THE ACTIVE TRANSPORT OF CORTISOL BY MOUSE FIBROBLASTS GROWING IN VITRO

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The rate of growth in vitro of both mouse lymphoma cells ML-388 (Gabourel and Aronow, 1962) and mouse fibroblasts L-929 (Pratt and Aronow, 1966; Berliner and Ruhmann, 1967) is inhibited by low concentrations of glucocorticoids. In order to further elucidate the mechanism of this growth inhibition, a study of the uptake and distribution of ^{3}H-cortisol in the mouse fibroblast, growing in vitro, was initiated as part of an effort to detect intracellular binding components for cortisol. Initial experiments demonstrated that cortisol, when added at a low concentration, did not readily enter the fibroblasts. Further experiments have shown that incubation of L-cells under any of the following conditions favors an increase in the amount of cortisol associated with the cells: (1) incubation at 0^{o}; (2) the presence of sulfhydryl-reacting reagents; (3) incubation under certain conditions of energy deprivation; (4) the presence of high concentrations of the steroid. These observations are consistent with the hypothesis that there exists in mouse fibroblast cells growing in vitro an outward active transport process which serves to keep the intracellular cortisol concentration very low.

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METHODS

Mouse fibroblasts, L strain, were grown as monolayer cultures in Roux bottles containing 70 ml of Joklik medium supplemented with 10% bovine serum in an atmosphere of 5% carbon dioxide in air as described previously (Pratt and Aronow, 1966). Cells were harvested intact in fresh growth medium, collected by centrifugation at 600 g, and resuspended in fresh culture medium at a cell density approximating 10^6 cells per ml. ^{14}C-Inulin (New England Nuclear) 3.54 mc/g, was added to the cell suspension at a final concentration of 10 μg/ml. Replicate aliquots of the cell suspension were distributed into stoppered flasks with an atmosphere of 5% CO_2 in air. ^{3}H-Cortisol, 33.8 c/mM (Nuclear Chicago), was added to some cultures to give a final concentration of 3.3×10^{-8} M, while others received the same amount of non-radioactive cortisol and ^{3}H-water, 1.1 μc/ml (New England Nuclear). Cells were incubated with shaking on a rotary shaker either in an ice bath or at 37°. At appropriate intervals, aliquots were removed and the cells were centrifuged at 500 g for 5 minutes. The supernatant solution was carefully removed with a fine-tipped pipette and set aside. The cell pellet was suspended directly in 5 ml of distilled water without any washing and the resulting suspension was sonicated for 10 seconds with a Bronwill Biosonik III at a setting of 30, such that there was complete cell disruption. Duplicate 1 ml samples were added to 10 ml of scintillation solution prepared according to the method of Bray (1960). Protein assays were performed on duplicate 1 ml samples of the sonicated cell suspension after the method of Oyama and Eagle (1956). 0.05 ml aliquots of the incubation medium (i.e., the supernatant retained after centrifugation at 500 g) were added to 10 ml of scintillator fluid, as were samples of the radioactive labeled cortisol, inulin and water.

All radioactive assays were carried out in a Packard Tri-Carb liquid scintillation spectrometer, Model 8810, with the channels set in such a way that one

channel counted ^{14}C exclusively. The lower energy channel, that used for assaying tritium, also detected carbon^{14}, and the ratio of ^{14}C counts appearing in the two channels was determined in each experiment using the ^{14}C-inulin sample only. These determinations permitted a correction of the apparent tritium count in the lower energy channel such that the absolute amount of ^{3}H-cortisol present in the sample could be calculated. The ^{14}C-inulin count in the cell pellet was multiplied by the ratio of cortisol tritium counts over the carbon^{14} inulin counts in the incubation medium to determine the amount of extracellular ^{3}H-cortisol present in the resuspended cell pellet. Thus, the ^{3}H-cortisol contributed by medium present in the cell pellet was subtracted from the total tritium counts to yield the amount of ^{3}H-cortisol associated with the cells themselves. Similar corrections were applied to the ^{3}H-water counts, thereby allowing a calculation of the intracellular water space. The actual ^{3}H-cortisol counts associated with the cells were divided by the ^{3}H-cortisol count per ml of medium, yielding an apparent volume of distribution for the cell-associated ^{3}H-cortisol. The results are expressed as apparent volumes of distribution per microgram of cellular protein nitrogen.

RESULTS

The apparent volume of distribution of cortisol as a function of time of incubation at 0^{o} or 37^{o} is presented in Figure 1. This represents a typical result of several such experiments. On incubation at 37^{o}, the apparent volume of distribution of cortisol is essentially constant over the range of time studied. However, when the cell suspensions are incubated with ^{3}H-cortisol in an ice bath, the apparent volume of distribution increases with time. In most experiments the increase in the amount of cortisol associated with the cells is greatest within the first hour. The cells remain intact during incubation in ice. Figure 1 also demonstrates that the increased apparent volume of distribution of cortisol seen on incubation in the cold is markedly

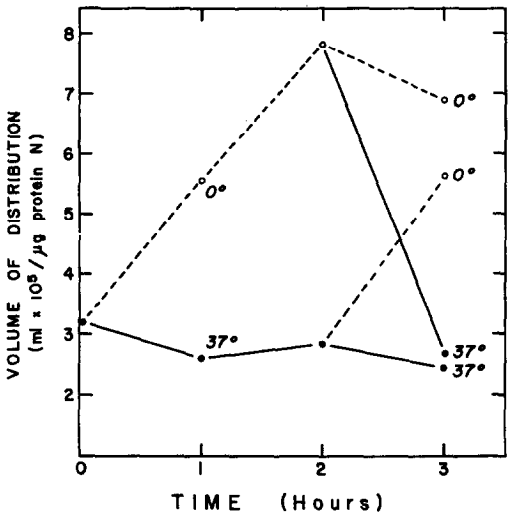

FIGURE 1. The effect of temperature on the apparent volume of distribution of ^{3}H-cortisol in L-cells. A suspension of L-cells was prepared and ^{14}C-inulin and ^{3}H-cortisol (final concentration 3.3 x 10^{-8} M) were added as described in the Methods. Replicate cultures were dispensed, one half being incubated at 37° and one half at 0°. After 2 hours of incubation, duplicate cultures were taken from the ice bath and placed at 37°, and duplicate 37° incubations were placed in an ice bath. The apparent volume of distribution of cortisol was determined on duplicate samples after the intervals indicated in the figure.

reduced by continuing the incubation at 37°. Apparently all of the cortisol which was allowed into the cell on incubation in the cold is removed upon raising the incubating temperature to 37°. Duplicate experiments carried out with tritiated water instead of tritiated cortisol demonstrated that the water space of the cells is not affected by change in temperature or the length of the incubation.

The data presented in Table I demonstrate that N-ethylmaleimide (a sulfhydryl-reacting reagent), high concentrations of cortisol or deprivation of cellular energy sources all yield increases in the apparent volume of distribution of cortisol after a one-hour incubation at 37°. In the experiment presented in Table I, cells were preincubated in the absence of glucose for 24 hours and the culture was then divided into two parts. Glucose was added to one series of replicate subcultures, and the other

TABLE I

Influence of High Cortisol Concentrations, N-Ethylmaleimide, Dinitrophenol and Cyanide on the Apparent Volume of Distribution of ^{3}H-Cortisol

Addition	Concentration	Apparent Volume of Distribution	
		Minus glucose	Plus glucose
	(M)	(ml x $10^{5}/\mu g$ protein-N)	
None		8.1	6.7
Cortisol (non-radioactive)	3.3×10^{-4}	13.3	12.9
N-ethylmaleimide	3.3×10^{-4}	15.5	15.8
Dinitrophenol	1.0×10^{-3}	13.4	9.3
Sodium cyanide	1.0×10^{-3}	15.6	7.9

L-cell monolayer cultures were prepared as described under Methods and grown for 24 hours before harvesting in Joklik medium without glucose. The cells were harvested, suspended in Earle's balanced salt solution without glucose and ^{14}C-inulin and ^{3}H-cortisol (3.3×10^{-8} M) were added. Replicate cell suspensions were then incubated at 37° with the appropriate additions, as presented in the table, for a total of 1 hour. Glucose (1 mg/ml) was added to half the cell suspensions after 1/2 hour of incubation. After 1 hour, the incubations were terminated and the apparent volume of distribution of ^{3}H-cortisol was determined as described in the Methods.

half of the same series remained glucose-free. The apparent volume of distribution of ^{3}H-cortisol in those cultures containing low levels of steroid in the absence of glucose is somewhat greater than in replicate cultures to which glucose has been added. Incubating the glucose-starved cells with 1 mM sodium cyanide or dinitrophenol produces a large increase in the volume of distribution of cortisol which can be partially overcome by glucose addition. The effects of N-ethylmaleimide or high concentrations of cortisol are not reversed by glucose addition. The effect of 0.33 mM cortisol could be interpreted as saturation of an exclusion process by high concentrations of steroid, thereby allowing large concentrations of hormone to remain in the cell.

DISCUSSION

The results reported in this work demonstrate that at low concentrations cortisol is excluded from fibroblast cells cultured at 37° and that the exclusion mechanism can be partially or completely overcome by incubation at low temperature, by the addition of sulfhydryl group reacting reagents, and after inhibition of the cells' energy supply. The effect of low temperature incubation (Fig. 1) was obtained in an experiment which included 10% beef serum in the suspension medium; however, identical experiments employing serum-free suspension medium (as in Table I) demonstrate qualitatively the same results. Therefore alterations in binding to components of the serum in the medium, such as transcortin, cannot be responsible for this effect. However, the level of these binding factors in the final suspension medium apparently is important in determining the absolute magnitude of the baseline apparent volume of distribution of cortisol on incubation at 37°.

The fact that at 0° the cells bind more cortisol than at 37° might be explained if there are cellular binding sites with negative temperature coefficients, as has been described for plasma proteins which bind cortisol (Westphal, 1967; Goldie, Hasham, Keane and Walker, 1968). However, our results with high external concentrations of cortisol and the effects obtained with metabolic inhibitors are not consonant with this hypothesis, but rather suggest that we are investigating an active process which serves to exclude cortisol from the cells.

The mechanism of exclusion of cortisol from L-cells is unknown. The obvious possibility of a permeability barrier is incompatible with the rapid efflux of cortisol when the temperature of the incubation is raised from that of the ice bath to 37°, or with the extrusion of cortisol which occurs when cells deprived of their energy supplies are given glucose. Thus, an energy-dependent, temperature-sensitive transport process seems to be the most logical explanation for the effects reported in this paper.

If this process does actively exclude cortisol from fibroblast cells, the possible ramifications are many. First, the authors know of no previous report of an active transport process for steroid hormones. Second, the presence or absence of such a process in fibroblasts might determine the level at which inhibition of growth by circulating cortisol occurs. Third, the affinity of diverse steroids for this process may be an important determinant of their potency as anti-inflammatory agents. Fourth, this transport process might be the mechanism of glucocorticoid secretion by certain cells, for example the adrenal cortex.

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