

SIGNALING PATHWAYS FOR TNF PRODUCTION INDUCED BY HUMAN AMINOACYL-tRNA SYNTHETASE-ASSOCIATING FACTOR, p43

Heonyong Park,^{†*} Sang Gyu Park,[†] Junghee Kim,[†] Young-Gyu Ko,^{†**} Sunghoon Kim^{†¶}

The p43 protein is associated with human macromolecular aminoacyl tRNA synthetase complex and secreted to up-regulate diverse proinflammatory genes including TNF. Here we focused on the p43-induced TNF production and determined its responsible signal pathway. The p43-induced TNF production was mediated by the activation of MAPK family members, ERK and p38 MAPK, and by I κ B degradation leading to the activation of NF κ B. We also studied the upstream molecules for ERK and p38 MAPK by using a variety of inhibitors. The inhibitors for protein kinase C (PKC) and phospholipase C (PLC) prevented the p43-induced TNF production. Interestingly, all of the effective drugs inhibited the ERK activity, while the drugs had no effects on p38 MAPK activity and I κ B degradation. Together, the p43-induced TNF production was controlled by NF κ B, p38 MAPK, and ERK that is dependent on the activities of PLC and PKC.

© 2002 Elsevier Science Ltd. All rights reserved.

The p43 protein is a protein associated with mammalian macromolecular aminoacyl tRNA synthetase complex consisting of at least eight different enzymes. Interestingly, the C-terminal domain of p43 was shown to be equivalent to EMAP II (endothelial monocyte-activating polypeptide II) that was first isolated from the methylcholanthrene A-induced fibrosarcoma.¹ EMAP II enhances cell migration in polymorphonuclear and mononuclear cells and its expression is predominant in inflammatory autoimmune lesions of the rat central nervous system.¹ EMAP II also elevates the calcium concentration in the cytoplasm, and subsequently activates the myeloperoxidase. Many inflammatory genes such as tumor necrosis factor (TNF), TNF receptor-1 and P, E-selectins, are up-regulated by EMAP II.¹

Since EMAP II is a potent proinflammatory cytokine and generated in vitro by proteolytic cleavage of p43 with caspase-7, p43 was thought to be a precursor of EMAP II. However, we have recently shown that p43 itself is secreted from the intact cells and induce proinflammatory genes,² suggesting that it is a real cytokine working at physiological condition. Among the p43-induced genes, the expression of TNF was robustly increased.² Thus, we decided to dissect the signaling pathways of the TNF production in response to p43.

TNF has been known to induce numerous proinflammatory activities and be secreted by a variety of cells, predominantly macrophages and monocytes.^{3,4} TNF is also considered as a pathogenic mediator in autoimmune diseases and inflammatory diseases, such as multiple sclerosis, endotoxic shock, rheumatoid arthritis and ulcerative colitis.^{5,6} Therefore TNF is an important molecule for inflammation and inflammatory diseases. Since the regulation of TNF production is differentiated depending upon different cell types and stimuli, determination of the signaling pathway for the p43-induced TNF production is essential for identifying the physiological and pathophysiological role of p43 in inflammation.

TNF expression is mediated mainly by mitogen-activated protein kinases (MAPKs) and NF κ B.⁷⁻¹⁰ In addition, the TNF production is also mediated by different signaling molecules such as protein kinase C (PKC), phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3-kinase).^{11,12} Therefore it is highly

From the [†]National Creative Research Initiatives Center for ARS Network, College of Pharmacy, Seoul National University, San 56-1, Shillim-dong, Kwanak-ku, Seoul, 151-742, Korea

Current addresses: *Department of Molecular Biology, Dankook University, Seoul 140-714, **Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Correspondence to: ¶Sunghoon Kim, National Creative Research Initiatives Center for ARS Network, College of Pharmacy, Seoul National University, San 56-1, Shillim-dong, Kwanak-ku, Seoul, 151-742, Korea. Tel: +82 2 880 8180; Fax: +82 2 875 2621; E-mail: sungkim@snu.ac.kr

Received ; received in revised form ; accepted for publication
1043-4666/02/\$-see front matter © 2002 Elsevier Science Ltd.
All rights reserved.

KEY WORDS: mitogen-activated protein kinase/endothelial monocyte-activating polypeptide II/NF- κ B/phospholipase C/protein kinase C

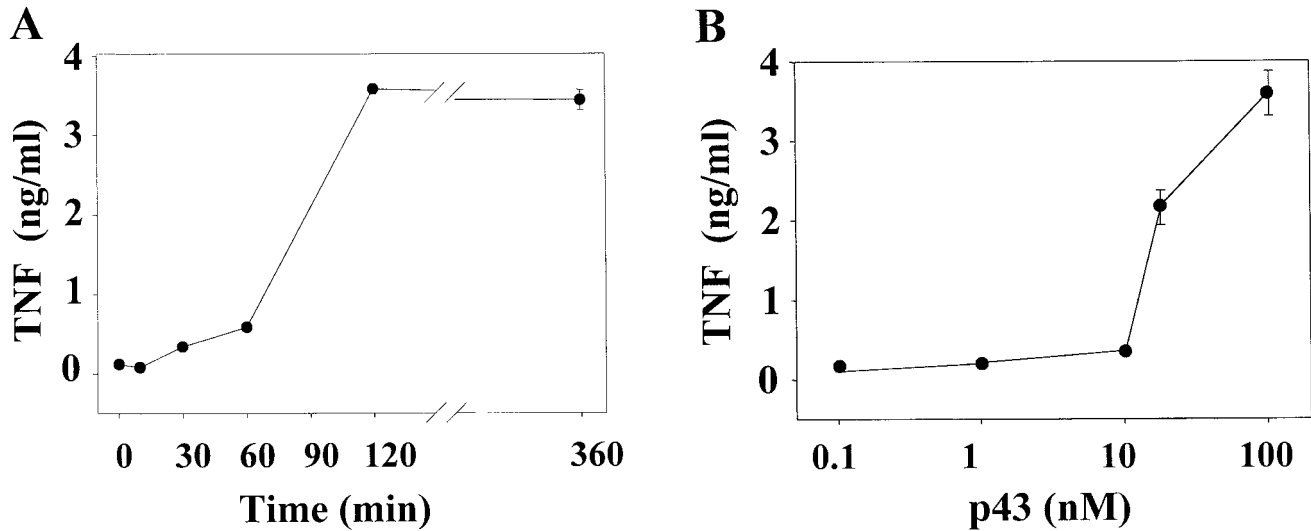


Figure 1. The p43 protein induces the TNF production.

(A) THP-1 cells were treated with 100 nM p43 for the indicated time points. Then TNF amounts in the media were measured by ELISA. (B) The TNF production was measured in the media of THP-1 cell culture treated with the indicated concentration of p43 for 2 h. Data are representative for the mean \pm SE obtained from three independent experiments.

possible for the signaling molecules, such as PKC, PLC and PI3-kinase, to be involved in the MAPK activation leading to the p43-induced TNF production. Here we have used monocyte and investigated the p43-dependent signal pathways leading to TNF production.

RESULTS

The p43 protein induces TNF production

To determine the optimal condition of TNF production, the dose- or time-dependent experiments of the TNF production were executed. In Figure 1, TNF was maximally produced by the treatment of 100 nM p43 for 2 h. As shown in Figure 1A, the TNF production was rapidly increased after 1 h. For dose curve, the TNF production was highly increased at 20 nM and appeared maximal at 100 nM of p43 (Fig. 1B). The time course of the p43-dependent TNF induction is very similar to that of the TNF production induced by lipopolysaccharide (LPS) in THP-1 cells.¹³ Previously we also examined the time-dependent TNF mRNA level by cDNA microarray, showing that mRNA was maximally expressed 30 min after the p43 treatment.² When the peripheral blood mononuclear cells were isolated from blood and used to test the p43-induced TNF production, 1 nM of p43 was sufficient to see the activity (data not shown), suggesting that p43 would also induce TNF at the physiological condition.

ERK and p38 MAPK were involved in the p43-induced TNF production

In THP-1 cells, p43 activates MAPK family members.² Since MAPK family members mediate TNF

production upon cellular exposure to LPS and other cytokines, we investigated whether these kinases are also involved in the p43-induced TNF production. JNK (c-Jun N-terminal protein kinase) is a regulating factor for TNF production among MAPK family members. However, because of low transfection efficiency to THP-1 cells and the lack of specific inhibitors, JNK studies were not feasible in the THP-1 cells. Therefore we dissected the pathway mediated by ERK (extracellular-signal regulated kinase) and p38 MAPK in this study. As shown in Figure 2, both of ERK and p38 MAPK inhibitor blocked the p43-induced TNF production. These results indicate that ERK and p38 MAPK are specific mediators for the p43-induced TNF production. The coordinated regulation of TNF production by ERK and p38 MAPK was reported previously¹⁴ in macrophage. By unknown mechanisms both ERK and p38 MAPK inhibit the Jun activity, leading to inhibition of TNF expression. Although previous reports are consistent with our current observation, the detailed mechanisms by which ERK and p38 MAPK regulate the p43-dependent TNF production remain to be investigated.

PLC and PKC were involved in the p43 induced TNF production

Since PLC and PKC are upstream molecules to control the TNF production,^{11,15} we investigated whether PLC and PKC molecules are the intermediate regulators for the p43-induced TNF production. We first tested whether p43 activates PLC. For monitoring the PLC activity, the cell lysates were immunoprecipitated with anti-PLC antibody after the treatment of

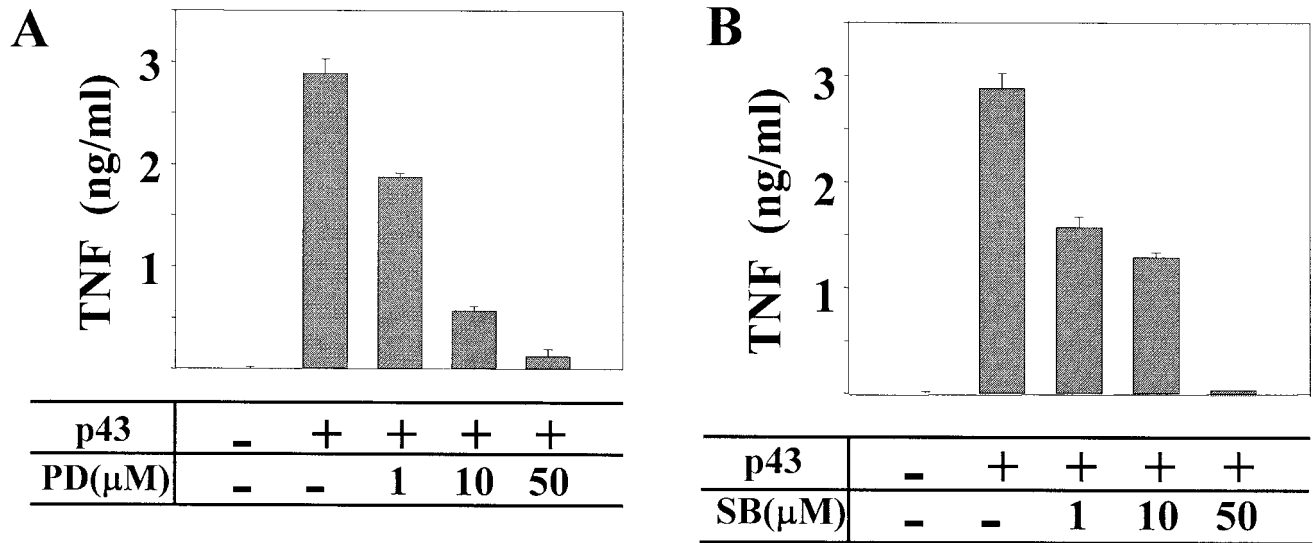


Figure 2. ERK and P38 MAPK are involved in the p43-induced TNF-production.

THP-1 cells were pre-incubated with PD (PD98059) (A), or SB (SB202190) (B) 1 h before the treatment with 100 nM of p43. The TNF amounts in the media were measured by ELISA. The bar graph shows the mean \pm SE ($n=3$).

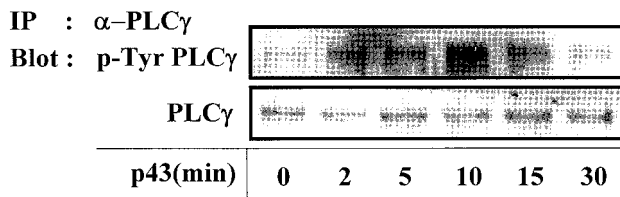


Figure 3. The p43 protein induces the phosphorylation of PLC γ .

The cells were treated with 100 nM of p43 for the indicated time points. The cell lysates were immunoprecipitated with the antibody for PLC γ and then blotted with the antibody specific for phosphotyrosine.

THP-1 cells with p43, and immunoblotted with phospho-tyrosine antibodies. The three different isoforms (β , δ and γ) of PLC were tested for the p43-induced phosphorylation and observed that the only PLC γ protein was phosphorylated by p43 stimulation (data not shown). In Figure 3, the phosphorylation of PLC γ was maximally enhanced 10 min after the treatment of p43 and reduced back to basal level at 30 min. Then, in order to investigate whether PLC and/or PKC regulate the p43-induced TNF production, we used general inhibitors, U-73122 and staurosporine, which block PLC and PKC, respectively. When THP-1 cells were pretreated with the PLC or PKC inhibitor, the TNF production was inhibited in either case, indicating that PKC and PLC control the p43-induced TNF production (Fig. 4A and B).

Since PKC is activated by diacylglycerol (DAG) produced by PLC,¹⁶ p43 could sequentially activate both PLC and PKC. We then asked whether the PKC activation by p43 affects the ERK activity leading to the TNF production. The pretreatment of the cells with U-73122 and staurosporine inhibited the p43-

induced activation of ERK (Fig. 4A and B). In contrast, either inhibitor has no effect on the p43-induced activation of p38 MAPK. Taken together, the PLC/PKC signaling molecules are upstream regulators only for the ERK activation, but not for the p38 MAPK activation.

The p43-dependent I κ B degradation mediates the p43-induced TNF production

Since p43 induces the degradation of I κ B leading to NF κ B activation,² we investigated whether the degradation of I κ B is responsible for the p43-induced TNF production. Since ALLN (N-acetyl-leuciny-leucinylnorleucinal) is a potent inhibitor of NF κ B activation working by the mechanism of preventing I κ B proteolysis,¹⁷ ALLN was used for blocking the degradation of I κ B. The pretreatment of cells with ALLN inhibited the p43-induced I κ B degradation and TNF production in a dose-dependent manner (Fig. 5A), indicating that the I κ B degradation may mediate the TNF production stimulated by p43. We also tested whether other blocking agents inhibit the I κ B degradation induced by p43. Other inhibitors like staurosporine, and U-73122, have no effect on the p43-induced I κ B degradation (Fig. 5B), suggesting that the I κ B degradation is independent of the ERK signaling pathway.

DISCUSSION

The p43 protein is a newly found cytokine up-regulating many inflammation-related gene products, such as MIP-1, IL-8, and MCP-1. Therefore, p43

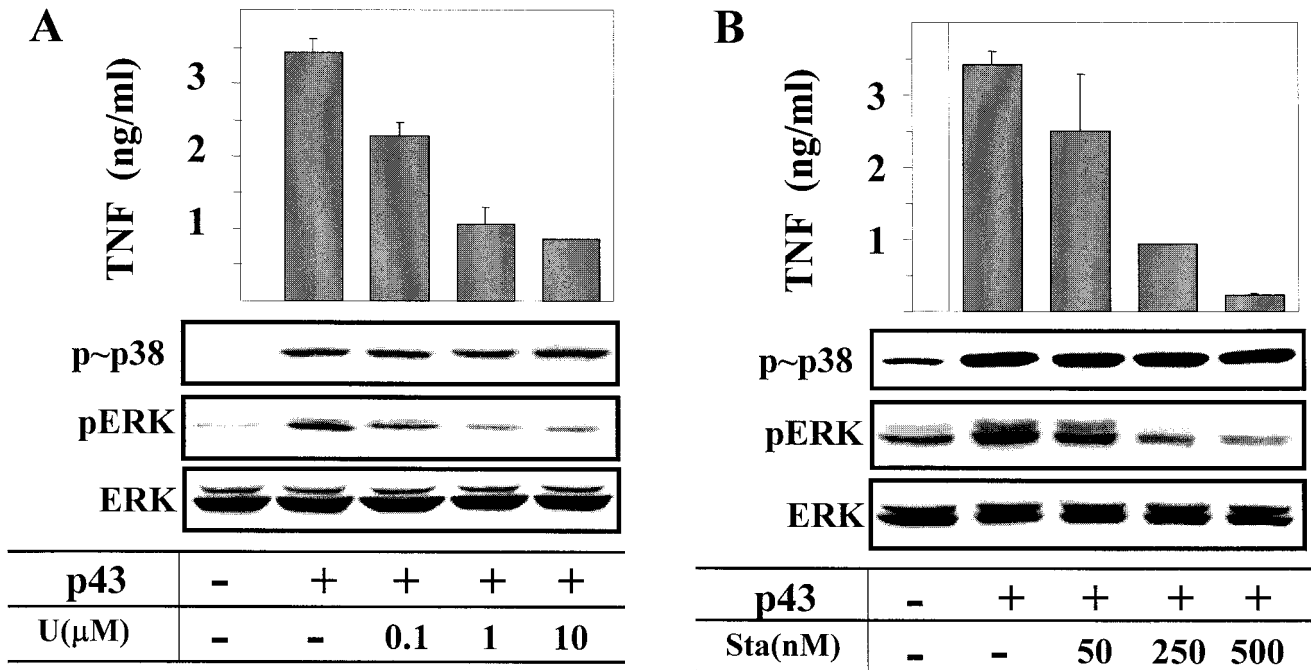


Figure 4. Inhibitors for PLC γ and PKC block the p43-induced production of TNF.

Cells were pretreated with U (U-73122) (A) and Sta (staurosporine) (B) 1 h before the treatment with 100 nM of p43. Then TNF amounts produced in the media were measured by ELISA. TNF amounts produced by p43 were showed in the bar graphs. The bar graphs show the means \pm SE ($n=3$). The effect of U (U-73122) (A) and Sta (staurosporine) (B) in ERK and p38 MAPK phosphorylation was monitored by western blots using antibodies specific for the phospho forms of ERK and p38 MAPK.

is suggested to act on the inflammation-related diseases such as atherosclerosis.^{2,18} Among the gene products up-regulated by p43, TNF was most strongly induced by p43. For this reason, p43 has been also suggested as an important factor in normal inflammation and inflammatory diseases related to TNF, such as inflammatory arthritis and multiple sclerosis. Since TNF is considered as a pathogenic mediator in autoimmune diseases and inflammatory diseases, we dissected the signaling pathways for the p43-dependent TNF production to search for a way of treating the TNF-related diseases. Interestingly, p43 increases the TNF production through the pathways responsible for activation of ERK, p38 MAPK and NF κ B. The identification of the signaling pathways induced by p43 will be utilized for the development of synergistically effective drug combination.

A plethora of reports address that PLC and PKC are involved in TNF production stimulated by a variety of cytokines and stimulus in different immune cells. Thus we can easily expect that PLC and PKC are responsible for the p43-dependent TNF production. However it is of note to demonstrate here that the signaling pathway regulated by PLC and PKC is responsible for ERK activation, which is not sufficient for inducing the production of TNF. Thus the current studies suggest that the ERK signaling pathway is coordinated with other signaling pathways eliciting

activation of p38 MAPK or NF κ B. The coordinated regulation of TNF production by ERK, p38 MAPK and NF κ B was reported in the glass fiber-stimulated TNF expression.¹⁴ In LPS induction of TNF, MAPK and NF κ B are concertedly participated in the production of TNF through the CRE and κ B sites.¹⁹ We now don't understand how ERK and p38 MAPK regulate the induction of TNF production, but the previous studies provide a possibility that ERK and p38 MAPK somehow stimulate the Jun activity, interacting with NF κ B in a coordinative manner, which requires the induction of TNF promoter.

Determination for the signaling pathway is also important for receptor studies as well as drug developments. The finding of the upstream molecules involved in the p43-dependent signal transduction provides an insight into the potential p43 receptor. However, the upstream molecules, PLC and PKC are involved in the only ERK signaling pathway regardless of two independent p38 MAPK and NF κ B signaling pathways. We still do not know whether the three independent stimulation of signaling molecules is caused by separate receptors or a divergent event triggered by a p43-specific receptor.

The signaling pathway determined in this study further supports the roles of p43 as a novel cytokine. Referring to its role as a cytokine, the signaling pathways determined in this study also suggest that p43

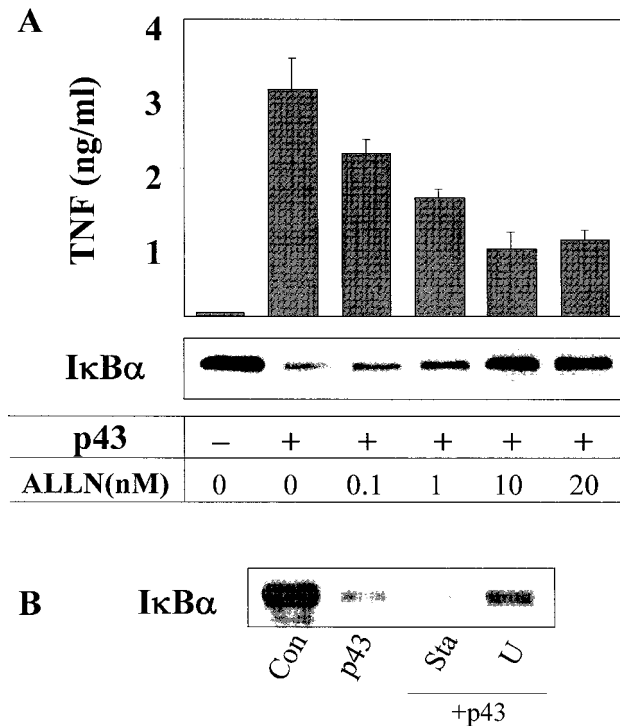


Figure 5. An inhibitor for I κ B α degradation, ALLN, prevented the p43-induced TNF production.

Cells were pretreated with indicated concentration of ALLN 1 h before the p43 treatment. 100 nM of p43 was applied for 1 h to detect I κ B α degradation, whereas cells were treated with 100 nM of p43 for 2 h to determine TNF production (panel A). In panel B, cells were treated with none or 100 nM of p43 for 1 h after the treatment of each inhibitor, Sta (staurosporine, 10 μ M, 1 h) and U (U-73122, 500 μ M, 1 h).

may be responsible for a variety of physiological and/or pathophysiological functions. Here, we observed that p43 activates NF κ B and MAP kinases, which regulate a variety of the physiological functions such as gene expression, cell proliferation and programmed cell death. Although the scope of the current study is limited to the TNF production, physiological roles of p43 are suggested to be diverse.

In conclusion, p43 up-regulates an inflammatory factor, TNF, via the three independent pathways involving p38 MAPK, ERK and NF κ B. Among them, the p43-activated ERK pathway was shown to include PLC and PKC. The results obtained in this work would also help to unravel other physiological roles of p43 that are yet to be found.

MATERIALS AND METHODS

Cell culture and materials

THP-1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 μ g/ml streptomycin and penicillin in a 5% CO₂ incubator at 37°C. The primary cultured cells used in this study were between

passages 2 and 4. Antibodies against phospho-ERK1/2, phospho-p38 were purchased from New England Biolabs. Anti-phosphotyrosine and anti-phospholipase C- γ antibodies was obtained from BD Transduction Laboratories.

Construction and purification of the p43 protein

The p43 protein was expressed as His-tag fusion protein in *Escherichia coli* BL21 (DE3) and purified by nickel affinity chromatography and Mono Q or S ion-exchange chromatography. To remove lipopolysaccharide (LPS), the protein solution was dialyzed in pyrogen free buffer (10 mM potassium phosphate buffer, pH 6.0, 100 mM NaCl). After dialysis, the protein was loaded to polymyxin resin (Bio-Rad) pre-equilibrated with the same buffer, incubated for 20 min and eluted. The concentration of the residual LPS was below 20 pg/ml when determined using Limulus Amebocyte Lysate QCL-1000 kit (Bio Whittacker).

Western blots

The cells treated with p43 were harvested by centrifugation at 600 $\times g$ for 5 min and lysed with 0.25 ml of lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM vanadate, 1 mM dithiothreitol, 1.0% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride). For p38 MAPK and I κ B α assay, cells were lysed by sonication in radioimmune precipitation buffer (RIPA). The proteins in the lysates were resolved by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) and probed with specific antibodies. The antibodies bound to specific proteins were reacted with the secondary antibody conjugated with horseradish peroxidase and then detected with an enhanced chemiluminescence substrate kit (Amersham).

PLC assay

Vehicle or p43 treated THP-1 cells were harvested by the centrifugation at 10 000 $\times g$ and washed twice with ice cold 1X phosphate buffered saline (PBS). Cells were lysed with the lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 10 mM NaF, 12 mM b-glycerophosphate, 1 mM Na₃VO₄, 10% glycerol, 1% Nonidet P-40, 5 μ g/ml aprotinin, 1 mM PMSF, 1 mM DTT) at 4°C for 20 min incubation. Equal amounts of proteins were incubated with 5 μ g of anti-PLC γ antibody (Santa Cruz) for 2 h. Then, protein G conjugated agarose was added and incubated for additional 2 h. The immunoprecipitates were washed three times with the lysis buffer, boiled for 10 min after mixing with Laemmli sample buffer and then resolved with 7.5% SDS-PAGE. PLC γ and phospho-PLC γ was detected by western blots using anti-PLC γ and PY20 antibodies (BD Transduction Laboratories).

TNF assay

THP-1 cells were treated with p43 as the indicated amounts and time points. For inhibitor assay, the cells were pre-incubated with vehicle control or each drug before the p43 treatment. Then the cells were removed by the centrifugation. TNF amounts in the media were measured by an ELISA using TNF assay kit (Pharmingen).

Acknowledgments

This work was supported by a grant from the National Creative Research Initiatives of the Ministry of Science and Technology of Korea.

REFERENCES

- Berger AC, Tang G, Alexander HR, Libutti SK (2000) Endothelial monocyte-activating polypeptide II, a tumor-derived cytokine that plays an important role in inflammation, apoptosis, and angiogenesis. *J Immunother* 23:519–527.
- Ko YG, Park H, Kim T, Lee JW, Park SG, Seol W, Kim JE, Lee WH, Kim SH, Park JE, Kim S (2001) A cofactor of tRNA synthetase, p43, is secreted to up-regulate proinflammatory genes. *J Biol Chem* 276:23028–23033.
- Vassalli P (1992) The pathophysiology of tumor necrosis factors. *Annu Rev Immuno* 10:411–452.
- Grell M, Douni E, Wajant H, Löhden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K, Scheurich P (1995) The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83:793–802.
- Feldmann M, Brennan FM, Elliott M, Katsikis P, Maini RN (1994) TNF alpha as a therapeutic target in rheumatoid arthritis. *Circ Shock* 43:179–184.
- Strieter RM, Kunkel SL, Bone RC (1993) Role of tumor necrosis factor-alpha in disease states and inflammation. *Crit Care Med* 21(10 Suppl):S447–S463.
- Ishizuka T, Terada N, Gerwins P, Hamelmann E, Oshiba A, Fanger GR, Johnson GL, Gelfand EW (1997) Mast cell tumor necrosis factor alpha production is regulated by MEK kinases. *Proc Natl Acad Sci USA* 94:6358–6363.
- Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW *et al.* (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372:739–746.
- Lafont V, Liautard J, Gross A, Liautard JP, Favero J (2000) Tumor necrosis factor-alpha production is differently regulated in gamma delta and alpha beta human T lymphocytes. *J Biol Chem* 275:19282–19287.
- Liu H, Sidiropoulos P, Song G, Pagliari LJ, Birrer MJ, Stein B, Anrather J, Pope RM (2000) TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. *J Immunol* 164:4277–4285.
- Sinji H, Akagawa KS, Tsuji M, Maeda M, Yamada R, Matsuura K, Yamamoto S, Yoshida T (1997) Lipopolysaccharide-induced biphasic inositol 1,4,5-triphosphate response and tyrosine phosphorylation of 140-kilodalton protein in mouse peritoneal macrophages. *J Immunol* 158:1370–1376.
- Foreback JL, Sarma V, Yeager NR, Younkin EM, Remick DG, Ward PA (1998) Blood mononuclear cell production of TNF-alpha and IL-8: engagement of different signal transduction pathways including the p42 MAP kinase pathway. *J Leukoc Biol* 64:124–133.
- Steer JH, Kroeger KM, Abraham LJ, Joyce DA (2000) Glucocorticoids suppress tumor necrosis factor-alpha expression by human monocytic THP-1 cells by suppressing transactivation through adjacent NF-kappa B and c-Jun-activating transcription factor-2 binding sites in the promoter. *J Biol Chem* 275:18432–18440.
- Ye J, Zeidler P, Young S, Martinez A, Robinson VA, Jones W, Baron P, Shi X, Castranova V (2001) Activation of mitogen-activated protein kinase p38 and extracellular signal-regulated kinase is involved in glass fiber-induced tumor necrosis factor-alpha production in macrophages. *J Biol Chem* 276:5360–5367.
- Yan Z, Yang DC, Jett M (1999) Cholera toxin induces tumor necrosis factor alpha production in human monocytes. *Mol Cell Biol Res Commun* 2:124–130.
- Hurley JH, Grobler JA (1997) Protein kinase C and phospholipase C: bilayer interactions and regulation. *Curr Opin Struct Biol* 7:557–565.
- Schow SR, Joly A (1997) N-acetyl-leucanyl-leucynyl-norleucinal inhibits lipopolysaccharide-induced NF-kappaB activation and prevents TNF and IL-6 synthesis in vivo. *Cell Immunol* 175:199–202.
- Berger AC, Alexander HR, Tang G, Wu PS, Hewitt SM, Turner E, Kruger E, Figg WD, Grove A, Kohn E, Stern D, Libutti SK (2000) Endothelial monocyte activating polypeptide II induces endothelial cell apoptosis and may inhibit tumor angiogenesis. *Microvasc Res* 60:70–80.
- Yao J, Mackman N, Edgington TS, Fan S-T (1997) Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. *J Biol Chem* 272:17795–17801.