

## 【S-5】

**Particle Toxicity; From Coal Dust to Nanoparticle**

Young Lim

*Department of Occupational & Environmental Medicine, St. Mary Hospital,  
The Catholic University of Korea, Seoul, Korea*

Health effect of particles was a big concern because it had been historically closely connected to industrial activities or materials, such as coal, asbestos, man-made mineral fibers, and ambient particulate matter. Recent debate on the issue of nanoparticle exposure and possible health effect is acceptable. Differences in size, shape, surface area, chemical composition and biopersistence require that the possible environmental and health impact be assessed for each type of nanomaterial because similar compounds may induce substantially different health effects. It has been recently shown not only that inhaled ultrafine particles exert respiratory effects, but that they may also translocate from the lung into the systemic circulation and this may result in cardiovascular and other extrapulmonary effects. We evaluated the characteristics of ultrafine particles (Tab.1) including silica, titanium dioxide, DEP and metal nanoparticles and their cytotoxicity (Fig.1). The toxicity of particles is dependent on the physical and chemical activity in addition to the biological feature. Also, this experiment is just the beginning and all the particles should be comprehensively assessed for the prevention of health effect.

Table1. Analysis of transition metals.

	Units: ppm									
	Co	Cr	Cu	Fe	Mn	Ni	Sc	Ti	V	Zn
TiO <sub>2</sub>	<5	<5	10	<50	<2	<5	<2	595,000	5	<5
DEP	<2	30	6	5600	22	14	1	10	5	12
SRM2975	<20	50	100	300	<10	<50	<5	50	20	400
Mini_U_silica	<2	4	<2	250	5	<2	<1	<100	<5	2

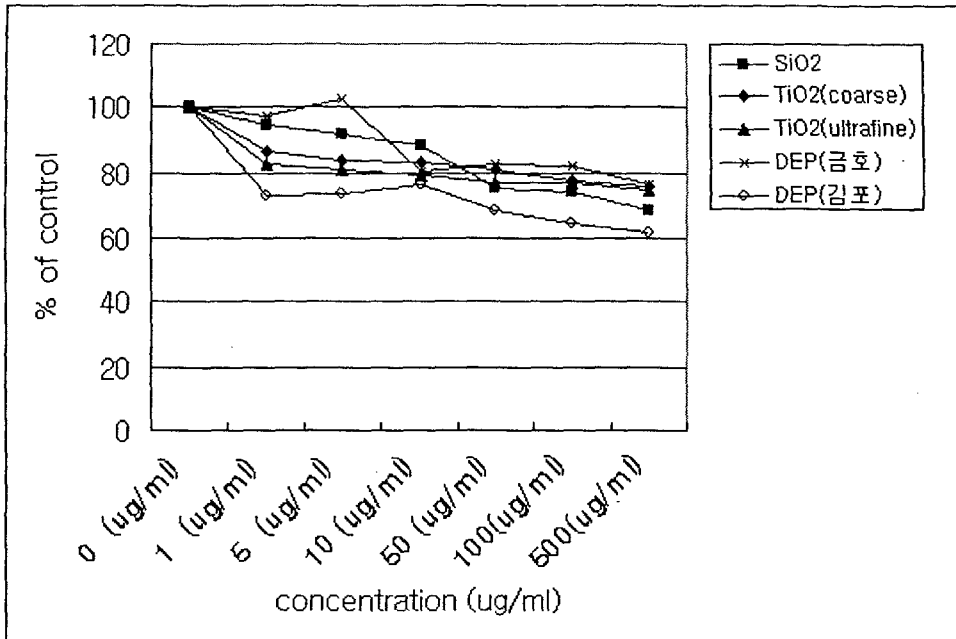


Fig.1. cytotoxicity of SiO<sub>2</sub>, TiO<sub>2</sub>(coarse size), TiO<sub>2</sub>(ultrafine size), F-DEP, NF-DEP using MTS assay in A549 cells.



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## Induction of nuclear factor- $\kappa$ B activation through TAK1 and NIK by diesel exhaust particles in L2 cell lines

Young-Pil Yun<sup>a</sup>, Jin-Deok Joo<sup>b</sup>, Joo-Yong Lee<sup>c</sup>, Hae-Yun Nam<sup>a</sup>, Young-Hoon Kim<sup>c</sup>,  
Kweon-Haeng Lee<sup>c,d</sup>, Cheol-Soo Lim<sup>e</sup>, Hyung-Jung Kim<sup>f</sup>,  
Yong-Gul Lim<sup>b</sup>, Young Lim<sup>a,\*</sup>

<sup>a</sup> Department of Occupational and Environmental Medicine, St. Mary's Hospital, The Catholic University of Korea, 62 Youido-dong, Youngdunpo-gu, Seoul 150-713, Republic of Korea

<sup>b</sup> Department of Anesthesiology, Saint Vincent's Hospital, The Catholic University of Korea, 93-6 Ji-dong, Paldal-gu, Suwon, Gyeonggi-Do, Republic of Korea

<sup>c</sup> Catholic Neuroscience Center, The Catholic University of Korea, Seoul 137-701, Republic of Korea

<sup>d</sup> Department of Pharmacology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea

<sup>e</sup> Environmental Research Complex, Kyungseo-dong, Seo-gu, Incheon 404-170, Republic of Korea

<sup>f</sup> Department of Internal Medicine, College of Medicine, Yonsei University, Seoul 120-7522, Republic of Korea

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### Abstract

Diesel exhaust particles (DEPs) are known to induce allergic responses in airway epithelial cells, such as the production of various cytokines via nuclear factor-kappa B (NF- $\kappa$ B). However, the intracellular signal transduction pathways underlying this phenomenon have not been fully examined. This study showed that DEP induced NF- $\kappa$ B activity via transforming growth factor- $\beta$  activated kinase 1 (TAK1) and NF- $\kappa$ B-inducing kinase (NIK) in L2 rat lung epithelial cells. DEP induced the NF- $\kappa$ B dependent reporter activity approximately two- to three-fold in L2 cells. However, this effect was abolished by the expression of the dominant negative forms of TAK1 or NIK. Furthermore, it was shown that DEP induced TAK1 phosphorylation in the L2 cells. These results suggest that TAK1 and NIK are important mediators of DEP-induced NF- $\kappa$ B activation.

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**Keywords:** DEP; NF- $\kappa$ B; TAK1; NIK; L2 cells

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**Abbreviations:** NF- $\kappa$ B, nuclear factor-kappa B; TAK1, transforming growth factor- $\kappa$  activated kinase 1; NIK, NF- $\kappa$ B-inducing kinase; IKK, I $\kappa$ B kinase; MAPKKK, mitogen-activated protein kinase kinase kinase

\* Corresponding author. Tel.: +82 2 3779 1401; fax: +82 2 782 6017.

E-mail address: [nlim@catholic.ac.kr](mailto:nlim@catholic.ac.kr) (Y. Lim).

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## 1. Introduction

Diesel exhaust particles (DEP) from diesel engine powered cars and trucks, which contain a broad spectrum of polycyclic aromatic hydrocarbons (PAH), are common atmospheric pollutants. Controlled human exposure studies have shown that DEP induces an acute inflammatory response in the airways and exert adverse effects on the pulmonary functions (Nel et al., 1998; Casillas et al., 1999). Inflammatory reactions stimulated by the inhalation of DEP are associated with bronchial hyper-responsiveness and the infiltration of eosinophils, neutrophils, macrophages and lymphocytes (Salvi et al., 1999). The biological responses to DEP appear to be mediated via the increased local production of cytokines and chemokines (Devalia et al., 1997; Diaz-Sanchez, 2000).

NF- $\kappa$ B represents a family of eukaryotic transcription factors that participate in regulating the immune response, cell growth and survival (Ghosh and Karin, 2002; Okamoto et al., 1997). In most cells, NF- $\kappa$ B for the most part is restricted in the cytoplasm by the inhibitory protein of I $\kappa$ B. However, I $\kappa$ B becomes phosphorylated by the I $\kappa$ B kinase (IKK) complex on two serine residues in the N-terminal region in response to proinflammatory cytokines, such as TNF (tumor necrosis factor) and IL-1 $\beta$  (Interleukin-1 $\beta$ ) (Traenckner et al., 1995). I $\kappa$ B phosphorylation results in its ubiquitination and proteolysis by the 26S proteasome, which allows the liberated NF- $\kappa$ B to translocate to the nucleus (Ghosh and Karin, 2002; Okamoto et al., 1997). The IKK complex consists of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit, IKK $\gamma$  (DiDonato et al., 1997; Zandi et al., 1997; Rothwarf et al., 1998). The kinase activity of both IKK $\alpha$  and IKK $\beta$  is induced by a wide variety of NF- $\kappa$ B inducers, such as TNF or IL-1 $\beta$  via the upstream kinases including the NIK and the extracellular signal-regulated kinases (Nakano et al., 1998; Ling et al., 1998; Lee et al., 1998; Yang et al., 2001). NIK was originally identified as a protein interacting with the TNF receptor-associated factor 2 component of the TNF receptor complex (Malinin et al., 1997). NIK physically interacts with IKK $\alpha$  and IKK $\beta$  via its C-terminal region and stimulates their catalytic activity (Ling et al., 1998; Regnier et al., 1997; Woronicz et al., 1997). Moreover, recent studies using NIK-deficient mice demonstrated that NIK plays an important role in the LT $\beta$ R (lymphotoxin  $\beta$  recep-

tor) signalling pathway but not in the TNF signalling pathway (Yin et al., 2001; Matsushima et al., 2001).

In this study, we have attempted to investigate if DEP could induce the activation of NF- $\kappa$ B in L2 cells, one of the rat lung epithelial cell lines, although the intracellular pathway leading to the activation of NF- $\kappa$ B is not fully understood in lung epithelial cell. We also examined the TAK1 and NIK signalling pathway to clarify the intracellular signal transduction pathway that regulates NF- $\kappa$ B activation. Furthermore, we demonstrated that TAK1 is activated by phosphorylation in the DEP-stimulated L2 cells.

## 2. Materials and methods

### 2.1. Plasmids

The expression plasmids of the wild-type TAK1, dominant negative TAK1 (TAK1K63W) and TAB1 were obtained from Dr. K. Matsumoto (Nagoya University, Nagoya, Japan). The expression plasmids of the wild type and dominant negative NIKs were obtained from Dr. T. Taniguchi (Tokyo University, Tokyo, Japan). An NF- $\kappa$ B-dependent luciferase-reporter plasmid (pNF- $\kappa$ B-luc), and the pRL-TK plasmid were purchased from Promega.

### 2.2. Cell culture and reagents

The L2 cell line was cultured in a RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The cells were grown at 37 °C in 5% CO<sub>2</sub>. The RPMI 1640, fetal bovine serum, penicillin and streptomycin were purchased from Gibco BRL.

### 2.3. DEP preparation

The DEP were collected using a turbo-charged inter-cooler 6-cylinder (11,000cc) heavy-duty diesel engine (model year 2000) operated mainly by urban buses. The engine was connected to a 430 kW dynamometer (ELIN) and operated with four different speeds, 1000, 1320, 1760, and 2200 rpm, under a combination of different loads, 10%, 50%, and 100%. The exhaust from the engine was discharged into a mini dilution tunnel (SPC472, AVL), and DEP were collected directly from the mini dilution tunnel. We simply attached the Teflon

filter to collect DEP just on the dilution tunnel, and extracted the filter by shaking. The exhausted DEP was suspended in Dulbecco's Phosphate Buffered Saline (DPBS), Gibco BRL buffer—vortexed and sonicated for 3 min. DEP stock solution was of 100 mg/ml concentration. In order to test dose-dependency, 12-well plates were titrated with the concentration of 0, 1, 10, 100, 250, 500 and 1000  $\mu\text{g/ml}$ .

#### 2.4. Transfection and luciferase assay

A confluent monolayer of L2 cells was trypsinized, and grown in a 12-well plate ( $3 \times 10^4$  cells/well). The plates were incubated at 37 °C in 5% CO<sub>2</sub>. After incubating the cells overnight, they were transiently transfected with the plasmids using a Tfx-20 transfection reagent, according to the manufacturer's protocol (Promega). The L2 cells were transfected with the NF- $\kappa$ B-luc reporter gene. The total amount of DNA was kept constant with pcDNA 3.1 for each transfection. After 24 h, the cells were exposed to DEP at 37 °C for 12 h. The cells were then lysed, and their luciferase activities were measured using a Dual-luciferase reporter system (Promega, Madison, WI). Luciferase activity was confirmed using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample. The results were expressed as the relative NF- $\kappa$ B activity compared with that of the controls.

#### 2.5. Detection of phosphorylated TAK1 and I $\kappa$ B $\alpha$ degradation

The L2 cells were incubated with or without 250  $\mu\text{g/ml}$  DEP at 37 °C for the indicated times and then lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and 1  $\mu\text{g/ml}$  each of aprotinin, leupeptin and pepstatin. The lysates were immunoblotted with anti-TAK1 polyclonal antibody and anti-I $\kappa$ B $\alpha$  polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

#### 2.6. Statistical analysis

The results are presented as mean  $\pm$  standard deviation. A statistical verification between the groups was performed using a Student's *t*-test. A *P*-value < 0.05 was considered significant.

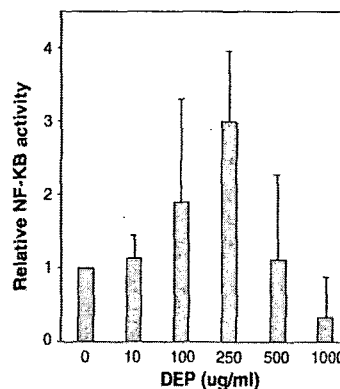


Fig. 1. Dose-dependent induction of NF- $\kappa$ B activity by DEP in L2 Cells. The activity of the NF- $\kappa$ B dependent reporter in the L2 cells was measured by luciferase assay after a treatment with increasing amounts of DEP. The numbers of the left column represent relative luciferase activity. Each bar shows the mean  $\pm$  S.D. of the values obtained in three experiments performed in duplicate.

### 3. Results

#### 3.1. DEP induces NF- $\kappa$ B dependent transcriptional activation in L2 cells

In order to determine the effect of DEP on NF- $\kappa$ B activation, the L2 cells were treated with various concentrations of the DEP. As shown in Fig. 1, within the range 100–250  $\mu\text{g/ml}$  of DEP, NF- $\kappa$ B activity was stimulated in a dose-dependent manner. However, it then decreased at higher DEP concentrations. The optimal time of DEP to induce NF- $\kappa$ B was then determined. As shown in Fig. 2, the NF- $\kappa$ B activity was stimulated by 250  $\mu\text{g/ml}$  DEP in a time-dependent manner and reached its maximum activation after 12 h. From these results, it was concluded that 250  $\mu\text{g/ml}$  DEP for 12 h was the optimal condition for NF- $\kappa$ B activation in the L2 cells, and was used in the following experiments.

#### 3.2. TAK1 and NIK are required for the DEP-induced NF- $\kappa$ B activation

TAK1 and NIK have been reported to mediate the NF- $\kappa$ B activation triggered by lipopolysaccharides. For this reason, these molecules were examined to determine if they were also involved in the NF- $\kappa$ B sig-

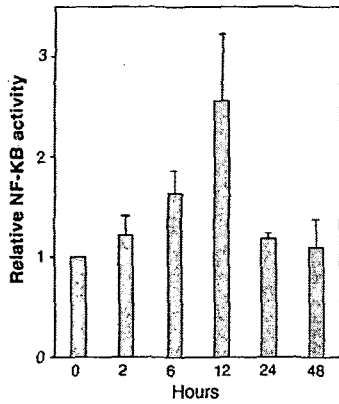


Fig. 2. Time-dependent induction of NF-κB activity by DEP in L2 Cells. The activity of the NF-κB dependent reporter was measured by luciferase assay after treatment of 250 μg/ml DEP in the L2 cells. The numbers of the left column represent the relative luciferase activity. Each bar shows the mean ± S.D. of the values obtained in three experiments performed in duplicate.

nalling pathway induced by the DEP. This study examined the effects of the expression of dominant negative mutants of TAK1 and NIK on the activation of the NF-κB signalling pathway by DEP in L2 cells. The L2 cells were co-transfected with the NF-κB-luc reporter gene and plasmids expressing the dominant negative mutants of TAK1 or NIK. Twenty-four hours after transfection, the transfected cells were treated with 250 μg/ml DEP for 12 h. When the cells were examined by a luciferase assay, the NF-κB activity was two to five times higher in the DEP treated cells than in the non-treated cells. However, the DEP-induced NF-κB activity was reduced by the expression of dominant negative mutants of NIK or TAK1 to the basal level (Fig. 3). This suggests that both TAK1 and NIK are needed for the DEP-induced NF-κB activation in L2 cells.

3.3. Phosphorylated TAK1 and IκBα degradation by DEP

The level of TAK1 phosphorylation was examined by western blotting; using phospho-TAK1-specific antibodies in the DEP-treated L2 cells. In the DEP-treated cells TAK1 was phosphorylated, as indicated by its slow migration pattern, demonstrating that TAK1 is activated by the DEP treatment (Fig. 4). TAK1 phos-

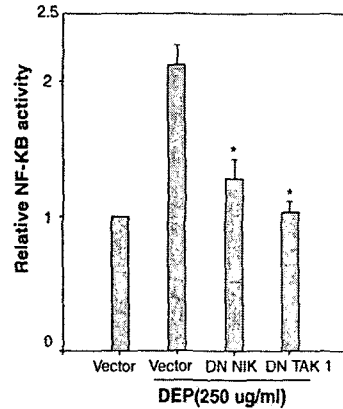


Fig. 3. Effect of the dominant negative forms of TAK1 and NIK on the DEP-induced NF-κB activation. The L2 cells were transiently transfected with the reporter plasmid together with the expression plasmids for the dominant negative forms of TAK1 and NIK. The cells were then treated with 250 μg/ml of DEP for 12 h. The activity of the NF-κB reporter was measured by luciferase assay. The numbers in the left column represent the relative luciferase activity. Each bar shows the mean ± S.D. of the values obtained in three experiments performed in duplicate. The statistical significance of the differences was evaluated by Student's *t*-test ( $P < 0.05$ ).

phorylation was evident 30 min after stimulation and continued for at least 90 min. This suggests that TAK1 plays an important role in the DEP-mediated activation of the NF-κB signalling pathway.

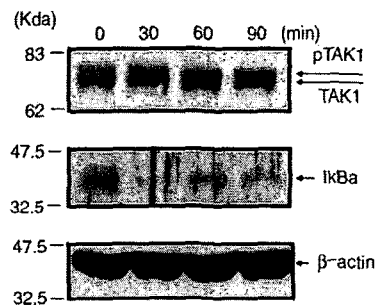


Fig. 4. TAK1 phosphorylation as a result of DEP stimulation. The L2 cells ( $1 \times 10^6$ ) were stimulated with 250 μg/ml DEP at 37 °C for the indicated times. The cells were then lysed and the endogenous TAK1 was immunoblotted with the anti-TAK1 antibody. IκBα degradation was immunoblotted with the anti-IκBα antibody. The proteins were separated on 8% SDS-PAGE gel. The pTAK1 represents the phosphorylated TAK1.

I $\kappa$ B $\alpha$  is a potent inhibitor of NF- $\kappa$ B, and the degradation of I $\kappa$ B $\alpha$  via the ubiquitin pathway is essential for the nuclear translocation of NF- $\kappa$ B. Therefore, this study examined the effect of DEP on the degradation of I $\kappa$ B $\alpha$ . The addition of 250  $\mu$ g/ml DEP resulted in the degradation of I $\kappa$ B $\alpha$  within 30 min (Fig. 4). This suggests that DEP acts via the NF- $\kappa$ B signalling pathway through the I $\kappa$ B $\alpha$  degradation.

#### 4. Discussion

The present study examined the effect of DEP on the activation of NF- $\kappa$ B. Activation of cells by inflammatory mediators such as TNF- $\alpha$  causes the rapid degradation of the regulatory protein, the I $\kappa$ B $\alpha$ , which allows the nuclear translocation of NF- $\kappa$ B, and the transcriptional activation of its target genes (Lentsch et al., 1998). This study showed that DEP stimulates the activation of NF- $\kappa$ B in the L2 cells. Similar to these findings, several studies have demonstrated that DEP can activate NF- $\kappa$ B in human bronchial epithelial cells (Takizawa et al., 1999; Takizawa et al., 2003; Bonvallot et al., 2001). Therefore, NF- $\kappa$ B appears to be a target of DEP action and may mediate the DEP-stimulated cytokine transcription.

Recently, it was reported that TAK1 and NIK are involved in NF- $\kappa$ B activation via the IL-1 signalling pathway. IL-1 has been implicated in the pathogenesis of pulmonary fibrosis including silicosis (Marano et al., 2002). Briefly, when the IL-1 binds to the cell-surface type-1 IL-1 receptor (IL-1RI), IL-1RI forms a complex with an IL-1-receptor accessory protein, resulting in the recruitment of MyD88 and the Ser/Thr kinase IRAK (IL-1 receptor-associated kinase) to the receptor. IRAK then dissociates from the receptor complex and interacts with TRAF6, which transduces the IL-1 signal to TAK1 (Robledo and Mossman, 1999; Cao et al., 1996; Henzel et al., 1996; Wesche et al., 1997). In order to determine the mechanism of NF- $\kappa$ B activation in L2 cells by DEP, TAK1 and NIK were investigated to determine if they participate in the signalling pathway induced by DEP. Initially, it was found that the expression of wild type TAK1 and NIK exhibited enhanced NF- $\kappa$ B activity with DEP stimulation (data not shown). The co-transfection of the dominant negative mutants of TAK1 or NIK was then performed. As expected, the dominant negative mutants of either TAK1 or NIK in-

hibited the DEP-induced NF- $\kappa$ B activity (Fig. 3). This suggests that TAK1 and NIK signalling is involved in the DEP-stimulated, NF- $\kappa$ B activation pathway in the L2 cells. It was also shown that DEP induces TAK1 phosphorylation. Previously, several groups reported that TAK1 is phosphorylated and this phosphorylation correlates well with the kinase activity when TAK1 is activated (Ninomiya-Tsuji et al., 1999; Sakurai et al., 1999). Accordingly, the phosphorylation of TAK1 by DEP stimulation again indicates that TAK1 plays an important role in the DEP-stimulated activation of the NF- $\kappa$ B signalling pathway.

In the cytosol, NF- $\kappa$ B is in an inactive form that is bound to an inhibitory molecule, I $\kappa$ B $\alpha$ . The degradation of I $\kappa$ B $\alpha$  by the ubiquitin-dependent proteasome is essential for NF- $\kappa$ B activation. It was demonstrated that the DEP treatment induced the I $\kappa$ B $\alpha$  degradation in rat lung epithelial cells, which indicates that DEP plays a major role in NF- $\kappa$ B activation and nuclear translocation via the I $\kappa$ B $\alpha$  degradation pathway.

In conclusion, NF- $\kappa$ B in L2 cells is activated by DEP stimulation. TAK1 and NIK signalling in L2 cells is involved in the DEP-stimulated NF- $\kappa$ B activation pathway. In particular, TAK1 is activated by phosphorylation in the DEP-stimulated L2 cells. This suggests that the direct contact of epithelial cells with DEP stimulates NF- $\kappa$ B activation via TAK1 and NIK activation dependent on I $\kappa$ B $\alpha$  degradation. Taken together, DEP stimulated NF- $\kappa$ B activation is regarded as one of the mechanisms for DEP induced cellular signalling.

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