BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **220,** 181–185 (1996)
ARTICLE NO. 0377

The Induction of Cyclo-oxygenase-2 in Human Pulmonary Epithelial Cell Culture (A549) Activated by IL-1β Is Inhibited by Tyrosine Kinase Inhibitors

P. Akarasereenont* and C. Thiemermann†,[1]

*†The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ, United Kingdom; and *Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*

Received January 24, 1996

Cyclo-oxygenase (COX) exists as two isoforms. In endothelial cells, the induction of COX (COX-2) elicited by endotoxin or inflammatory cytokines is mediated by tyrosine kinase. Here we have investigated whether the induction of COX-2 elicited by IL-1β in human pulmonary epithelial cells (A549) is mediated by tyrosine kinase. The activity of COX-2 was assessed by measuring the accumulation of PGE_2 by radioimmunoassay. The expression of COX-2 protein was detected by immunoblot using specific antibodies to COX-2. Untreated A549 cells contained no COX-2 protein and released low levels of PGE_2 (<0.3 ng/ml for 24h). A549 cells treated with IL-1β (0.01 to 10 ng/ml) contained COX-2 protein and released greater amounts of PGE_2. The increased COX-2 protein and activity in response to IL-1β (10 ng/ml) was inhibited by the tyrosine kinase inhibitors tyrphostin (AG126; 0.015 to 15 μM) or erbstatin (0.004 to 4 μM). Thus, the induction of COX-2 by IL-1β in epithelial cells is mediated by tyrosine kinase.

Cyclo-oxygenase (prostaglandin endoperoxide synthase, EC 1.14.991, COX) converts arachidonic acid to prostaglandin H_2 (PGH_2)[1] which is then further metabolized to various prostaglandins, prostacyclin and thromboxane A_2[2]. COX exists in at least two isoforms[3]. COX-1 is expressed constitutively in endothelial cells[4] and is probably responsible for the production of prostaglandins under physiological conditions[5]. COX-2 is induced by pro-inflammatory stimuli, including mitogens[6], cytokines[7] and bacterial lipopolysaccharide (LPS)[8,9] in cells *in vitro* and in inflammed sites *in vivo*[10].

The regulation of the different isoforms of COX in pulmonary tissue is not known, although human pulmonary macrophages express COX-2 after stimulation with LPS[11]. Recently, we and others[12,13] have shown that some cytokines can induce COX in human pulmonary epithelial cell. The induction of COX-2 is mediated by tyrosine kinase in endothelial cells and macrophages[14]. We have, therefore, investigated whether a similar mechanism exists in human pulmonary epithelial cells (A549) by using the specific tyrosine kinase inhibitors, erbstatin and tyrphostin (AG126).

METHODS AND MATERIAL

Cell culture. Human pulmonary epithelial cells (A549; The European Collection of Animal Cell Culture; Salisbury, U.K.) were cultured in 96-well plates with Dulbecco's Modified Eagle's Medium (DMEM; 200 μl/well) containing 4 mM L-glutamine. All agents, which were dissolved in distilled water or DMSO (final concentration less than 0.1%; v/v), were sterilised by filtration through a filter (pore size: 0.22 micron) before being added to the cells under sterile conditions. Cells were incubated at 37°C in a humidified incubator.

Measurement of the release of COX metabolites. The increase in COX activity following the activation of A549 with IL-1β (0.01–10 ng/ml) was assessed by measuring the amounts of for prostaglandin E_2 (PGE_2) in the cell supernatant by radioimmunoassay[15]. In experiments to investigate the effects of tyrosine kinase inhibitors on the release of COX metabolites from endogenous arachidonic acid, cells were treated with IL-1β (10 ng/ml) together with the tyrosine kinase inhibitors erbstatin (0.004–4 μM) or tyrphostin (AG126; 0.015–15 μM) for 24 h, and the medium was subsequently removed for radioimmunoassay. In separate experiments designed to measure the effect of tyrosine kinase inhibitors (concentrations as

[1] To whom correspondence should be addressed. FAX: 0171-2511685.

0006-291X/96 $18.00

above) on COX activity, to eliminate any effects of other enzymes induced or activated by IL-1β involved in arachidonic cascade during IL-1β stimulation, cell were treated with IL-1β (10 ng/ml) together with the respective tyrosine kinase inhibitors (as above) for 12 h, after which time the cells were washed and fresh medium containing arachidonic acid (30 μM) was added for 15 min at 37°C. The formation of COX metabolites was then assessed by radioimmunoassay in the cell culture supernatant.

Immunoblot (Western blot) analysis. A549 which were untreated (control), treated with IL-1β alone (10 ng/ml), treated with IL-1β (10 ng/ml) plus erbstatin (4 μM) or with IL-1β (10 ng/ml) plus tyrphostin (15 μM) were cultured in 6-well plates (37°C; for 24 h). After incubation, cells were extracted and a specific COX-2 antibody was used to detect the expression of COX-2 protein as previously described[4].

Measurement of cell viability. Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[16].

Positive controls for loss of cell viability were provided by experiments designed to determine limiting concentrations of DMSO. Cell viability was 85±1% with 0.1%, 70±4% with 1% and 61±5% with 10% DMSO (v/v), relative to the control untreated cells over a 24 h incubation period.

Statistical analysis. Results are shown as mean ± s.e. mean from triplicate determinations (wells) from 3 separate experimental days (n = 9). Student's paired or unpaired *t*-tests, as appropriate, were used to determine the significance of differences between means and *p*-values of less than 0.05 were taken as statistically significant.

RESULTS

Effect of tyrosine kinase inhibitors on the release of COX metabolites from endogenous stores of arachidonic acid by A549 activated by IL-1β. IL-1β enhanced the accumulation of PGE_2 in A549 from < 0.3 ng/ml in untreated cells to 12.8±0.4 ng/ml after treatment with IL-1β (10 ng/ml) for 24 h (Figure 1A). This figure also shows the dose-dependent increase in the formation of PGE_2 by IL-1β in A549 cells. This accumulation of PGE_2 was inhibited by erbstatin (33% inhibition at 4 μM) or tyrphostin (50% inhibition at 15 μM) in a dose-dependent manner. The minimal concentrations necessary to achieve a significant inhibition were 0.4 and 1.5 μM for erbstatin and tyrphostin, respectively (Figure 2A and 3A).

Effect of tyrosine kinase inhibitors on COX activity in A549 activated by IL-1β. The increase in COX activity afforded by IL-1β, measured in the presence of exogenous arachidonic acid, was almost thirty-fold that of the control cells at 24 h (Figure 1B). This figure also shows the dose-dependent increase in COX activity by IL-1β in A549 cells. Figure 2B and 3B show the small, but dose-dependent inhibition by erbstatin or tyrphostin of this increase in COX activity.

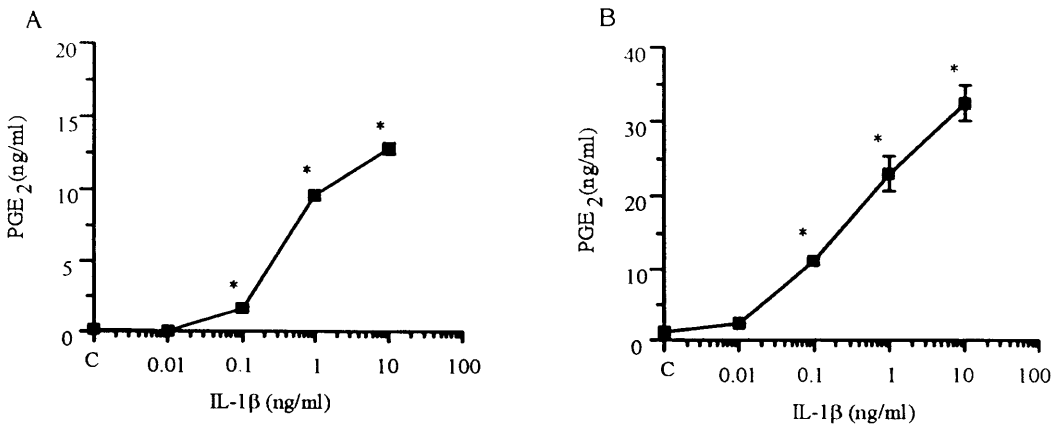

FIG. 1. Dose-dependent increase in the accumulation of COX metabolites (panel A) and the increase of COX activity (panel B) in IL-1β-activated A549 cells at 24 h. The accumulation of COX metabolites (PGE_2) was measured from the supernatant medium from IL-1β-activated A549 cells for 24 h. The increase of COX activity in IL-1β-activated A549 cells at 24 h was measured by the formation of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean ± s.e. mean from 9 determinations from at least 3 separate experimental days. * $p < 0.05$ when compared to untreated cells at 24 h (C).

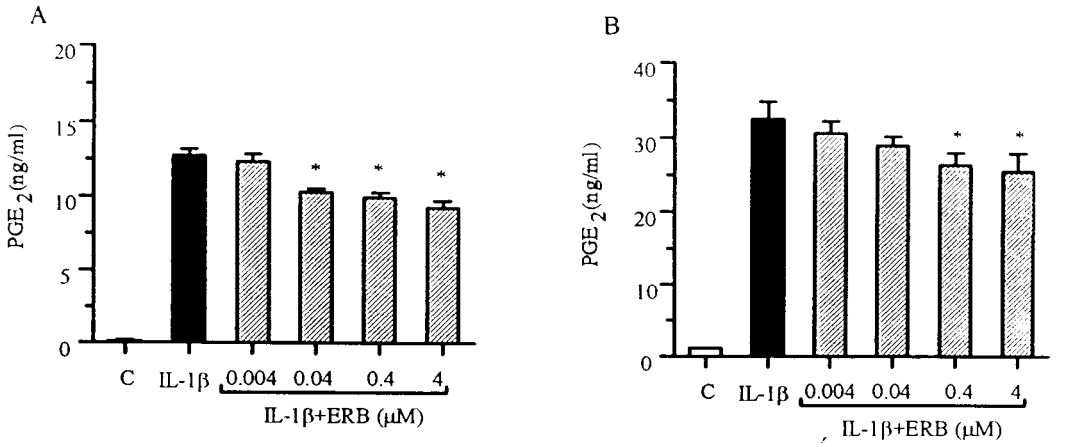

FIG. 2. Dose-dependent inhibition of the accumulation of COX metabolites (panel A) and the increase of COX activity (panel B) by erbstatin (ERB) in IL-1β-activated A549 cells at 24 h. The accumulation of COX metabolites (PGE_2) was measured from the supernatant medium from IL-1β-activated A549 cells for 24 h. The increase of COX activity in IL-1β-activated A549 cells at 24 h was measured by the formation of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean ± s.e. mean from 9 determinations from at least 3 separate experimental days. * $p < 0.05$ when compared to untreated cells at 24 h (C).

Effect of tyrosine kinase inhibitors on COX-2 protein IL-1β-treated A549. Untreated A549 cells contained no COX-2 protein (Figure 4; lane 1). In contrast, A549 activated with IL-1β (10 ng/ml) contained a protein of approximately 70-kDa, which was recognised by a specific antibody to COX-2 (Figure 4; lane 2). This induction of COX-2 protein by IL-1β in A549 was inhibited by erbstatin (4 μM; at 24 h; Figure 4; lane 3) or tyrphostin (15 μM; at 24 h; Figure 4; lane 4).

DISCUSSION

IL-1β (0.01–10 ng/ml) caused a concentration-dependent increase in PGE_2 accumulation in the medium of the A549 cells, and the appearance within the cells of COX-2 protein at 24 h. Untreated

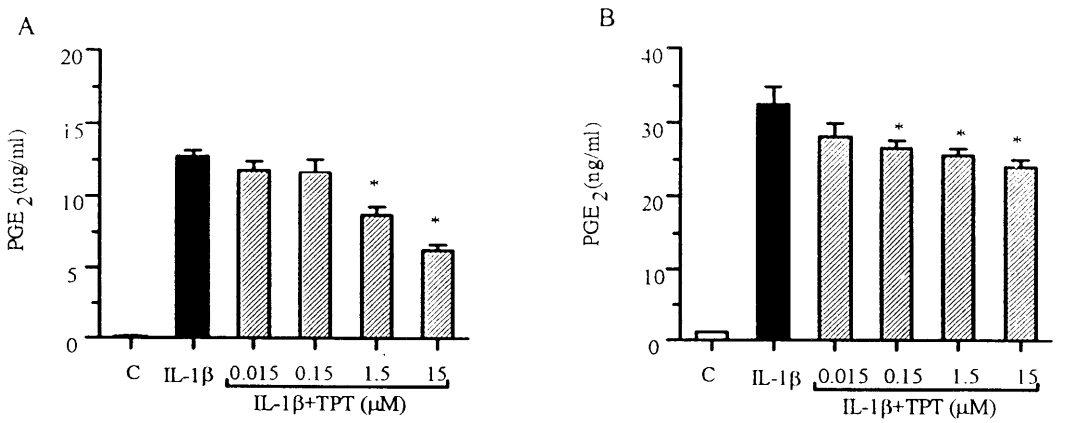

FIG. 3. Dose-dependent inhibition of the accumulation of COX metabolites (panel A) and the increase of COX activity (panel B) by tyrphostin (TPT) in IL-1β-activated A549 cells at 24 h. The accumulation of COX metabolites (PGE_2) was measured from the supernatant medium from IL-1β-activated A549 cells for 24 h. The increase of COX activity in IL-1β-activated A549 cells at 24 h was measured by the formation of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean ± s.e. mean from 9 determinations from at least 3 separate experimental days. * $p < 0.05$ when compared to untreated cells at 24 h (C).

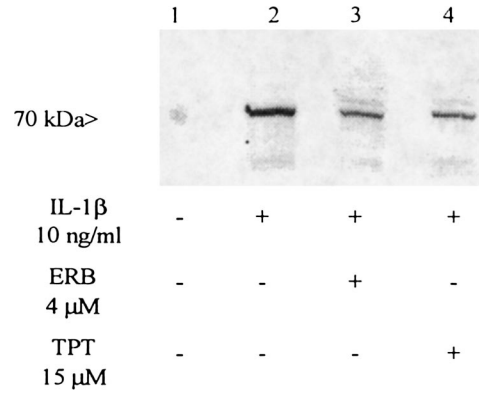

FIG. 4. The figure shows Western blots using polyclonal antibodies to COX-2 of cell extracts from IL-1β-treated and untreated A549 cells. Equal amounts of protein were loaded in all lanes of A549 cells (30 μg/lane). Control untreated A549 cells at 24 h (lane 1) contained no COX-2 protein. In contrast, IL-1β-activated (10 ng/ml for 24 h) A549 cells contained COX-2 protein (lane 2). The induction of COX-2 protein by IL-1β in A549 cells was inhibited by erbstatin (ERB; 4 μM; lane 3) and tyrphostin (TPT; 15 μM; lane 4). Similar results were obtained using cell extracts from 3 separate batches of cells.

A549 cells contained no COX-2 protein and did not release PGE_2 (<0.3 ng/ml for 24 h). The model employed in this study can, therefore, be used to study the signal transduction mechanism(s) of the expression of COX-2 caused by endotoxin or cytokines in human pulmonary epithelial cells. We have recently discovered that the induction of COX-2 by LPS in endothelial cells and macrophages involves the activation of tyrosine kinase[14]. Here, we have used the tyrosine kinase inhibitors, erbstatin and tyrphostin (AG126), as pharmacological tool to study the signal transduction mechanism in human epithelial cells activated by IL-1β. Similarly, in human pulmonary epithelial cells (A549), the induction of COX-2 protein and activity in response to IL-1β (10 ng/ml) was inhibited by either erbstatin (0.004–4 μM) or tyrphostin (0.015–15 μM). Interestingly, the inhibition by erbstatin or tyrphostin of the increase in COX-2 activity (exogenous substrates) is not as pronounced as the inhibition by these tyrosine kinase inhibitors of PGE_2 accumulation in the supernatant (endogenous substrates) or the increase in COX-2 protein which was inhibited by more than 50 percent by either erbstatin or tyrphostin. The finding that the increase in PGE_2 formation from endogenous arachidonic acid is more susceptable to inhibition with tyrosine kinase inhibitors than the formation of PGE_2 from exogenous arachidonic acid may well suggest that the activation of tyrosine kinases is not only involved in the expression of COX-2, but also in the induction of phospholipase A_2, afforded by endotoxin. Indeed, endotoxin and cytokines cause the induction of phospholipase A_2 in a variety of other cells including macrophages and fibroblasts[17,18,19,20]. In conclusion, this study demonstrates that the expression of COX-2 protein and activity caused by IL-1β in A549 epithelial cells involves the activation of tyrosine kinase. We propose that inhibitors of tyrosine kinase and/or other agents which interfere with the expression of COX-2 may be useful agents for the therapy of human lung disorders associated with inflammation.

ACKNOWLEDGMENTS

The authors are indebted to Ms. Elizabeth Wood for supplying the cultured cells and Ms. Ivana Vojnovic for technical assistance.

REFERENCES

1. Hamberg, M., Svensson, J., and Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3400–3404.
2. Smith, W. L., and Marnett, L. J. (1991) *Biochem. Biophys. Acta* **1083,** 1–17.
3. Xie, W. L., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2692–2696.

4. Mitchell, J. A., Akarasereenont, P., Thiemermann, C., Flower, R. J., and Vane, J. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11693–11697.
5. Vane, J. R. (1994) *Nature* **367,** 215–216.
6. O'Banion, M. K., Winn, V. D., and Young, D. A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4888–4892.
7. Maier, J. A. M., Hla, T., and Maciag, T. (1990) *J. Biol. Chem.* **265,** 10805–10808.
8. Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D., and Hwang, D. (1992) *J. Biol. Chem.* **267,** 25934–25938.
9. Akarasereenont, P., Mitchell, J. A., Thiemermann, C., and Vane, J. R. (1995) *Eur. J. Pharmacol.* **273,** 121–128.
10. Vane, J. R., Mitchell, J. A., Appleton, I., Tomlinson, A., Bishop-Baily, D., Croxtall, J., and Willoughby, D. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2046–2050.
11. Hempel, S., Monick, M. M., and Hunninghake, G. W. (1994) *J. Clin. Invest.* **93,** 391–396.
12. Mitchell, J. A., Belvisi, M. G., Akarasereenont, P., Robbins, R. A., Kwon, O. J., Barnes, P. J., and Vane, J. R. (1994) *Br. J. Pharmacol.* **113,** 1008–1014.
13. Newman, S. P., Flower, R. J., and Croxtall, J. D. (1994) *Biochem. Biophys. Res. Commun.* **202,** 931–939.
14. Akarasereenont, P., Mitchell, J. A., Appleton, I., Thiemermann, C., and Vane, J. R. (1994) *Br. J. Pharmacol.* **113,** 1522–1528.
15. Salmon, J. A. (1978) *Prostaglandins* **15,** 383–397.
16. Mosmann, T. (1983) *J. Immunol. Meth.* **65,** 55–63.
17. Pruzanski, W., and Vadas, P. (1991) *Immunol. Today*. **12,** 143–146.
18. Pernas, P. et al. (1991) *Biochem. Biophys. Res. Commun.* **178,** 1298–1305.
19. Crowl, R. M., Stoller, T. J., Conroy, R. R., and Stoner, C. R. (1991) *J. Biol. Chem.* **266,** 2647–2651.
20. Glaser, K. B., Mobilio, D., Chang, J. Y., and Senko, N. (1993) *Trend. Pharmacol. Sci.* **14,** 92–98.