygen species, NFAT, asbestos

Abbreviations: NFAT – nuclear factor of activated T cells; UV – ultraviolet; AP-1 – activated protein-1; TPA – 12-*O*-tetradecanoylphorbol-13-acetate; PKC – protein kinase C; NAC – N-acety-L-cyteine; NADPH – β-nicotinamide adenine dinucleotide phosphate; SOD – superoxide dismutase; FBS – fetal bovine serum; MEM – Eagle's minimal essential medium; ROS – reactive oxygen species

Introduction

Asbestos is a common environmental contaminant. Exposure to asbestos is associated with the development of pulmonary fibrosis, pleural disease and malignancies such as lung cancer [1]. Extensive investigations over the last two decades have revealed some of pathogenic mechanisms of asbestos-related pulmonary diseases [2–4]. However, no single mechanism can fully account for all the complex biological abnormalities. Considerable evidence suggests that free radicals, especially ROS, play an essential role in some biological effects caused by asbestos [5]. Asbestos-mediated ROS can damage various cellular components, cause DNA strand breaks, protein modification, and lipid peroxidation [6]. ROS can also modify cellular function by stimulating signal transduction cascades. Recent studies have revealed that ROS are implicated in activation of nuclear transcription factors (such as NFκB, AP-1), expression of pro-inflammatory cytokines and activation of MAPK and PKC [7–11].

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The nuclear factor of activated T cells (NFAT) was originally described as a transcriptional factor expressed in activated but not resting T cells [12–15]. The induction of NFAT in T cells required a calcium-activated signaling pathway and was blocked by cyclosporin A (CsA) and FK506 [16–22]. Over the last decade, studies from several laboratories have indicated that the pre-existing/cytoplasmic component of NFAT was a mixture of proteins belonging to a novel family of transcription factors [23–25]. The first member of this family (NFATp, later renamed $NFAT_1$) was purified from cytoplasmic extracts of a murine T cell cloned by affinity chromatography using the distal NFAT site of the murine IL-2 promoter [20, 26] and cloned from murine (Ar-5) and human (Jurkat) T cell cDNA libraries [26, 27]. Other distinct proteins belonging to the same family, such as NFATc, $NFAT_3$ and $NFAT_4$, were also isolated and cloned [28–31]. There are three functional domains in NFAT family proteins: the Rel-similarity domain (RSD), which is responsible for DNA-binding activity and interaction with AP-1; the NFAT-homology region (NHR), which regulates intracellular localization; and the transcriptional activation domain (TAD) [32]. The activation of NFAT in T cells includes dephosphorylation, nuclear translocation and an increase in affinity for DNA binding [16]. Stimuli that elicit calcium mobilization result in rapid dephosphorylation of NFAT proteins and their translocation to the nucleus. These dephosphorylated proteins show increased affinity for DNA binding [16].

Growing evidence indicates that NFAT is not only a T cell-specific transcriptional factor, but also is expressed in a variety of lymphoid cells and in non-lymphoid tissue [16, 33, 34]. The involvement of NFAT in various responses in the tissues other than lymphoid cells is reasonably well established for the production of IL-1, IL-2, IL-3, IL-4, IL-5, Il-6, IL-8, IL-10, IL-13, IL-18, TNF-α, IFN-γ, and GM-CSF in a variety of cell types [16]. NFAT is also believed to play an important role in cancer development [35, 36]. Since the asbestos is a potent carcinogen, the objective of the present study was to determine if activation of NFAT occurred in the cellular response to asbestos and if so to investigate the molecular mechanism by which asbestos leads to NFAT activation.

Materials and methods

Reagents

Crocidolite asbestos was obtained from NIEHS. This asbestos, originally was from Kalahari desert, South Africa, had a median fiber length of 10 μm, diameter of 0.21 μm, surface area of 9.8 m^2/g. TPA, N-acety-L-cyteine (NAC), superoxide dismutase (SOD), catalase and sodium formate were purchased from Sigma (St. Louis, MO, USA); luciferase assay substrate was obtained from Promega (Madison, WI, USA); fetal bovine serum (FBS), Eagle's minimal essential medium (MEM), and Dulbecco's modified Eagle's medium (DMEM) were from BioWhittaker (Walkersville, MD, USA). Cyclosporin A (CSA) was purchased from Alexis Biochemicals (San Diego, CA, USA).

Cell culture

Mouse embryo fibroblast PW cells and its transfectant, PW NFAT $mass_1$, were cultured in DMEM with 10% FBS, 2 mM L-glutamine and 25 mg of gentamicin/ml [37].

Assay for NFAT activity in vitro

Confluent monolayers of PW NFAT $mass_1$ were trypsinized, and 5×10^3 viable cells suspended in 100 μl of medium were added into each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then exposed to asbestos for the indicated times and doses. The cells were extracted with lysis buffer and luciferase activity was measured as previously described [37]. The results were expressed as NFAT activity relative to control [37].

Statistical analysis

The significance of the difference in the NFAT activities was determined with the ANOVA test. The results are expressed as mean ± S.D.

Results

Induction of NFAT transactivation in PW cells by asbestos

To study the regulation of NFAT transcription activity in cellular response to asbestos, we used a well characterized NFAT-luciferase reporter stable transfectant, PW NFAT mass1 [37]. The results showed that exposure of PW cells to asbestos resulted in marked NFAT activation, which is at similar level induced by vanadate (Fig. 1). This NFAT activation by asbestos appeared to be time- and dose-dependent (Figs 1b and 1c). The maximum induction of NFAT activity by asbestos occurred nearly 24 h after cells were exposed to asbestos (Fig. 1b). These results demonstrate that asbestos is able to stimulate NFAT activation in PW cells.

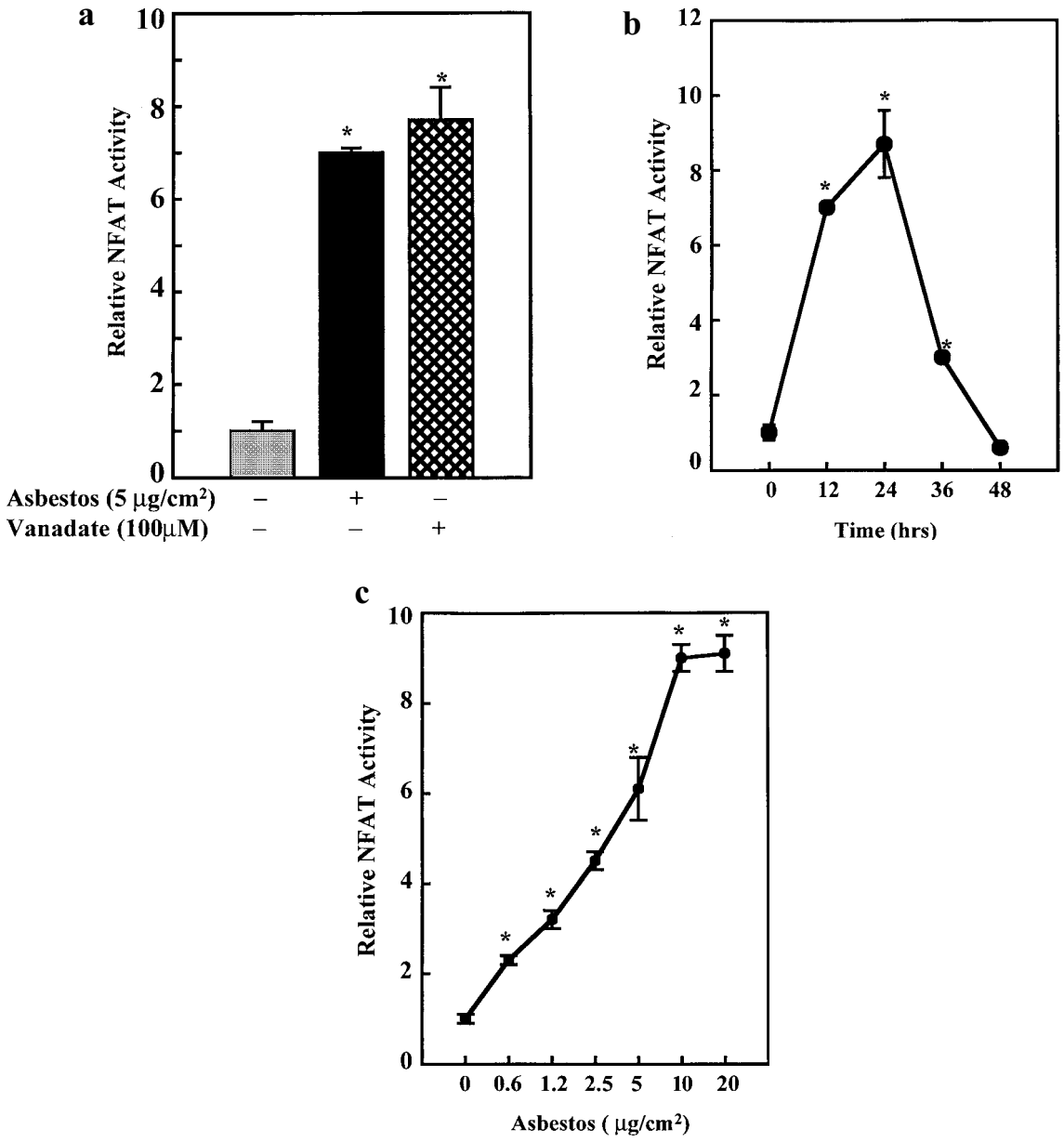

Fig. 1. Induction of NFAT-dependent transcription by asbestos in PW cells. 8×10^3 cells of PW NFAT mass$_1$ were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were treated with (a) asbestos (5 μg/cm^2) or vanadate (100 μM) for 24 h; (b) for a time course study, the cells were exposed to 5 μg/cm^2 of asbestos for various times; (c) for a dose-response study, the cells were exposed to different concentrations of asbestos as indicated for 24 h. Then, NFAT activity was determined by the luciferase activity assay. The results are presented as NFAT-dependent transcription activity relative to control. Each bar indicates the mean and standard deviation of four repeat assay wells. *Indicates a significant increase from control ($p < 0.05$).

Co-incubation of cells with TPA enhances asbestos-induced NFAT activation

TPA is a strong typical chemical tumor promoter [38]. To investigate possible interactive effects of TPA on asbestos-induced NFAT activation, asbestos and TPA were co-incubated with PW NFAT mass$_1$ cells. The results showed that asbestos alone increased NFAT induction by 4.2 fold in PW cells (Fig. 2). TPA not only caused a 7.1 fold increase in NFAT activity by itself (Fig. 2), but also had a additive effect on asbestos-induced NFAT activity (Fig. 2). These effects occurred in time-dependent manner (Fig. 2). The maximum induction of NFAT activity by asbestos plus TPA occurred at 12 h after exposure (Fig. 2b).

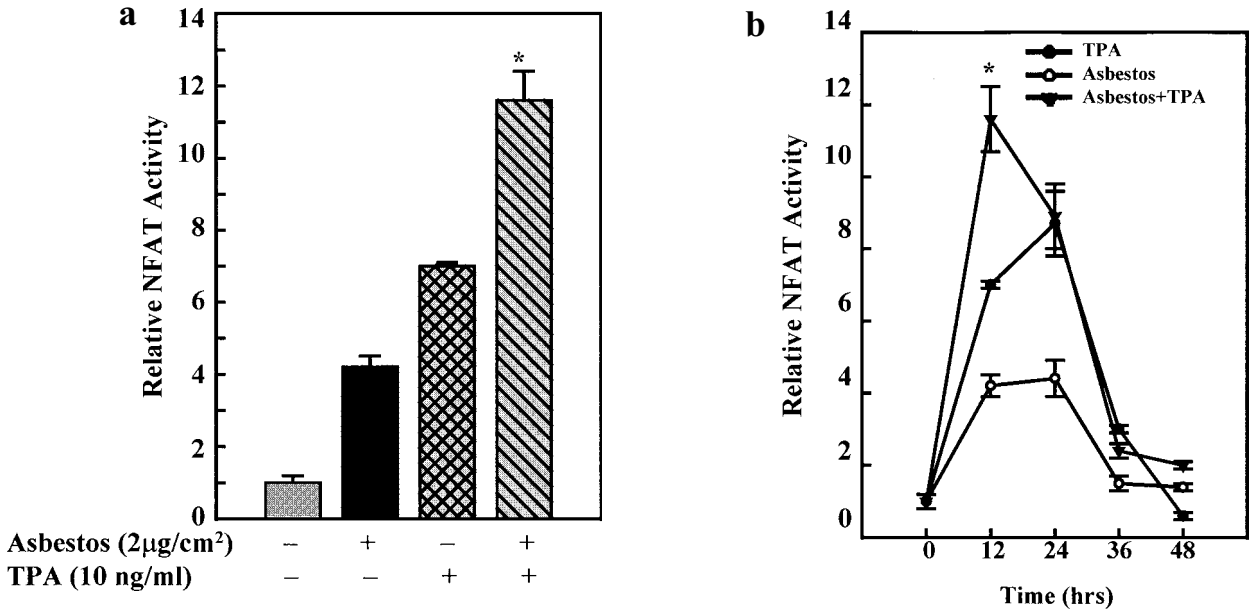

Fig. 2. TPA enhances Asbestos-induced NFAT activity. 8×10^3 cells of PW NFAT mass$_1$ were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were exposed to (a), asbestos (2 μg/cm^2), TPA (10 ng/ml), or asbestos (2 μg/cm^2) plus TPA (10 ng/ml) for 24 h; (b), asbestos (2 μg/cm^2), TPA (10 ng/ml), or asbestos (2 μg/cm^2) plus TPA (10 ng/ml) for various time indicated. Then, NFAT activity was determined by the luciferase activity assay. The results are presented as NFAT-dependent transcription activity relative to control. Each bar indicates the mean and standard deviation of four repeat assay wells. *Indicates a significant increase from TPA and asbestos ($p < 0.05$).

Induction of NFAT transactivation by asbestos or TPA+asbestos is through a cyclosporin A (CSA)-dependent pathway

Previous studies demonstrated that in T cells the major NFAT activation pathway appears to involve in a calcium/calmodulin-dependent phosphatase, calcineurin [15, 16]. To test the role of calcineurin in asbestos-induced NFAT-dependent transcription activity in PW cells, CSA, a widely used pharmacological inhibitor of calcineurin, was used. Pretreatment of cells with CSA resulted in a dramatic (95%) inhibition of NFAT transactivation induced by asbestos and an 84% inhibition of the induction by TPA plus asbestos ($p < 0.05$) (Fig. 3). This inhibition was observed in all time points studied (Fig. 4). These data suggest that activation of calcineurin is required for asbestos-induced or asbestos+TPA-induced NFAT activation, suggesting that asbestos activates the NFAT transcription activity in mouse embryo fibroblasts through a pathway that is similar to that in T cells.

Hydrogen peroxide is involved in NFAT activation by asbestos

Previous studies have indicated that reactive oxygen species (ROS) are involved in asbestos-induced biological activities [42]. If NFAT activation is responsible for some of the biological effects caused by asbestos, ROS generation may play a role in asbestos-induced NFAT activation. To test this hypothesis, the N-acety-L-cyteine (NAC, a general antioxidant) was used to pretreat cells before exposure to asbestos. NAC pretreatment blocked asbestos-induced NFAT activation (Fig. 5), revealing that ROS may be involved in asbestos-induced NFAT activation. To further clarify which ROS play a major role in NFAT activation, catalase, superoxide dismutase (SOD) and sodium formate were used. Pre-incubation of cells with sodium formate (an $^{\bullet}OH$ radical scavenger) did not exhibit any inhibitory effects on asbestos-induced NFAT activation (Fig. 5). In contrast, addition of catalase (a specific scavenger of H_2O_2) impaired asbestos-induced NFAT activity totally, while SOD (a specific $O_2^{\bullet-}$ scavenger) slightly enhanced asbestos-induced NFAT activity (Fig. 5). These observations indicate that H_2O_2 plays an essential role in asbestos-induced NFAT activation.

Discussion

Increasing evidence indicates that ROS is involved in asbestos-induced cellular damage [5, 6]. There are at least two principal mechanisms for asbestos-mediated ROS production. The first involves the iron content of the fiber augmenting ·OH formation through iron catalyzed reactions ($H_2O_2 + Fe^{2+} = Fe^{3+} + {}^{\bullet}OH + OH^-$) [39]. The second mechanism im-

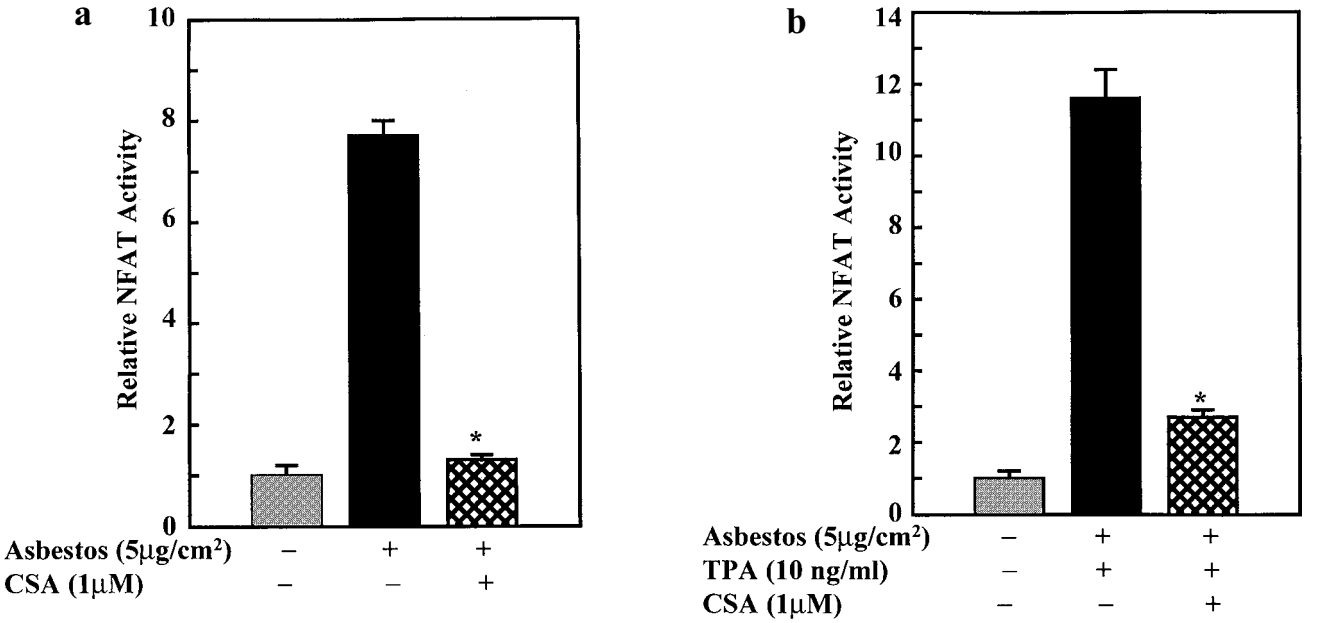

Fig. 3. Blocking asbestos- or asbestos plus TPA-induced NFAT activation by CSA. PW NFAT mass$_1$ were seeded to each well of 96-well plates and cultured until 90% confluent. The cells were then treated with CSA (1 μM) for 30 min and sequentially were exposed to (a), asbestos (5 μg/cm^2) or (b), asbestos (5 μg/cm^2) plus TPA (10 ng/ml). After being cultured for 24 h, NFAT activity was determined by the luciferase activity assay. The results are presented as NFAT-dependent transcription activity relative to control. Each bar indicates the mean and S.D. of assays from triplicate wells. *Indicates a significant decrease from asbestos alone (a) or asbestos plus TPA (b), respectively ($p < 0.05$).

plicates the release of ROS upon activation of inflammatory cells such pulmonary alveolar macrophages and neutrophils [40]. Pulmonary epithethial cells, mesothelial cells and fibroblasts are all susceptible to the toxicity of asbestos [41]. The molecular targets of asbestos and its second messengers, ROS, include critical biological macromolecules such as li-

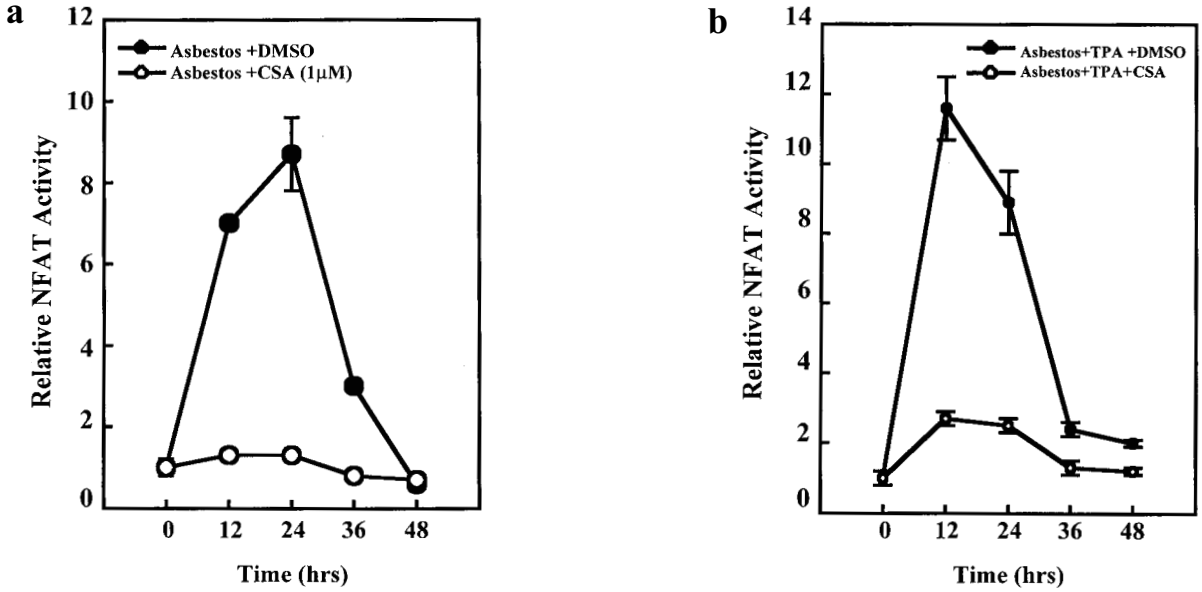

Fig. 4. Time course studies of inhibitory effects of CSA on asbestos- or asbestos plus TPA-induced NFAT activity. PW NFAT mass$_1$ were seeded to each well of 96-well plates and cultured until 90% confluent. The cells were then treated with CSA (1 μM) for 30 min and sequentially were exposed to (a) asbestos (5 μg/cm^2) or (b) asbestos (5 μg/cm^2) plus TPA (10 ng/ml). The NFAT activity was determined by the luciferase activity assay at various times indicated. Each bar indicates the mean and S.D. of assays from triplicate wells.

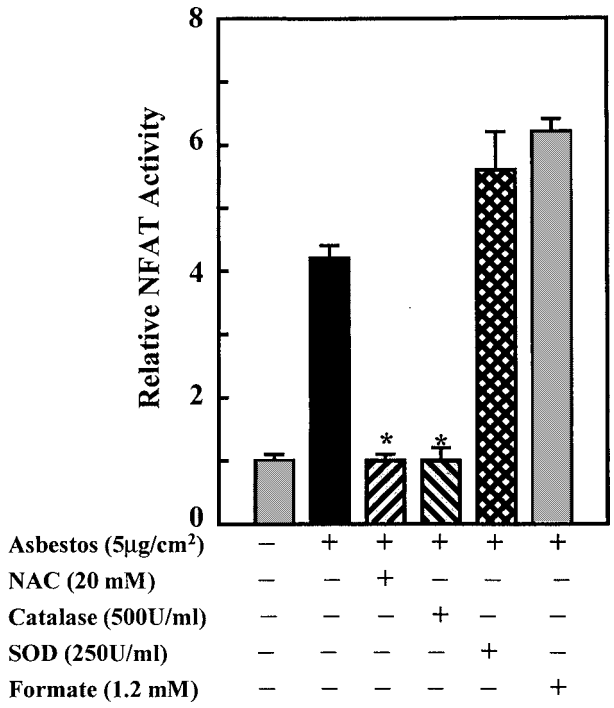

Fig. 5. Effects of free radical modifiers on NFAT activation by asbestos. PW NFAT mass$_1$ cells suspended in 10% FBS DMEM were added to each well of 96-well plates and cultured overnight. The cells were pretreated with different free radical modifiers at the concentrations indicated. The cells were then exposed to asbestos (5 μg/cm^2) for 24 h. The NFAT activity was determined by the luciferase activity assay. The results are presented as relative NFAT activity. Each column and bar indicates the mean and S.D. from triplicate assays. *Indicates a significant decrease from asbestos alone ($p < 0.05$).

pid membranes, DNA and signal transduction protein kinases [5, 6, 42]. Asbestos-mediated lipid peroxidation is one of mechanism by which asbestos modifies cell membrane structure and function [5]. ROS can also cause cellular toxicity by damaging DNA, via altered DNA bases, DNA-SB formation, chromosal aberration and sister chromotid exchange, and modify cellular function by stimulating signal transduction cascades [43, 44]. Previous studies revealed that H_2O_2 can rapidly increase in membrane bound tyrosine kinase (TK) and activate protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) signaling pathways, which subsequently can activate transcription factors, such as AP-1 and NFkB [10, 45]. Asbestos-mediated ROS generation also acts as a tumor promoter to augment cellular proliferation, which is considered to play important role in the development of malignancy [46]. The expression of cytokines and growth factors modulated by ROS is also an important mechanism for asbestos-induced pulmonary toxicity. Accumulating evidence suggests that TNF-α and transforming growth factor β (TGFβ) stimulated by ROS are involved in asbestos-induced pulmonary toxicity [9, 42]. The present study found that pretreatment of cells with NAC or catalase blocked asbestos-induced NFAT activation, while pre-incubation of cells with sodium formate or SOD did not exhibit any inhibitory effects on asbestos-induced NFAT activation. These results demonstrate that H_2O_2 is involved in asbestos-induced NFAT activation. It might be noted that pre-incubation of cells with sodium formate slightly increased in asbestos-induced NFAT activity (Fig. 5), suggesting that ·OH radical may mediate an inhibitory pathway by which regulates NFAT activation at certain level.

Five different members of the NFAT family have been identified. The classical members of this family comprise NFAT1 (also known as NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), and NFAT4 (NFATx or NFATc3). Recently, a novel NFAT isoform with special characteristics was cloned and named NFAT5 [47]. All five NFAT members share a Rel-like homology region and recognize similar promoter regions of targeted genes [32]. NFAT isoforms are expressed in different tissues. It has been reported that NFAT$_1$ and NFAT$_2$ mRNAs have been detected in brain, heart, skeletal muscle, testis, placenta, pancreas, small intestine, prostate, colon, skin tumors, as well as in lung [16, 33]. NFAT expression or NFAT-derived transactivation has also been described in several types of nonlymphoid cells, including mast, endothetial, neuronal, vascular smooth muscle and liver-derived Chang (CHL) cells [16]. In this study, exposure of mouse embryo fibroblast PW cells to asbestos can induce NFAT expression. Taken together with our previous observation that vanadium also induces NFAT expression in PW cells [37], it is demonstrated that NFAT is expressed in embryo fibroblasts.

Investigation of signal transduction pathways leading to activation of transcription factors is one of major tasks for understanding the molecular mechanisms by which carcinogens induce cancer development. NFAT is regulated tightly in response to elevations of both intracellular calcium ion (Ca^{2+}) and diacylglycerol following activation of phospholipase C (PLC) in T cells, [16]. Increased intracellular calcium stimulates the activation of calmodulin (16). It is believed that binding of calmodulin to a region near the c-terminus of calcineurin displaces the auto-inhibiting domain and exposes the calcineurin active site [16]. Activated calcineurin subsequently dephosphorylates the cytoplasmic NFAT proteins, leading to NFAT nuclear translocation [16, 17, 48]. It has also been reported that phosphorylation of NFAT is regulated by several protein kinases, including GSK$_3$ and JNK$_2$ [16, 49–51]. The results obtained from the present investigation showed that asbestos alone induced an increase of NFAT activity. Pretreatment of cells with CSA resulted in impairment of NFAT transactivation induced by asbestos. These data demonstrate that calcineurin activation is required for asbestos-induced NFAT transactivation. In addition, co-stimulation studies showed that TPA augmented the NFAT-

mediated transcription in response to asbestos, resulting in an additive effect. The enhancement of asbestos-induced NFAT activity by TPA may be through activation of the PLC pathway. It has been reported that treatment of mouse splenocytes by CSA also leads to a change in mitochondrial transmembrane potential ($\Delta\Psi_m$) [52]. Thus, calcineurin-dependent pathway may not be the only mechanism involved in inhibition of asbestos-induced NFAT activity by CSA.

Asbestos-containing compounds exert potent toxic and carcinogenic effects, such as cell transformation [5, 6]. Previous studies have indicated that expression of IL-8, TNF-α and other cytokines is associated with the initiation and control of effective immune and inflammatory responses as well as cancer development [35, 36, 50]. NFAT is a transcription factor, which has been reported to play an essential role in regulation of these cytokines [16, 50]. Therefore, we hypothesize that NFAT is involved in asbestos-induced inflammation and may subsequently be involved in the carcinogenic effects of asbestos.

In conclusion, the data from present study demonstrate that NFAT is expressed in embryonic fibroblast cells, and asbestos is able to induce NFAT activation in these PW cells. H_2O_2 appear to be the mediator for asbestos-induced NFAT activation through CSA-sensitive pathways. These findings may elucidate the molecular mechanisms of carcinogenicity by asbestos.

Acknowledgements

This research was partially supported by grant numbers ES00260 and CA13687 from the NIH.

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