Br. J. Pharmac. (1981), **74**, 322–324

THE INHIBITION BY HYDROCORTISONE OF PROSTAGLANDIN BIOSYNTHESIS IN RAT PERITONEAL LEUCOCYTES IS CORRELATED WITH INTRACELLULAR MACROCORTIN LEVELS

ROSA CARNUCCIO, M. DI ROSA, R.J. FLOWER[1] & A. PINTO

Universita Degli Studi di Napoli, Istituto di Farmacologia Sperimentale, Via Leopoldo Rodino 22, 80138 Napoli, Italy

Hydrocortisone inhibits prostaglandin generation by rat peritoneal leucocytes by releasing the polypeptide phospholipase inhibitor, macrocortin. The susceptibility of these cells to hydrocortisone is directly correlated with their intracellular macrocortin content. Cells depleted of the peptide by prior incubation with steroid cannot respond to the steroid, until a fresh intracellular store has been synthesized. *In vitro*, this process requires 4–5 h. Cells remain sensitive to the inhibitory action of the peptide at all times.

Introduction Both aspirin-like drugs and glucocorticoids inhibit prostaglandin synthesis by leucocytes (Bray & Gordon, 1976). The former directly inhibit the prostaglandin forming cyclo-oxygenase (Vane, 1971) but the glucocorticoids appear to work by inhibiting the release by phospholipase A_2 of substrate fatty acids, since their effect is readily reversed by the addition of exogenous arachidonate (Di Rosa & Persico, 1979). We have recently demonstrated that this phospholipase inhibition is caused by polypeptide ('Macrocortin') selectively released from leucocytes by steroids (Carnuccio, Di Rosa & Persico, 1980; Blackwell, Carnuccio, Di Rosa, Flower, Parente & Persico, 1980). This release process requires a short induction period and may be inhibited by RNA/protein synthesis inhibitors. The same peptide seems to be responsible for the steroid-induced inhibition of thromboxane A_2 release from perfused lungs (Blackwell *et al.*, 1980) and a protein with similar properties has been detected by other workers in rabbit leucocytes (Hirata, Schiffman, Venkatasubramanian, Salomon & Axelrod, 1980).

We now report that the ability of hydrocortisone to block prostaglandin synthesis in leucocytes depends absolutely upon the intracellular macrocortin content of the target leucocytes and present some data on the temporal relationship between depletion and resynthesis of this peptide.

Present address: Dept. of Prostaglandin Research, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

Methods The basic experimental strategy was to measure hydrocortisone-induced inhibition of prostaglandin production in leucocytes before, and at various times after, the intracellular macrocortin stores had been depleted (by a prior incubation with hydrocortisone for 150 min). In separate experiments the macrocortin content of these cells was determined by measuring the arachidonate-reversible inhibition of prostaglandin synthesis in fresh leucocytes caused by cell extracts.

Resident peritoneal leucocytes (80% mononuclear cells) collected from Wistar rats were used throughout this study. The harvesting procedure has been described earlier (Carnuccio *et al.*, 1980).

Prostaglandin production was assessed by incubating cells at a density of 10^7/ml in Krebs bicarbonate solution enriched with bovine serum albumin (100 μg/ml) together with heat-killed bacteria (*B. pertussis*, 800–1000 organisms/cell) for 2 h at 37°C (Carnuccio *et al.*, 1980). Prostaglandins were assessed by bioassay on a superfused rat stomach strip, synthetic prostaglandin E_2 (PGE_2, Upjohn) being used as a reference standard. Hydrocortisone (sodium phosphate salt, final concentration 2 μM) or macrocortin containing extracts (see below) were added to the cells as required.

To estimate the intracellular macrocortin content we first washed the cells, disrupted them by repeated (× 4) freeze-thawing and then heated the extracts to 70°C for 5 min. This procedure does not inactivate macrocortin but precipitates many other proteins and also limits proteolysis. After heating, the extracts were centrifuged for 1 h at 100,000 *g* and the supernatants finally dialysed against 500 volumes of fresh Krebs bicarbonate solution to remove residual steroid and adjust the ionic balance. These solutions were then assessed for inhibitory activity as described.

When cells previously incubated with hydrocortisone to deplete macrocortin stores, were tested for their ability to generate prostaglandins in the presence of hydrocortisone, the polypeptide inhibitor was first removed by washing the cells (3 × 1 vol of fresh Krebs solution). This procedure fully reverses the effect of macrocortin.

0007-1188/81/10 0322-03 $01.00

Results When incubated with heat-killed bacteria alone, rat peritoneal leucocytes released 9.9 ± 0.6 ng ($n = 9$) PGE_2 equivalent /10^6 cells in 120 min. In the presence of 2 μM hydrocortisone this was reduced by 43.0 ± 3% ($n = 9$). Macrocortin extracts from an identical number of untreated cells reduced the prostaglandin production by 48.0 ± 5% ($n = 5$). Very little inhibitory activity was found in extracts from cells which had been previously incubated with hydrocortisone for 150 min (3.0 ± 1%; $n = 3$), showing that virtually all the macrocortin had been secreted by this time.

Figure 1 shows the temporal relationship between macrocortin content and sensitivity of the cells to the steroid. After 150 min with hydrocortisone, the cells had secreted virtually all their macrocortin, and when the inhibitor was removed by washing and the cells incubated again with heat killed bacteria, hydrocortisone was no longer able to inhibit prostaglandin generation. One hour later, there was a small increase in macrocortin content of the cells and they were slightly more sensitive to the steroid. After a further 60 min had elapsed the intracellular levels had again reached control values and the cells were fully sensitive to hydrocortisone (41 ± 6%; $n = 8$: inhibition not significantly different from control).

To eliminate the possibility that the cells varied in their sensitivity to macrocortin, preparations of the inhibitor from non-depleted cells were tested on the cells at all time points. At 150 min, when the cells no longer responded to hydrocortisone (< 5% inhibition, see Figure 1) macrocortin containing cell extracts were fully active (≃ 56% inhibition) as inhibitors of prostaglandin synthesis.

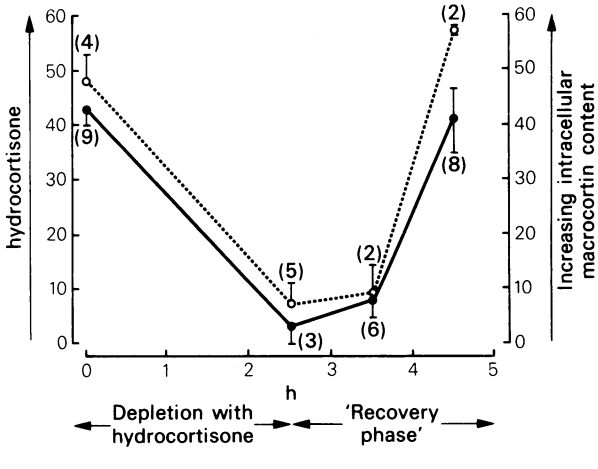

Figure 1 The temporal relationship between intracellular macrocortin content (○), estimated by the inhibitory action of cell extracts on prostaglandin synthesis, and sensitivity of leucocyte prostaglandin production to hydrocortisone 2 μM (●). Each point is the x̄ of n experiments; vertical lines show s.e.mean. After depletion of macrocortin from the cells by 150 min incubation with steroid, they were washed and their sensitivity to hydrocortisone inhibition of prostaglandin synthesis and intracellular macrocortin content assessed at different times as described in the text. Ordinates represent % inhibition of prostaglandin synthesis.

Discussion In previous studies we, and other workers, have demonstrated that the acute inhibitory effect exerted by steroids on prostaglandin production was not mediated by an action on the cyclo-oxygenase but by an action on the enzymes which supply the substrate arachidonate (Gryglewski, Panczenco, Korbut, Grodzinska & Ocetkiewicz, 1975; Hong & Levine, 1976; Nijkamp, Flower, Moncada & Vane, 1976; Danon & Assouline, 1978; Blackwell, Flower, Nijkamp & Vane, 1978; Di Rosa & Persico, 1979). This effect was not a direct action of the steroids, but was mediated by a 'second messenger' polypeptide released from the cells by the hormone: an action which required a short induction time and undisturbed protein/RNA synthesis for its manifestation (Flower & Blackwell, 1979; Carnuccio *et al.*, 1980; Blackwell *et al.*, 1980).

In this paper we show that the sensitivity of the rat peritoneal leucocytes depends absolutely upon their intracellular macrocortin content and that when intracellular stores are depleted the cells are almost refractory to the steroid. The observed lack of effect of steroids following the macrocortin depletion cannot be due to changes in sensitivity of the cells to the peptide since, even when hydrocortisone is ineffective macrocortin continues to be an effective inhibitor of prostaglandin generation. *In vitro*, under the conditions described in this paper, depletion takes approximately 150 min. In experiments described by us in a previous publication, the intracellular content of macrocortin *in vivo* was depleted within 1 h of the injection of 1 mg/kg dexamethasone into rats (Blackwell *et al.*, 1980). Similarly, the re-synthesis of macrocortin *in vivo* was complete after 2 h, but *in vitro* the re-synthesis takes approximately 4.5 h. We cannot say for certain whether the re-synthesis is a steroid stimulated event or whether there is an internal feedback system which initiates re-synthesis of the peptide when intracellular levels fall below a certain point.

We have pointed out (Blackwell *et al.*, 1980) that by preventing the action of the phospholipase A_2, steroids not only reduce the generation by leucocytes of prostaglandins but of other pro-inflammatory lipids such as the leucotrienes and other hydroxy-acids and that this action may well contribute to the enigmatic anti-inflammatory action of these potent drugs. The finding that this anti-phospholipase action of steroids is absolutely dependent upon intracellular

macrocortin has some relevance to the understanding of the mechanism of action and suggests that spontaneously arising (or drug-induced) defects in the release mechanism could confer a resistance to the therapeutic actions of steroids.

References

BLACKWELL, G.J., CARNUCCIO, R., DI ROSA, M., FLOWER, R.J., PARENTE, L. & PERSICO, P. (1980). Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature*, **287**, 147–149.

BLACKWELL, G.J., FLOWER, R.J., NIJKAMP, F.P. & VANE, J.R. (1978). Phospholipase A_2 activity of guinea pig isolated perfused lungs: stimulation and inhibition by anti-inflammatory steroids. *Br. J. Pharmac.*, **62**, 79–89.

BRAY, M.A. & GORDON, D. (1976). Effect of anti-inflammatory drugs on macrophage prostaglandin synthesis. *Br. J. Pharmac.*, **57**, 466P.

CARNUCCIO, R., DI ROSA, M. & PERSICO, P. (1980). Hydrocortisone induced inhibitor of prostaglandin biosynthesis in rat leucocytes. *Br. J. Pharmac.*, **68**, 14–16.

DANON, A. & ASSOULINE, G. (1978). Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. *Nature*, **273**, 552–554.

DI ROSA, M. & PERSICO, P. (1979). Mechanism of inhibition of prostaglandin biosynthesis by hydrocortisone in rat leucocytes. *Br. J. Pharmac.*, **66**, 161–163.

FLOWER, R.J. & BLACKWELL, G.J. (1979). Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. *Nature*, **278**, 456–459.

GRYGLEWSKI, R.J., PANCZENKO, B., KORBUT, R., GRODZINSKA, L. & OCETKIEWICZ, A. (1975). Corticosteroids inhibit prostaglandin release from perfused mesenteric blood vessels of rabbit and from perfused lungs of sensitized guinea-pigs. *Prostaglandins*, **10**, 343–355.

HIRATA, F., SCHIFFMAN, E., VENKATASUBRAMANIAN, K., SALOMON, D. & AXELROD, J. (1980). A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. natn. Acad. Sci. U.S.A.*, **77**, 2533–2536.

HONG, S-C.L. & LEVINE, L. (1976). Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory steroids. *Proc. natn. Acad. Sci. U.S.A.*, **73**, 1720–1734.

NIJKAMP, F.P., FLOWER, R.J., MONCADA, S. & VANE, J.R. (1976). Partial purification of rabbit aorta contracting substance – releasing factor and inhibition of its activity by anti-inflammatory steroids. *Nature, Lond.*, **263**, 479–482.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as mechanism of action for aspirin-like drugs. *Nature, New Biol.*, **231**, 232–235.

(*Received July 14, 1981.*)