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INHIBITORY EFFECTS OF DICLOFENAC AND INDOMETHACIN ON INTERLEUKIN-1-INDUCED CHANGES IN PGE_2 RELEASE

A NOVEL EFFECT ON FREE ARACHIDONIC ACID LEVELS IN HUMAN SYNOVIAL CELLS

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Abstract—The inhibitory effects of two non-steroidal anti-inflammatory drugs (NSAIDS), diclofenac and indomethacin, on interleukin-1 (IL-1)-induced changes in arachidonic acid (AA) release and prostaglandin E_2(PGE_2) production by human synovial cells was investigated. Both diclofenac and indomethacin potently inhibited IL-1α-induced PGE_2 release, with IC_{50} values of 1.6 ± 0.02 nM and 5.5 ± 0.1 nM, respectively. A novel effect on IL-1α-mediated changes in AA levels was observed using cells labelled with radioactive AA. Both drugs at micromolar concentrations (10–30 μM) showed an apparent inhibition of IL-1α-induced increases in radioactivity associated with free AA. Concomitant with this inhibition, there was an increase in radioactivity associated with phosphatidylethanolamine (PE) and triglyceride (TG). As the drugs had no effect on IL-1α-induced decreases in radioactivity associated with phosphatidylcholine (PC), this result was interpreted as being due to an enhanced acylation of AA into PE and TG. These results suggest that whilst at nanomolar concentrations, diclofenac and indomethacin can inhibit IL-1α-induced PGE_2 output, at micromolar concentrations an effect on free AA levels is also evident. This may have consequences for the release of other mediators such as leukotrienes, whose synthesis also involves the level of free AA.

The mechanism by which non-steroidal anti-inflammatory drugs (NSAIDS) such as diclofenac and indomethacin exert their anti-inflammatory effects involves decreasing the release of prostaglandins by inhibition of the enzyme cyclo-oxygenase. Prostaglandins have been implicated in many of the features of both acute and chronic inflammation and inhibition of their synthesis provides symptomatic relief in diseases like rheumatoid arthritis. However, differences between NSAIDS have been observed which suggest that their effects may not solely be due to inhibition of cyclo-oxygenase [1, 2]. Several groups have shown other effects of NSAIDS including inhibition of phospholipases [3, 4] and effects on acyltransferase [5]. Such effects would result in changes in the level of free arachidonic acid (AA), which would thereby alter the release of other inflammatory mediators such as leukotrienes.

We have been studying the induction of prostaglandin E_2 (PGE_2) release by the pro-inflammatory cytokine interleukin-1 (IL-1) in human synovial cells. IL-1 is one of the most potent inducers of PGE_2 release yet described, and this effect is thought to underlay some of its inflammatory effects *in vivo*. We have shown that the mechanism involves increases in free AA with concomitant changes in phospholipids such as phosphatidycholine (PC) [6], and secondly an induction of cyclo-oxygenase [7].

Using this system we now go on to describe the effects of two NSAIDS: diclofenac and indomethacin, on two aspects of the IL-1 response. Firstly, the induction of PGE_2 release, and secondly the changes in phospholipid and AA metabolism. The aim of the study is both to determine the effects of diclofenac and indomethacin on what is considered a key pro-inflammatory activity of IL-1, and secondly to examine for any novel effects of these drugs on the release and metabolism of AA in IL-1-stimulated human cells.

MATERIALS AND METHODS

Synovial cells. Human adherent synovial cells were prepared as described previously [8] and grown to confluence in either 24 well macrowell plates (6×10^4/cm^2, Linbro) or 25 cm^2 tissue culture flasks (Nunc). Second to fifth passage cells were used in all experiments. The medium used was Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Uxbridge, U.K.) supplemented with 10% heat inactivated fetal calf serum and containing 100 units/ml penicillin/streptomycin (Gibco). Cells were maintained at 37° in a humidified atmosphere at 5% CO_2.

PGE_2 release. Second to fifth passage cells were grown to confluence in 24 well macrowell plates and incubated with 0.5 ng/ml human recombinant IL-1α (a gift from P. Lomedico, Hoffman La Roche, Mijdrecht, The Netherlands) in the presence or

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absence of diclofenac (sodium salt, Ciba Geigy, Summit, NJ, 10^{-10}–10^{-7} M) or indomethacin (Sigma Chemical Co., Poole, U.K., 10^{-10}–10^{-7} M). After 24 hr, PGE_2 levels were determined in cell supernatants by radioimmunoassay as described previously [9]. The antiserum used cross-reacted with other eicosanoids as follows: PGE_1 26%, PGF_2 1.1%, TxB_2 <0.3%, 6-oxo-PGF_1 0.03% and with AA 0.06%.

Arachidonic acid release. To label membrane phospholipids with radioactive AA, cells grown to confluence in 25 cm^2 Nunc flasks ($6 \times 10^4/cm^2$) were incubated for 24 hr with 3 ml of media containing 0.5 μCi 5, 6, 8, 9, 11, 12, 14, 15-^{3}H AA (Amersham, Bucks., U.K. act. 210 Ci/mmol). After washing, cells were stimulated with IL-1α (0.5 ng/ml) for 4 hr, in the presence or absence of increasing concentrations of diclofenac or indomethacin (0.1–30 μM). Incubations were terminated by adding 1 ml of ice-cold 10 mM HCl, and lipids were extracted using the technique of Bligh and Dyer [10]. The resulting organic phase was analysed by thin layer chromatography (TLC) on DC-alufoilen Kieselgel 60 plates (Merck, Darmstadt, F.R.G.) using three solvent systems: for phospholipids, chloroform/methanol/acetic acid (40/10/20 v/v/v), for prostaglandins, chloroform/methanol/acetic acid (90/90/5 v/v/v) and for glycerides, hexane/diethylether/acetic acid (50/50/1 v/v/v). Lipids were generally dried under nitrogen and resuspended in 80 μl of chloroform. Ten microlitres of this extract were subjected to TLC in each system. Lipid standards were run with each sample to act as carriers and to help in visualization which was achieved using iodine vapour. Plates were sectioned and immersed in 2 ml methanol for 1 hr. Eight millilitres of liquid scintillation fluid (LKB) was then added and radioactivity determined using liquid scintillation spectrometry.

RESULTS

Inhibition of PGE_2 release

Diclofenac and indomethacin could both dose-dependently inhibit IL-1α-induced PGE_2 release from synovial cells (Fig. 1). Diclofenac proved more potent than indomethacin, with an IC_{50} value of 1.6 ± 0.02 nM, as compared to 5.5 ± 0.1 nM for indomethacin. In the absence of either drug, the cells produced 52–56 ng/ml PGE_2 in response to IL-1α (0.5 ng/ml), as compared to <3 ng/ml in media treated controls.

Effects on AA release

Both drugs had a complex range of effects on IL-1α-mediated changes in AA-associated radioactivity in synovial cells. After 4 hr incubation, IL-1α (0.5 ng/ml) increased the level of radioactivity associated with AA from 421 ± 20 dpm in media alone treated cells, to 1712 ± 317 dpm, as shown in Fig. 2. Concomitant with this increase there was a drop in PC-associated radioactivity from 8802 ± 1035 dpm to 6084 ± 388 dpm (Table 1) and in phosphatidylethanolamine (PE)-associated radioactivity, from 4103 ± 724 dpm to 2872 ± 327 dpm. Increasing concentrations of diclofenac had an interesting effect on the response. At the higher doses examined (10 and 30 μM) diclofenac decreased the level of AA, so that at 30 μM it had reached non-IL-1α-treated control levels of 457 ± 120 dpm (Fig. 2). However, concomitant with the decrease in free AA levels, there was an increase in radioactivity associated with PE and triglyceride (TG), as also shown in Fig. 2. After treatment with 30 μM diclofenac, PE had reached levels of 5472 ± 721 dpm as compared to 2872 ± 327 dpm in IL-1α-treated controls. In addition TG radioactivity also increased, reaching 2732 ± 219 dpm as compared to 1392 ± 210 dpm. No major effects were seen on other lipids examined (Table 1), such as PC, which remained decreased at 6023 ± 1270 dpm in cells treated with 30 μM diclofenac.

When indomethacin was examined for effects on IL-1α-mediated changes in radioactive AA levels, a very similar result to that seen with diclofenac was obtained (Fig. 3 and Table 2). In these experiments, more radioactivity was incorporated into the cells. This was likely to be a consequence of donor variation. However, the relative proportions of radioactivity in the major lipid species was the same as in experiments with diclofenac. Like diclofenac, indomethacin could dose-dependently inhibit the IL-1α-mediated increase in free AA, whilst increasing radioactivity associated with PE and TG. The effect was evident at concentrations of 1, 10 and 30 μM. Diclofenac did not have an effect at 1 μM (Fig. 2), whereas indomethacin at this dose could decrease the IL-1α-induced increase in free AA from 2812 ± 317 dpm to 2017 ± 132 dpm. There was also an increase in PE-associated radioactivity to 5629 ± 432 dpm from 4872 ± 297 dpm, and TG-associated radioactivity to 3491 ± 372 dpm from 2126 ± 193 dpm. At doses of 10 and 30 μM the effect was more pronounced. As with diclofenac, indomethacin had no major effect on IL-1α-induced changes in other lipid species such as PC (Table 2).

DISCUSSION

IL-1 induces a complex series of events leading to an increase in PGE_2 output from synovial cells. The mechanism involves phospholipase activation [6, 11, 12] and an increased *de novo* synthesis of cyclo-oxygenase [7, 13]. Other enzymes such as acyltransferase may be involved [6]. We report here how diclofenac and indomethacin effect the response. Both drugs proved to be potent inhibitors of IL-1-induced PGE_2 output, with IC_{50} values of 1.6 ± 0.02 nM for diclofenac and 5.5 ± 0.1 nM for indomethacin. This level of potency compares favorably with that reported by Robinson *et al.* [14] who studied spontaneous PGE_2 release from primary culture synovial cells, and Hamilton *et al.* [15] who examined mononuclear cell factor-stimulated synovial cells. The effect was several orders of magnitude more potent than that for aspirin, which we reported previously as having an IC_{50} value of 4800 ± 23 nM [16].

At higher concentrations (μM), both drugs inhibited the IL-1α-induced increases in free AA.

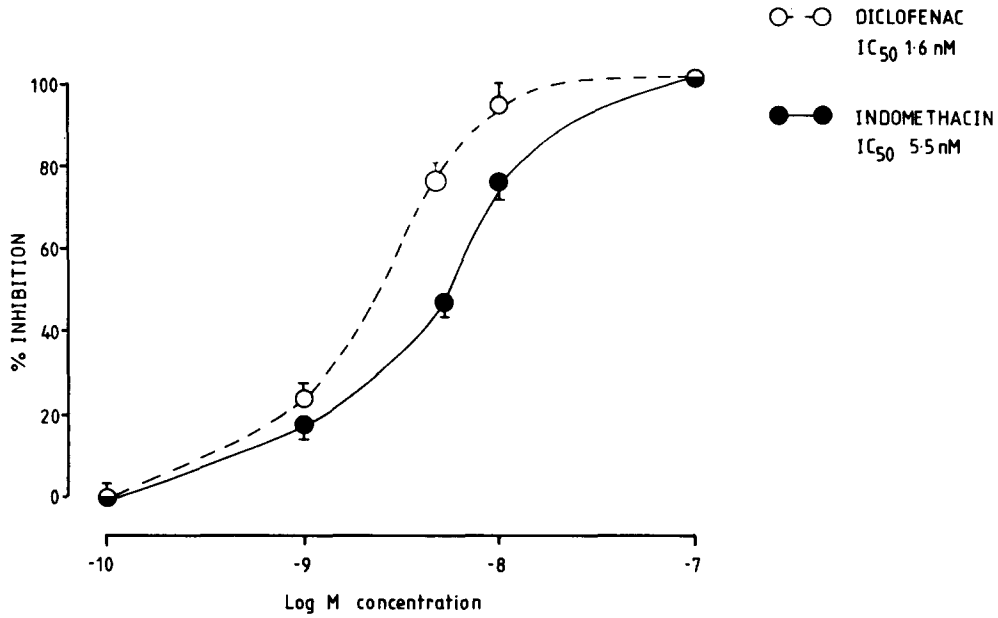

Fig. 1. Effect of diclofenac and indomethacin on IL-1-induced PGE_2 release in human synovial cells. Cells grown to confluence in macrowells (1.2×10^5 per 500 μl well) were incubated with IL-1α (0.5 ng/ml) and increasing concentrations of diclofenac (○) or indomethacin (●) for 24 hr, PGE_2 levels being determined in the supernatant by radioimmunoassay. Results are expressed as % inhibition where 0% corresponds to 54 ± 2 ng/ml PGE_2, and represent mean ± SE from three separate experiments, each in triplicate.

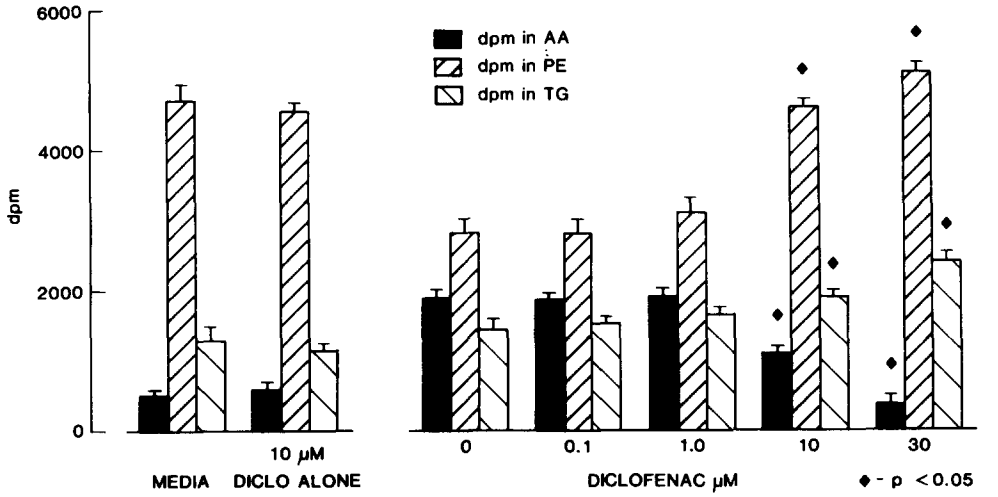

Fig. 2. Effect of diclofenac on IL-1α-mediated changes in AA, PE and TG-associated radioactivity in human synovial cells. Cells grown to confluence in 25 cm^2 flasks (6×10^4 per cm^2) and labelled with 0.5 μCi [^{3}H]AA for 24 hr, were stimulated with IL-1α (0.5 ng/ml) in the presence of increasing concentrations of diclofenac. After 4 hr lipids were extracted, separated and analysed using TLC and liquid scintillation spectrometry. Results are shown for three lipid species: AA, PE and TG. All histobars except controls show results for cells treated with IL-1α (0.5 ng/ml), the histobar with 0 μM diclofenac corresponding to cells treated with IL-1α alone. Total radioactivity recovered over all experiments was 174,123 ± 5320 dpm, which was dissolved in 80 μl of chloroform. 10 microlitres of this extract was subjected to TLC, the radioactivity in the figure corresponding to that lipid species indicated. Results represent the mean ± SE of three separate experiments, each being carried out in duplicate. (◆) indicates a significant difference ($P < 0.05$) from cells treated with diclofenac alone.

This was not solely due to an inhibition of phospholipase, an effect previously reported for indomethacin [3], as the IL-1α-mediated decrease in radioactivity associated with PC, which would reflect phospholipase activity, was unaffected. A more likely intepretation was an increase in reacylation of AA into PE and TG, both of which showed significant increases. This is consistent with the hypothesis

Table 1. Effect of diclofenac on IL-1α-mediated changes in PC, PI, PS, MG, DG and PGE_2-associated radioactivity in human synovial cells

Lipid	Media alone	+IL-1α (0.5 ng/ml)	+IL-1α (0.5 ng/ml) + Diclofenac (μM) 0.1	1	10	30
PC	8802 ± 1035	6084 ± 388	6472 ± 388	5307 ± 1294	5955 ± 1035	6023 ± 1270
PI	3107 ± 517	3365 ± 68	3207 ± 102	3706 ± 421	4120 ± 627	3272 ± 270
PS	1812 ± 225	2971 ± 517	2187 ± 326	2937 ± 394	3017 ± 106	2921 ± 470
DG	225 ± 50	227 ± 10	217 ± 22	218 ± 22	217 ± 10	ND
MG	103 ± 27	103 ± 57	103 ± 37	107 ± 92	221 ± 70	209 ± 82
PGE_2	125 ± 62	407 ± 37	ND	ND	ND	ND

Cells grown to confluence in 25 cm^2 flasks (6×10^4 per cm^2) and labelled with 0.5 μCi [^{3}H]AA for 24 hr, were stimulated with IL-1α (0.5 ng/ml) in the presence of increasing concentrations of diclofenac. After 4 hr lipids were extracted, separated and analysed using TLC and liquid scintillation spectrometry. Results are shown for six lipid species: PC, PI, PS, DG MG and PGE_2. Total radioactivity recovered over all experiments was 174, 123 ± 5320 dpm, which was dissolved in 80 μl of chloroform. Ten microlitres of this extract was subject to TLC, the radioactivity in the table corresponding to that lipid species indicated. Results represent the mean ± SE of three separate experiments, each being carried out in duplicate. ND = not detectable.

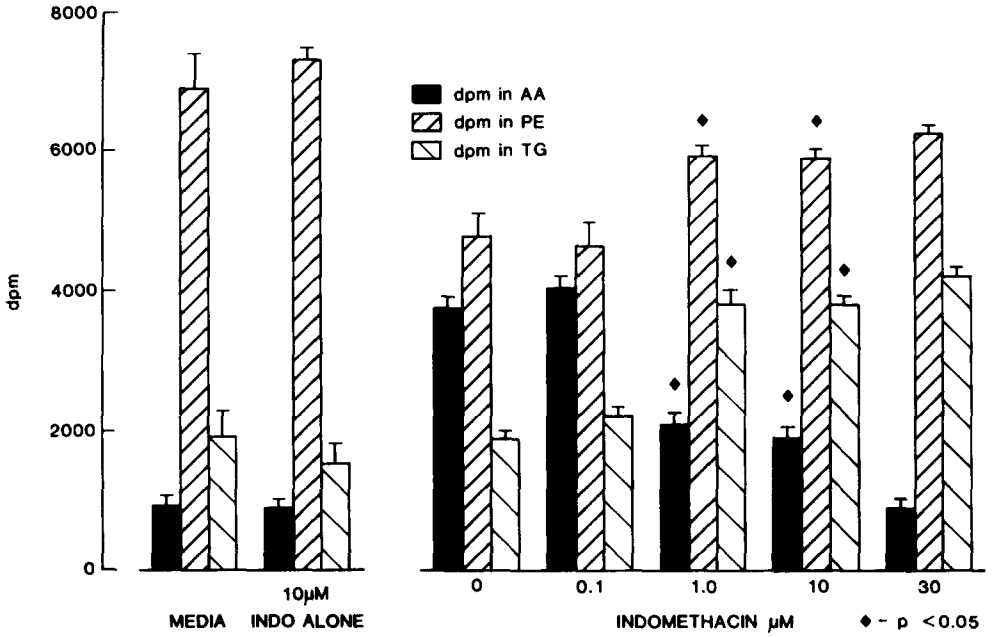

Fig. 3. Effect of indomethacin on IL-1α-mediated changes in AA, PE and TG-associated radioactivity in human synovial cells. Cells grown to confluence in 25 cm^2 flasks (6×10^4 per cm^2) and labelled with 0.5 μCi [^{3}H]AA for 24 hr, were stimulated with IL-1α (0.5 ng/ml) in the presence of increasing concentrations of indomethacin. After 4 hr lipids were extracted, separated and analysed using TLC and liquid scintillation spectrometry. Results are shown for three lipid species: AA, PE and TG. All histobars except controls show results for cells treated with IL-1α (0.5 ng/ml), the histobar with 0 μM indomethacin corresponding to cells treated with IL-1α alone. Total radioactivity recovered over all experiments was 287,708 ± 7228 dpm, which was dissolved in 80 μl of chloroform. Ten microlitres of this extract was subjected to TLC, the radioactivity in the figure corresponding to that lipid species indicated. Results represent the mean ± SE of three separate experiments, each being carried out in duplicate. (◆) indicates a significant difference ($P < 0.05$) from cells treated with indomethacin alone.

that the level of AA in cells is determined by the balance between its release by phospholipases and its reacylation by acyltransferase [17]. Whilst phospholipase would be still active in this system (PC is still decreased), an enhanced acylation into PE and TG would be reflected in a net decrease in free AA levels. Interestingly, Kothari *et al.* [18] have shown a similar effect in A23187-stimulated leukocytes. They also showed a decrease in AA by diclofenac, along with increases in PE and TG, and suggested that this may explain the inhibitory effects of diclofenac on leukotriene release in this system. From our studies it would appear that diclofenac and indomethacin (both belonging to the acetic acid class of NSAIDS) have a similiar effect in IL-1α-stimulated synovial cells. No change would be observed

Table 2. Effect of indomethacin on IL-1α-mediated changes in PC, PI, PS, MG, DG and PGE_2-associated radioactivity in human synovial cells

Lipid	Media alone	+IL-1α (0.5 ng/ml)	+IL-1α (0.5 ng/ml) + Indomethacin (μM) 0.1	1	10	30
PC	8210 ± 1216	5999 ± 972	6124 ± 273	6327 ± 371	6210 ± 279	6110 ± 821
PI	4122 ± 827	2932 ± 771	2991 ± 721	3106 ± 721	3010 ± 634	3122 ± 110
PS	1901 ± 292	1321 ± 476	1017 ± 292	1729 ± 721	1874 ± 291	2176 ± 322
DG	ND	ND	210 ± 57	357 ± 56	ND	ND
MG	ND	273 ± 22	217 ± 57	ND	ND	ND
PGE_2	207 ± 129	673 ± 10	ND	ND	ND	ND

Cells grown to confluence in 25 cm^2 flasks (6×10^4 per cm^2) and labelled with 0.5 μCi [^{3}H]AA for 24 hr, were stimulated with IL-1α (0.5 ng/ml) in the presence of increasing concentrations of indomethacin. After 4 hr lipids were extracted, separated and analysed using TLC and liquid scintillation spectrometry. Results are shown for six lipid species: PC, PI, PS, DG, MG and PGE_2. Total radioactivity recovered over all experiments was 287,708 ± 7228 dpm, which was dissolved in 80 μl of chloroform. Ten microlitres of this extract was subjected to TLC, the radioactivity in the table corresponding to that lipid species indicated. Results represent the mean ± SE of three separate experiments, each being carried out in duplicate. ND = not detectable.

in leukotriene output here however as the cells lack lipoxygenase enzymes [19].

In conclusion therefore, this result implies that at micromolar concentrations, diclofenac and indomethacin have additional effects on the PGE_2 biosynthetic pathway other than cyclooxygenase inhibition. These effects involve modulation of the level of free AA via enhanced acylation of AA into PE and TG. Such effects on AA metabolism may have consequences for the overall anti-inflammatory effect of these agents *in vivo*, (where indomethacin for example reaches concentrations of 5–10 μM [20]), as other inflammatory mediators such as leukotrienes, whose synthesis also involves free AA levels, may also be affected.

REFERENCES

1. Abramson S, Edelson H, Kaplan NB, Given W, Korchak HM and Weissman G, Differing effects of nonsteroidal anti-inflammatory agents on neutrophil functions *in vitro* and *in vivo*. *Arth Rheum* **25**: 37A, 1982.
2. Goodwin JS, Mechanism of action of nonsteroidal anti-inflammatory agents. *Am J Med* **77**: 57–64, 1984.
3. Flower RJ, Drugs which inhibit prostaglandin biosynthesis. *Pharmacol Rev* **26**: 33–67, 1974.
4. Jesse RL and Franson RC, Modulation of purified phospholipase A_2 activity from human platelets by calcium and indomethacin. *Biochim Biophys Acta* **598**: 467–470, 1979.
5. Kroner EE, Bernhard A, Peskar F, Fischer H and Ferber E, Control of arachidonic acid accumulation in bone-marrow derived macrophages by acyltransferases. *J Biol Chem* **256**: 3690–3697, 1981.
6. O'Neill LAJ and Lewis GP, Effect of interleukin-1 on free arachidonic acid levels in human synovial cells. *Biochem Soc Trans* **16**: 286, 1988.
7. O'Neill LAJ, Barratt ML and Lewis GP, Induction of cyclo-oxygenase by interleukin-1 in human synovial cells. *FEBS Lett* **212**: 35–39, 1987.
8. Gordon D and Lewis GP, Effects of piroxicam on mononuclear cells: comparison with other anti-arthritics. *Inflammation* **8**: S87–102, 1984.
9. Jose PJ, Page DA, Wolstenholme BE, Williams TJ and Dumonde DC, Bradykinin-stimulated prostaglandin E_2 production by endothelial cells and its modulation by anti-inflammatory compounds. *Inflammation* **6**: 363–378, 1981.
10. Bligh EG and Dyer WG, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911–913, 1959.
11. Chang J, Gilman SC and Lewis AJ, Interleukin-1 activates phospholipase A_2 in rabbit chondrocytes: a possible signal for IL-1 action. *J Immunol* **136**: 1283–1287, 1986.
12. Godfrey RW, Johnson WJ and Hoffstein ST, Recombinant tumour necrosis factor and interleukin-1 both stimulate human rheumatoid synovial cell arachidonic acid release and phospholipid metabolism. *Biochem Biophys Res Commun* **142**: 235–241, 1987.
13. Raz A, Wyche A, Siegel N and Needleman P, Regulation of fibroblast cyclo-oxygenase synthesis by interleukin-1. *J Biol Chem* **263**: 3022–3029, 1988.
14. Robinson D, Maguire MB and Levine L, Prostaglandins in rheumatic diseases. *Ann NY Acad Sci* **256**: 318–329, 1975.
15. Hamilton JA, Clarris BJ, Fraser JRE and Niall MC, Peripheral blood mononuclear cells stimulate prostacyclin levels of human synovial fibroblast-like cells. *Rheum Int* **5**: 121–125, 1985.
16. O'Neill LAJ and Lewis GP, Effect of anti-rheumatic drugs on interleukin-1 activity in human synovial cells. *Biochem Soc Trans* **16**: 892, 1988.
17. Irvine RF, How is the level of free arachidonic acid controlled in mammalian cells? *Biochem J* **204**: 3–16, 1982.
18. Kothari HU, Lee WH and Ku EC, An alternate mechanism for regulation of leukotriene production in leukocytes: studies with an anti-inflammatory drug, sodium diclofenac. *Biochim Biophys Acta* **921**: 502–511, 1987.
19. Davidson ER, Rae SE and Smith, MJH, Leukotriene B_4, a mediator of inflammation present in synovial fluid in rhematoid arthritis. *Ann Rheum Dis* **42**: 677–679, 1983.
20. Emori HW, Champion GD, Bluestone R and Paulus HE, Simultaneous pharmacokinetics of indomethacin in serum and synovial fluid. *Ann Rheum Dis* **32**: 433–435, 1973.