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Dermatophagoides pteronyssinus 2 regulates nerve growth factor release to induce airway inflammation via a reactive oxygen species-dependent pathway

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Ye Y, Wu H, Lin C, Hsieh C, Wang J, Liu F, Ma C, Bei C, Cheng Y, Chen C, Chiang B, Tsao C. *Dermatophagoides pteronyssinus* 2 regulates nerve growth factor release to induce airway inflammation via a reactive oxygen species-dependent pathway. *Am J Physiol Lung Cell Mol Physiol* 300: L216–L224, 2011. First published November 18, 2010; doi:10.1152/ajplung.00165.2010.—Group 2 allergen of *Dermatophagoides pteronyssinus* 2 (Der p2) induces airway inflammation without protease activity, and elevated nerve growth factor (NGF) levels are also found in this inflammation. How the allergen Der p2 regulates NGF release via reactive oxygen species (ROS) to induce inflammation remains unclear. In the present study, intratracheal administration of Der p2 to mice led to inflammatory cell infiltration, mucus gland hyperplasia, and NGF upregulation in the bronchial epithelium, as well as elevated ROS and NGF production in bronchoalveolar lavage fluids. In addition, Der p2 caused fibrocyte accumulation and mild fibrosis. p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) inhibitors inhibited Der p2-induced NGF release in LA4 lung epithelial cells and MLg lung fibroblasts. Pretreatment with an antioxidant, tiron, reduced the Der p2-induced ROS production, NGF expression and release, p38 MAPK or JNK phosphorylation, and airway inflammation. These results suggest that Der p2 allergen-induced airway inflammation and elevated NGF release were through increasing ROS production and a MAPK-dependent pathway. The use of an antioxidant, tiron, may provide a new therapeutic modality for the treatment of allergic asthma.

mitogen-activated protein kinase; mite; antioxidant

HYPERSENSITIVITY TO HOUSE dust mite (HDM) allergens is one of the most common allergic responses. Estimates suggest that ~5% of HDM proteins are thought to be allergens and >50% of children and adolescents with asthma are sensitized to HDM (12, 24). Current studies (12) have focused on the proteolytic activity of HDM-derived allergens in the pathogenesis of allergic disease. *Dermatophagoides pteronyssinus* 1 (Der p1) and Der p3 exert cysteine protease activity that causes epithelial cell desquamation and cytokine release and that disturbs the bronchial epithelial monolayer (12, 36). In contrast, other major allergens of Der p have been identified to induce asthma without protease activity, such as Der p2 and Der p5.

Der p2 is used as a model to induce the development of asthma (16, 32, 44, 58). It is known to be homologous to the human epididymis-specific protein HE1 and MD2 and promotes proinflammatory cytokine release via activating nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) in primary cultured airway smooth muscle cells and epithelial cells (14, 50). However, its relative signals to the development of asthma are still unclear.

Nerve growth factor (NGF) is thought to have a protective effect against oxidative stress and promotes cell growth in neurons (38, 42). Several studies (19, 20, 22) have reported that NGF is involved in allergic diseases, which are characterized by airway inflammation, bronchial responsiveness, and airway remodeling. High levels of NGF are detected in bronchoalveolar lavage fluids (BALF) and serum from asthmatic patients, suggesting that NGF is regulated in airways and that the level of NGF expression is associated with the severity of the allergic disease (6, 35, 49).

In addition to neurons and inflammatory cells, NGF affects the contraction, migration, differentiation, and proliferation of airway structural cells (20, 22). It is also synthesized in airway structural cells such as epithelial cells (18, 28, 52), pulmonary fibroblasts (2, 48), and smooth muscle cells (21, 37). After bronchial epithelium and infiltrating inflammatory cells in the submucosa had been exposed to allergen ovalbumin, they showed intense NGF immunoreactivity (7, 23). Connective tissues on bronchial biopsy sections in humans are similarly affected (49). Although a recent study (8) on human nasal turbinate sections of 25 patients with persistent allergic rhinitis and sensitization to Der p reported strong NGF immunostaining in mucous cells of the epithelial lining and in the submucosal glands, the mechanism of how Der p2 without protease activity influences asthma development via NGF regulation is still unclear.

Lung epithelium is not only the primary site of lung damage but also participates in inflammatory responses by reactive oxygen species (ROS; Refs. 25, 41). Animal models and human studies (56) have reported that excessive ROS production leads to airway inflammation, hyper-responsiveness, microvascular hyperpermeability, tissue injury, and remodeling. However, little is known about the possible mechanisms through which Der p2 causes asthmatic inflammation by inducing ROS production from bronchial epithelium.

In the present study, we intratracheally administered Der p2 to mice to examine NGF expression in bronchial epithelium

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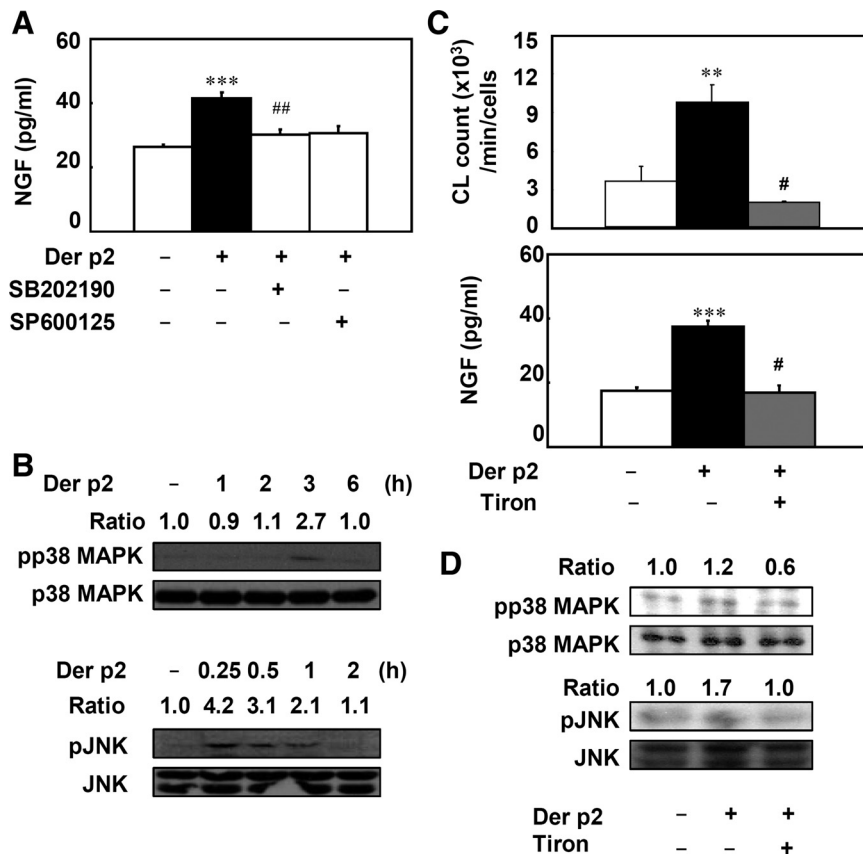


Fig. 1. Effects of *Dermatophagoides pteronyssinus* 2 (Der p2) on nerve growth factor (NGF) release, reactive oxygen species (ROS) production, p38 MAPK, and JNK phosphorylation in LA4 cells. LA4 cells were pretreated with 25 μ M of SB202190 or SP600125 for 30 min before 100 μ g/ml of Der p2 treatment for 24 h. A: culture medium was collected and NGF content was determined using an ELISA kit. B: cell lysates were harvested at indicated times for detection of p38 MAPK or JNK phosphorylation levels using Western blot. C: cells were treated with 2 mM of tiron for 1 h before Der p2 treatment. Cells were collected for detection of ROS, and culture medium was harvested to determine NGF content. Cells were treated with 2 mM of tiron for 1 h before Der p2 treatment. D: cell lysates were harvested for detection of phospho-p38 MAPK or phospho-JNK levels by Western blot after Der p2 treatment at 3 h or 15 min, respectively. Ratios are shown in optical density (OD) of phospho-p38 MAPK/total p38 MAPK or phospho-JNK/total JNK. Data are means $\pm$ SE from 3 individual experiments. CL, chemiluminescence. ** P < 0.01 and *** P < 0.001, compared with medium controls. # P < 0.05, compared with Der p2-treated group.

and detect cellular changes, ROS production, and NGF release in BALF. Lung structural cell lines were treated with Der p2 combined with MAPK-related inhibitors to investigate the possible mechanisms of NGF release induced by Der p2. Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), a water-soluble and cell-permeable superoxide scavenger (27, 40, 59) and an efficient nontoxic chelator of various metals (17), has been widely used in oxidative stress-related physiologic researches (3, 4, 10, 26, 30, 33, 54, 55). In the in vitro and in vivo systems, we also administered tiron before Der p2 to further investigate the effects of tiron on Der p2-induced ROS production and NGF release during airway inflammation.

MATERIALS AND METHODS

Animals. Five-week-old C57BL/6 female mice were purchased from the Animal Center of National Cheng Kung University Medical College. The experimental procedures were done in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

Allergen administration and assessment of blood eosinophilia and airway inflammation. The mice intratracheally received 25 μ g Der p2 or were intraperitoneally injected with 300 mg/kg tiron (Fluka Chemical, Milwaukee, WI) for 1 h before Der p2 treatment (intracheal) for 7 days. Der p2 was donated by B. L. Chiang (Graduate Institute of Clinical Medicine, National Taiwan University, Taipei, Taiwan). Lipopolysaccharide contamination of Der p2 was excluded (≤ 0.01 EU/ μ g; Limulus amoebocyte lysate test kit). After mice had been injected with an overdose of pentobarbital (Sigma-Aldrich, St. Louis, MO), they were given a BAL. BAL was performed (2 washes of 1 ml of ice-cold endotoxin-free saline each) as described previously (13).

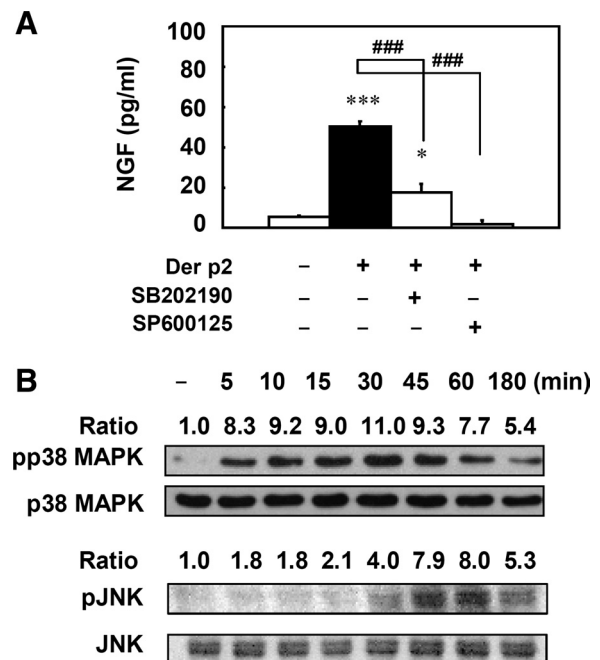


Fig. 2. Der p2-induced NGF release via p38 MAPK and JNK pathways in MLg cells. Cells were pretreated with 25 μ M of SB202190 or SP600125 for 30 min before 100 μ g/ml of Der p2 treatment for 24 h. A: culture medium was collected for determination of NGF content using an ELISA kit. B: cell lysates were harvested at indicated time for the detection of phospho-p38 MAPK or phospho-JNK levels by Western blot. Ratios are shown in OD of phospho-p38 MAPK/total p38 MAPK or phospho-JNK/total JNK. Data are means $\pm$ SE from 3 individual experiments. * P < 0.05 and *** P < 0.001, compared with medium controls. ### P < 0.05, compared with Der p2-treated group.

The BALF were separated (2,000 rpm, 10 min) and stored at -70°C . Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Shandon cytospin 3; Thermo Fisher Scientific, Waltham, MA). To examine the cell differentials, we stained the smears with Liu's stain solution (Tonyar Biotech, Taipei, Taiwan). Approximately 200 cells were counted in each of the 4 different random locations.

Cell lines. The MLg cell line (ATCC CCL-206) and the LA4 cell line (ATCC 50767) were purchased from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan).

Cell culture. The MLg cells were cultured in MEM (Gibco, Grand Island, NY), which contained 10% FBS and 0.1% gentamicin. The LA4 cells were cultured in DMEM (Gibco), which contained 2.5 mM of L-glutamine, 50% nutrient mix F-12 Ham (Sigma-Aldrich), 15% FBS, and 0.1% gentamicin. Various doses of MAPK inhibitors, SB202190 and SP600125 (Tocris Bioscience, Ellisville, MO), or tiron were added before Der p2 treatment. Cells were harvested at indicated times, and the supernatant of each well was collected and frozen at -20°C .

Determining NGF release. The level of NGF was determined using an immunoassay (NGF E_{max} ImmunoAssay System; Promega Madison, WI, USA). The optical density was detected at 450 nm using an ELISA reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA).

Western blot analysis. Cells (2×10^6) were lysed using a Triton X-100-based lysis buffer containing 1% Triton X-100, 150 mM of NaCl, 10 mM of Tris (pH 7.5), 5 mM of EDTA, 5 mM of NaN₃, 10

mM of NaF, and 10 mM of sodium pyrophosphate. Cell extract was separated using SDS-PAGE and then transferred to a PVDF membrane (Millipore, Billerica, MA). After they had been blocked, the blots were developed using a series of antibodies as indicated. Rabbit antibodies specific for phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), phospho-MKK3 (Ser189/207), phospho-MKK4 (Thr 261), phospho-apoptosis signal-regulating kinase 1 (ASK1) (T845), p38 MAPK, SAPK/JNK, MKK3, MKK4, and ASK1 were used (Cell Signaling Technology, Beverly, MA). The blots were then hybridized using horseradish peroxidase-conjugated goat anti-rabbit IgG (Calbiochem, San Diego, CA) and developed using a reagent (Western Lightning Chemiluminescence Reagent PLUS; PerkinElmer Life Sciences, Boston, MA). The optical density of phospho-protein/total protein was determined using VisionWorks LS software (Upland, CA).

Histological analysis. The lungs of the mice were removed after the trachea had been ligatured and had been intratracheally filled with a fixative with 10% (vol/vol) ethanol formalin (Sigma-Aldrich). The lung tissues were fixed with 10% (vol/vol) neutral buffered formalin. Specimens were dehydrated and embedded in paraffin. After section of the specimens, they were placed on slides, deparaffinized, and stained sequentially with standard hematoxylin-eosin, periodic acid-Schiff (PAS), or HT15 Trichrome stain (Masson) kit Sigma (Sigma-Aldrich). The samples were mounted on glass slides, depleted of paraffin at 60°C for 10 min and gradually resuspended in xylene, ethanol, 3% H_2O_2 (80% methanol; vol/vol), and 0.01 M sodium citrate buffer (pH 6.0) at 95°C . After the sections had been cooled,

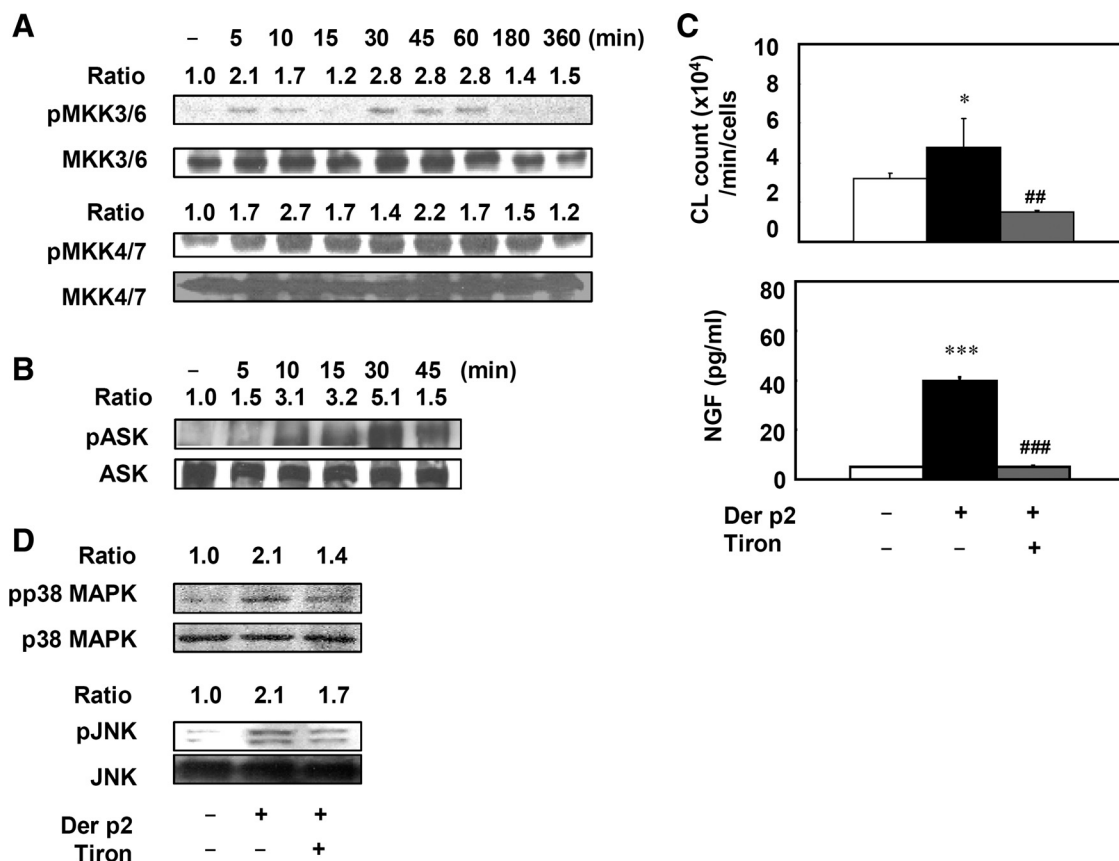


Fig. 3. Involvement of MKK3/6, MKK4/7, apoptosis signal-regulating kinase 1 (ASK1), and ROS production in Der p2-induced NGF release in MLg cells. Cells were treated with 100 $\mu\text{g}/\text{ml}$ of Der p2, and cell lysates were harvested at indicated times for the detection of phospho-MKK3/6 and -MKK4/7 (A) and phospho-ASK levels (B). C: pretreatment with 1 mM of tiron inhibited Der p2-induced ROS production and NGF release. D: levels of phospho-p38 or -JNK were also detected after treatment with 1 mM of tiron before 100 $\mu\text{g}/\text{ml}$ of Der p2 treatment at 45 min or 3 h, respectively. Ratios are shown in OD of phospho-protein/total protein. Data are means $\pm$ SE from 3 individual experiments. * $P < 0.05$ and *** $P < 0.001$, compared with medium controls. ## $P < 0.01$ and ### $P < 0.001$, compared with Der p2-treated group.

they were blocked with 10% milk and subjected to immunofluorescence staining overnight at 4°C with a 1:250 dilution of sheep polyclonal anti-NGF antibody (Abcam, Cambridge, UK), a 1:100 dilution of goat polyclonal anti-collagen-1 (C-18) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a 1:100 dilution of rat anti-mouse CD45-conjugated Alexa Fluor 488 antibody (BioLegend, San Diego, CA), or a 1:100 dilution of goat polyclonal eosinophil peroxidase antibody (Santa Cruz Biotechnology). Subsequently, anti-sheep IgG conjugated with FITC (Jackson ImmunoResearch, Laboratories, West Grove, PA), Alexa Fluor 488 donkey anti-goat IgG, or Alexa Fluor 594 donkey anti-goat IgG (Invitrogen) were used. Slides were observed with a light microscope.

Determining ROS production. Leukocytes in BALF or MLg and LA4 cells were suspended in 200 µl of appropriate medium buffer and determined by enhanced chemiluminescence (CL) analyzer system (Tohoku Electronic, Miyagi, Japan). Luminol (2×10^{-3} M; Sigma-Aldrich) was then added to the sample at 150 s and enhanced CL was measured. CL counts were detected continually for a total of 1,200 s, and the mean CL counts of 10-s intervals were recorded.

Statistics. A one-way ANOVA test was used to examine cell subpopulations, NGF release, and ROS production between the Der p2-treated group and the medium control group. Statistical significance was set at $P < 0.05$.

RESULTS

Der p2-induced NGF release in LA4 cells via ROS production and a MAPK- dependent pathway. Der p2 significantly increased NGF expression for 24 h in LA4 epithelial cells (Fig. 1A). The NGF levels in cells pretreated with p38 MAPK inhibitor SB202190 and JNK inhibitor SP600125 for 30 min before 24-h Der p2 treatment were significantly lower than in cells treated with only Der p2. Der p2-induced phospho-p38 MAPK levels were higher at 3 h, and Der p2-induced phospho-JNK levels increased between 15 and 60 min (Fig. 1B). ROS production significantly increased at 30 min and so did NGF release at 24 h posttreatment (Fig. 1C). Pretreatment with tiron for 1 h

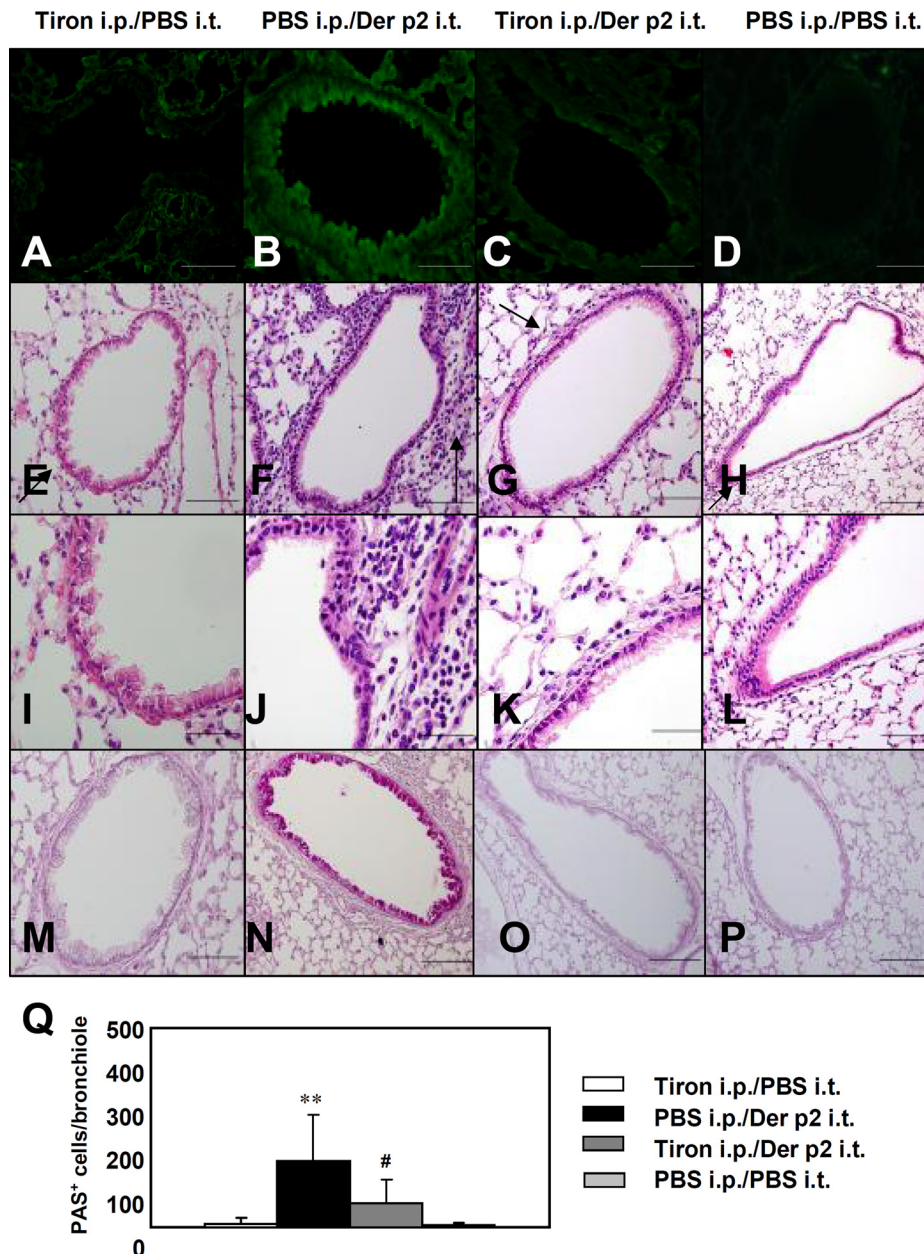


Fig. 4. Effect of tiron on Der p2-induced NGF expression, cell infiltration, and mucus gland hyperplasia. Five to seven mice in each group intratracheally received 25 µg Der p2 or were intraperitoneally injected with 300 mg/kg tiron 1 h before Der p2 treatment (intracheal) for 7 days. After 7 days, the lung tissue was removed and fixed with 10% formaldehyde for the detection of NGF expression by immunofluorescence (A-D, $\times 100$ magnification), immune cell infiltration by hematoxylin-eosin staining (E-H, magnification = $\times 200$; I-L, $\times 400$ magnification), mucus gland hyperplasia by periodic acid-Schiff (PAS) staining (M-P, $\times 100$ magnification), or quantification of PAS+ cells (Q). ** $P < 0.01$, compared with tiron (ip)/PBS (it)-treated group. # $P < 0.05$, compared with PBS (ip)/Der p2 (it)-treated group.

decreased the Der p2-induced ROS production, NGF release, phospho-p38 MAPK level at 3 h, and phospho-JNK level at 15 min (Fig. 1D).

Der p2-induced NGF release in MLg cells via a MAPK-dependent pathway. Like LA4 cells, Der p2 significantly induced NGF expression in MLg fibroblasts (Fig. 2A). Pretreatment with SB202190 and SP600125 for 30 min significantly inhibited NGF release. Der p2 induced substantial phospho-p38 MAPK between 5 and 30 min posttreatment; the level declined markedly from 45 min; and phospho-JNK expression increased between 45 min and 3 h (Fig. 2B).

ROS production and the upstream signals, phospho-MKK3/6, -MKK4, and -ASK1 were involved in Der p2-regulated NGF release in MLg cells. The level of Der p2-induced phospho-MKK3/6 expression was slightly higher between 5 and 10 min and substantially higher between 30 and 45 min posttreatment; phospho-MKK4/7 expression was markedly

higher between 5 and 45 min posttreatment and then declined (Fig. 3A). Phospho-ASK1 expression was noticeably higher between 10 and 15 min posttreatment, peaked at 30 min, and then declined (Fig. 3B). ROS production was significantly increased at 30 min and NGF release 24 h posttreatment (Fig. 3C). Pretreatment with tirion for 1 h inhibited Der p2-induced ROS production, NGF release, phospho-p38 MAPK level at 45 min, and phospho-JNK level at 3 h (Fig. 3D).

Tiron inhibited Der p2-induced NGF expression, cell infiltration, and mucus gland hyperplasia. After the mice had been given intratracheal instillations of 25 μ g Der p2 for 7 days, immunofluorescence analysis showed that the levels of NGF expression were more obvious in lung airway epithelium in Der p2-treated mice (Fig. 4B). As shown in Fig. 4F, lung sections in the Der p2-treated group showed apparent cell infiltration (hematoxylin-eosin stain; indicated by the arrow and amplified in Fig. 4J). Mucus gland hyperplasia was ob-

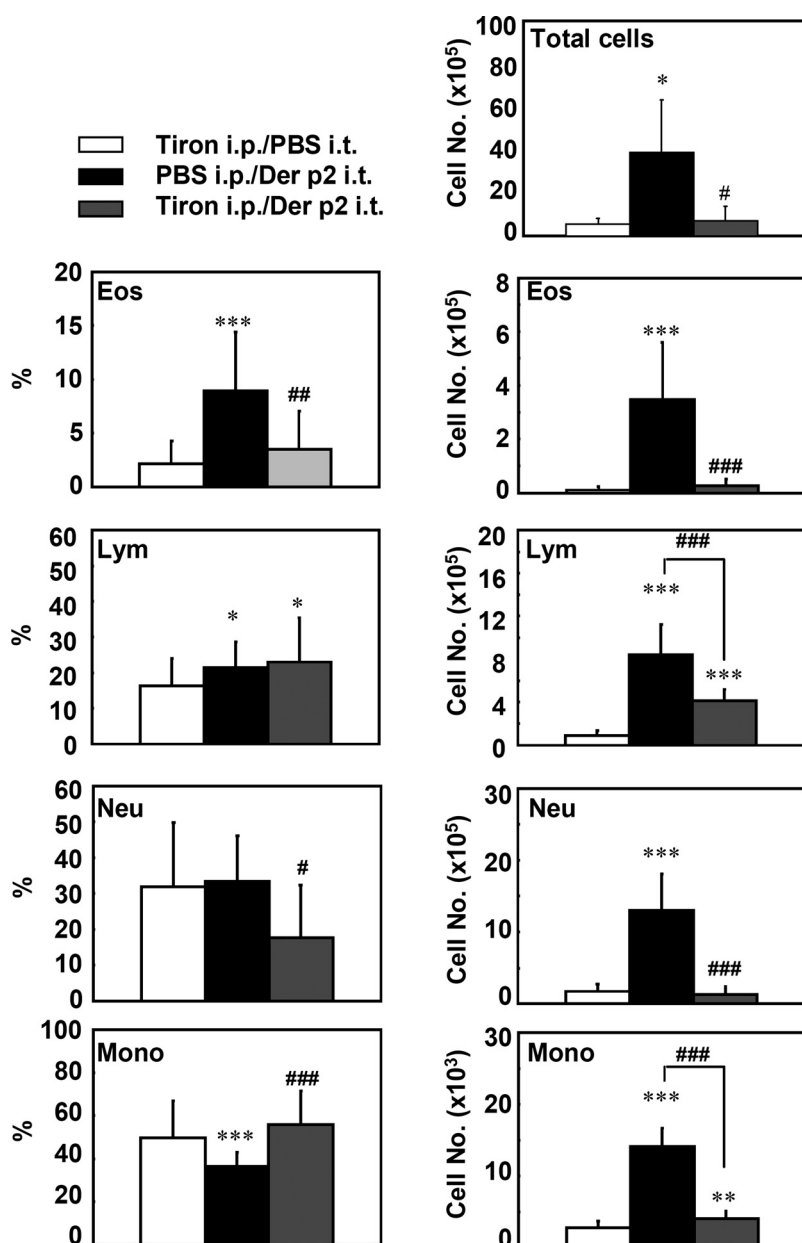


Fig. 5. Effect of tiron on Der p2-induced cell infiltration in bronchoalveolar lavage fluids. Five to seven mice in each group intratracheally received 25 μ g Der p2 or were intraperitoneally injected with 300 mg/kg tiron for 1 h before Der p2 treatment (intratracheal) for 7 days. Total infiltrated cells, eosinophils (Eos), lymphocytes (Lym), neutrophils (Neu), and monocytes were determined in bronchoalveolar lavage fluids by a bright field microscope. Percentages (left) and numbers (right) of 4 subpopulations are shown. Data are means $\pm$ SE of 3 individual experiments. * P < 0.05, ** P < 0.01, and *** P < 0.001, compared with Tiron (ip)/PBS (it)-treated group. # P < 0.05, ## P < 0.01, and ### P < 0.001, compared with PBS (ip)/Der p2 (it)-treated group.

served in the airway epithelium of Der p2-treated mice, determined by PAS staining (Fig. 4N). Before administration of PBS (intracheal), there was no difference in histological morphology between pretreatment with tiron (intraperitoneal) and

of PBS (intraperitoneal) for 1 h. Moreover, tiron-treated mice significantly produced lower NGF expression (Fig. 4C) and had improved cell infiltration (Fig. 4G, indicated by the arrow and amplified in Fig. 4K) and mucus gland hyperplasia (Fig.

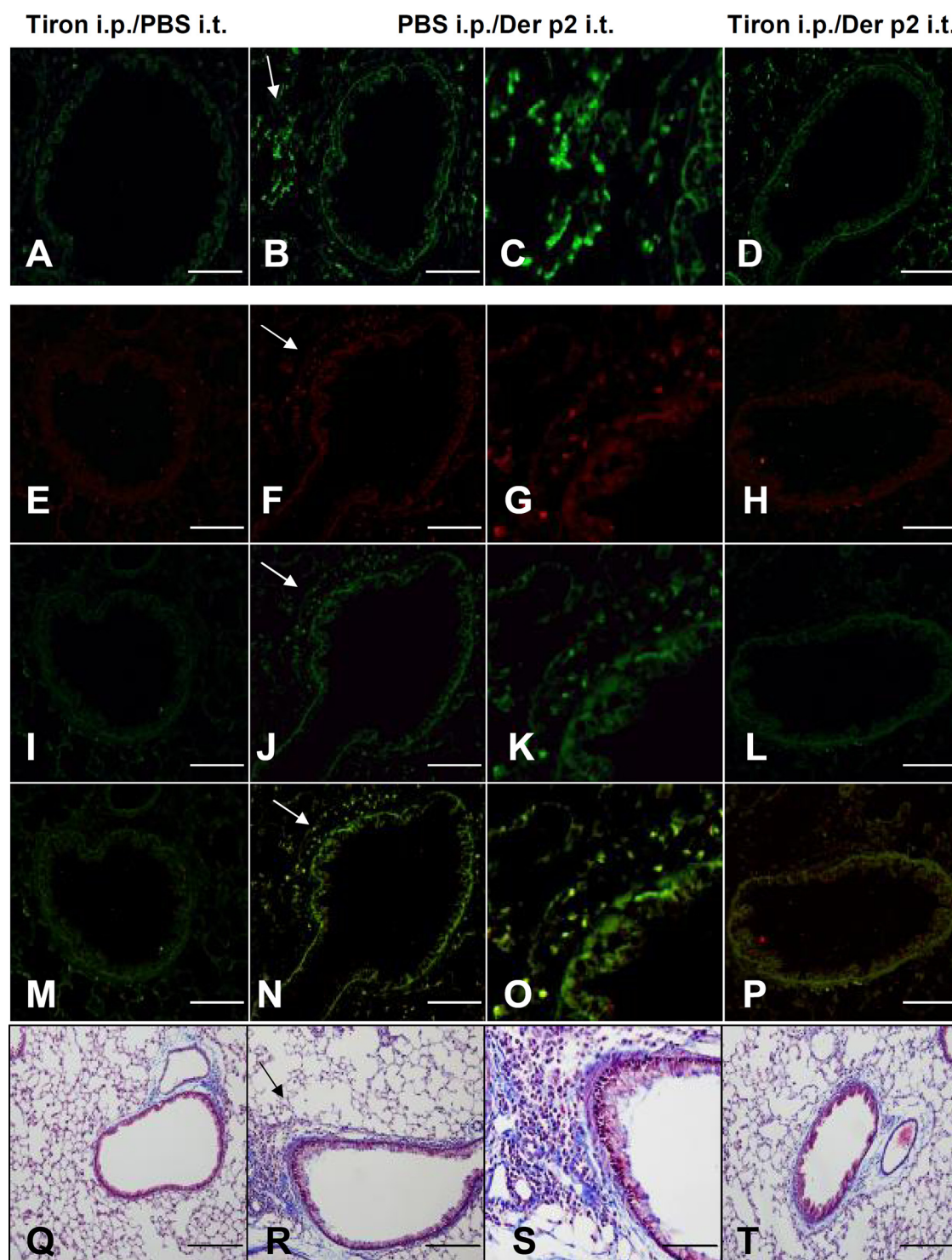


Fig. 6. Effect of tiron on Der p2-induced infiltration of eosinophils and fibrocytes and airway fibrosis. Five to seven mice in each group intratracheally received 25 μ g Der p2 or were intraperitoneally injected with 300 mg/kg tiron 1 h before Der p2 treatment (intracheal) for 7 days. After 7 days, the lung tissue was removed and fixed with 10% formaldehyde for detection of infiltration of eosinophils and fibrocytes, and fibrosis by immunofluorescence. Eosinophil peroxidase expressions were shown in A-D ($\times 200$ magnification). Arrows indicate eosinophil infiltration and are amplified in Fig. C. Collagen-1⁺ cells (E-H), CD45⁺ cells (I-L), and collagen-1⁺ CD45⁺-positive cells (M-P) were shown ($\times 200$ magnification). Arrows indicate fibrocyte infiltration and are amplified in Fig. G, K, O. Fibrosis was determined by Trichrome staining (Q-T) [$\times 100$ magnification (Q-T); $\times 200$ magnification (S)].

40) in bronchial epithelium compared with Der p2-treated mice. A significant increase of percentage of PAS⁺ cells had been quantified in Der p2-treated mice, which was decreased by tiron treatment (Fig. 4Q).

Tiron inhibited Der p2-induced cellular changes in BALF. There were significantly more total cells, including eosinophils, lymphocytes, neutrophils, and monocytes, in the BALF of the Der p2-treated group than in that of the PBS-treated group (Fig. 5). The markedly increased percentages of eosinophils and lymphocytes were also observed, whereas a decreased percentage was shown in the monocytes of the Der p2-treated mice. In contrast, tiron-treated mice produced lower numbers and percentages of cell subpopulations, especially in eosinophils and neutrophils. However, there was a significantly increased percentage of monocytes after tiron treatment. There was no difference in cellular changes between pretreatment with tiron (intraperitoneal) and of PBS (intraperitoneal) for 1 h before administration of PBS (intratracheal; data not shown).

Tiron inhibited Der p2-induced eosinophil and fibrocyte infiltration as well as partially improved airway fibrosis. Der p2-treated mice increased infiltration of eosinophils with expression of eosinophil peroxidase in submucosa layer (Fig. 6B, indicated by the arrow and amplified in Fig. 6C) and fibrocytes with coexpression of collagen-1 and CD45 in the subepithelial zone (Fig. 6N, indicated by the arrow and amplified in Fig. 6O). Moreover, tiron-treated mice significantly improved eosinophil (Fig. 6D) and fibrocyte infiltration (Fig. 6P) compared with Der p2-treated mice (Fig. 6, B and N). We also found that Der p2-induced airway fibrosis determined by Trichrome staining (Fig. 6R, indicated by the arrow and amplified in Fig. 6S). However, it did not cause severe symptoms. A slight decrease was also observed in tiron-treated mice (Fig. 6T).

Tiron inhibited Der p2-induced ROS production and NGF release. Der p2-treated mice also exhibited greater ROS production (Fig. 7A) and NGF release (Fig. 7B) than the PBS-treated mice. Compared with Der p2-treated group, there were significantly less NGF release and ROS production in the tiron-treated group (Fig. 7).

DISCUSSION

In the present study, Der p2 induced NGF expression in bronchial epithelium and increased NGF release and ROS production in BALF. In addition to controlling ROS, tiron also blocked NGF release and relieved airway inflammation. In our investigation of whether epithelial cells and fibroblasts are the major sources of NGF expression related to ROS production and MAPK signals, we found that p38 MAPK or JNK inhibitors blocked the Der p2-induced NGF release in LA4 and MLg cells. Der p2 induced ROS production and activation of p38 MAPK and JNK, which were both inhibited by antioxidant. The upstream signals phospho-MKK3/6, -MKK4, and -ASK1 were also involved.

Increased levels of ROS caused epithelium injury, further leading to the loss of the barrier function. As a result, various stimuli achieved access to the airway tissue and thereby caused the inflammation to worsen. ROS are not only cytotoxicants but are also secondary messengers in intracellular signal transduction and control the action of several signaling pathways. ASK1 is a MAPK kinase and a kinase of the JNK and p38 MAPK pathways and is preferentially activated in response to

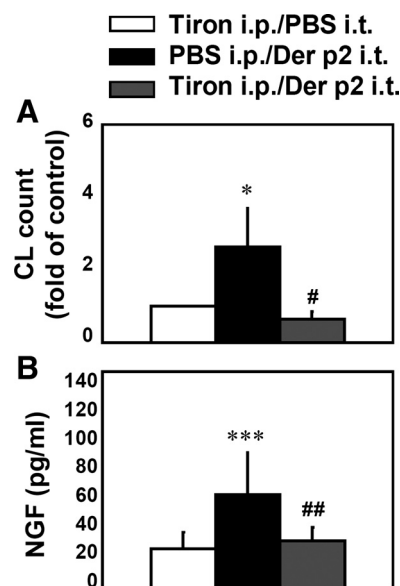


Fig. 7. Effect of tiron on Der p2-induced ROS production and NGF release. Five to seven mice in each group intratracheally received 25 μ g Der p2 or were intraperitoneally injected with 300 mg/kg tiron 1 h before Der p2 treatment (it) for 7 days. ROS production (A) and NGF release (B) were measured by CL analyzer and an ELISA kit. Data are means $\pm$ SE from 3 individual experiments. * P < 0.05 and *** P < 0.001, compared with Tiron (ip)/PBS (it)-treated group. # P < 0.05 and ## P < 0.01, compared with PBS (ip)/Der p2 (it)-treated group.

oxidative stress (45, 46). The ADP-stimulated NADPH oxidase activates the ASK1/MKK4/JNK pathway in alveolar macrophages (43). The ASK1-p38 MAPK/JNK cascade regulates AP-1 activation in nitric oxide-stimulated human bronchial epithelial cells (31). However, few studies have mentioned that mites stimulate ASK1 activation. In our study, we showed that Der p2 directly activated ASK1 and ROS production in lung structure cells (Fig. 3).

ASK1 is an important factor of NGF withdrawal-induced JNK activation and neuronal apoptosis (34). Rapid activation of antioxidant defenses by NGF rapidly suppresses ROS during neuronal apoptosis (38). These results indicate that the ROS/ASK1/p38 MAPK and JNK pathway occurred in cells lacking NGF supplement. However, NGF-stimulated pheochromocytoma PC12 cells transiently increase the intracellular concentration of ROS. This increase is blocked by the antioxidant (57). NGF causes intracellular ROS generation in rat peritoneal mast cells at lower concentrations, which implies that this may contribute to the activation of cytokine production (9). In our study, we clarified that Der p2 directly regulates NGF release via ROS generation, which further participates in airway inflammation. Tiron also inhibited Der p2-induced ROS production, NGF release, and the activation of p38 MAPK and JNK, as well as relieved immune cells infiltration, indicating that Der p2-induced airway inflammation was related to oxidative stress.

Airway eosinophilia was accompanied by enhanced NGF levels in BALF in a mouse model of allergic asthma (7). NGF is also a chemoattractant for eosinophils and activates these cells (19). Increased NGF that secretes from airway epithelial cells promotes the survival of eosinophils during ovalbumin-induced allergic inflammation (7, 28, 32). Pretreatment with

NGF blocking antibodies in mice before the allergen challenge not only reduces the number of infiltrated eosinophils but also decreases Th2 cytokine levels (51). Der p2 not only caused an elevated level of NGF but also produced eosinophil infiltration. Recruitment and activation of neutrophils, monocytes, and lymphocytes also play a role in exposure to HDM allergen-induced bronchoalveolar inflammatory responses (53). Consistent with previous studies (58, 60), we found that Der p2 infiltrated more inflammatory cells in airway. Although increased NGF secretion and the changes of numbers and percentages of cell infiltration were observed, the actions of NGF induced by Der p2 on these immune cells were still obscure.

Activated inflammatory cells, such as eosinophils and neutrophils, produce high concentrations of ROS in the lungs of asthmatic inflammation (1). In our study, ROS was generated in BALF by Der p2 stimulation. Tiron treatment not only reduced ROS production but also decreased the cell number of inflammatory cells. Noticeably, it also decreased percentages of eosinophils and neutrophils (Fig. 5). Thus, in addition to lung epithelium, inflammatory cells are also to be considered one of major sources of the oxidative reactions involved in this inflammatory process.

Dermatophagocyte extracts alter confluent A549 growth via protease activity, which has discontinuous tight junctions and stimulates the secretion of factors that deregulate fibroblast growth (11). NGF stimulates fibronectin-induced fibroblast migration (39) and exerts a direct profibrogenic effect on lung fibroblasts, indicating a role of NGF in tissue repair and fibrosis (47). In addition, the ability of fibrocytes to differentiate into mature mesenchymal cells has been confirmed in animal models of wound healing or fibrotic diseases, which suggested a causal relation between fibrocyte accumulation and ongoing tissue fibrogenesis in response to tissue damage (5). However, how Der p2 without protease activity directly affects fibroblasts is still unknown. Der p2-treated mice exhibited fibrocyte infiltration in the subepithelial zone and mild subepithelial fibrosis. We also found that fibroblasts were one major source of NGF, which might participate in Der p2-induced inflammation. Antioxidants are thought to be as potential therapeutics for lung fibrosis (15). Tiron also blocked slightly decreased fibrosis after Der p2 stimulation for short-term periods.

Antioxidants are able to reduce airway inflammation in asthmatic animal models. Antioxidant treatment of asthma, such as *N*-acetylcysteine, has been one subject of therapeutic strategies, but it has had limited success in clinical settings (29). Thus novel antioxidant drugs are needed for further investigation. Molecular targeted therapy against redox-sensitive signals might be an alternative therapeutic approach (29). Tiron is a useful protective agent against oxidative stress, and it also offers protective ability by chelating redox-active transition metal ions (40). It has been used widely in concentrations of 1–10 mM in vitro (3, 4, 10, 33) and 500–600 mg/kg (intraperitoneal) in vivo (30, 54, 55) to determine the role of superoxide anion in modulating a variety of biological responses. In our study, tiron at lower concentrations is an effective agent for improving airway inflammation. Moreover, it inhibits NGF-related signals induced by Der p2. Therefore, the effects of tiron might be an available therapeutic agent for asthma.

Taken together, these results indicated that both ROS production and NGF release might be related to Der p2-induced asthmatic inflammation and that ROS-mediated MAPK signals were partially involved. In addition, tiron improved Der p2-induced lung inflammation and blocked NGF-related signals.

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GRANTS

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DISCLOSURES

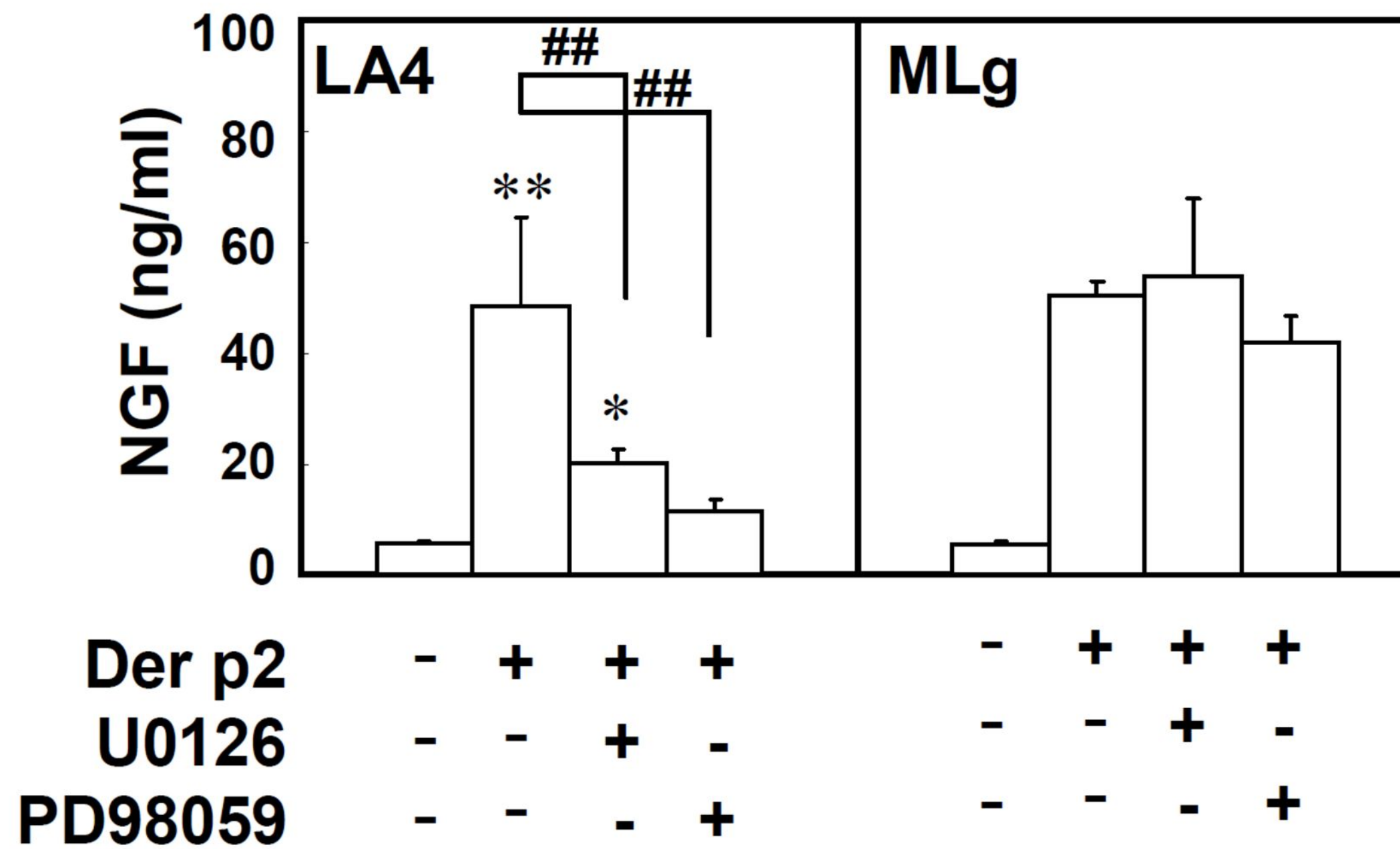
No conflicts of interest, financial or otherwise are declared by the author(s).

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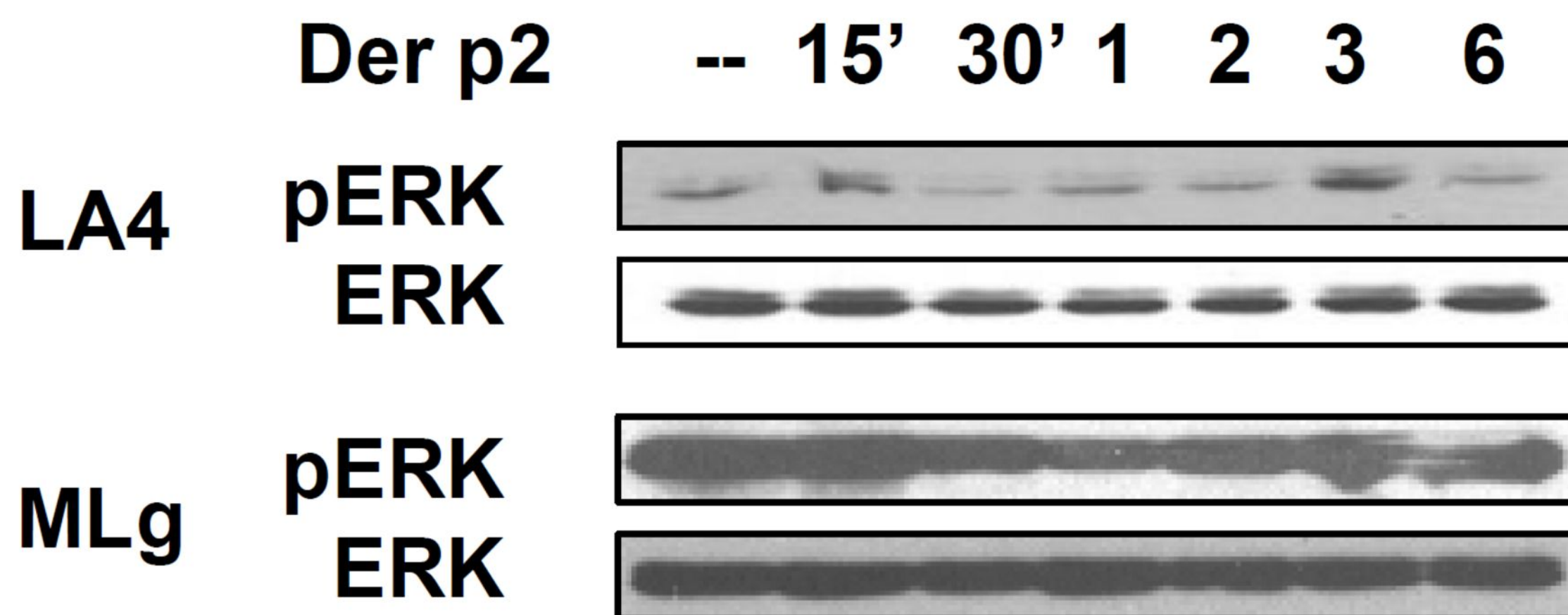
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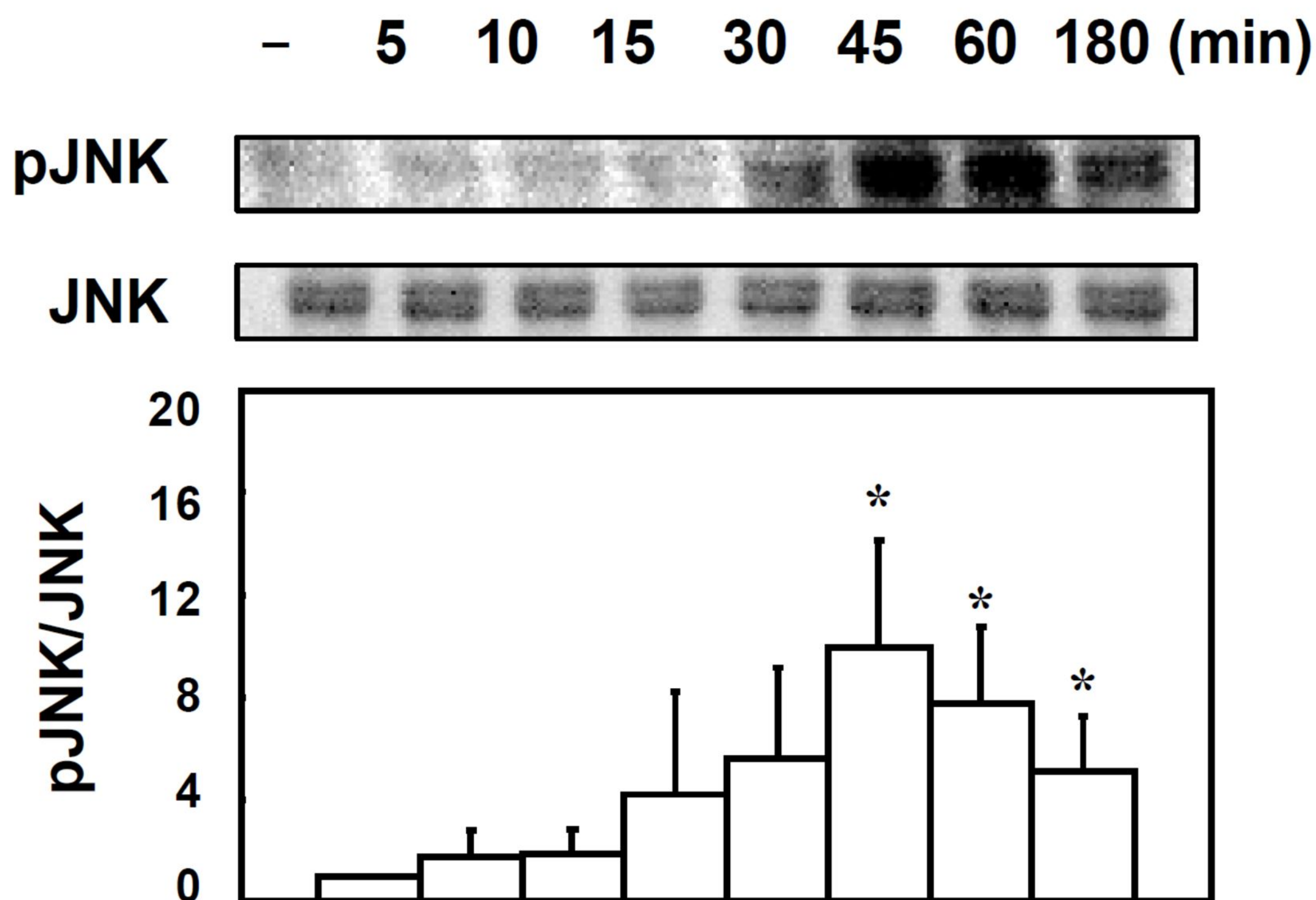
A



B



Supplementary figure 1 The effects of Der p2 on NGF release and ERK phosphorylation in LA4 and MLg cells. Cells were pretreated with 25 μ M of U0126 or PD98059 for 30 min prior to 100 μ g/ml of Der p2 treatment for 24 h. The culture medium was collected and the NGF content was determined using an ELISA kit (A). The cell lysates were harvested at indicated times for the detection of ERK phosphorylation levels using Western blot (B). Ratios are shown in OD of phospho-pERK/total ERK. Data are expressed as mean $\pm$ SE of the data from three individual experiments. * $P < 0.05$ and ** $P < 0.01$ compared with medium controls. ## $P < 0.01$ compared with Der p2-treated group.



Supplementary figure 2 Der p2 activated JNK phosphorylation in MLg cells in a time-dependent manner. The cell lysates were harvested at indicated time for the detection of phospho-JNK levels by Western blot. Ratios are shown in OD of phospho-JNK/total JNK. Data are expressed as mean $\pm$ SE of the data from three individual experiments. * $P < 0.05$ as compared with medium controls.