Biochimica et Biophysica Acta, 731 (1983) 415–420
Elsevier

BBA 71685

EVIDENCE FOR CARRIER-MEDIATED TRANSPORT OF GLUCOCORTICOIDS BY HUMAN PLACENTAL MEMBRANE VESICLES

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(Received November 22nd, 1982)

Key words: Reproduction; Hormone action; Steroid transport; Glucocorticoid; (Human placenta)

Glucocorticoid uptake by isolated placental membrane vesicles has been studied in an attempt to identify a membrane-mediated carrier mechanism. A preliminary communication from this laboratory has reported that uptake of the glucocorticoid corticosterone by these vesicles was a time-dependent, saturable, osmotically sensitive process (Fant, M.E., Harbison, R.D. and Harrison, R.W. (1979) J. Biol. Chem. 254, 6218–6221), but did not conclusively demonstrate a carrier mechanism. Further studies of labeled corticosterone uptake by placental vesicles are described herein which indicate that steroid uptake by these vesicles is a carrier-mediated process. We found that corticosterone uptake was temperature-sensitive, and an apparent phase-transition effect on the rate of uptake was seen to occur at approximately 16°C. Treatment of the vesicles with phospholipase A_2 and the sulfhydryl group attacker, *p*-chloromercuriphenylsulfonate, inhibited corticosterone uptake. In contrast to our previous findings in intact cells, neuraminidase treatment of membranes did not inhibit steroid uptake, perhaps indicating a species variation. Lastly, it was possible to show that corticosterone movement across the membrane exhibited countertransport, a phenomenon common only to carrier-mediated transport mechanisms. These studies show that placental vesicles accumulate corticosterone by a carrier-mediated mechanism.

Introduction

Glucocorticoid action on target cells is largely mediated via a soluble cytoplasmic protein receptor and involves the binding of that glucocorticoid-receptor complex to target cell nuclei; a variety of evidence supporting this contention has been recently reviewed [1]. However, studies by us using intact, cultured AtT-20 cells have suggested that specific plasma membrane components facilitate the entry of glucocorticoids into target cells. For example, we found that glucocorticoid uptake by intact AtT-20 cells was inhibited by prior treatment of the cells with neuraminidase or phospholipase A_2 [2–4]. This effect suggested that a necessary step prior to steroid-receptor binding was affected, since the binding capacity of the intracellular cytosol receptor was unaffected [2]. We also found that glucocorticoid uptake by these cells was temperature-sensitive to an extent that could not be explained by temperature-dependent

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Trivial names: corticosterone, 11β,21-dihydroxy-pregn-4-ene-3,20-dione; cortisol, 11β,17,21-trihydroxypregn-4-ene-3,20-dione; 11-dehydrocorticosterone, 21-hydroxypregn-4-ene-31,11,20-trione; 11-ketoprogesterone, pregn-4-ene-3,11,20-trione; triamcinolone acetonide, 9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 17,17-acetal with acetone; dexamethasone, 9α-fluoro-11β,17,21-trihydoxy-3,20-dione; aldosterone, 11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al; testerone, 17β-hydroxyandrost-4-en-3-one; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol.

binding characteristics of the intracellular receptor, but was best explained by postulating a prior membrane step [5]. Recently, we have shown that the uptake process requires intact sulfhydryl groups and that glucocorticoid-specific sites exist on isolated AtT-20 cell membranes [6,7].

This paper describes the properties of a membrane vesicle preparation which we have found suitable for the explicit examination of glucocorticoid-membrane interactions [8]. A similar preparation has been used by others to study amino acid transport [9,10]. This paper describes features of glucocorticoid uptake by these vesicles, including phenomena, such as countertransport, which indicate that a carrier mechanism is present.

Materials and Methods

[1,2-^{3}H]Corticosterone (60 Ci/mmol) and α-amino[*Me*-^{3}H]isobutyric acid (10 Ci/mmol) were obtained from New England Nuclear Corp. Unlabeled steroids and other chemicals were obtained from Sigma Chemical Co., unless otherwise noted.

Preparation of vesicles and measurement of steroid uptake

These experiments were performed after review and approval of the protocol by the Vanderbilt Committee on the Protection of Human Subjects. The 5′-nucleotidase activity of membrane vesicles, prepared as previously described [8,11], was routinely increased 19-fold while the activity of choline acetyltransferase, a soluble tissue constitutent, was reduced 80% [11]. Electron micrographs of vesicle preparations showed predominantly microvillous structures with an average diameter of 0.1–0.2 μM [11]. Only vesicles maintained at −70°C for 1 week or less were used in these experiments.

Miscellaneous

Radioactivity was determined in a Beckman LS-7000 scintillation spectrometer. Protein was quantitated by a method using Coomassie blue [12].

Results

Characterization of the filter assay of steroid uptake

Since this vesicle preparation has not been used extensively to analyze steroid uptake, the characteristics of the assay system were carefully examined. Vesicles were incubated at 37°C with labeled α-amino[*Me*-^{3}H]isobutyric acid or corticosterone and the amount of uptake determined by filtration on mixed-ester cellulose filters (Millipore Corp.). The uptake of α-aminoisobutyric acid by these vesicles is known to be concentration- and time-dependent [10] and was used as one test of the filtration procedure. Uptake of α-aminoisobutyric acid was linearly related to vesicle concentration over a 10-fold range, and extrapolation of the results showed that at zero vesicle concentration there was negligible amino acid uptake. When corticosterone uptake was evaluated, nonspecific factors, probably steroid adsorption to the filters, accounted for approximately 20% (range, approx. 10–30%) of the radioactivity in each filtered sample. Nonspecific adsorption was estimated by including controls containing a 1000-fold excess of unlabeled steroid vs. labeled. After this correction corticosterone uptake was linearly related to vesicle volume. In order to assess vesicle recovery after filtration, vesicles whose protein was labeled by reductive methylation [13] were diluted in phosphate-buffered saline and incubated at 25°C for 60 min as for an uptake experiment. Various amounts (10–100 μl) of vesicles were then treated with 5% (w/v) trichloroacetic acid and the precipitates recovered by filtration to determine the total amount of vesicle radioactivity in each sample. Duplicate aliquots of vesicles in phosphate-buffered saline were simply filtered and washed routinely as for an uptake experiment. The total amount of trichloroacetic acid-precipitable radioactivity and the amount retained after routine filtration were essentially identical throughout. It was also found that recovery was unaffected by treatment of vesicles with neuraminidase, phospholipase A_2 or *p*-chloromericuriphenylsulfonate. The effects of these agents on steroid uptake will be described later in this paper.

Effect of extravesicular steroids on efflux

Incubation of vesicles with 14 nM labeled corticosterone at 25°C led to an accumulation of radioactivity which reached apparent equilibrium after 2 h [8]. At 120 min several aliquots of vesicles were filtered, washed with 10 ml phosphate-

buffered saline and placed in 50 ml of phosphate-buffered saline at 25°C. This treatment resulted in a 5000-fold dilution relative to the volume of vesicles retained on the filter and proved to be a simple method of assessing efflux from the vesicles under conditions of extreme dilution. Smith et al. [9] reported that this method could be used without incurring loss of vesicles from the filter, and experiments by us with vesicles labeled by reductive methylation showed that there was no loss of vesicle protein by this procedure (data not shown). At various times filters were removed and the remaining radioactivity determined after repeat filtration. The loss was linear with time and had a $t_{1/2}$ of 45 min (data not shown).

The abilities of various steroids to affect efflux were tested by adding the steroids to beakers containing phosphate-buffered saline. Preliminary experiments showed that these studies were best performed if the vesicles were loaded by incubation with 140 nM labeled corticosterone. Efflux from vesicles loaded at this higher concentration was fractionally slower ($t_{1/2}$, approx. 60 min) than efflux from vesicles loaded with 14 nM corticosterone. Efflux was accelerated when 14 μM unlabeled corticosterone was added to the phosphate-buffered saline efflux medium (Fig. 1). This effect was also seen when 11-dehydrocorticosterone, 11-ketoprogesterone, dexamethasone and testosterone were used, but estradiol, cortisol and triamcinolone acetonide were much less active. The inability of steroids in the latter group to accelerate efflux is consistent with their low activity in inhibiting corticosterone uptake [8]. α-Aminoisobutyric acid uptake by vesicles incubated with similar concentrations of corticosterone was unaffected. This and the selectivity of the steroid effect on efflux suggests that the effect is specific and not due to physical damage of the vesicles by the steroid concentrations employed. The ability of extravesicular corticosterone and certain other steroids to accelerate the efflux of intravesicular corticosterone is similar to examples of counter-transport described for glucose [14] and glycine [15].

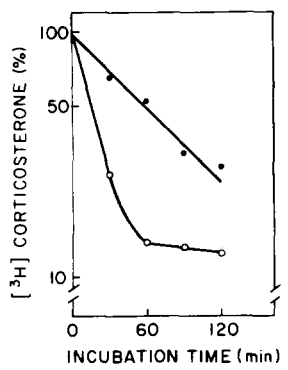

Fig. 1. Effect of unlabeled corticosterone on labeled corticosterone efflux from placental vesicles. The vesicles were labeled for 2 h with 140 nM labeled corticosterone. Efflux was begun by placing filtered, labeled vesicles in beakers containing 500 ml of phosphate-buffered saline with (○) or without (●) the addition to the beakers of 14 μM corticosterone. Duplicate sets of filters were removed at the indicated times and washed with 10 ml ice-cold phosphate-buffered saline (10 mM Na_2HPO_4/154 mM NaCl, pH 7.5).

Earlier studies by us showed glucocorticoid uptake by intact AtT-20 cells to be highly temperature-dependent [5]. Interestingly, the ability of vesicles to accumulate corticosterone at low temperature (4°C) was also found to be much less than that observed at 25 or 37°C (data not shown). In order to verify and characterize further the temperature-dependent nature of this system, temperature-shift experiments were done in which uptake was initiated at 37°C and the incubation mixture rapidly cooled to 4°C after various incubation periods. A typical example of several such experiments (Fig. 2) shows that uptake was rapidly halted at lower temperatures and that uptake remained blocked for up to 1 hour at 4°C.

It has been suggested that membrane-mediated processes may demonstrate a significant alteration in their activation energy with increasing temperature as the membrane lipid environment undergoes one or more phase transitions [16–18]. Glucocorticoid uptake by AtT-20 cells shows evidence of such a phase transition [5], providing some of the earliest evidence of steroid-membrane interactions during intact cell uptake. Accordingly, we evaluated the effect of temperature (from 4 to 37°C) on the rate of corticonsterone uptake. The results, displayed as an Arrhenius plot [19], showed that at approximately 16°C the reaction undergoes a transition such that at temperatures greater than 16°C the effect of temperature on uptake is more profound (Fig. 3). This observation is similar to those made on the effect of temperature on (Na^+

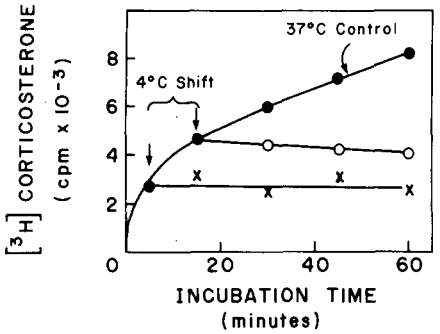

Fig. 2. Effect of temperature shift on uptake. Vesicles (approx. 100 μg protein/ml) were incubated at 37°C in phosphate-buffered saline containing 14 nM [^{3}H]corticosterone alone, or with 14 μM unlabeled corticosterone to correct for nonspecific adsorption. At 5 (×) and 15 (○) min, aliquots were placed in an ice-water bath. Uptake was determined by filtering 100 μl of the incubate on pre-wet 0.45-μM mixed ester cellulose filters and washing with 10 ml ice-cold phosphate-buffered saline.

+ K^+)ATPase [15,16] and intact cell glucocorticoid uptake [5], and may indicate similar mechanisms in the mouse and human cell membrane.

Effects of enzymatic treatment on uptake

Pronase, a mixture of proteolytic enzymes, neuraminidase, which hydrolyzes sialic acid groups and phospholipase A_2, which excises the middle triacylglycerol of phospholipids, were tested for effects on vesicle steroid uptake. Experiments done using vesicles labeled by reductive methylation

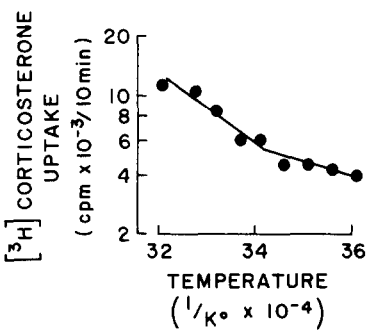

Fig. 3. Arrhenius plot of the effect of temperature on uptake. Vesicles were incubated for 10 min at various temperatures from 4 to 37°C in phosphate-buffered saline containing 14 nM [^{3}H]corticosterone alone, or with 14 μM corticosterone to correct for nonspecific adsorption. Uptake was determined by filtration.

TABLE I

THE EFFECT OF VARIOUS TREATMENTS ON CORTICOSTERONE OR α-AMINOISOBUTYRIC ACID UPTAKE

Vesicles were treated for 30 min with 25 μg/ml of pronase, neuraminidase or phospholipase A_2. These vesicles were then diluted into phosphate-buffered saline containing 75 μM [^{3}H]corticosterone alone, or with 14 μM corticosterone to correct for nonspecific adsorption, or 75 μM α-amino-[*Me*^{3}H]isobutyric acid, incubated at 25°C for 10 min and filtered.

	Uptake (% of control)		
	Pronase	Neuraminidase	Phospholipase A_2
Corticosterone	100	146	12.5
α-Aminoisobutyric acid	111	119	15.5

were used to verify that the recovery of vesicles was unchanged in every instance.

We found that pronase treatment did not reduce uptake (Table I) and that neuraminidase treatment caused a slight increase in the amount of steroid retained by the vesicles. In contrast, phospholipase A_2 caused a marked reduction in uptake (12% of control). These results suggest that the glucocorticoid-uptake mechanism is dependent on an intact phospholipid matrix.

Discussion

Several observations made previously by us [2–8] and others [21] have suggested that glucocorticoid uptake is a membrane-mediated process, but direct evidence of a carrier mechanism has been lacking. This paper reports the most direct examination of this question to date. The experiments were made possible by isolation of active plasma membrane vesicles free of detectable cytoplasmic contamination. Significantly, it was possible to use the placenta, a human glucocorticoid target tissue which has been shown to contain cytosolic glucocorticoid receptors [22], for these studies. This paper describes evidence which indicates that glucocorticoid uptake by these vesicles is carrier-mediated.

The presence of carrier-mediated steroid transport by the vesicles is indicated by two observations. The first observation was that the extent of vesicular steroid at equilibrium was proportional

by exposure of the vesicles to sucrose. This experiment suggested that the absolute amount of steroid associated with the vesicles was dependent on the vesicle volume. This phenomenon is observed in cases of facilitated diffusion or simple diffusion and indicated that the steroid was present in the vesicle as free solute and not bound to fixed sites. However, simple diffusion seemed an unlikely mechanism in view of the saturability and specificity of the uptake process [8]. The second observation was that efflux of steroid from loaded vesicles was enhanced by steroids in the extravesicular medium (Fig. 1). Steroids which effectively competed for uptake were found to also accelerate efflux, suggesting that the phenomenon resulted from interaction with the uptake mechanism. Furthermore, efflux was increased by these steroids best when the vesicles were loaded at high concentrations of corticosterone. This latter characteristic rules out negative cooperativity as an explanation for these findings, since acceleration should have been observed at all ligand concentrations. These two observations are important because several other characteristics of vesicle uptake, such as saturability, steroid specificity and temperature-dependence, could be explained by simple binding phenomena. Volume dependence and countertransport cannot. These considerations led us to conclude that the acceleration of efflux was a form of countertransport and that free steroid was taken up by these vesicles via a carrier mechanism.

Glucocorticoid specificity of steroid uptake by these vesicles was clearly present and different from that of the placental cytosol receptor. Dexamethasone and triamcinolone acetonide are the most potent competitors for the cytosol receptor [22], but dexamethasone was a relatively weak competitor of uptake and triamcinolone acetonide was impotent. The fact that 11-dehydrocorticosterone and 11-ketoprogesterone inhibit uptake effectively is interesting, since these steroids have no known biologic effects in the human, but are probably effective competitiors for the 11β-ol dehydrogenase activity which the vesicles are known to contain [8,19]. However, the two functions, transport and metabolism, can be readily separated since the dehydrogenase enzyme is very stable and is active in placental tissue stored at 4°C for several days, whereas vesicles prepared from such placenta do not transport corticosterone (Sloshberg, S. and Harrison, R.W., unpublished data).

The uptake mechanism was not affected by treatment of the vesicles by pronase, but was drastically inhibited by treatment with phospholipase A_2. These results are similar to our previous observations of the effect of these enzymes on glucocorticoid uptake by AtT-20 cells [2,3]. The results suggest that any protein moieties associated with the uptake mechanism are sensitive to alteration of the membrane phospholipid matrix. The enhanced uptake consistently observed after neuraminidase treatment remains to be explained. It is possible that this represents an unmasking of previously inaccessible transport sites. Lastly, the destruction of transport activity by *p*-chloromercuriphenylsulfonate indicates that the transport mechanism is dependent on intact sulfhydryl groups. These data and the data summarized in the preceding paragraphs show that placental vesicles contain a glucocorticoid-specific carrier transport mechanism which is sensitive to its lipid environment and is sulfhydryl-group dependent.

For several years various investigators have addressed the question of whether glucocorticoid uptake occurred by a membrane-mediated mechanism. Studies from this laboratory using the AtT-20 mouse pituitary cell as a model [2–7] and by others using liver cells [21] and pituitary [23] and liver [24] membranes have suggested that uptake is membrane-mediated. Other investigators have found no evidence to support such a mechanism [25]. It seems possible that such divergent results represent differences between species as well as steroid classes. Further studies will be necessary to define the physiological role of the placental membrane transport mechanism.

Acknowledgements

The authors would like to thank the Obstetrical faculty and staff and the Pathology Department of the Vanderbilt Medical Center for their cooperation in obtaining the placenta. We are grateful to Ms. Bettye Ridley for typing this manuscript. Supported in part by NIH grant HD-13477 and the

Vanderbilt University Center for Population Research and Reproductive Biology. M.F. would like to thank Dr. Vivian Allen (presently at the Boston Children's Hospital, Boston, MA) for support.

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