GLUCOCORTICOID TIGHT BINDING IN RAT LIVER AND THYMUS PARTICLES

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Summary: Glucocorticoid binding to certain cell particles of rat liver and thymus following treatments in vivo and in vitro consists in part of a very "tight binding" that resists hot and cold perchloric acid extractions. This binding is found in thymus nuclei and in liver cytoplasmic particles, but not in liver nuclei nor in thymus mitochondria or microsomes. The existence of "tight binding" coincides with the ability of the same particles to bind free corticoid directly in incubations in vitro. The difference in the cellular location of this binding suggests that different methods of glucocorticoid activation exist in the anabolic target tissue, liver, and the catabolic target tissue, rat thymus.

Glucocorticoids, like other steroids, are assumed to act on target tissues by first binding to a cytosol receptor protein forming a corticoid-receptor complex which when activated binds in turn to cell nuclei (1-4). The steroid-receptor complexes are reportedly very labile and mild conditions are routinely used to prevent dissociation of the complex during experimental procedures (5). It was surprising, therefore, to observe that following injections of rats with labelled glucocorticoid that in some cases the label remained in association with protein pellets of liver and thymus cell particulates even after hot and cold perchloric acid (PCA) extractions. It was decided to investigate this phenomenon in further detail.

METHODS

Male Holtzman rats were injected i.p. with 1 μC of [^{3}H]9-a-fluoroprednisolone (9FP) +4 mg unlabelled 9FP carrier per animal. Liver and thymus were removed at various times after treatment and separated into cell fractions as previously described (6). Microsomes were purified by recentrifugation through 1.1 M sucrose-TKM buffer (50 mM Tris buffer, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5). Mitochondria were purified in a discontinuous 25-55% sucrose-TKM buffer gradient. Liver nuclei were purified by the method of

Grunicke, et al (7), and thymus nuclei by the method of Blöbel and Potter (8). The 9FP, a highly activated synthetic glucocorticoid, was provided by the Cancer Chemotherapy National Service Center of the National Cancer Institute, tritiated by New England Nuclear Co., and repurified in the laboratory by silica gel thin layer chromatography (chloroform/ethanol, 9:1), spec. act. 10 mC/μM.

RESULTS AND DISCUSSION

In the course of certain experiments with [^{3}H]9FP treatments *in vivo*, it was found that following purification of nuclei and mitochondria, most of the label was extracted in the first cold 10% cold PCA extraction, with a gradual reduction occurring during subsequent PCA extractions. The remaining protein pellets of liver nuclei and thymus mitochondria contained no radioactivity. However, the protein pellets of thymus nuclei and liver mitochondria were highly labelled, and these had many times more label than was previously extracted by hot PCA. The large number of samples (over 24 accumulated coincidental to studies of DNA changes in these fractions) and the pattern of extraction of label indicated that protein labelling where it was found was not an artifact of contamination.

The PCA-resistant binding in all the cell particulates of rat liver and thymus following treatments with [^{3}H]9FP *in vivo* at 30, 60 and 90 min was investigated and is shown in Fig. 1. The radioactivity remained with protein pellets of thymus nuclei, but not of liver nuclei, and with all the liver cytoplasmic particles, but not with the corresponding thymus particles. The similarity of results with purified liver mitochondria and microsomes and washed lysosomes indicated that incomplete separation of liver cytoplasmic particulates was not a relevant factor. While the specific activity of the protein pellet was low in some cases, the actual cpm recovered was not insignificant and was consistent in the cell fractions in which it was observed.

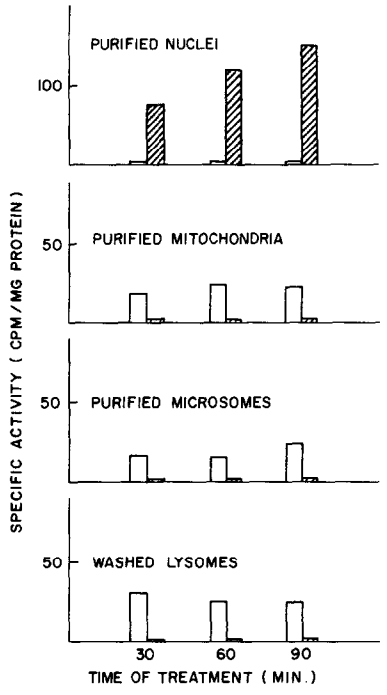

Fig. 1. Tight binding in vivo of [^{3}H] FP to rat liver and thymus particles. Treatment of animals (groups of 3) and purification of cell fractions is described in Methods. Purified cell particles were suspended in 0.125 M sucrose-TKM buffer. Aliquots were precipitated in cold 10% PCA. The precipitate was washed x 2 with cold 10% PCA, then extracted with 10% PCA at 75° for 15 min. The remaining pellet was washed with cold PCA then suspended in 5 mM Tris buffer, final pH 6.5. Samples in duplicate were counted in 10 ml Aquasol in a Beckman scintillation counter.

Liver fractions, blank bars
Thymus fractions, dashed line bars

Most of the labelled corticoid could be extracted from protein pellets with ethyl acetate and the radioactivity recovered as 9FP by silica gel thin layer chromatography (chloroform/ethanol, 9:1). While quantitative estimations of recovery were not made, it appeared that most of the corticoid had not been metabolized and the PCA-resistant binding was non-covalent. The precise nature of this binding was not further analyzed and is referred to as "tight binding."

Experiments in vitro in which [^{3}H]9FP was incubated directly with purified cell fractions resulted in somewhat higher labelling in all fractions, but generally confirmed results of experiments in vivo, that is that "tight

Table 1. Tight binding *in vitro* of free glucocorticoid to rat liver microsomes.

Purified microsomes in 0.33 M sucrose-TKM buffer were incubated with [^{3}H] 9FP ($2x10^5$ cpm/sample) for 30 min at 22°. In some cases microsomes were preincubated for 15 min at 22° with 10^{-3}M pCMS (p-chloromercuriphenylsulfonic acid) or with unlabelled 9FP (100-fold excess). All samples were in duplicate.

A. Microsomes were diluted in 0.25 M sucrose following incubations, centrifuged, then repurified through 1.1. M sucrose-TKM buffer to remove unbound radioactivity.

B. Aliquots were taken directly after incubations for precipitation and extractions with hot and cold 10% PCA as described in Fig. 1.

	Bound [^{3}H]9FP (cpm)		
		Additions	
Methods	None	pCMS	unlabelled 9FP
A) Repurified microsomes	21,000	9000 (-59%)*	7800 (-62%)
B) PCA precip. protein	5,000	3400 (-30%)	4900

*Change from controls where significant.

binding" to protein pellets was found in thymus nuclei and liver cytoplasmic particles, but not significantly to liver nuclei nor thymus cytoplasmic particles. The tight binding in the protein pellets represented only a small part of the total binding present in the intact cell particulates prior to the PCA extractions. This varied from 10-25% in thymus nuclei (4 experiments), 20-40% in liver mitochondria (4 experiments), and 20-30% in liver microsomes (3 experiments).

One such experiment on binding of free [^{3}H]9FP *in vitro* to liver microsomes is shown in Table 1. The "tight binding" was about 25% of the total binding in the intact repurified microsomes. Additions of unlabelled 9FP to incubation mixtures blocked about 60% of the binding of [^{3}H]9FP to whole

microsomes, but the binding to the PCA-precipitable protein was not affected. Apparently specific corticoid binding (as measured by the extent of inhibition of binding in the presence of excess unlabelled corticoid) is limited to a weaker type of binding that is removed in the PCA extractions. Similar results were obtained in binding in vitro of [^{3}H]9FP to thymus nuclei and liver mitochondria. Binding to both whole cell particles and to the PCA-extracted pellets was blocked in the presence of mercurial agents, again indicating that true binding is involved and that observed results are not due to contamination.

It is difficult to evaluate the importance of this kind of "tight binding" especially since it is apparently non-specific. It is interesting that it exists at all in view of the reported lability of the steroid-receptor complexes in other tissues (5). It is also interesting that where it occurs - in thymus nuclei and in liver cytoplasmic particles - corresponds to the ability of these particles to bind free glucocorticoids directly in vitro without prior formation of a corticoid-receptor complex in cytosol fractions. Binding of free corticoid to liver mitochondria in vitro that we observed has been reported previously by other investigators (9,10). Bottoms recently reported (11) that free corticoid added in vitro to pig thymus nuclei decreased RNA polymerase activity while the enzyme activity in liver nuclei was unaffected by similar treatment. These results are consistent with our observations that free corticoid binds directly to thymus but not to liver nuclei in vitro. Binding of free corticoid to liver microsomes which has not previously been studied is included in this report, and we have reported elsewhere in more detail (12,13) on binding of free corticoid to all of these cell particulates.

While there is no necessary connection between these two sets of data - the existence of the PCA resistant binding and the ability of these same particles to bind free corticoids in vitro - they suggest that there is something unusual about the nature of binding in these particles. The difference

in cellular location of this binding, nuclear in one case, cytoplasmic in the other case, may mean that different methods of glucocorticoid activation exist in the different cell types. Such differences might in turn be related to the opposite biological effects of corticoid treatments - anabolic in liver and catabolic in thymus.

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