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Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory corticosteroids

(prostaglandins/phospholipids/anti-inflammation/serum)

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ABSTRACT **Serum stimulates the production of prostaglandins by transformed mouse fibroblasts. Hydrocortisone (cortisol) inhibits this stimulation. The half-maximal inhibition occurs at 6×10^{-9} M. Studies with cells labeled with [^{3}H]arachidonic acid in their lipids show that the stimulation by serum results in the release of arachidonic acid from the cellular lipids, mostly phospholipids. Hydrocortisone inhibits this release but does not inhibit the production of prostaglandins from exogenously supplied arachidonic acid. This inhibition of arachidonic acid release from phospholipids may be the mechanism for the anti-inflammatory action of corticosteroids.**

Prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$) are present in the culture medium of a methylcholanthrene-transformed mouse fibroblast, MC5-5 (1, 2). This cell line proves to be a useful model for the study of the effect of vasoactive substances on the production of prostaglandins. We have shown that mechanical manipulation and vasoactive substances such as bradykinin, arachidonic acid, serum, and thrombin stimulate the production of prostaglandins. Cofactors for *in vitro* microsomal prostaglandin synthetase systems, such as hydroquinone and glutathione, also stimulate prostaglandin synthesis by the cells. Indomethacin, a nonsteroid anti-inflammatory drug, inhibited all of the above-mentioned stimulations of prostaglandin formation.

Prostaglandins have been implicated in inflammation (3). Nonsteroid, aspirin-like anti-inflammatory drugs such as aspirin and indomethacin inhibit *in vitro* microsomal prostaglandin biosynthesis, and this inhibition has been postulated to be the basis of their therapeutic effects (4). On the other hand, hydrocortisone (cortisol), an anti-inflammatory steroid, did not inhibit *in vitro* prostaglandin synthetase systems in some studies (4, 5); but in another study fluocinolone, at high concentrations, did inhibit (6). Corticosteroids do inhibit prostaglandin formation by mouse fibrosarcoma cells (7) and by rheumatoid synovia (8). Lewis and Piper, based on their study on the infusion of adrenocorticotropic hormone into rabbit adipose depots, concluded that inhibition of release of prostaglandins from the tissue was the mechanism of some actions of corticosteroids in inflammation, in gastric mucosa, and in the central nervous system (9). Gryglewski *et al.* (10), working with perfused guinea pig lungs, suggested that corticosteroids act by reducing the availability of the substrate for the prostaglandin synthetase. The mechanism involving inhibition of release would be less likely if tissue storage of prostaglandins were low, as has been suggested by Ramwell and Shaw (11). In this paper, we report studies on the effect of hydrocortisone on prostaglandin synthesis in MC5-5 cells and provide evidence for the mechanism of the action of corticosteroids in inhibiting prostaglandin synthesis.

Abbreviations: PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

MATERIALS AND METHODS

Cell and Cell Culture. MC5-5, a methylcholanthrene-transformed mouse BALB/3T3 cell line (12) was generously provided to us by Dr. G. J. Todaro. Cells were grown in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum as previously described (1, 2).

Exponentially growing MC5-5 cells were used for all experiments. Cells treated with trypsin were seeded at 2 to 5×10^5 cells per 60 mm Falcon tissue culture dish in 4 ml of the serum-supplemented medium and grown for 1 day. For experiments that require changes to fresh medium for incubation, the dishes were washed two times with 2 ml of serum-free medium and 2 ml of the experimental medium was then added and the plates were incubated in a humidified incubator at 37° in an atmosphere of 95% air and 5% CO_2. After a desired time interval, medium was withdrawn, chilled, and stored at −20°. When preincubation with the drugs was required, the drugs were added to the culture for the desired length of time. The densities of the cells were not the same from experiment to experiment, but within each series of experiments, identically prepared cell dishes were used. Duplicate plates were used for all experiments. The analyses of the prostaglandin levels and radioactivity from duplicate dishes agreed within 5% of the mean value. PGE_2 and $PGF_{2\alpha}$ were measured serologically with either anti-PGE_2 or anti-$PGF_{2\alpha}$ as previously described (1, 2).

Cells radioactively labeled in phospholipids were prepared as follows. MC5-5 cells were inoculated at 5×10^5 cells per dish (60 mm) containing 4 ml of serum-supplemented medium and 10 μCi of [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid, specific activity 72 Ci/mmol, and grown for 24 hr. About 60% of the radioactivity was incorporated into the cells. Analysis of the cell lipids indicated that 95% of the radioactivity was associated with phospholipids, 5% was associated with triglycerides, and less than 0.3% was present as free arachidonic acid.

For thin-layer chromatography, Eastman Kodak chromatogram sheets with silica gel adsorbent and fluorescent indicator were used. For the analysis of radioactive material released from the cells, aliquots of the medium were directly applied to the sheets and developed with a solvent system described by Lands and Samuelsson (13). For analysis of the composition of cell lipids containing [^{3}H]arachidonic acid, the lipids were extracted with chloroform/methanol (2:1 vol/vol), and aliquots of the extract were applied to the sheets and developed by two-dimensional chromatography with the solvent systems described by Ames (14). For analysis of radioactive prostaglandins and their metabolites, the upper layer of the solvent system ethylacetate/2,2,4-trimethylpentane/acetic acid/water (90:50:20:100 vol/vol) was used. Carrier lipids were applied on the chromatogram sheet with radioactive samples. The lipids were located with iodine vapor. Pieces of the sheet

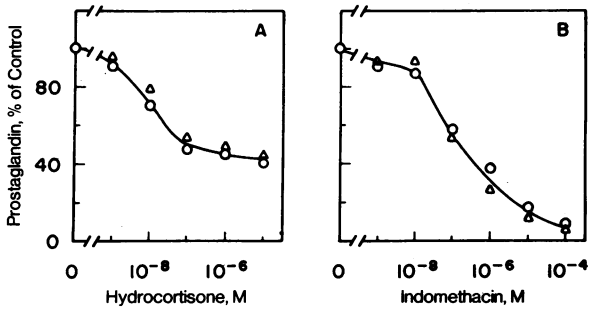

FIG. 1. Effect of hydrocortisone and indomethacin concentration on prostaglandin production. Cells (0.7×10^6 cells per dish) were rinsed and incubated with 2 ml of serum-supplemented medium containing the indicated concentration of hydrocortisone or indomethacin for 3 hr. The media were withdrawn and assayed for PGE_2 (○) and $PGF_{2\alpha}$ (△). Duplicate dishes were used for each concentration and the average values, expressed as percent of control (16 ng of PGE_2 and 1.6 ng of $PGF_{2\alpha}$ per ml of medium), are plotted.

containing the lipid of interest were cut and counted directly in toluene-based scintillation fluid.

Hydrocortisone, dexamethasone, deoxycorticosterone, corticosterone, estradiol-17β and estrone were obtained from Sigma (St. Louis, Mo.); cholesterol from Supelco (Bellefonte, Pa.); dehydroisoandrosterone (3β-hydroxyandrost-5-en-17-one) from Calbiochem (Los Angeles, Calif.); and epitestosterone (17-α-hydroxyandrost-4-en-3-one) from Mann Research (New York, N.Y.). Indomethacin was a gift from Dr. F. A. Kuehl, Jr., of Merck and Co. (Rahway, N.J.). Powdered minimal essential medium (F-11) was purchased from Grand Island Biological Company (Grand Island, N.Y.) and fetal bovine serum from Microbiological Associates (Bethesda, Md.). [*5,6,8,9,11,12,14,15*-^{3}H(N)]Arachidonic acid, [*5,6,8,11,12,14,15*-^{3}H(N)] PGE_2, and [*5,6,8,9,11,12,14,15*-^{3}H(N)]$PGF_{2\alpha}$ were purchased from New England Nuclear (Boston, Mass.). Ethanol was used as a solvent for the steroids and indomethacin, and when present in the medium at 0.1% had no effect on prostaglandin production by the cells.

RESULTS

Prostaglandins E_2 and $F_{2\alpha}$ accumulate in the culture medium of MC5-5 cells (1, 2). When the dishes containing growing cells were rinsed and incubated with medium containing serum, the prostaglandin levels in the medium increased with time for several hours before reaching a constant value. These maximum levels were proportional to the cell number in the dishes up to 2×10^6 cells per dish.

As shown in Fig. 1A, hydrocortisone inhibits the production of prostaglandins by MC5-5 cells. In this experiment, cells were incubated for 3 hr with fresh serum-supplemented medium containing various concentrations of hydrocortisone. The inhibition of PGE_2 and $PGF_{2\alpha}$ production was found to be dependent on hydrocortisone concentration and reached a maximum of 50% at 10^{-7} M. The half-maximal inhibition occurred at 6×10^{-9} M. In a similar study, indomethacin, a known inhibitor of prostaglandin synthetase, inhibited 50% at 10^{-7} M (Fig. 1B). At the highest concentration tested, 10^{-4} M, inhibition was essentially complete (95%). In the following experiments a concentration of 5×10^{-6} M was used for both hydrocortisone and indomethacin. At this concentration of drugs, no toxicity to the cells was observed, as judged by their growth rate.

When the production of prostaglandins was measured for a shorter period of time (30 min), only 10 to 20% inhibition by hydrocortisone was obtained. Preincubation of the cells with hydrocortisone increased the extent of inhibition. A 2 hr preincubation was necessary to reach the 60% level of inhibition. However, if the cells were pretreated with hydrocortisone for 1 or 2 days, the inhibition was almost complete. The continuous presence of hydrocortisone in the medium after pretreatment was required, since the extent of inhibition was reduced if hydrocortisone was not present. Similar studies with indomethacin indicated that even a 10 min preincubation was sufficient to cause complete inhibition of prostaglandin production.

Table 1. Effects of steroids on the formation of prostaglandins

Steroid	PGE_2, %	$PGF_{2\alpha}$, %
None	100	100
Dexamethasone	29	32
Hydrocortisone	41	44
Corticosterone	61	71
Deoxycorticosterone	82	52
Estrone	84	85
Estradiol-17β	88	95
Cholesterol	94	90
Epitestosterone	104	94
Dehydroisoandrosterone	116	102

MC5-5 cells at 1.3×10^6 per dish were preincubated for 2 hr with the indicated steroids at 5×10^{-6} M. The dishes were then rinsed and incubated with serum-supplemented medium containing the steroids for 1 hr, and the prostaglandins produced were assayed as described in *Materials and Methods*. The levels of prostaglandin are expressed as percent of control (PGE_2, 13 ng/ml; $PGF_{2\alpha}$, 1.0 ng/ml) and the average values from duplicate dishes are shown.

Not all of the steroids tested inhibited prostaglandin production; only steroids with anti-inflammatory activity did (Table 1). In these experiments, cells were pretreated for 2 hr with the steroids at 5×10^{-6} M and then incubated for 1 hr with fresh serum-supplemented medium containing the steroids. Dexamethasone is the most potent, with 71% inhibition of PGE_2 production, followed by hydrocortisone (59%) and corticosterone (39%). Deoxycorticosterone, estradiol-17β, and estrone are only slightly inhibitory (10–20%), while cholesterol, epitestosterone, and dehydroisoandrosterone did not show significant effects. The order of the extent of inhibition by corticosteroids follows that of their relative anti-inflammatory potency. Similar results were also observed when $PGF_{2\alpha}$ levels were determined, with the exception of deoxycorticosterone, which showed activity between that of hydrocortisone and corticosterone.

We had previously shown that serum stimulates the production of prostaglandins (1, 2). With the use of cells labeled in phospholipids with [^{3}H]arachidonic acid, we found that the presence of serum also stimulated the release of the prostaglandin precursor, arachidonic acid, from the cells. The data in Fig. 2 show that a significant amount of radioactivity was released into the medium, which after 3 hr amounted to about 20% of the total radioactivity incorporated into the cells. Analysis of the radioactive material by thin-layer chromatography indicated that arachidonic acid was the major component and accounted for 92% of the radioactivity, while the radioactivity with an R_F value corresponding to PGE_2 was about 4% of the total radioactivity released. The $PGF_{2\alpha}$ and the phospholipid spots contained minor amounts of radioactivity (0.6% and 1%, respectively). Minor amounts of radioactivity, 1% and 1.5%, were associated with two unidentified spots; one running with the solvent front, and the other immediately behind arachidonic acid. The radioactivity associated with arachidonic

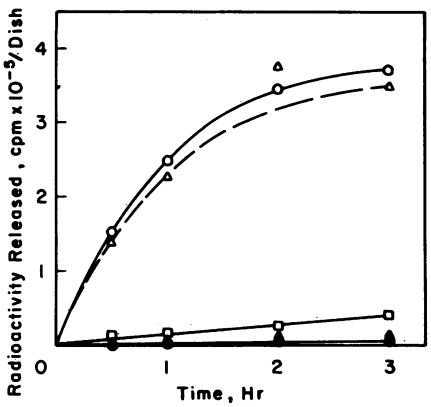

FIG. 2. Effect of serum on the release of radioactive material from labeled cells. Radioactive labeled cells (5×10^5 cells per dish) were rinsed two times with 4 ml of serum-free medium and then incubated with 2 ml of serum-supplemented medium. Aliquots of 0.3 ml were withdrawn at the indicated times. For total radioactivity released (○), aliquots of 20 μl were counted for radioactivity. For the analysis of radioactivity in arachidonic acid (△), PGE_2 (□), $PGF_{2\alpha}$ (●), and phospholipid (▲), aliquots of 20 μl were used for the analysis by thin-layer chromatography. The initial radioactivity incorporated in the cells was 1.85×10^6 cpm per dish.

acid, PGE_2, and $PGF_{2\alpha}$ increased with time of incubation. The increases with time of these values for PGE_2 and $PGF_{2\alpha}$ are comparable to those for the same compounds released from unlabeled cells and assayed by immunological methods.

The effects of hydrocortisone and indomethacin on arachidonic acid and on prostaglandin production were examined using these ^3H-labeled cells. The cells were pretreated with hydrocortisone or indomethacin for 2 hr and then incubated with fresh serum-supplemented medium containing the same inhibitor for another 2 hr. The results, summarized in Table 2, show that in the presence of serum-supplemented medium 19% of the total incorporated radioactivity was released. When the cells were treated with indomethacin, the released radioactivity was only slightly reduced (to 16%). However, when the cells were treated with hydrocortisone, the released radioactivity was markedly reduced (to 9.7%).

Table 2. Effect of hydrocortisone and indomethacin on release of radioactivity from labeled cells

Test medium	Radioactivity (cpm × 10^{-5} per dish)		
	Initial activity in cells	Released into medium	Remaining in cells
MEM(−)	19.7	0.212	18.4
MEM(+)	20.0	3.87 (19%)*	16.2
MEM(+), hydrocortisone	20.1	1.97 (9.7%)*	18.0
MEM(+), indomethacin	20.2	3.15 (16%)*	17.0

MEM(−) and MEM(+) are minimal essential medium containing 0 and 10% fetal bovine serum, respectively. MC5-5 cells (1×10^6 cells per dish) labeled in phospholipids with [^3H]arachidonic acid were rinsed and incubated with 2 ml of the desired medium for 2 hr. In the presence of hydrocortisone or indomethacin the dishes were preincubated with the drugs at 5×10^{-6} M for 2 hr. The media were withdrawn and counted for radioactivity. The radioactivity remaining in the cells after the incubation was obtained by counting the trypsinized cell suspension. The initial radioactivity in the cells was obtained by subtracting the total radioactivity used for labeling from that remaining in the medium after 1 day of incorporation. Average values from duplicate dishes are shown.

* Expressed as percent of the initial radioactivity in the cells.

The radioactive materials released under the above conditions were analyzed by thin-layer chromatography. As shown in Fig. 3, in serum-supplemented medium, 96% of the released radioactivity appeared as arachidonic acid, 4% as PGE_2, <1%

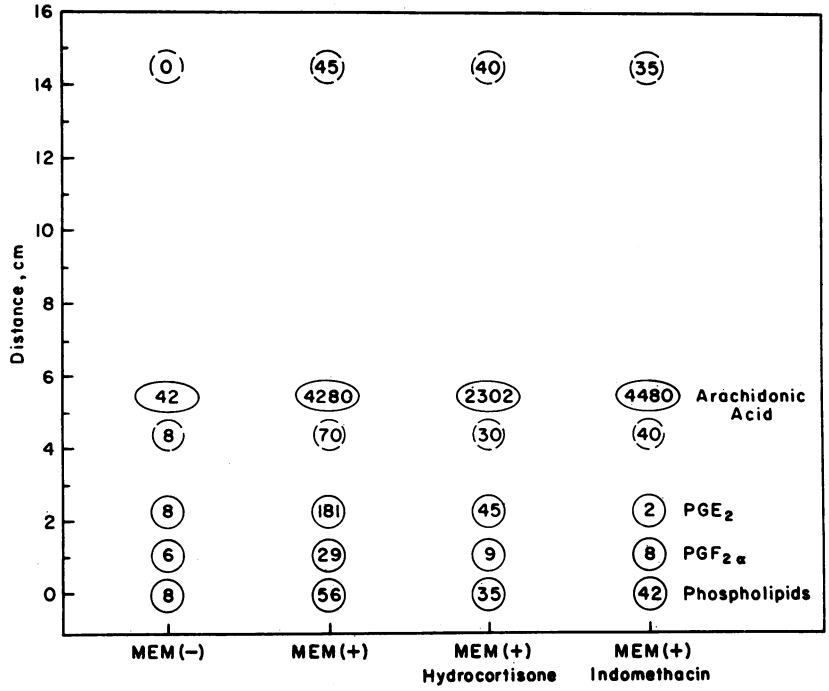

FIG. 3. Thin-layer chromatograms of radioactive materials released after incubation with serum-free medium [MEM(−)], serum-supplemented medium [MEM(+)], and serum-supplemented medium containing hydrocortisone or indomethacin (both 5×10^{-6} M). Aliquots (40 μl) from incubation mixtures described in Table 2 were used for the analysis. The number in each spot shows the radioactivity in cpm of the average values from duplicate dishes. The values are corrected for a background of 25 cpm.

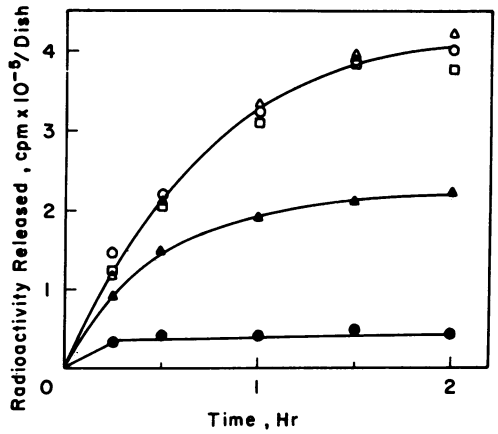

FIG. 4. Effect of hydrocortisone, dehydroisoandrosterone, and estradiol-17β on the release of radioactivity from cells. ^{3}H-Labeled cells (9×10^5 cells per dish) were incubated with 2 ml of serum-free medium (●) or serum-supplemented medium (○), or serum-supplemented medium containing hydrocortisone (▲), dehydroisoandrosterone (△), or estradiol-17β (□) at 5×10^{-6} M. At the indicated times, aliquots of 50 μl were withdrawn from dishes and counted for the radioactivity. The average values from duplicate dishes were plotted. The initial total radioactivity incorporated in the cells was 3.1×10^6 cpm per dish.

as $PGF_{2\alpha}$, and 1% remained at the origin. Incubation of the cells with serum-supplemented medium containing hydrocortisone resulted in a 50% reduction of the released radioactive arachidonic acid. A simultaneous reduction of radioactive PGE_2 and $PGF_{2\alpha}$ was also observed. Although indomethacin did not cause any significant change in radioactive arachidonic acid, it completely inhibited the appearance of radioactive PGE_2 and $PGF_{2\alpha}$. Indomethacin inhibited the conversion of arachidonic acid to PGE_2 and $PGF_{2\alpha}$, whereas hydrocortisone exerted its effect by inhibiting the release of arachidonic acid from phospholipids. Inhibition of the release of arachidonic acid would result in the reduction of prostaglandin production since arachidonic acid, the precursor for prostaglandin biosynthesis, limits the production of prostaglandins (1, 2). Inhibition of arachidonic acid release from phospholipids was observed only with hydrocortisone; no significant effects were found with other steroids tested, including estradiol-17β and dehydroisoandrosterone (Fig. 4).

We have previously demonstrated that exogenously supplied arachidonic acid stimulates the production of prostaglandins by MC5-5 cells (1, 2). The levels of prostaglandins formed were found to be proportional to the concentration of arachidonic acid, at least up to 50 μg/ml. If hydrocortisone inhibits only the release of arachidonic acid from phospholipids and does not inhibit the prostaglandin synthetase, the ability of the cells to form prostaglandins from exogenous arachidonic acid should not be affected. As shown in Table 3, the level of PGE_2 increased from 2 ng/ml to 42 ng/ml and that of $PGF_{2\alpha}$ increased from 0.3 ng/ml to 4.1 ng/ml in serum-free medium in the presence of 5 μg of arachidonic acid. Indomethacin completely inhibited the formation of prostaglandins in the presence or absence of the added arachidonic acid, whereas hydrocortisone inhibited prostaglandin production in the absence of arachidonic acid, but not in its presence. Dehydroisoandrosterone, which previously was shown not to inhibit the production of prostaglandin or release of radioactive arachidonic acid, had no effect on the conversion of exogenous arachidonic acid into prostaglandins. Similar results were found when serum-supplemented medium was used as the incubation medium. In the absence of added arachidonic acid, hydrocortisone, after preincubation with the cells, inhibited between 70 and 80% of the prostaglandin production. In the presence of added arachidonic acid, prostaglandin levels were increased. Hydrocortisone did not inhibit the formation of prostaglandins from arachidonic acid. The inhibition of prostaglandin production by hydrocortisone in serum-supplemented medium containing arachidonic acid was as expected if the hydrocortisone inhibited only the production of prostaglandins by the cells from serum-supplemented medium alone.

Table 3. Effects of hydrocortisone and indomethacin on prostaglandin formation in the presence and absence of exogenous arachidonic acid

Treatment	PGE_2, ng/ml	$PGF_{2\alpha}$, ng/ml
MEM(−)	2.0	0.30
Indomethacin	0.10	0.0
Hydrocortisone	0.88	0.17
Dehydroisoandrosterone	1.9	0.42
MEM(−), arachidonic acid, 5 μg/ml	42	4.1
Indomethacin	0.80	0.45
Hydrocortisone	38	4.4
Dehydroisoandrosterone	42	4.7
MEM(+)	20[a]	1.2
Indomethacin	1.0	0.0
Hydrocortisone	5.9[b]	0.22
Dehydroisoandrosterone	21	0.95
MEM(+), arachidonic acid, 5 μg/ml	35[c]	2.3
Indomethacin	1.2	0.0
Hydrocortisone	21 (21)*	1.2 (1.3)‡
Dehydroisoandrosterone	30	2.1
MEM(+), arachidonic acid, 10 μg/ml	57[d]	2.7
Indomethacin	3.0	0.0
Hydrocortisone	35 (43)†	1.6 (1.7)‡
Dehydroisoandrosterone	51	2.9

MEM(−) and MEM(+) are minimal essential medium containing 0 and 10% fetal bovine serum, respectively. MC5-5 cells (1.3×10^6 cells per dish) were preincubated with drugs for 2 hr and then incubated with test media containing the indicated amount of arachidonic acid and drugs (5×10^{-6} M) for 1 hr. Average values for prostaglandins from duplicate dishes are given.

[a] Arachidonic acid stimulates prostaglandin production less in serum-supplemented medium than it does in serum-free medium. This is probably due to a tight binding of arachidonic acid to serum proteins.

* Expected value obtained from c − a + b.

† Expected value obtained from d − a + b.

‡ Expected values obtained similarly as described for * and †.

The possibility that hydrocortisone accelerated the catabolism of prostaglandins and thereby reduced prostaglandin levels was also investigated. Dishes of cells growing in serum-supplemented medium in the presence and absence of hydrocortisone were incubated with 2 μCi of high specific activity [^{3}H]PGE_2 (210 Ci/mmol) or [^{3}H]$PGF_{2\alpha}$ (178 Ci/mmol) for 1 day. The media were analyzed by thin-layer chromatography for PGE_2 and $PGF_{2\alpha}$ and their metabolites, 15-keto-PGE_2, 13,14-dihydro-15-keto-PGE_2, 15-keto-$PGF_{2\alpha}$, and 13,14-dihydro-15-keto $PGF_{2\alpha}$. No change in the profile of radioactive substances was observed. Thus, hydrocortisone does not affect the degradation of prostaglandins.

Inhibition of prostaglandin production by indomethacin and hydrocortisone cannot be due to their effects on the release of preformed prostaglandins because intracellular prostaglandins

in hydrocortisone- and indomethacin-treated cells were found to be lower than those in the control cells, 70% and 8%, respectively.

DISCUSSION

Inhibition of prostaglandin synthesis in MC5-5 cells by anti-inflammatory corticosteroids at low concentrations is in agreement with the inhibition found in rheumatoid synovia (8) and mouse fibrosarcoma cells (7). The results presented here demonstrate that the inhibition by hydrocortisone is due to block of the release of arachidonic acid from phospholipids and not due to inhibition of prostaglandin synthetase. Two lines of evidence support this conclusion: (*i*) Hydrocortisone reduces the release of arachidonic acid from the phospholipids of the cells; and (*ii*) no inhibition of prostaglandin formation is observed when exogenously supplied arachidonic acid is used as substrate for the formation of prostaglandins by the cells. Hydrocortisone does not affect the catabolic rate of the prostaglandins.

Many cells and most organs in culture medium produce prostaglandins, yet very little is known about the mechanisms underlying prostaglandin production. Our studies demonstrate that the release of arachidonic acid from the phospholipids of the cells (in addition to the presence of this compound originally in serum), presumably by activation of phospholipases either directly or indirectly, may be an important factor in prostaglandin biosynthesis. Prostaglandins may play a role in the mediation of the inflammatory response (3). Our findings that corticosteroids inhibit the release of arachidonic acid from phospholipids with a resultant reduction of prostaglandin formation suggests strongly that this mechanism may be the basis for their anti-inflammatory effects. Gryglewski *et al.* (10) reached similar conclusions from their study with perfused guinea pig lungs. This mechanism of corticosteroid action may explain the failures of previous attempts to inhibit the formation of prostaglandins from arachidonic acid by microsomal enzyme preparations (4, 5). One exception was the observation by Greaves and McDonald-Gibson (6), who found partial inhibition of prostaglandin formation by fluocinolone, but not hydrocortisone, with skin homogenates as the source of enzyme. The same investigators, however, reported later that no inhibition was observed when microsomal enzyme preparations were used (15).

The anti-inflammatory action of corticosteroids is thought to be mediated in part by stabilization of plasma and lysosomal membranes (16), although studies with phagocytic cells and bactericidal systems have failed to support this concept (17, 18). The inhibition of arachidonic acid release by corticosteroids could result from stabilization of the cellular membranes, thereby rendering the phospholipids less accessible to phospholipases; however, the inhibition of release could also result directly from inhibition of phospholipase activity or indirectly from the corticosteroids' effect on cellular metabolism.

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