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Differential regulation of cyclo-oxygenase-2 and 5-lipoxygenase-activating protein (FLAP) expression by glucocorticoids in monocytic cells

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1 The objective of the present study was to determine the effects of dexamethasone on key constituents of prostaglandin and leukotriene biosynthesis, cyclo-oxygenase-2 (COX-2) and 5-lipoxygenase activating protein (FLAP). The human monocytic cell line THP-1 was used as a model system. mRNA and protein levels of COX-2 and FLAP were determined by Northern and Western blot analyses, respectively.

2 Low levels of COX-2 and FLAP mRNA were expressed in undifferentiated THP-1 cells, but were induced upon differentiation of the cells along the monocytic pathway by treatment with phorbol ester (TPA, 5 nM). Maximal expression was observed after two days.

3 Coincubation of the undifferentiated cells with dexamethasone ($10^{-9}-10^{-6}$ M) and phorbol ester prevented induction of COX-2 mRNA, but did not affect the induction of FLAP mRNA.

4 Dexamethasone downregulated COX-2 mRNA and protein in differentiated, monocyte-like THP-1 cells. In contrast, FLAP mRNA and protein were upregulated by dexamethasone in differentiated THP-1 cells. After 24 h, FLAP mRNA levels were increased more than 2 fold. Dexamethasone did not change 5-lipoxygenase mRNA expression.

5 Release of prostaglandin E_2 (PGE_2) and peptidoleukotrienes was determined in cell culture supernatants of differentiated THP-1 cells by ELISA. Calcium ionophore-dependent PGE_2 synthesis was associated with COX-2 expression, whereas COX-1 and COX-2 seemed to participate in arachidonic acid-dependent PGE_2 synthesis. Very low levels of peptidoleukotrienes were released from differentiated THP-1 cells upon incubation with ionophore. Treatment with dexamethasone did not significantly affect leukotriene release.

6 These data provide evidence that prostaglandin synthesis is consistently downregulated by glucocorticoids. However, the glucocorticoid-mediated induction of FLAP may provide a mechanism to maintain leukotriene biosynthesis through more efficient transfer of arachidonic acid to the 5-lipoxygenase reaction, in spite of inhibitory effects on other enzymes of the biosynthetic pathway.

Keywords: Cyclo-oxygenase-2; FLAP; phospholipase A_2; prostaglandin; leukotriene; glucocorticoid; monocytic cells; THP-1

Introduction

Glucocorticoids are potent anti-inflammatory drugs because they interfere with the biosynthesis of many proinflammatory mediators in multiple cells involved in inflammatory reactions (Schleimer, 1993; Geley *et al*., 1996). Part of their action is attributed to their interference with eicosanoid synthesis. Prostanoid biosynthesis has been investigated in considerable detail and it has been shown that glucocorticoids reduce the expression of key enzymes of prostanoid synthesis, phospholipases and cyclo-oxygenase-2 (reviewed by Goppelt-Struebe, 1997). In contrast, there is little evidence for the regulation of leukotriene biosynthesis by glucocorticoids. Treatment of various human cells with glucocorticoids differentially affected their capacity to synthesize leukotrienes, whereas prostaglandin production was reduced in all cases, indicating responsiveness of the cells. Reduced leukotriene C_4 (LTC_4) synthesis was demonstrated in human basophils (Marone *et al*., 1993; Hamasaki *et al*., 1994), inhibition (Abe *et al*., 1996) or no change (Azevedo *et al*., 1995) was seen in macrophages whereas no change (Schleimer *et al*, 1989), a decrease (Shindo *et al*., 1996) or even an increase (Thomas *et al*, 1995) was detected in human neutrophils. These conflicting results may relate to different activation states and concomitant changes of enzyme expression of the cells investigated. Glucocorticoids bind to intracellular receptors and interfere with gene expression (Schleimer, 1993). Therefore, *de novo* protein biosynthesis appears to be a prerequisite for their action.

In the first steps of leukotriene biosynthesis, liberated arachidonic acid is transferred to 5-lipoxygenase (5-LO) by 5-lipoxygenase activating protein (FLAP) (Reid *et al*., 1990; Mancini *et al*., 1993; Abramovitz *et al*., 1993). Upon activation, 5-LO translocates to the nuclear envelope where FLAP is localized (Woods *et al*., 1993; 1995). FLAP binds free arachidonic acid, presents it to 5-LO and thus facilitates 5-LO substrate interaction. FLAP mRNA and protein expression are subject to regulation in monocytic cells (e.g. Bennett *et al*., 1993; Flair & Pritchard, 1994) and thereby such mechanisms are involved in the regulation of leukotriene biosynthesis.

The human monocytic cell lines U937 and THP-1 provide model systems to investigate glucocorticoid effects *in vitro* (Hoff *et al*., 1992; Abrink *et al*., 1994). In their undifferentiated state these cell lines express no or very low levels of arachidonic acid metabolizing enzymes (Nolfo & Rankin, 1990). Upon differentiation along the monocytic lineage by treatment with phorbol ester they acquire monocyte-like properties (Schwende *et al*., 1996). Effects of glucocorticoids on cyclo-oxygenase (COX) isozyme expression have shown that both enzymes, COX-1 and COX-2, were regulated, but the most pronounced effects were observed on the induced COX-2 ex-

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pression, consistent with findings in macrophages (Hoff *et al*., 1993). THP-1 cells were chosen in the present study because they more closely approximate to mature monocytes when compared to U937 cells, also reflected by their higher levels of LTC_4 synthase activity (Nickolson *et al*., 1993; Goppelt-Struebe, 1995). We demonstrate here that FLAP and COX-2, key constituents of cyclo-oxygenase and 5-lipoxygenase pathways, are significantly upregulated during monocytic differentiation, but are differentially regulated by glucocorticoids.

Methods

Cell culture

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were grown in RPMI 1640 (Life Technologies, Eggenstein, Germany) containing 5% (v/v) foetal calf serum, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1% L-glutamine. Cells were differentiated with 5 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma, München, Germany) for the times indicated.

Northern blot analysis

Total cellular RNA was prepared by the guanidine isothiocyanante technique essentially as described by Chomszynski and Sacchi (1987). RNA (10 μg) was subjected to electrophoresis, transferred to nylon membranes and hybridized by standard techniques as described previously (Stroebel & Goppelt-Struebe, 1994). Specific COX-1 and COX-2 cDNA probes were 2.767- and 1.156-kilobase EcoRI fragments from the 5′-end of mouse cDNA, respectively (DeWitt *et al*., 1993). FLAP cDNA was kindly provided by P. Vickers, formerly at the Merck Frosst Center for Therapeutic Research in Quebec, Canada. DNA/RNA hybrids were detected by autoradiography with Kodak X-Omat AT film. Quantitative analysis was performed by densitometric scanning of the autoradiographs (Bioprofil, Fröbel, Germany). All values were corrected for differences in RNA loading by use of ethidium bromide fluorescence of 28S rRNA or hybridization with an 28S specific cDNA as indicated.

Reversed phase polymerase chain reaction (RT-PCR)

RNA (0.3 μg) were reverse transcribed with oligo-dT primers. DNA corresponding to 0.02 μg RNA were amplified as follows: 5-LO upstream primer 5′ACC ATT GAG CAG ATC GTG GAC ACG C; downstream primer 5′ GCA AGT CCT GCT CTG TGT AGA ATG GG; annealing temperature 72°C 40 cycles; FLAP upstream primer: 5′ GGC CAT CGT CAC CCT CAT CAG CG, downstream primer: 5′ GCC AGC AAC GGA CAT GAG GAA CAG G; annealing temperature 71°C, 24 cycles; GAPDH upstream primer 5′TCG GAG TCA ACG GAT TTG GTC GTA; downstream primer 5′ ATG GAC TGT GGT CAT GAG TCC TTC; annealing temperature 66°C, 23 cycles. Amplified cDNA bands were detected by ethidium bromide staining and the volumes evaluated by densitometry. cDNA bands of 5-LO and FLAP were corrected for GAPDH expression.

Western blot analysis

COX-2 and FLAP proteins were detected in a fraction of crude nuclear membranes. After stimulation of the cells, THP-1 cells were pelleted and resuspended in 20 mM Tris/HCl, pH 8, 10% glycerol, 10 mM EDTA, 1 mM phenylmethylsulphonylfluoride. After sonication and removal of cellular debris by low speed centrifugation ($600 \times g$), cellular membranes were pelleted ($100{,}000 \times g$) and resuspended in 20 mM Tris/HCl, pH 8. Proteins (10 μg/lane) were separated by SDS polyacrylamide gel electrophoresis and transferred to nylon membranes. A specific antibody against COX-2 was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The antibody against FLAP was kindly provided by Dr J. Evans (Merck Frosst, Canada). Protein bands were detected with the ECL Western blotting analysis system from Amersham (Braunschweig, Germany).

Determination of PGE_2 and peptidoleukotrienes

PGE_2 and peptidoleukotrienes (pLT) were determined in cell culture supernatants essentially as described previously (Schaefer *et al*., 1996). Release was determined in 4 independent experiments with triplicate biological samples each assayed in duplicate. The antibody directed against pLT recognized LTC_4 and the metabolites LTD_4 and LTE_4 with equal sensitivity (Reinke *et al*, 1991). Crossreactivity for arachidonic acid was tested between 10^{-8} and 10^{-4} M arachidonic acid. Antibodies crossreacted with 10^{-4}M arachidonic acid, but not with concentrations of 10^{-5} M and below. Detection limit was 3 pg/well (30 pg ml^{-1}) for pLT and PGE_2.

Statistical analysis

Statistical significance was calculated by the non-paired two-tailed Student's t test. A P value <0.05 was considered to indicate a statistically significant difference between two sets of data.

Results

Expression of FLAP and COX-2 during monocytic differentiation

Low levels of FLAP mRNA expression were detectable in undifferentiated THP-1 cells. Upon differentiation with low concentrations of phorbol ester (5 nM TPA), FLAP mRNA expression was induced, maximal expression being observed between 24 to 40 h (Figure 1). Concomitant incubation of THP-1 cells with TPA and dexamethasone (10^{-6} M) did not change the time course or intensity of FLAP mRNA expression (Figures 1 and 2). Of the cyclo-oxygenase isoenzymes, only COX-2 mRNA was significantly induced upon differentiation. Dexamethasone interfered with COX-2 mRNA induction at concentrations as low as 10^{-9} M (Figure 2).

Effect of dexamethasone on FLAP expression in differentiated THP-1 cells

Undifferentiated THP-1 cells represent rather immature monocytic cells. Upon incubation with phorbol ester, these cells become more monocyte-like. Therefore, the effect of dexamethasone on FLAP and COX-2 expression was also investigated in THP-1 cells that had been differentiated with TPA (5 nM) for 40 h. When differentiated THP-1 cells were treated with fresh medium, they showed a further increase in FLAP mRNA expression (163 ± 9% after 24 h, with TPA-differentiated cells set to 100%, $n=4$). Levels remained elevated for at least 48 h (Figure 3). Treatment of differentiated

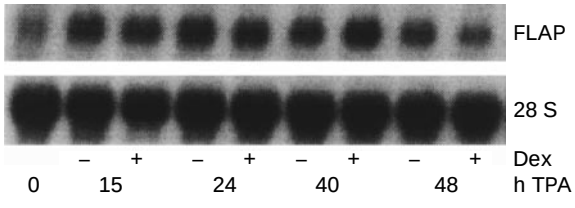

Figure 1 Upregulation of FLAP during monocytic differentiation. Undifferentiated THP-1 cells were incubated with TPA (5 nM) and/or dexamethasone (Dex, 10^{-6} M) for the times indicated. Northern blot analysis with specific cDNAs for FLAP and 18 S rRNA was performed as described in Methods. The blot is representative of 3 independent experiments with similar results.

THP-1 cells with dexamethasone (10^{-6} M) increased FLAP expression (258 ± 62% within 24 h, $n=4$, $P<0.05$ compared to medium-treated cells). Enhancement was concentration-dependent with 10^{-8} M dexamethasone being effective (Figure 4). COX-2 expression, in contrast, was downregulated by dexamethasone within the first hours of incubation. COX-1 expression was very low in these cells and not significantly affected by either TPA or dexamethasone.

Expression of 5-LO mRNA was not detectable by Northern blot analysis. Therefore, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed (Janssen-Timmen *et al*., 1995). Dexamethasone did not significantly affect the expression of 5-LO mRNA, whereas FLAP mRNA expression was elevated (data not shown). The latter result was comparable with the results obtained by Northern blot analysis.

Differential regulation of COX-2 and FLAP at the protein level

The changes in FLAP and COX-2 mRNA expression were also observed at the protein level (Figure 5): upon differentiation with TPA, FLAP and COX-2 protein were increased in the nuclear membrane fraction. Further treatment of differentiated cells with dexamethasone for 24 h caused COX-2 protein to decrease but FLAP protein to increase (Figure 5).

Effect of dexamethasone on eicosanoid release

In order to detect changes at the product level, differentiated THP-1 cells were incubated with medium alone or with medium supplemented with dexamethasone (10^{-6} M). Cells were

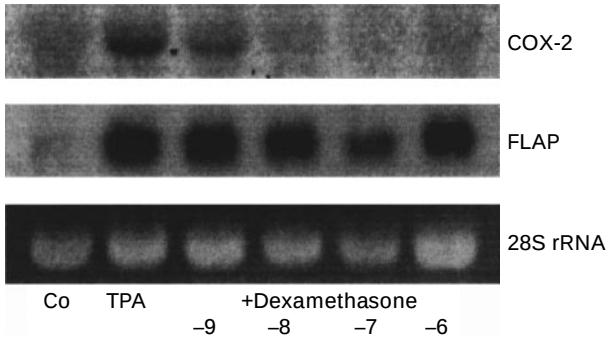

Figure 2 Interference by dexamethasone of COX-2 induction at low concentrations. Undifferentiated THP-1 cells (Co) were incubated with TPA (5 nM) and dexamethasone (10^{-6}–10^{-9} M) for 24 h. Thereafter, RNA was extracted and Northern blot analysis performed as described in Methods.

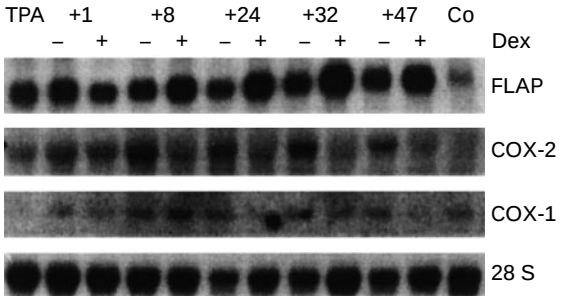

Figure 3 Effect of dexamethasone on gene expression in TPA-differentiated THP-1 cells. Undifferentiated THP-1 cells (Co) were differentiated with TPA for 40 h. They were further incubated with fresh medium in the presence (+) or absence (−) of dexamethasone (Dex, 10^{-6} M) for the times (h) indicated. RNA was extracted and Northern blot analysis was performed as described in Methods. The autoradiogram is representative of 3 experiments with similar results.

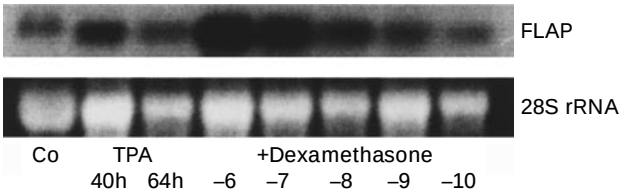

Figure 4 Concentration-dependent induction of FLAP mRNA by dexamethasone in differentiated THP-1 cells. THP-1 cells (Co) were differentiated with TPA for 40 h. They were further incubated with fresh medium in the presence or absence of dexamethasone (10^{-6}–10^{-10} M) for 24 h. Data shown are a Northern blot hybridization with a cDNA specific for FLAP and the ethidium bromide fluorescence of the 28S rRNA.

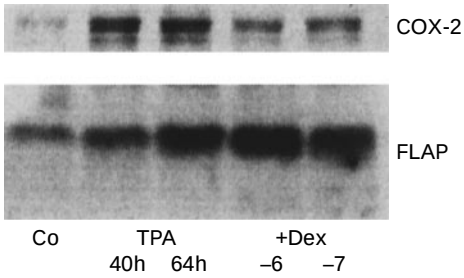

Figure 5 Differential modulation of COX-2 and FLAP protein expression in differentiated THP-1 cells. THP-1 cells (Co) were differentiated with TPA for the times indicated. Dexamethasone (10^{-6} and 10^{-7}M) was added between 40 and 64 h. Crude membrane preparations containing nuclear membranes were fractionated by SDS polyacrylamide gel electrophoresis. COX-2 and FLAP proteins were detected by specific antibodies.

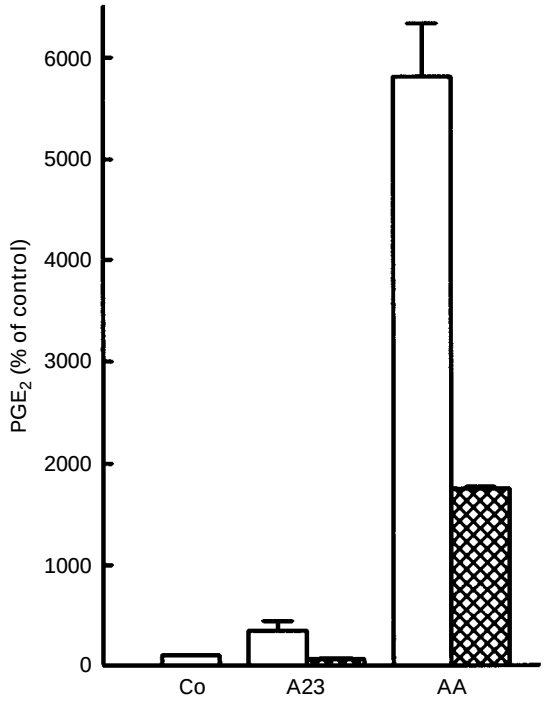

Figure 6 Release of PGE_2 from differentiated THP-1 cells. THP-1 cells were differentiated with TPA for 40 h. They were further incubated with fresh medium in the presence (hatched columns) or absence (open columns) of dexamethasone (10^{-6} M) for 24 h. Cells were left untreated (Co) or stimulated with ionophore A23187 (A23; 10 μg ml^{-1}) or arachidonic acid (AA; 10 μM) for 30 min. PGE_2 was determined in the cellular supernatants by ELISA. Data are means ± s.d. of 4 independent experiments.

then stimulated for 30 min with ionophore A23187 (10 μg ml^{-1}), to activate endogenous synthesis of eicosanoids, or with exogenous arachidonic acid (10^{-5} M). Stimulation with ionophore increased PGE_2 synthesis about 3 fold (Figure 6). PGE_2 levels of dexamethasone-treated cells fell below basal levels of unstimulated cells (58.8 $\pm$ 10.4% of basal levels; $n=4$, $P<0.001$ in each experiment). With exogenous arachidonic acid as substrate, large amounts of PGE_2 were formed (>6 ng ml^{-1}). In contrast to the ionophore-stimulated PGE_2 synthesis, arachidonic acid-stimulated PGE_2 synthesis was only partially inhibited in dexamethasone-treated cells (34.0 $\pm$ 4.5% of arachidonic acid-stimulated PGE_2 release, $n=3$).

Comparatively low but significant levels of peptidoleukotirenes (pLT) were identified in the supernatants of differentiated THP-1 cells. Basal levels were close to the detection limit of about 30 ng ml^{-1} (103 $\pm$ 56 pg ml^{-1}, 4 independent experiments with triplicate samples). Increased levels of pLT were found when differentiated THP-1 cells were treated with ionophore A23187 (10 μg ml^{-1}), but did not reach statistical significance (121 $\pm$ 7%, compared to basal levels, $n=3$). Incubation of differentiated THP-1 cells with dexamethasone for 24 h before treatment did not significantly alter the amount of stimulated pLT released.

Discussion

Inhibition of prostanoid synthesis in monocytic cells is considered to be part of the anti-inflammatory potency of glucocorticoids. A strong effect of the glucocorticoid dexamethasone was also observed in the cellular system investigated, the human monocytic cell line THP-1. When undifferentiated cells were differentiated along the monocytic pathway by treatment with phorbol ester, concomitant treatment with dexamethasone completely prevented induction of COX-2 mRNA and protein, whereas the low levels of COX-1 expression were not significantly altered. Induction of COX-1 as described in Smith *et al*. (1993) was not detected by us in previous experiments (Hoff *et al*., 1994) and was not seen in the present experiments. The reason for this discrepancy is unclear at present. However, results are consistent with data obtained previously showing that COX-2 is a major target of glucocorticoid action in monocytic cells (Fu *et al*., 1990; Hoff *et al*., 1993).

The functional activity of 5-LO, the enzyme catalyzing the first step in leukotriene synthesis from arachidonic acid, critically depends on the 5-lipoxygenase activating protein, FLAP. FLAP was first considered to act as a docking protein, directing the cytosolic 5-LO towards cellular membranes, primarily nuclear membranes (Ford-Hutchinson, 1991; Woods *et al*., 1995). However, current evidence suggests that FLAP acts as an arachidonic acid binding protein presenting arachidonic acid to 5-LO (Abramovitz *et al*., 1993; Mancini *et al*., 1993). Expression of FLAP was shown in a variety of cells to correlate well with their capacity to synthesize leukotrienes (Reid *et al*., 1990). In undifferentiated THP-1 cells, we found very low levels of FLAP mRNA expression in accordance with previous results showing that undifferentiated THP-1 cells were unable to form leukotrienes (Nolfo & Rankin, 1990). However, upon differentiation with TPA FLAP mRNA expression was strongly increased. Induction was transient with a decrease visible after 72 h. Also *in vivo*, monocyte differentiation seems to involve an increase in FLAP expression as shown for the maturation of monocytes to alveolar macrophages (Coffey *et al*., 1994).

Coincubation of THP-1 cells with dexamethasone together with TPA did not affect FLAP mRNA induction. In contrast, differentiated THP-1 cells were sensitive to dexamethasone: dexamethasone increased both mRNA and protein expression of FLAP in differentiated THP-1 cells. We have shown previously that the number of glucocorticoid receptors is upregulated during monocytic differentiation of U937 cells (Koehler *et al*., 1990). Higher numbers of receptors may be necessary to mediate the effect of dexamethasone on FLAP expression that on COX-2 expression. In line with this argument, COX-2 expression was sensitive to glucocorticoid action in undifferentiated THP-1 cells and much lower concentrations of dexamethasone were needed to inhibit COX-2 expression than FLAP expression in differentiated cells. Depending on the gene and the cellular system, glucocorticoids may affect transcriptional as well as posttranscriptional mechanisms (Goppelt-Struebe, 1997). In human neutrophils a direct upregulation of FLAP mRNA by dexamethasone was observed (Pouliot *et al*., 1994). The molecular mechanism of dexamethasone action in THP-1 cells was not investigated, but it is tempting to speculate that glucocorticoid receptors directly interfere with the glucocorticoid responsive element located in the promotor region of the FLAP gene (Kennedy *et al*., 1991).

Inhibition of COX-2 by dexamethasone was clearly reflected at the product level. Ionophore-induced PGE_2 synthesis was almost completely inhibited in cells treated with dexamethasone. When the cells were incubated with high concentrations of exogenous arachidonic acid to assay for PGE_2 synthesis independent of phospholipases, inhibition by dexamethasone was not complete suggesting that under these conditions, COX-1 may also contribute to the high PGE_2 synthesis rate.

Undifferentiated THP-1 cells were shown to be unable to form any products of the 5-lipoxygenase pathway (Nolfo & Rankin, 1990) and even differentiated THP-1 cells released very low concentrations of pLTs upon stimulation. However, induction of FLAP by dexamethasone did not significantly change pLT release. This may be due to the counteracting effects of dexamethasone on activity and/or expression of phospholipases A_2. In differentiated THP-1 cells, we observed reduced mRNA levels of the cytosolic phospholipase A_2 ($cPLA_2$) after dexamethasone treatment (unpublished observation) in line with reduced phospholipase activity determined previously in glucocorticoid-treated differentiated U937 cells (Koehler *et al*., 1989). Additional effects of glucocorticoids on the activation of $cPLA_2$ were reported in macrophages and may also play a role in monocytic cells (Gewert & Sundler, 1995). It is not known yet which type of phospholipase A_2 is responsible for the liberation of arachidonic acid for leukotriene synthesis in THP-1 cells. In a recent paper, Marshall *et al*. (1997) provided evidence that not $cPLA_2$ but a low molecular weight PLA_2 may provide arachidonic acid for leukotriene synthesis in human monocytes. Expression of this type of PLA_2 has also been shown to be inhibited by glucocorticoids (Nakano *et al*., 1990; Vervoordeldonk *et al*., 1996). Our data are thus in line with the interpretation that inhibition of phospholipase activity opposes induction of FLAP, leading to an inhibition of pLT synthesis in some experiments and a stimulation in others. The situation may be further complicated by glucocorticoid effects on LTC_4 synthase, an enzyme that is regulated by phosphorylation (Ali *et al*., 1994). Partial inhibition of LTC_4 synthase was observed in basophilic leukemia cells (Hamasaki *et al*., 1994), while the regulation of this enzyme by glucocorticoids in monocytic cells is unknown.

Taken together our data indicate that glucocorticoids induce FLAP expression that should result in an increased leukotriene production, but seems to be counterbalanced by glucocorticoid effects on other enzymes in the biosynthetic pathway. These data may help to explain the varying effects of glucocorticoids on leukotriene synthesis observed *ex vivo*.

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