Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1986 Pages 160-165

GLUCOCORTICOIDS AND THE ISOLATED RAT HEPATOCYTE

C.J. Lovell-Smith and P. Garcia-Webb

Department of Clinical Biochemistry
Queen Elizabeth II Medical Centre
Nedlands 6009, Perth
Western Australia, Australia

Received January 9, 1986

Following incubation at 37°C with tritiated glucocorticoids isolated hepatocytes prepared from non-adrenalectomized rats show rapid uptake of label. Uptake is non-saturable, and non-linear over the first 60 sec of exposure to steroids. HPLC separation of aqueous extracts of cells and incubation medium shows that polar metabolites of the natural steroid, corticosterone, appear within 10 sec, whereas the synthetic glucocorticoid, dexamethasone, is not altered. Our results suggest that diffusion is the most important process by which glucocorticoids enter liver cells, and that the predominant fate of corticosterone is rapid metabolism.

It is believed that glucocorticoids, in common with other steroid hormones, exert their effects via interaction with nuclear DNA. These intranuclear events have been widely studied (1,2). However, the passage into the cell and the subsequent metabolism of glucocorticoids is less clearly understood.

It is generally accepted that steroids pass into the cell by diffusion and, after binding to specific cytoplasmic receptors, migrate to the nucleus. Both aspects of this view have been challenged. A number of authors have suggested that the uptake of tritiated glucocorticoids by rat liver cells involves a carrier-mediated, energy-dependent process (3-6). Following the intraperitoneal injection of tritiated cortisol into adrenalectomized rats, much of the labelled material associated with liver cytoplasmic macromolecules has been shown to be metabolites of cortisol (7,8).

We have developed a new technique that allows the separation of intact corticosterone from its more polar metabolites following the brief incubation of isolated hepatocytes with labelled steroid. Taking advantage of this technique, we have examined the early passage into and metabolism of glucocorticoids by isolated hepatocytes prepared from non-adrenalectomized rats.

0006-291X/86 $1.50

MATERIALS AND METHODS

[1,2,6,7-^{3}H] corticosterone and [1,2,4,6,7-^{3}H] dexamethasone were obtained from Amersham Australia Pty. Ltd; the specific activities were 3.48 and 3.85 TBq/mmol, respectively. Aliquots of the steroid solutions were dissolved in 5 µL of ethylene glycol, which was taken up in protein-free Krebs-Ringer bicarbonate buffer (pH 7.4) containing 25 mM Hepes (KRH buffer).

Corticosterone and dexamethasone were purchased from Sigma Chemical Co., USA.

Hepatocytes were isolated from normal male Wistar-Furth rats, 160-250 g, fed ad libitum, as described previously (9,10). After washing, cells were suspended in KRH Buffer containing fatty-acid and globulin-free bovine serum albumin (30 g/L). Suspensions contained 4-10x10^6 cells/ml. Viability, assessed by trypan-blue exclusion, was routinely 95% or better.

UPTAKE OF GLUCOCORTICOIDS: Aliquots (100 µL) of the cell suspension were added to tubes containing 61 pmol of tritiated steroid (3 x 10^5 dpm) in KRH buffer. The final incubation volume was 1.2 ml. After incubation at 37°C, 0.9 ml of the incubation mixture was rapidly filtered on a Millipore 1225 sampling manifold (Millipore Corporation, USA), using Whatman GF/C glass-fibre filter discs, which were then washed twice with 5 ml of ice-cold KRH buffer (6). Experiments with U-[^{14}C]-sucrose showed no detectable trapping of extracellular water on the filter discs.

Filter discs were placed in 7 ml of scintillation fluid for counting. The fluid contained 2 g PPO and 0.5 g POPOP per litre of toluene:Triton-X100 (2:1, v/v).

HPLC OF CELL EXTRACTS AND INCUBATION MEDIUM: Aliquots of cells were incubated and separated as above, except that the amount of radioactive steroid was increased to 2 x 10^6 dpm. The filters, with the trapped cells, were placed in 2 ml of ice-cold KRH buffer, and sonicated for 15 sec (Branson Sonifier Cell Disrupter B15, Branson Sonic Power, USA). After centrifugation, the supernatant was removed and the filters washed twice more. The cell-free filtrates were also collected and placed on ice. Both filtrates and pooled cell-extracts were then prepared for HPLC by Sep-pak separation, using C18 cartridges (Waters Associates, USA). Steroids were eluted in 2 ml of methanol, which was dried under nitrogen and the residue dissolved in water. The methanol fraction contained 80-90% of the initial radioactivity.

Samples (45 µL) were injected onto a reverse-phase column (10 µm, RP-18; Hewlett-Packard). The column was eluted with 45% methanol in water at 1.5 ml/min. Radioactivity in the fractions (0.75 ml) was assessed by liquid scintillation counting.

RESULTS

Tritiated corticosterone and dexamethasone were rapidly taken up during incubation with isolated rat hepatocytes. In the first 60 sec the rate of uptake of corticosterone appeared greater than that of dexamethasone (Fig. 1). Cellular uptake of both tritiated steroids was non-linear from the earliest measurable time point (5 sec) in repeated experiments.

The uptake of either steroid by rat hepatocytes could not be saturated by increasing the concentration of steroid in the incubation buffer (Table 1). The uptake of tritiated corticosterone was unaffected by incubation in the presence of dexamethasone up to a final concentration of 50 µmol/L. The converse was also true (Table 2).

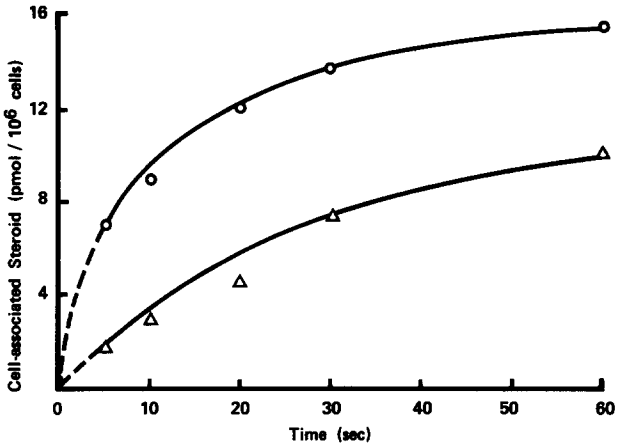

Fig. 1 Isolated hepatocytes were added to tritiated steroid and separated by rapid filtration as described in "Methods". Points shown are the mean of duplicate tubes. A typical experiment is represented.

o-o Cell-associated ^{3}H-corticosterone; pmol/10^6 cells
Δ-Δ Cell-associated ^{3}H-dexamethasone; pmol/10^6 cells

Steroids were extracted from either hepatocytes or the incubation medium after incubation at 37°C. Polar metabolites were separated from less polar unchanged steroids by reverse phase HPLC. Tritiated metabolites of corticosterone were detected in hepatocyte extracts after only 10 sec incubation, and in extracts of the incubation medium after one minute (Fig. 2A). The rate of metabolism was extremely rapid; after 10 min exposure of cells to tritiated corticosterone, the metabolite peak extracted from the incubation medium contained over 25% of the total tritiated compounds recovered by Sep-pak separation (Fig. 2B). Dexamethasone was not metabolised in similar experiments. There was negligible metabolism of corticosterone following incubation with KRH buffer alone, or with buffer that had previously been exposed to hepatocytes.

Table 1

Steroid Concentration (nM)	Uptake of Steroid at 15 sec (pmol/10^6 cells)	
	Corticosterone (mean ± SD; n = 4)	Dexamethasone (mean ± SD; n = 3)
1.3	0.15 ± 0.02	0.05 ± 0.006
51	4.9 ± 0.4	2.2 ± 0.3
101	9.7 ± 0.4	4.1 ± 0.7
1001	69.2 ± 10.6	36.5 ± 5.6

Table 2

A. Dexamethasone Concentration (nM)	Corticosterone Uptake at 15 sec (pmol/10^6 cells)
0	0.15
1	0.16
50	0.16
1000	0.16
50,000	0.13

B. Corticosterone Concentration (nM)	Dexamethasone Uptake at 15 sec (pmol/10^6 cells)
0	0.047
1	0.061
50	0.056
1000	0.049
50,000	0.048

Results shown are mean values of duplicate tubes. A typical experiment is presented.

DISCUSSION

Our results show for the first time that intracellular metabolites of the endogenous rat glucocorticoid, corticosterone, can be detected after only 10 sec exposure of the rat hepatocyte to the steroid. This early metabolism by the liver has not been reported previously. Experiments with adrenalectomized rats have demonstrated protein-bound

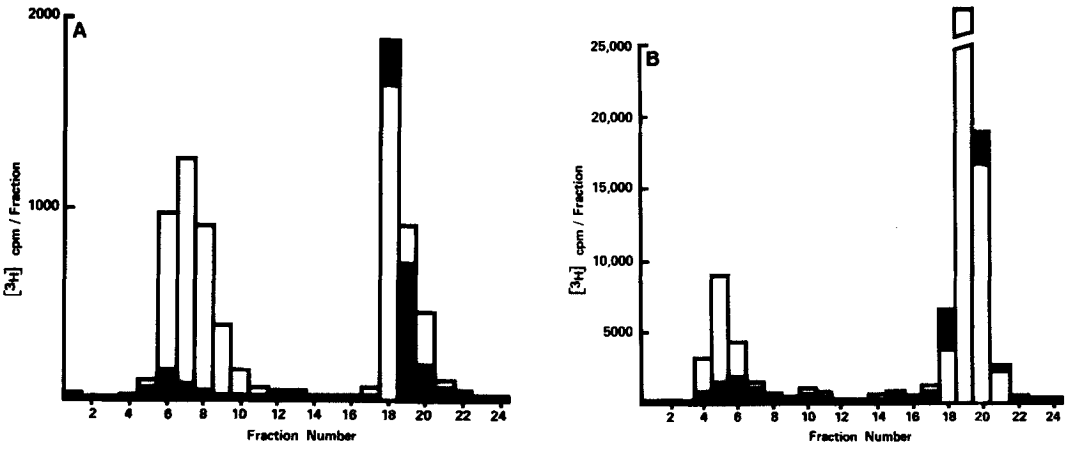

Fig. 2A HPLC chromatogram of water-soluble extracts from filtered cells, 10 sec (■) and 10 min (□) after exposure to ^{3}H-corticosterone.

Fig. 2B HPLC chromatogram of tritiated compounds found in the incubation medium, 1 min (■) and 10 min (□) after cells were exposed to ^{3}H-corticosterone. Corticosterone elutes in fractions 18-22; fraction 19 contained 31,000 cpm (1 min) and 46,700 cpm (10 min).

metabolites in extracts of rat liver obtained 5 min after an intraperitoneal injection of tritiated cortisol (7,8). Similar experiments with tritiated corticosterone have demonstrated both protein-bound and non-bound metabolites after 10 min (11). Our experiments using isolated hepatocytes and high pressure liquid chromatography make it possible to look for the presence of corticosterone metabolites at a much earlier stage. Adaptations of the technique currently under study should enable specific metabolites to be identified.

An early linear phase together with saturability are two of the features expected of a carrier-mediated cell uptake system. A linear phase of steroid uptake in the first minute has been claimed by a number of workers (3,5,6). However, on inspection, the data presented by these workers appears to demonstrate curvilinear uptake. We found no evidence of a linear phase of uptake between 5 and 60 sec. There was no evidence of saturability since the uptake of either steroid increased as the concentration increased. Furthermore the uptake of one steroid by the isolated rat hepatocyte was not affected by the presence of a large concentration of the second steroid.

The lack of linearity during uptake, the absence of saturation and the lack of competition between corticosterone and dexamethasone all strongly suggest that most glucocorticoid influx into the hepatocyte occurs by passive diffusion down a concentration gradient. It is possible that the different uptake rates we observed for corticosterone and dexamethasone reflect differences in the steroid permeability coefficients (12).

Conventional thought is that unmetabolised steroids plus receptor protein bind to DNA to produce intranuclear events. The data presented here suggest that corticosterone is metabolised very soon after entering the hepatocyte. A protective cellular system would be required to ensure that some intact corticosterone is able to bind to cytoplasmic receptors. Such a system might simply involve differences in corticosterone binding affinities of the receptor and metabolising enzymes. Alternatively, given that cortisol metabolites have been shown to be associated with macromolecules, it is possible that metabolites of steroids may act as effectors in rat liver cells (7,13). Thus, the mechanism by which glucocorticoids migrate to the nucleus requires further investigation.

From the results reported here dexamethasone enters the cell more slowly than corticosterone. Moreover, dexamethasone is not metabolised at a rate at all comparable

to that of corticosterone. The availability of intact dexamethasone for binding to intracellular receptors may well be greater than that for the more rapidly metabolised corticosterone. This fact needs to be taken into account when conclusions concerning migration of dexamethasone to the nucleus are applied to the migration of corticosterone.

REFERENCES

1. Rousseau, G.G. (1984) Biochem. J. 224, 1-12.
2. Cake, M.H. and Litwack, G. (1975) Biochemical action of hormones. Litwack, G. (ed), vol. 3, pp. 317-390, Academic Press, New York.
3. Jonkers, G.H., Timmermans, H.A.T., de Jong, F.H., Lamberts, S.W.J., and Birkenhager, J.C. (1980) FEBS Letts. 111, 403-406.
4. Suyemitsu, T., and Terayama, H. (1975). Endocrinology 96, 1499-1508.
5. Rao, M.L., Rao, G.S., Eckel, J., and Breuer, H. (1977) Biochim. Biophys. Acta 500, 322-332.
6. Rao, M.L., Rao, G.S., Holler, M., Breuer, H., Schattenberg, P.J., and Stein, W.D. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 573-584.
7. Voight, J., Grote, H., and Sekeris, C.E. (1981) Biochim. Biophys. Acta 674, 306-318.
8. Grote, H., Voight, J., and Sekeris, C.E. (1983) Biochem. J. 212, 305-312.
9. Bonser, A.M., Garcia-Webb, P., and Bhagat, C.I. (1983) Biochim. Biophys. Acta 762, 390-397.
10. Bonser, A.M., and Garcia-Webb, P. (1984) Biochem. Biophys. Res. Communs. 119, 706-712.
11. Carlstedt-Duke, J., Gustafsson, J.-A., Gustafsson, S.A., and Wrange, O. (1977) Eur. J. Biochem. 73, 231-238.
12. Giorgi, E.P., and Stein, W.D. (1981) Endocrinology 108, 688-697.
13. Voight, J., and Sekeris, C.E. (1980) Biochim. Biophys. Acta 633, 422-435.