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A COMPARISON OF THE EFFECT OF GLUCOCORTICOIDS ON GLUCOSE UPTAKE AND HEXOKINASE ACTIVITY IN LYMPHOSARCOMA P1798*

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SUMMARY

Exposure of cortisol-sensitive lymphosarcoma P1798 cells to $1 \cdot 10^{-6}$ M dexamethasone for 3 h resulted in a marked depression in the uptake of labeled 2-deoxyglucose. The majority of the trichloroacetic acid-soluble radioactivity was present as deoxyglucose 6-phosphate, with a small amount present as the free sugar. Approximately a 60 % reduction was seen in both intracellular deoxyglucose and deoxyglucose 6-phosphate following exposure to the hormone. The inhibitory effect of glucocorticoids on glucose uptake was compared with their effect on tumor hexokinase activity after treatment both *in vivo* and *in vitro*. No change in tumor hexokinase activity could be detected at times when there was a significant inhibition of glucose uptake. No detectable glucokinase activity was found in untreated tumors. From these experiments it may be concluded that glucocorticoids are acting to decrease the entry of glucose into sensitive P1798 cells. Several possible modes of corticosteroid action on glucose uptake are discussed.

INTRODUCTION

Administration of glucocorticoids to animals or their addition *in vitro* markedly inhibits glucose metabolism in both normal[1,2] and malignant[3] lymphoid tissues. A similar effect of glucocorticoids has been seen in adipose tissue[4], mouse ear strip preparations[5], cultured chick duodenum[6] and mouse fibroblasts grown in culture[7]. This inhibitory effect appears to be exerted on an early step in glucose metabolism, and both glucose uptake and phosphorylation have previously been implicated as a site of adrenal corticoid action in one or more of these tissues[2,3,8].

Studies in our laboratory[3,9] have indicated that glucocorticoids inhibit the entry of labeled glucose into cortisol-sensitive cells isolated from lymphosarcoma P1798. This inhibition of glucose entry is rapid, appears to be related to the physiological effects of cortisol on tumor regression, and precedes the effects of the hormone on nucleic

Abbreviation: dexamethasone, 9-fluoro-11β,17,21-trihydroxy-16-α-methyl-pregna-1,4-diene-3,20-dione.

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acid synthesis[3,10]. Studies utilizing labeled 2-deoxy-D-glucose, which is not metabolized past 2-deoxy-D-glucose 6-phosphate in lymphosarcoma P1798, have demonstrated that the inhibitory effect of the hormone is dose-dependent and specific for both glucocorticoids and the steroid-responsive tumor[3]. The present investigation was designed to differentiate an effect of glucocorticoids on the cell membrane, and hence on glucose penetration, from one on the hexokinase system, and thereby on intracellular glucose phosphorylation. A preliminary report of this work has been presented[9].

MATERIALS AND METHODS

Biochemicals

Uniformly labeled D-[$^{14}C_6$]glucose (65.7 mC/mmole) was purchased from Amersham/Searle Corp. and 2-deoxy-D-[1-^{14}C]glucose (2.29–7.85 mC/mmole) or uniformly labeled with ^{14}C (10 mC/mmole) was purchased from either New England Nuclear Corp. or International Chemical and Nuclear Corp., respectively. Unlabeled sugars, including 2-deoxyglucose and 2-deoxyglucose 6-phosphate, $NADP^+$, ATP and glucose-6-phosphate dehydrogenase (Type XV from Baker's yeast) were purchased from Sigma Chemical. The Glucostat reagent was obtained from Worthington Biochemical. Cortisol and dexamethasone were obtained as commercial preparations from Merck, Sharp and Dohme.

Experiments in vivo

CDF (DBA/2 × BALB/C) mice were bred in our laboratory and lympho sarcoma P1798 was transplanted as described previously[11]. Mice bearing 16-day-old cortisol-sensitive tumors were treated with 75 mg/kg cortisol intraperitoneally for 8 h; 30 min prior to sacrifice 1 μC of uniformly labeled D-[$^{14}C_6$]glucose was given to each animal. The tumors were removed, frozen in a dry ice–acetone bath, and kept frozen on dry ice until the assays were performed. The control and treated samples were then homogenized in ice-cold 0.001 M EDTA–0.1 M Tris buffer (pH 7.4, 1:2, w/v) with a glass hand homogenizer. The homogenates were centrifuged at 20000 × *g* for 30 min and the fat-free supernatant removed by aspiration and used for the determination of both hexokinase activity and glucose uptake.

Glucose uptake was measured following the addition of 1 ml of cold 10% trichloroacetic acid to 1 ml of the above supernatant. After centrifugation at 500 × *g* for 5 min, the radioactivity in the trichloroacetic acid-soluble fraction was determined as described previously[3]. Hexokinase activity (EC. 2.7.1.1) was initially assayed by the method of SALAS *et al.*[12] using a glucose-6-phosphate dehydrogenase-dependent spectrophotometric method. However, this method was found to be unsatisfactory for assaying crude P1798 hexokinase activity, because there was considerable formation of NADPH even when ATP and glucose were absent from the assay system. Therefore, the assay was modified to allow for the measurement of glucose disappearance rather than NADPH formation. A mixture containing 0.3 I.U. of glucose-6-phosphate dehydrogenase, 0.5 mM $NADP^+$, 5 mM ATP (pH adjusted to 7.4), 0.5 mM EDTA (disodium salt), 5 mM $MgCl_2$, 50 mM Tris buffer (pH 7.4), 0.5 mM glucose and 0.1 ml of crude extract in a total volume of 3.0 ml was used to determine the hexokinase activity. Samples without ATP were run as blanks, and samples with 0.1 M glucose were used to measure glucokinase activity[12] (EC 2.7.1.2). After incubation in a shaking

water bath at room temperature (20–24°), the reaction was stopped by chilling on ice. Then 1 ml of ice-cold 0.5 M $HClO_4$, followed by 1 ml of ice-cold 0.5 M KOH, was added to each beaker. After standing in an ice bath for 15 min, the samples were centrifuged at 20000 × *g* for 15 min. The glucostat reagent[13] was used with 2.0 ml of the final supernatant to determine the amount of glucose utilized. The results are expressed as μmoles of glucose phosphorylated per min per g of tumor at room temperature.

Experiments in vitro

Cell suspensions were isolated and incubated in RPMI (Roswell Park Memorial Institute) 1640 culture medium[14] as previously described[3]. After the incubation of aliquots of control or treated suspensions with the labeled hexose, the cells were washed twice with 3 ml of ice-cold medium and resuspended in 1 ml of 5 % trichloroacetic acid. The amount of radioactivity in the trichloroacetic acid-soluble fraction was determined as described previously[3]. Chromatography of deoxyglucose and its phosphorylated metabolite was performed by the method of SMITH AND GORSKI[15].

Hexokinase activity was measured in comparable aliquots of the tumor suspensions by the method of HATANAKA *et al.*[16]. After incubation the suspensions were centrifuged at 500 × *g* for 5 min and the culture medium removed by aspiration. Approx. $4 \cdot 10^8$ cells were resuspended in 2.0 ml of ice-cold 25 mM Tris buffer (pH 7.4) and disrupted using a small glass hand homogenizer. The assay mixture was composed of 0.1 ml broken cells in Tris buffer (5 mM final concentration), 2 mM $MgCl_2$, 0.5 mM 2-deoxy[^{14}C]glucose and 1 mM ATP in a total volume of 0.5 ml. The mixture was incubated at 37° in a Dubnoff metabolic shaking incubator and at the appropriate times the reaction stopped by immersing the beakers in ice. Carrier 2-deoxyglucose (2.5 μmoles) was added and the phosphorylated sugar was precipitated with the barium–zinc reagent of Somogyi[16]. Blank values were determined in samples run without ATP. The results are expressed as the amount of labeled 2-deoxyglucose remaining in the supernatant or as nmoles of sugar phosphorylated per min per mg protein at 37°. Protein was determined by the biuret method[17].

RESULTS

The effect of dexamethasone *in vitro* on the uptake and metabolism of 2-deoxyglucose by sensitive P1798 cells is shown in Fig. 1. After a 3-h exposure to dexamethasone there was a marked reduction in total trichloroacetic acid-soluble radioactivity (—67 %), which is in agreement with previous data[3]. Dexamethasone, a synthetic glucocorticoid has been shown to be 2–3 times as potent an inhibitor of glucose uptake as cortisol *in vitro* at $1 \cdot 10^{-6}$ M (see ref. 3), and was used in this and other experiments to maximize any differences that might exist between uptake and phosphorylation. Approx. 95 % of the total trichloroacetic acid-soluble radioactivity was present as either deoxyglucose or deoxyglucose 6-phosphate, and following exposure to dexamethasone there was a significant reduction in the amounts of intracellular deoxyglucose (—53 %) and deoxyglucose 6-phosphate (—69 %). Furthermore, most of the radioactivity was found in the phosphorylated sugar.

Since these data could not differentiate an effect on transport from one on phosphorylation, studies were undertaken to determine the hexokinase activity in lymphosarcoma P1798 following hormone administration. The characteristics of the hexoki-

nase assay described under MATERIALS AND METHODS *in vivo* are shown in Fig. 2. Disappearance of glucose is linear with time and with the amount of enzyme added. A complete dependence on ATP is evident and there is essentially no difference in activity using 0.1 M glucose in place of 0.5 mM glucose. This indicates that no significant glucokinase activity is present in these lymphoid tumors. Therefore, in subsequent experiments each sample was assayed in triplicate for a 5- or 6-min incubation at room temperature using 100 μl of the 20000 $\times$ *g* supernatant and 0.5 mM glucose.

A comparison of the effect of cortisol treatment on glucose uptake and hexokinase activity in sensitive P1798 tumors is shown in Table I. Although in two separate experiments there was an average inhibition of glucose uptake of 43 % at 8 h after

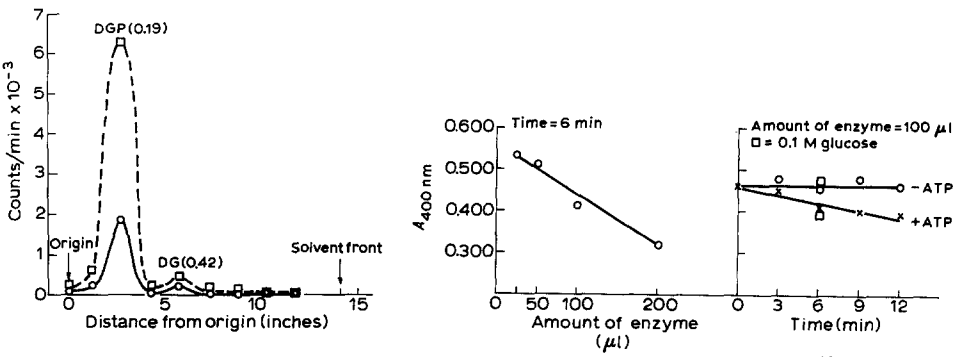

Fig. 1. Effect of dexamethasone on the uptake and metabolism of 2-deoxy-D-[1-^{14}C]glucose by P1798 cells. Sensitive cell suspensions were incubated for 3 h with and without the hormone. 0.5 μC of isotope was added to 2-ml aliquots of the cell suspensions 15 min prior to the end of the incubation. Chromatography of the trichloroacetic acid-soluble fraction was performed as described under MATERIALS AND METHODS. 2-Deoxy-D-glucose 6-phosphate is abbreviated DGP and 2-deoxy-D-glucose is DG. R_F values are shown in parentheses. □ --- □, control; ○—○, $1 \cdot 10^{-6}$ M dexamethasone.

Fig. 2. Properties of the "*in vivo*" hexokinase assay system. For details see MATERIALS AND METHODS. The values represented by ○ and × were obtained in an assay medium containing 0.5 mM glucose.

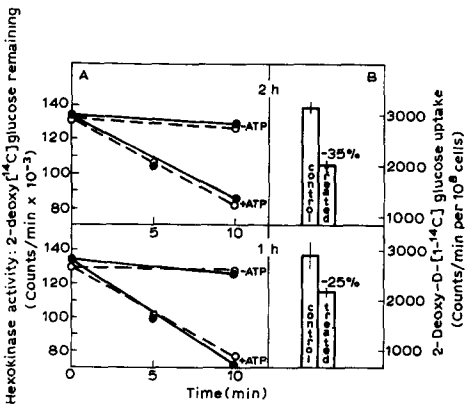

Fig. 3. A comparison of the effects of dexamethasone ($1 \cdot 10^{-6}$ M) *in vitro* on labeled deoxyglucose uptake and hexokinase activity in sensitive P1798 cells. Panel A shows the hexokinase activity 1 and 2 h after exposure to the hormone. ●—●, control; ○—○, treated. Panel B shows the uptake of labeled deoxyglucose at the same time points. The vertical lines represent the standard error of the mean. For other details see MATERIALS AND METHODS.

treatment, there was essentially no change in the hexokinase activity. In several other experiments no differences in hexokinase activity could be seen at 2, 4 or 6 h after treatment (data not shown). However, a significant inhibition of glucose uptake occurred as early as 4 h after treatment[3].

TABLE I

THE EFFECT OF CORTISOL TREATMENT ON D-[^{14}C]GLUCOSE UPTAKE AND HEXOKINASE ACTIVITY IN THE STEROID-SENSITIVE P1798

Mice bearing 16-day-old cortisol-sensitive tumors were treated with 75 mg/kg cortisol intraperitoneally for 8 h. 30 min prior to sacrifice 1 μC of D-[^{14}C]glucose was given to each animal. There were three animals in each group and the average values for two experiments are shown. Hexokinase activity was determined at 20–24° on the 20000 × *g* supernatant fraction.

	Uptake of [^{14}C]glucose (counts/min per ml)	*Hexokinase activity (μmoles/min per g tissue)*
Control	9500	1.53
Treated	4100 (−43 %)	1.57

TABLE II

THE EFFECT OF DEXAMETHASONE ON HEXOKINASE ACTIVITY IN CORTISOL-SENSITIVE P1798 CELLS

Conditions identical to Fig. 3.

Treatment	*Time of exposure (h)*	*Specific activity (nmoles/min per mg protein)*
Control	1	10.9
Dexamethasone		10.8
Control	2	10.1
Dexamethasone		9.7

To obtain more definitive data concerning the relationship of glucose uptake to hexokinase activity an *in vitro* assay system was used. As shown in Fig. 3, in comparison to untreated controls, exposure to dexamethasone for 1 h inhibited the uptake of labeled deoxyglucose into whole cells by 25 %, and by 35 % at 2 h. At both times there was no change in the hexokinase activity in broken cell preparations from the control and treated suspensions. This isotopic assay, using labeled 2-deoxyglucose, was considerably less complicated and somewhat more reproducible than the coupled hexokinase assay used above. Both control and treated hexokinase preparations showed a complete dependence on the presence of ATP in the assay systems and were linear with time. A calculation of the specific activity of hexokinase in control and treated suspensions using the 10-min values (with and without ATP) also revealed no significant change in activity after exposure to the hormone (Table II). Thus, compared to the controls glucocorticoid treatment did not influence hexokinase activity expressed on a per cell basis or per mg of protein. It appears then, that both *in vivo* and *in vitro*, glucocorticoids significantly depress glucose uptake without affecting tumor hexokinase activity.

DISCUSSION

These results demonstrate that glucocorticoids inhibit the transport of glucose into cortisol-sensitive P1798 cells by either a direct or indirect mechanism. FAIN[18] has previously shown that dexamethasone decreases the uptake and metabolism of glucose in adipose tissue and he suggested that the uptake of hexoses into adipose tissue may be the rate-limiting step in their metabolism. Studies by BLECHER[19] utilizing phospholipase C-treated fat cells have indicated that the inhibitory action of cortisol on glucose metabolism in these cells required an intact cell membrane. PLAGER *et al.*[8] have also suggested that the "anti-insulin" action of cortisol on mouse ear strip preparations was consistent with an effect on glucose passage through the cell membrane, or on the hexokinase system, with the former mechanism favored. In contrast, EMEL'YANTSEV AND SVESHNIKOVA[20] have reported an apparent inhibitory effect of cortisone on the hexokinase activity of rat blood lymphocytes. This apparent inhibitory effect may reflect the action of cortisone on glucose uptake, since intact cells were used in their assay procedure. Our data prove conclusively that the hexokinase activity of the P1798 tumor is not altered following glucocorticoid treatment either *in vivo* or *in vitro*.

Several possibilities exist to explain the action of corticosteroids on the entry of glucose into lymphoid cells. YOUNG[21] has reported that cortisol in thymocytes causes a small, but reproducible drop in intracellular ATP levels, with a concomitant increase in ADP and AMP. Thus, an effect of glucocorticoids on intracellular glucose phosphorylation as suggested by MUNCK[2] might be brought about by the reduced availability of ATP for the conversion of glucose to glucose 6-phosphate, rather than by a direct effect of the hormone on the amount or activity of tumor hexokinase. A block between glucose and glucose 6-phosphate would conceivably influence both the uptake and subsequent metabolism of glucose. Preliminary experiments in our laboratory revealed a 10–20 % decrease in ATP concentration in treated P1798 cells within 3 h after exposure to the hormone, but it was not possible to measure significant changes in ATP levels at 1 h or earlier. The inhibitory effect of glucocorticoids on glucose uptake is seen as early as 1 h after exposure to the hormone[3]. It is, therefore, not clear at present whether the reduction in ATP content is the cause of, or results from, decreased glucose uptake.

Phosphorylation may also be important in the active transport of glucose and its analogue deoxyglucose into P1798 cells. Studies by VAN STEVENINCK[22] in yeast have indicated that a membrane phosphorylating system, which utilizes a polyphosphate donor, is associated with an active transport mechanism for glucose. This concept contradicts the previous assumption of a passive, carrier-mediated transport of glucose followed by intracellular phosphorylation by the hexokinase–ATP system. A phosphotransferase system has also been described in bacteria[23,24]. It is comprised of two enzymes and a heat-stable protein and is involved in transmembrane sugar transport. Thus, an effect of glucocorticoids on membrane phosphorylation cannot be ruled out.

Transport changes could also depend on the synthesis of specific RNA species which direct the production of one or more crucial proteins. These proteins may be specific membrane carrier molecules, enzymes directly involved in energy production or nucleotide triphosphatases. This hypothesis has been suggested by MILLS AND SPAZIANI[25] to explain the stimulatory effect of testosterone on glucose and amino acid uptake into rat sex accessory tissues. Further support for this concept comes from the

work of SMITH AND GORSKI[15], who have shown that the stimulatory effect of another steroid hormone, estradiol, on glucose uptake into uterine tissue is abolished by cycloheximide, implying the requirement for prior protein synthesis. It is of interest in this regard that KONO[26] has recently reported that the insulin–effector system present in isolated fat cells is a rapidly renewable peptide element located on the surface of the cell.

Although the action of corticosteroids on glucose uptake appears to precede its inhibitory effect on total RNA and protein synthesis, it is quite likely that the pulse labeling technique employed in our experiments is unable to detect changes in one or more specific proteins comprising a very small fraction of the total cell protein[27]. It is also difficult to utilize inhibitors of protein synthesis, such as cycloheximide, to determine the effect of cortisol on the turnover of a critical protein, because both the inhibitor and cortisol depress the uptake of glucose into P1798 cells[9]. However, the similarity of the time-course of cortisol and cycloheximide action on glucose uptake suggests that protein synthesis may be important in the control of glucose transport in P1798 cells. Further studies are in progress to examine this possibility and the nature of the sugar transport system in both the cortisol-sensitive and cortisol-resistant lines of lymphosarcoma P1798.

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