Original Article

Effects of phosphodiesterase inhibitors on secretions of human monokines

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ABSTRACT

The purpose of this study was to evaluate the effect of newly developed selective phosphodiesterase (PDE) inhibitors, KF19514 (type I/IV) and cilostazol (type III), and theophylline on the secretions of tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) from human peripheral monocytes stimulated by lipopolysaccharide (LPS). Human blood monocytes were incubated with LPS in the absence or presence of KF19514, cilostazol or theophylline. TNFα and IL-1β in the cell-free supernatants were measured with enzyme-linked immunosorbent assay. KF19514 showed significant inhibition on the release of TNFα (% inhibition $\pm$ SEM was 82.8 $\pm$ 7.4% at 1 μmol/L) and IL-1β (34.4 $\pm$ 7.5% at 10 μmol/L). In addition, KF19514 inhibited the expression of TNFα mRNA. Cilostazol inhibited the release of TNFα significantly (60.2 $\pm$ 8.9% at 30 μmol/L) but not IL-1β. Theophylline inhibited slightly but significantly the release of TNFα at a therapeutic concentration (17.4 $\pm$ 5.1% at 100 μmol/L). These results suggest that theophylline may not only have a bronchodilating action but also an anti-inflammatory property in the treatment of bronchial asthma, and that KF19514 may have an anti-inflammatory action on at least the transcriptional level.

INTRODUCTION

Tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) have been found in the broncho-alveolar lavage fluid[1–3] and in bronchial biopsy specimens[4] obtained from patients with bronchial asthma. These cytokines have a role in airway inflammation through leukocyte recruitment into the airway by inducing leukocyte adhesion molecules on endothelial cells[5,6] and airway epithelial cells.[7] Peripheral blood monocytes secrete these cytokines by both immunological[8] and non-immunological stimulation, such as that by lipopolysaccharide (LPS).[9,10] Thus, an in vitro system that employs the secretions of TNFα and IL-1β from LPS-stimulated blood monocytes could be used to evaluate the anti-inflammatory properties of anti-asthmatic drugs.

Phosphodiesterase (PDE) inhibitors modulate monokine secretion via the elevation of cyclic nucleotides.[11–14] KF19514 is a newly synthesized type I/IV dual PDE inhibitor. It is a derivative of KF17625 and has a more potent bronchodilator action than KF17625.[15] IC$_{50}$ values for PDE I, PDE II, PDE III, PDE IV, and PDE V in vitro have been shown to be 0.27, >10, >10, 0.40 and >10 μmol/L, respectively.[16] Cilostazol, a derivative of cilostamide,[17–19] is a type III selective PDE inhibitor.[20] This drug is clinically used in Japan for the treatment of obstructive arteriosclerosis.

In this study, we examined the effect of these selective PDE isoenzyme inhibitors on the secretion of TNFα and

IL-1β from human peripheral blood monocytes (BMo) stimulated by LPS. In addition, we evaluated the effect of theophylline.

METHODS

Reagents and culture materials

Complete medium (MEM) consisted of alpha-modified Eagle's medium + 1% fetal calf serum + penicillin (100 units/mL) + streptomycin (100 μg/mL; all from Gibco, Paisley, UK). KF19514, 5-phenyl-3-(3-pyridyl)methyl-3H-imidazo[4,5-C][1,8] naphthyridin-4(5H)-one was donated by Kyowa Hakko Kogyo, Shizuoka, Japan. Cilostazol, 6-(4-[1-cyclohexyl-1H-tetrazol-5-yl] butoxy)-3,4-dihydro-2(1H)-quinolinone was donated by Otsuka Pharmaceutical company Ltd, Osaka, Japan. Theophylline was obtained from Sigma (St Louis, MO, USA). KF19514 and theophylline were dissolved in phosphate buffered saline (PBS; Gibco), whereas cilostazol was dissolved in N,N-Dimethylformamide (Gibco) and further diluted by adding MEM to the stock solution.

Isolation of blood monocytes

Blood monocytes were purified by adherence.[9] In brief, blood was drawn from normal volunteers and mononuclear cells (MNC) were obtained by Ficoll-Hypaque centrifugation (LSM, Organon Teknika Corp, NC, USA). The concentration of BMo was adjusted to 4×10^5/mL using non-specific esterase staining (Sigma) for MNC. Following this, 1 mL of cell suspension was poured into culture wells and incubated (37°C, 5% CO_2) for 1 h in order for BMo to adhere to the bottom of the well. After the incubation, non-adherent cells were removed by washing three times with PBS to purify the BMo. Trypan blue dye exclusion showed 100% viability of the cells before incubation.

Time course of secretion of TNFα and IL-1β by lipopolysaccharide stimulation

For the time course study, 400 000 BMo, purified as described above, were incubated in the absence and presence of 1 μg/mL LPS from *Escherichia coli* (serotype 026:B6, Sigma) for 0, 2, 6, 12 and 18 h in two subjects. The supernatant was harvested after centrifugation (600g, 10 min) at each incubation time and frozen at −20°C until the assay was undertaken.

Blood monocytes stimulation

A total of 400 000 BMo were incubated in the absence and presence of 1 μg/mL LPS with or without KF19514, cilostazol or theophylline at the following concentrations: KF19514 at 1, 10 and 100 μmol/L; cilostazol at 1, 10 and 30 μmol/L; and theophylline at 10, 100 and 1000 μmol/L. After 18 h incubation (37°C, 5% CO_2), the supernatant was harvested after centrifugation and frozen at −20°C until the assay was undertaken .

In order to verify that KF19514, cilostazol or theophylline did not influence the cell viability during the incubation, colorimetric assay[21] using tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma), was performed. There was no difference in cell viability among these conditions.

Measurement of TNFα and IL-1β

TNFα and IL-1β were assayed with an enzyme linked immunosorbent assay using human TNFα and IL-1β kits (Otsuka Pharmaceutical Company, Osaka, Japan). The assay procedure is briefly described as follows: the monoclonal antibody specific for each cytokine is coated onto the microtiter plate provided in the kit. Standards with known amounts of the cytokine and samples are pipetted into the wells and left at 4°C for 24 h. After washing, an enzyme-linked polyclonal antibody specific to the cytokine is added to the wells and left at room temperature for 2 h. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and the intensity of the color is measured by densitometry. A curve is prepared as a standard and, by comparing the optical density of the samples to this curve, the concentration of the cytokine in the sample is determined.

Northern analysis

In order to evaluate whether KF19514 inhibits the expression of TNFα mRNA, Northern analysis[22] was performed. Ten million BMo were cultured in large wells under the following conditions: MEM only, MEM + LPS (1 μg/mL), and MEM + LPS (1 μg/mL) + KF19514 (100 μmol/L). After 2 h incubation, the cells in each well were denatured with guanidine isothiocyanate and RNA was purified by phenol-chloroform.[23] After electrophoresis, the RNA was transferred to nitrocellulose filters. The filters were hybridized with human TNF cDNA probe

(820 bp EcoRI fragment of TNFα cloned in a pUC vector)[24] labeled with [^{32}P]dCTP (Amersham, UK) by nick translation and the hybridized signals were detected by autoradiography.

Statistical analysis

The paired two-tailed Student's t-test was performed for statistical analysis. Differences were considered significant for $P < 0.05$. Values are indicated as mean ± SEM.

RESULTS

Time course of secretion of TNFα and IL-1β from LPS-stimulated BMo

The secretions of TNFα and IL-1β from LPS-stimulated BMo were evaluated after 0, 2, 6, 12 and 18 h of incubation. Neither cytokine was detected at any period without LPS-stimulation. As shown in Fig. 1, the patterns of secretions for TNFα and IL-1β were different from each other. Thus, 40% of the maximal secretion obtained at 18 h culture was noted at 2 h for TNFα, whereas the secretion of IL-1β was not detected at 2 h incubation. However, the maximum level of secretion for both cytokines was observed by 12 h culture and remained at a plateau for up to 18 h. The means of the actual amount of the cytokines secreted after 18 h stimulation by LPS were 524.5 pg/mL for TNFα and
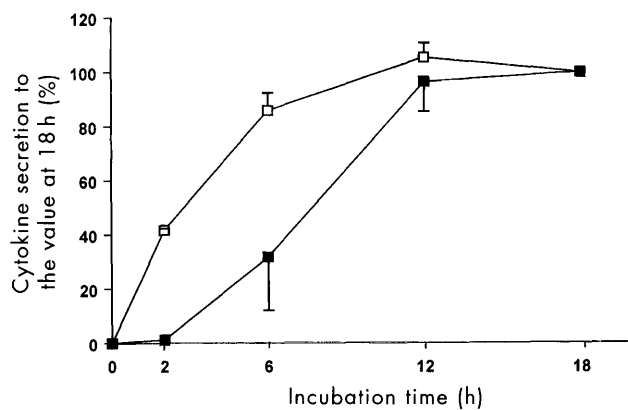
2355 pg/mL for IL-1β in subject 1, and 189 pg/mL and 598.5 pg/mL in subject 2.

Effects of KF19514, cilostazol and theophylline on the secretion of TNFα from LPS-stimulated BMo

As shown in Fig. 2, KF19514, cilostazol and theophylline dose-dependently inhibited the secretion of TNFα from LPS-stimulated BMo. The mean of the actual amount of TNFα secreted after 18 h-stimulation by LPS was 693 ± 84 pg/mL. IC_{50} values of each agent were as follows: KF19514, < 1 μmol/L; cilostazol, 28 μmol/L; theophylline, > 100 μmol/L. Thus, KF19514 had the most potent inhibitory action. Although theophylline showed the least inhibitory effect on a molar basis, a significant inhibition was observed at 100 μmol/L of theophylline, which is in the upper range of the therapeutic concentration (18 μg/mL).

Effects of KF19514, cilostazol and theophylline on the secretion of IL-1β from LPS-stimulated BMo

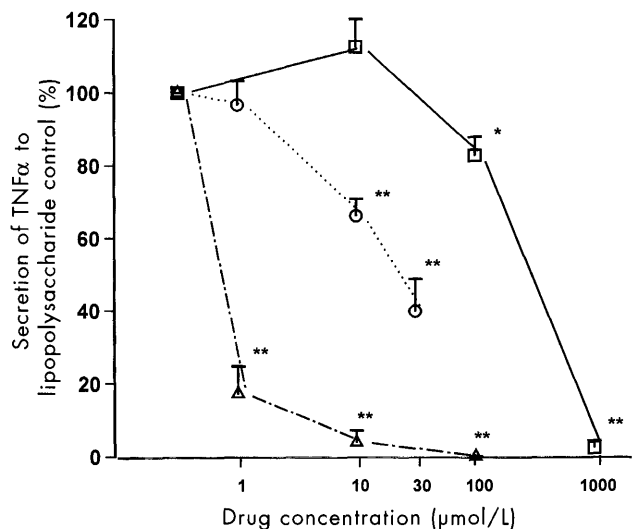As shown in Fig. 3, KF19514 also inhibited the secretion of IL-1β from BMo dose-dependently. The mean of the

Fig. 1   Time course of secretion of TNFα (□) and IL-1β (■) from human blood monocytes stimulated by lipopolysaccharide (LPS). The cytokines in the supernatant obtained after 0, 2, 6, 12 and 18 h culture were assayed. Each plot is the mean of % secretion of the amount secreted after 18 h culture. Bars show the ranges of two subjects.

Fig. 2   The effects of theophylline (□; $n = 11$), cilostazol (○; $n = 6$) and KF19514 (△; $n = 6$) on the secretion of TNFα from human blood monocytes stimulated by lipopolysaccharide (LPS). Results are expressed as mean ± SEM of % secretion of TNFα to positive control stimulation. *$P < 0.01$ and **$P < 0.001$ denote significant difference from the control level.

actual amount of IL-1β secreted after 18 h stimulation by LPS was 1372 ± 191 pg/mL. The IC$_{50}$ value of KF19514 was 55 μmol/L. However, neither cilostazol nor theophylline showed statistically significant inhibition on the secretion of IL-1β from BMo.

Effect of KF19514 on the expression of the TNFα gene by LPS-stimulated BMo

In order to evaluate whether KF19514 inhibits the expression of TNFα mRNA, we performed Northern analysis in two subjects. RNA was obtained from 10 million BMo in each of the following culture conditions: MEM alone, MEM containing LPS, and MEM containing LPS and KF19514 (100 μmol/L). Ten micrograms of RNA were obtained in each condition. The autoradiogram for TNFα mRNA in the lane obtained from cells cultured with LPS and KF19514 was weaker than that obtained from cells cultured with LPS. No gene expression was noted in the lane without LPS-stimulation. This result indicated that KF19514 partially inhibited the gene transcription for TNFα in the BMo stimulated by LPS (Fig. 4).
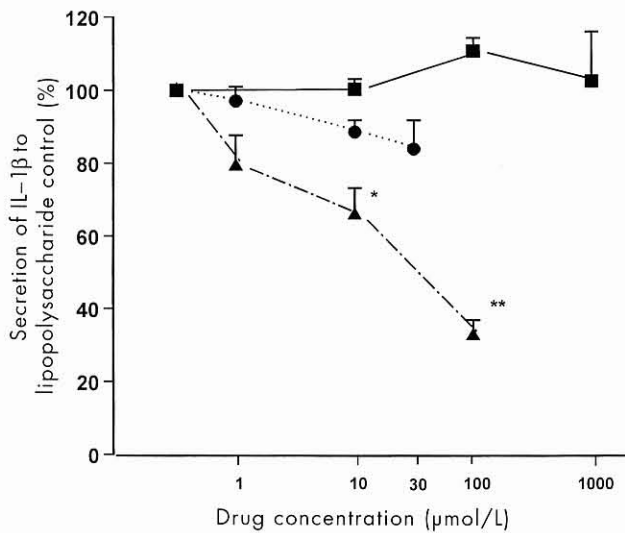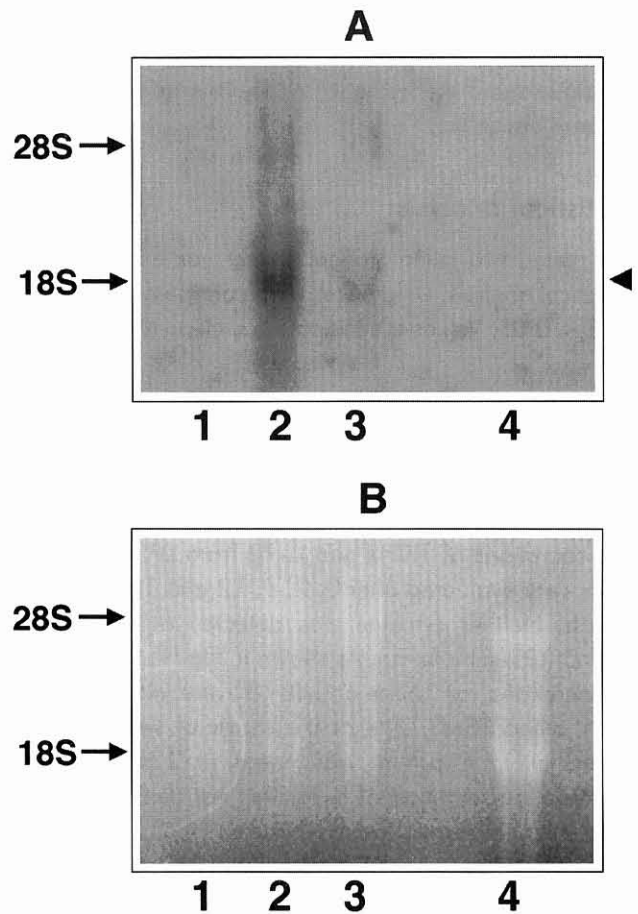
Fig. 4 Northern analysis for TNFα RNA extracted from monocyte culture with medium alone (lane 1), with LPS (lane 2), with LPS and KF19514 (lane 3) and rRNA from calf liver (lane 4), were hybridized with hTNFcDNA probe. An equal amount of RNA in each lane was confirmed with the assessment of 28S and 18S rRNA stained with ethidium bromide after electrophoresis (Panel B). Solid arrows indicate the positions of 18S and 28S rRNA. Closed arrow head indicates the position of TNFαmRNA.

Fig. 3 The effects of theophylline (■; n = 11), cilostazol (●; n = 6) and KF19514 (▲; n = 6)) on the secretion of IL-1β from human blood monocytes stimulated by lipopolysaccharide (LPS). Results are expressed as mean ± SEM of % secretion of IL-1β to positive control stimulation. *P < 0.01 and **P < 0.001 denote significant difference from the control level.

DISCUSSION

In the present study, we demonstrated that KF19514 significantly inhibited the secretions of both TNFα and IL-1β from BMo stimulated by LPS, and that cilostazol and theophylline significantly inhibited the secretion of TNFα, but not IL-1β, in the same system. In addition, we showed that 100 μmol/L of KF19514 decreased the TNFα mRNA signal.

The rank order of the inhibitory effect on the secretion of TNFα was KF19514 > cilostazol > theophylline. KF19514 showed an inhibitory effect on the release of

TNFα similar to that of type IV PDE isoenzyme inhibitors, rolipram,[25-28] nitraquazone,[26] Ro-20-1724,[27,28] and CP-77059.[27] When compared with zardaverine,[29] a type III/IV dual PDE inhibitor, the inhibitory effect of KF19514 was quite similar. By contrast, cilostazol showed a weaker inhibitory effect than did KF19514, which was equal to that of other type III PDE isoenzyme inhibitors, namely cilostamide,[27] milrinon,[26] CI-930,[26] motapizone[29] and SK&F94836.[28] Thus, it is suggested that in BMo a type IV PDE isoenzyme may have a major role in the regulation of the secretion of TNFα, and that the type IV PDE inhibitory action of KF19514 may be responsible for the inhibitory effect on the secretion of TNFα. In addition, strong inhibition on the secretion of TNFα by KF19514 is at least partially due to the inhibition on the expression of TNFα mRNA, which is similar to the effect of rolipram.[27]

KF19514 inhibits both PDE I and PDE IV, and 70% inhibition on the secretion of IL-1β was observed at 100 μmol/L, which is 350 times the IC_{50} for PDE I. It has been reported that the secretion of IL-1β from LPS-stimulated MNC is downregulated by cGMP.[14] Therefore, one possible mechanism is that complete inhibition of PDE I by 100 μmol/L of KF19514 may downregulate the secretion of IL-1β via a possible increase in cGMP, although BMo contains PDE I at a ratio of 1:10 of PDE IV.[30] However, we can not exclude the possibility that complete inhibition of PDE IV by KF19514 may inhibit the secretion of IL-1β because rolipram, a cAMP-elevating but not a cGMP-elevating agent, has been reported to inhibit the secretion of IL-1β.[27]

Cilostazol has been generally prescribed for the treatment of obstructive arteriosclerosis in Japan. A recent study has shown that cilostazol has bronchodilator and bronchoprotective effects in normal subjects at a serum concentration of 6.7 μmol/L.[31] However, all subjects complained of mild to severe headaches at that concentration. Although, as shown in this study's results, 10 μmol/L of cilostazol demonstrated a significant inhibition on the secretion of TNFα from LPS-stimulated BMo, TNFα inhibition by cilostazol is not clinically feasible.

Theophylline inhibited the secretion of TNFα dose-dependently and a significant inhibition was observed at 100 μmol/L which can be converted into a serum concentration of 18 μg/mL. Thus, our findings suggested that we may be clinically using the inhibitory action of theophylline in the treatment of bronchial asthma. Recently, Spatafora *et al.* reported that 100 μmol/L of theophylline inhibited the secretion of TNFα from BMo stimulated by LPS.[32] Although their inhibitory rate was much greater than that in the present study, they evaluated the inhibitory effect on the secretion after 2 h stimulation with LPS. Given that, as shown in the time-course study, the amount of TNFα after 2 h stimulation with LPS is only 40% of the maximal secretion which was observed after 12 h stimulation and lasted up to 18 h, the greater rate of inhibition in their study may have resulted from the shorter period of stimulation by LPS.

In conclusion, we showed that 100 μmol/L of theophylline had a significant inhibition on the secretion of TNFα from purified BMo stimulated by LPS. Therefore, we suggest that theophylline may have not only a bronchodilating action but also an anti-inflammatory property when used in the treatment of bronchial asthma. In addition, we demonstrated that KF19514 showed a potent inhibition on the secretion of TNFα and a moderate inhibition on the secretion of IL-1β from purified BMo stimulated by LPS, and that 100 μmol/L of KF19514 clearly decreased the TNFα mRNA signal. Therefore, KF19514 may have an anti-inflammatory property, although its clinical efficacy remains to be studied.

REFERENCES

1 Mattoli S, Mattoso VL, Soloperto M, Allegra L, Fasoli A. Cellular and biochemical characteristics of bronchoalveolar lavage fluid in symptomatic nonallergic asthma. *J. Allergy Clin. Immunol.* 1991; **87**: 794–802.

2 Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J. Allergy Clin. Immunol.* 1992; **89**: 958–67.

3 Marini M, Avoni E, Hollemborg J, Mattoli S. Cytokine mRNA profile and cell activation in bronchoalveolar lavage fluid from nonatopic patients with symptomatic asthma. *Chest* 1992; **102**: 661–9.

4 Ackerman V, Marini M, Vittori E, Bellini A, Vassali G, Mattoli S. Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest* 1994; **105**: 687–96.

5 Ebisawa M, Bochner BS, Georas SN, Schleimer RP. Eosinophil transendothelial migration induced by cytokines. I. Role of endothelial and eosinophil adhesion molecules in IL-1 beta-induced transendothelial migration. *J. Immunol.* 1992; **149**: 4021–8.

6   Howarth PH, Bradding P, Quint D, Redington AE, Holgate ST. Cytokines and airway inflammation. *Ann. N. Y. Acad. Sci.* 1994; **725**: 69–82.

7   Tosi MF, Stark JM, Smith CW, Hamedani A, Gruenert DC, Infeld MD. Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. *Am. J. Respir. Cell Mol. Biol.* 1992; **7**: 214–21.

8   Borish L, Mascali JJ, Rosenwasser LJ. IgE-dependent cytokine production by human peripheral blood mononuclear phagocytes. *J. Immunol.* 1991; **146**: 63–7.

9   Martinet Y, Yamauchi K, Crystal RG. Differential expression of the tumor necrosis factor/cachectin gene by blood and lung mononuclear phagocytes. *Am. Rev. Respir. Dis.* 1988; **138**: 659–65.

10 Bernaudin JF, Yamauchi K, Wewers MD, Tocci MJ, Ferrans VJ, Crystal RG. Demonstration by *in situ* hybridization of dissimilar IL-1 beta gene expression in human alveolar macrophages and blood monocytes in response to lipopolysaccharide. *J. Immunol.* 1988; **140**: 3822–9.

11 Strieter RM, Remick DG, Ward PA *et al.* Cellular and molecular regulation of tumor necrosis factor-alpha production by pentoxifylline. *Biochem. Biophys. Res. Commun.* 1988; **155**: 1230–6.

12 Taffet SM, Singhel KJ, Overholtzer JF, Shurtleff SA. Regulation of tumor necrosis factor expression in a macrophage-like cell line by lipopolysaccharide and cyclic AMP. *Cell Immunol.* 1989; **120**: 291–300.

13 Hurme M. Modulation of interleukin-1 beta production by cyclic AMP in human monocytes. *Febs Lett.* 1990; **263**: 35–7.

14 Endres S, Fulle HJ, Sinha B *et al.* Cyclic nucleotides differentially regulate the synthesis of tumour necrosis factor-alpha and interleukin-1 beta by human mononuclear cells. *Immunology* 1991; **72**: 56–60.

15 Suzuki F, Kuroda T, Kawakita T *et al.* New bronchodilators. 3. Imidazo[4, 5-c][1, 8]naphthyridin-4 (5H) –ones. *J. Med. Chem.* 1992; **35**: 4866–74.

16 Manabe H, Akuta K, Sejimo H *et al.* Anti-inflammatory and bronchodilator properties of KF19514, a phosphodiesterase 4 and 1 inhibitor. *Eur J. Pharmacol.* 1997; **332**: 97–107.

17 Hidaka H, Hayashi H, Kohri H *et al.* Selective inhibitor of platelet cyclic adenosine monophosphate phosphodiesterase, cilostamide, inhibits platelet aggregation. *J. Pharmacol. Exp. Ther.* 1979; **211**: 26–30.

18 Umekawa H, Tanaka T, Kimura Y, Hidaka H. Purification of cyclic adenosine monophosphate phosphodiesterase from human platelets using new-inhibitor Sepharose chromatography. *Biochem. Pharmacol.* 1984; **33**: 3339–44.

19 Shintani S, Toba Y, Suzuki S, Ninomiya S, Umezato M, Hiyama T. General pharmacological properties of cilostazol, a new antithrombotic drug. Part I. Effects on the central nervous system. *Arzneimittelforschung.* 1985; **35**: 1157–62.

20 Okuda Y, Kimura Y, Yamashita K. Cilostazol. *Cardiovas. Drug Rev.* 1993; **11**: 451–65.

21 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* 1983; **65**: 55–63.

22 Ohno I, Tanno Y, Yamauchi K, Takishima T. Gene expression and production of tumour necrosis factor by a rat basophilic leukaemia cell line (RBL-2H3): with IgE receptor triggering. *Immunology* 1990; **70**: 88–93.

23 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 1987; **162**: 156–9.

24 Pennica D, Nedwin GE, Hayflick JS *et al.* Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* 1984; **312**: 724–9.

25 Molnar-Kimber K, Yonno L, Heaslip R, Weichman B. Modulation of TNF alpha and IL-1 beta from endotoxin-stimulated monocytes by selective PDE isozyme inhibitors. *Agents Actions* 1993; **39**, Spec No: C77–9.

26 Semmler J, Wachtel H, Endres S. The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor-alpha production by human mononuclear cells. *Int. J. Immunopharmacol.* 1993; **15**: 409–13.

27 Prabhakar U, Lipshutz D, Bartus JO *et al.* Characterization of cAMP-dependent inhibition of LPS-induced TNF alpha production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor. *Int. J. Immunopharmacol.* 1994; **16**: 805–16.

28 Verghese MW, McConnell RT, Strickland AB *et al.* Differential regulation of human monocyte-derived TNF alpha and IL-1 beta by type IV cAMP-phosphodiesterase (cAMP-PDE) inhibitors. *J. Pharmacol. Exp. Ther.* 1995; **272**: 1313–20.

29 Schade FU, Schudt C. The specific type III and IV phosphodiesterase inhibitor zardaverine suppresses formation of tumor necrosis factor by macrophages. *Eur. J. Pharmacol.* 1993; **230**: 9–14.

30 Schudt C, Tenor H, Hatzelmann A. PDE isoenzymes as targets for anti-asthma drugs. *Eur. Respir. J.* 1995; **8**: 1179–83.

31 Fujimura M, Kamio Y, Saito M, Hashimoto T, Matsuda T. Bronchodilator and bronchoprotective effects of cilostazol in humans in vivo. *Am. J. Respir. Crit. Care Med.* 1995; **151**: 222–5.

32 Spatafora M, Chiappara G, Merendino AM, D'Amico D, Bellia V, Bonsignore G. Theophylline suppresses the release of tumour necrosis factor-alpha by blood monocytes and alveolar macrophages. *Eur. Respir. J.* 1994; **7**: 223–8.