ELSEVIER

European Journal of Pharmacology 436 (2002) 47–51

www.elsevier.com/locate/ejphar

Short communication

Ambroxol inhibits platelet-derived growth factor production in human monocytic cells

Mitsuyoshi Utsugi [a], Kunio Dobashi [a,*], Yasuhiko Koga [a], Ken Masubuchi [a], Yasuo Shimizu [a], Katsuaki Endou [a], Tsugio Nakazawa [b], Masatomo Mori [a]

[a] *First Department of Internal Medicine, Gunma University Faculty of Medicine, School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma, 371-8511, Japan*

[b] *Gunma University Faculty of Health Sciences, Maebashi, Japan*

Received 27 September 2001; received in revised form 13 December 2001; accepted 18 December 2001

Abstract

Several growth factors, including platelet-derived growth factor (PDGF), have been implicated in the mechanism of lung and airway remodeling. We investigated the effect of ambroxol, *trans*-4-[(2-amino-3,5-dibromobenzyl) amino] cyclohexanol hydrochloride, on the lipopolysaccharide-induced PDGF production in human monocytic cells, THP-1. Ambroxol inhibited the lipopolysaccharide-induced PDGF-AB production via PDGF-A mRNA expression. Lipopolysaccharide activated p44/42 extracellular signal-regulated kinase (ERK), and ambroxol attenuated the lipopolysaccharide-induced p44/42 ERK activation. Furthermore, mitogen-activated protein kinase kinase (MEK)-1-specific inhibitor, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD 98059), blocked the lipopolysaccharide-induced p44/42 ERK activation and PDGF production. These findings indicate that ambroxol inhibits the lipopolysaccharide-induced PDGF production due to the suppression of p44/42 ERK activity.

Keywords: Ambroxol; PDGF (Platelet-derived growth factor); ERK (p44/42 extracellular signal-regulated kinase); Monocytic cell, human

1. Introduction

Structural remodeling of the lung tissue is believed to be the consequence of repeated cycles of epithelial injury and the repair process associated with chronic pulmonary disease (Crouch, 1990; Reed, 1999). Several growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), secreted by inflammatory or resident structural cells, have been implicated in the mechanism of structural changes associated with lung and airway remodeling (Kelley, 1990). PDGF is a mitogen and chemoattractant for mesenchymal cells, such as fibroblasts and smooth muscle cells. Two different PDGF chains, termed A and B, encoded by different genes, have been identified to lead to three different PDGF isoforms: the AA and BB homodimers, and the AB heterodimer (Ross, 1989). PDGF is also synthesized and secreted by a wide variety of human cells, including monocytes and macrophages, in response to inflammatory stimuli, such as lipopolysaccharide (Allam et al., 1992).

Many extracellular stimuli elicit specific biological responses through the activation of mitogen-activated protein (MAP) kinase cascades (Whitmarsh and Davis, 1996). P44/42 extracellular signal-regulated kinase (ERK), one of the major subgroups of MAP kinases, is activated by mitogenic stimuli and plays a central role in cell proliferation and differentiation (Cowley et al., 1994; Force and Bonventre, 1998). In addition, recent studies have suggested that p44/42 ERK also plays an important role in the signal cascade of induction of various inflammatory cytokines and chemical mediators (Rose et al., 1997), including PDGF (Day et al., 1999).

Ambroxol, *trans*-4-[(2-amino-3,5-dibromobenzyl)amino]cyclohexanol hydrochloride, has been extensively therapeutically employed for treatment of chronic lung diseases due to its mucolytic and surfactant stimulating properties. However, a number of studies have recently shown that ambroxol also displays an antioxidant action (Lee et al., 1999) and anti-inflammatory properties which may be attributed to the free radical scavenging and antioxidative effects of this drug (Bianchi et al., 1990; Gibbs et al., 1999).

* Corresponding author. Tel.: +81-27-220-8123; fax: +81-27-220-8136.
E-mail address: dobashik@med.gunma-u.ac.jp (K. Dobashi).

0014-2999/02/$ - see front matter
PII: S0014-2999(01)01609-0

In the present study, we therefore examined whether or not ambroxol can inhibit the lipopolysaccharide-induced PDGF production in human monocytic cells. Furthermore, we investigated if the p44/42 ERK pathway is involved in the lipopolysaccharide-induced PDGF production, and if ambroxol affects this kinase activity.

2. Materials and methods

2.1. Reagents

Ambroxol was dissolved in distilled water at a concentration of 0.01 M, after which this stock solution was diluted to experimental concentrations with appropriate buffers. Lipopolysaccharide (from *Escherichia coli*, serotype 01 11:B4) was purchased from Sigma (St. Louis, MO). Mitogen-activated protein kinase kinase (MEK)-1-specific inhibitor, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD 98059) (Alessi et al., 1995), and p38 MAP kinase-specific inhibitor, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB 203580), were obtained from Calbiochem–Novabiochem (La Jolla, CA) and dissolved in dimethylsulfoxide (DMSO, Sigma).

2.2. Cell culture and stimulation

Human monocytic THP-1 cells (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium (GIBCO, Life Technologies, Rockville, MD) with 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol supplemented with 10% fetal bovine serum (Equitech-Bio, Ingram, TX), and they were maintained in humidified 5% CO_2 at 37 °C. Subconfluent cells were washed and resuspended in fresh RPMI-1% fetal bovine serum and cultured overnight, then they were stimulated with 10 ng/ml lipopolysaccharide. Ambroxol was added either before (-6 to -1 h), at the same time (0 h) as, or after ($+1$ to $+2$ h) the addition of lipopolysaccharide.

2.3. Quantification of PDGF-AB

The concentrations of PDGF-AB in the culture supernatants were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (AN'ALYZA; genzyme TECHNE, Minneapolis, MN), following the manufacturer's instructions.

2.4. Preparation of complementary RNA (cRNA) probes

A human PDGF-A cDNA fragment containing residues 542-1029 (Gen Bank accession no. X03795) (Betsholtz et al., 1986) was amplified by PCR. The synthesized sense and antisense PCR primers for PDGF-A were 5′ -CCGTAGGG-AGTGAGGATTCT-3′ and 5′ -GCTGCTTTAGGTGGGTT-TTA-3′ . The PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI). Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was cloned as previously described (Dobashi et al., 2001). The cRNA probes were synthesized using [α-^{32}P] UTP (ICN Biomedicals, Aurora, OH) and T7 RNA polymerase (Promega).

2.5. Northern blot analysis

Northern blots were performed as previously described (Dobashi et al., 2001). Briefly, 15 μg of total RNA per lane was size-fractionated and transferred onto nylon membranes.

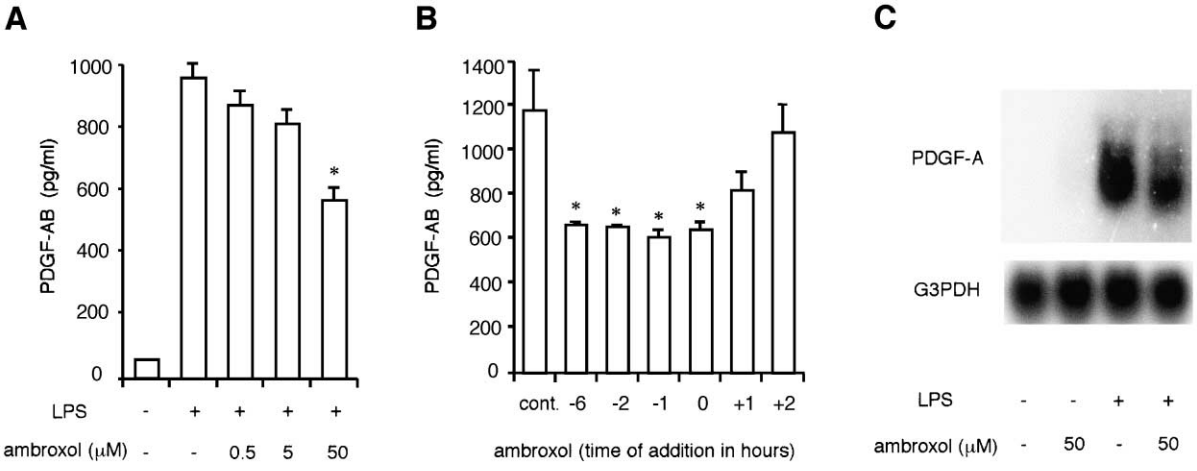

Fig. 1. Effect of ambroxol on the lipopolysaccharide-induced PDGF production in THP-1 cells. (A) THP-1 cells were pretreated with or without ambroxol for 1 h, and then they were stimulated with lipopolysaccharide (10 ng/ml) for 24 h. (B) Cells were stimulated with lipopolysaccharide in the absence (cont.) or presence of ambroxol (50 μM) added at the start (0 h), or 1 to 6 h before the start, or 1 to 2 h after start of stimulation. PDGF-AB protein in culture supernatants was evaluated by ELISA. Values represent means ± S.E.M. of five (A) or four (B) experiments. * $P < 0.05$ compared with the lipopolysaccharide-stimulated THP-1 cells. (C) THP-1 cells were pretreated with or without ambroxol for 1 h and stimulated with or without lipopolysaccharide for 12 h; then total RNA was extracted and hybridized with a human PDGF-A cRNA probe. The blots were stripped, and then a human G3PDH probe was used as a loading control.

The membranes were hybridized to a human PDGF-A cRNA probe at 60 °C overnight. The autoradiographic signal was visualized by exposure to X-ray film at − 70 °C. After the detection of PDGF-A mRNA, the probe was stripped off, and blots were rehybridized with a human G3PDH cRNA probe as a control.

2.6. Measurement of p44/42 ERK activity

P44/42 ERK activity was measured using commercially available kits (nonradioactive p44/42 MAP Kinase Assay Kit; New England Biolabs., Beverly, MA), according to the manufacturer's instructions.

2.7. Statistical analysis

All values are expressed as means ± S.E.M. of the indicated numbers of experiments. Data were compared by Student's *t*-test with the Bonferroni correction for multiple comparisons. A P value of $< 0.05/m$ (where m is the number of comparisons) was considered to be statistically significant in the Bonferroni method.

3. Results

3.1. Effect of ambroxol on the lipopolysaccharide-induced PDGF production in THP-1 cells

As shown in Fig. 1A, pretreatment with ambroxol for 1 h caused a significant dose-dependent inhibition of PDGF-AB protein production from THP-1 cells stimulated with lipopolysaccharide ($P < 0.05$). Moreover, ambroxol was inhibitory when added to the cultures before or simultaneously with the stimulator; this effect was, however, progressively lost when ambroxol was added after the addition of lipopolysaccharide (Fig. 1B).

At the mRNA level, consistent with protein data, pretreatment with ambroxol for 1 h suppressed the lipopolysaccharide-induced PDGF-A mRNA expression (Fig. 1C).

3.2. Effects of PD 98059 and ambroxol on the lipopolysaccharide-induced p44/42 ERK activity in THP-1 cells

Lipopolysaccharide strongly activated p44/42 ERK, which was maximal at 10 min and sustained for up to 80

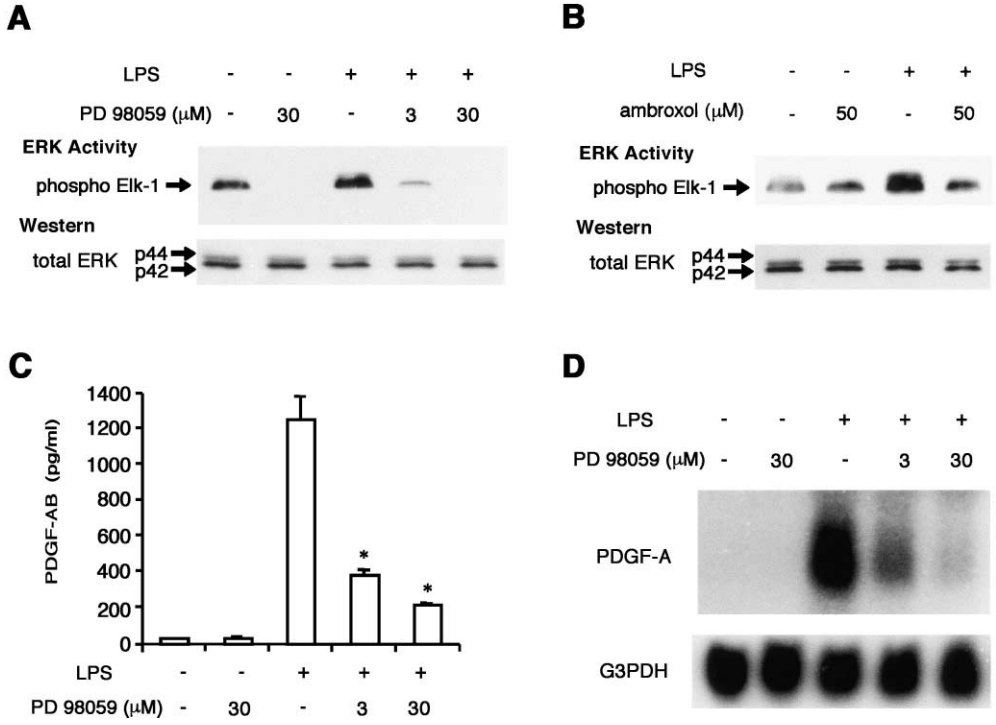

Fig. 2. Effects of PD 98059 (A) and ambroxol (B) on the lipopolysaccharide-induced p44/42 ERK activation. P44/42 ERK activity was measured in an in vitro kinase reaction using Elk-1 as substrate and probing for anti-phospho-Elk-1 in Western blots. (A) THP-1 cells were pretreated with 0.1% DMSO (control vehicle) or PD 98059 for 15 min, and then they were stimulated with or without lipopolysaccharide (10 ng/ml) for 10 min. (B) Cells were pretreated with or without ambroxol for 1 h, and then they were stimulated with or without lipopolysaccharide for 10 min. The corresponding bottom panels are Western blots using anti-p44/42 ERK specific antibody to demonstrate total p44/42 ERK contents. (C and D) Effect of PD 98059 on lipopolysaccharide-induced PDGF production. THP-1 cells were cultured with 0.1% DMSO (control vehicle) or PD 98059 for 15 min before stimulation with lipopolysaccharide. (C) After 24 h, PDGF-AB protein in culture supernatants was evaluated by ELISA. Values represent means ± S.E.M. of four experiments. * $P < 0.05$ compared with lipopolysaccharide-stimulated THP-1 cells. (D) After 12 h, total RNA was extracted, and expression of PDGF-A mRNA was evaluated by Northern blotting, as described in the legend of Fig. 1.

min after the addition of lipopolysaccharide (data not shown). PD 98059, a specific inhibitor of MEK-1, blocked the lipopolysaccharide-induced p44/42 ERK activation in a dose-dependent manner (Fig. 2A). Furthermore, ambroxol attenuated the lipopolysaccharide-induced p44/42 ERK activation (Fig. 2B).

3.3. Effect of PD 98059 on the lipopolysaccharide-induced PDGF production in THP-1 cells

In order to determine if the lipopolysaccharide-induced PDGF production requires the p44/42 ERK pathway, we examined the effect of PD 98059 on the lipopolysaccharide-induced PDGF production. PD 98059 dose-dependently blocked the lipopolysaccharide-induced PDGF-AB protein production ($P < 0.05$, Fig. 2C) and PDGF-A mRNA expression (Fig. 2D). On the other hand, SB203580, p38 MAP kinase inhibitor, did not significantly inhibit the lipopolysaccharide-induced PDGF-AB production (data not shown).

4. Discussion

One key finding of the present study is that ambroxol inhibits the lipopolysaccharide-induced PDGF-AB production and PDGF-A mRNA expression in the human monocytic cell line THP-1. Another was that the p44/42 ERK pathway is required for the lipopolysaccharide-induced PDGF production, and the inhibitory effect of ambroxol on PDGF production is due to the suppression of this kinase activity.

Ambroxol is used as an expectorant in the treatment of chronic bronchitis. Recently, this drug has been shown to exhibit some anti inflammatory effect: Ambroxol inhibits the production of interleukin-1 and tumor necrosis factor-α (TNF-α) from human mononuclear cells (Bianchi et al., 1990), and the production of histamine, leukotrienes, interleukin-4, and interleukin-13 from human leukocytes and mast cells (Gibbs et al., 1999). In the present study, we also found that ambroxol suppresses the lipopolysaccharide-induced PDGF production via the expression of PDGF-A mRNA in THP-1 cells. In addition, we considered that the inhibitory effect of ambroxol on PDGF production was not attributed to ambroxol toxicity, because this effect was progressively lost when ambroxol was added after the addition of lipopolysaccharide.

A recent report has shown that PDGF-A promotor activity stimulated by angiotensin II is mediated via the p44/42 ERK pathway (Day et al., 1999). In the present study, we demonstrated that lipopolysaccharide activated p44/42 ERK, and that selective blockade of this kinase activation blocked LPS-induced PDGF production, suggesting that p44/42 ERK activation is required for lipopolysaccharide-induced PDGF production in THP-1 cells. Furthermore, we demonstrated that ambroxol suppressed the lipopolysaccharide-induced p44/42 ERK activity and PDGF production, as did selective blockade of p44/42 ERK activation. These findings indicate that the pivotal role of ambroxol in the lipopolysaccharide-induced PDGF production is due to the suppression of the p44/42 ERK pathway.

From the data presented here, we conclude that ambroxol inhibits the lipopolysaccharide-induced PDGF production through the p44/42 ERK pathway in the THP-1 monocytic cells. Ambroxol is known to have high affinity to lung tissue, resulting in 16-fold higher tissue concentrations compared with blood (Mezzetti et al., 1990). Moreover, inhalation is expected to further elevate tissue concentrations of this drug in lung and airway. Therefore, ambroxol may have wider clinical applications not only simply as an expectorant but also as anti-inflammatory properties for the treatment of lung and airway remodeling disease, such as pulmonary fibrosis and chronic bronchial asthma, than in the past.

Acknowledgements

The authors would like to thank Kunihiko Iizuka and Tamotsu Ishizuka (Gunma University) for the valuable discussion.

References

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., Saltiel, A.R., 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. 270, 27489–27494.

Allam, M., Martinet, N., Gallati, H., Martinet, Y., 1992. Differential expression of PDGF A- and B-chain genes and production of AA and AB dimers by activated human blood monocytes. Biochimie 74, 1097–1101.

Betsholtz, C., Johnsson, A., Heldin, C.H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L., Knott, T.J., Scott, J., 1986. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. Nature 320, 695–699.

Bianchi, M., Mantovani, A., Erroi, A., Dinarello, C.A., Ghezzi, P., 1990. Ambroxol inhibits interleukin 1 and tumor necrosis factor production in human mononuclear cells. Agents Actions 31, 275–279.

Cowley, S., Paterson, H., Kemp, P., Marshall, C.J., 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77, 841–852.

Crouch, E., 1990. Pathobiology of pulmonary fibrosis. Am. J. Physiol. 259, L159–L184.

Day, F.L., Rafty, L.A., Chesterman, C.N., Khachigian, L.M., 1999. Angiotensin II (ATII) inducible platelet-derived growth factor A-chain gene expression is p42/44 extracellular signal-regulated kinase-1/2 and Egr-1-dependent and mediated via the ATII type 1 but not type 2 receptor. Induction by ATII antagonized by nitric oxide. J. Biol. Chem. 274, 23726–23733.

Dobashi, K., Aihara, M., Araki, T., Shimizu, Y., Utsugi, M., Iizuka, K., Murata, Y., Hamuro, J., Nakazawa, T., Mori, M., 2001. Regulation of LPS induced IL-12 production by IFN-gamma and IL-4 through intracellular glutathione status in human alveolar macrophages. Clin. Exp. Immunol. 124, 290–296.

Force, T., Bonventre, J.V., 1998. Growth factors and mitogen-activated protein kinases. Hypertension 31, 152–161.

Gibbs, B.F., Schmutzler, W., Vollrath, I.B., Brosthardt, P., Braam, U., Wolff, H.H., Zwadlo-Klarwasser, G., 1999. Ambroxol inhibits the release of histamine, leukotrienes and cytokines from human leukocytes and mast cells. Inflammation Res. 48, 86–93.

Kelley, J., 1990. Cytokines of the lung. Am. Rev. Respir. Dis. 141, 765–788.

Lee, C.S., Jang, Y.Y., Han, E.S., 1999. Depressant effects of ambroxol on lipopolysaccharide- or fMLP-stimulated free radical production and granule enzyme release by alveolar macrophages. Pulm. Pharmacol. Ther. 12, 275–284.

Mezzetti, M., Colombo, L., Marini, M.G., Crusi, V., Pierfederici, P., Mussini, E., 1990. A pharmacorinetic study on pulmonary tropism of ambroxol in patients under thoracic surgery. Surg. Intensive Care 13, 179–185.

Reed, C.E., 1999. The natural history of asthma in adults: the problem of irreversibility. J. Allergy Clin. Immunol. 103, 539–547.

Rose, D.M., Winston, B.W., Chan, E.D., Riches, D.W., Gerwins, P., Johnson, G.L., Henson, P.M., 1997. Fc gamma receptor cross-linking activates p42, p38, and JNK/SAPK mitogen-activated protein kinases in murine macrophages: role for p42MAPK in Fc gamma receptor-stimulated TNF-alpha synthesis. J. Immunol. 158, 3433–3438.

Ross, R., 1989. Platelet-derived growth factor. Lancet 8648, 1179–1182.

Whitmarsh, A.J., Davis, R.J., 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J. Mol. Med. 74, 589–607.