ESTRADIOL STIMULATION OF GLUCOSE TRANSPORT
IN RAT UTERUS

by

DANIEL ALAN MEIER, B.S.

A DISSERTATION

IN

MEDICAL BIOCHEMISTRY

Submitted to the Graduate Faculty of
Texas Tech University Health Sciences Center
in Partial Fulfillment of
the Requirements for
the Degree of

DOCTOR OF PHILOSOPHY

Approved

August, 1985

T3
1985
No. 51
copied

ACKNOWLEDGEMENTS

I am indebted to Dr. Charles W. Garner for his direction of this dissertation and to the other members of my committee, Drs. James C. Hutson, Gwynne H. Little, K. John Morrow, and Douglas M. Stocco, for their helpful criticism.

CONTENTS

ACKNOWLEDGEMENTS.....	ii
ABSTRACT.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
1. INTRODUCTION.....	1
2. CHARACTERIZATION OF THE GLUCOSE TRANSPORTER.....	10
Introduction.....	10
Materials and Methods.....	11
Results.....	15
Discussion.....	26
3. CHARACTERIZATION OF ESTRADIOL STIMULATION OF GLUCOSE TRANSPORT.....	31
Introduction.....	31
Materials and Methods.....	32
Results.....	35
Discussion.....	52
4. GLUCOSE TRANSPORT AND ESTROGEN RECEPTORS.....	56
Introduction.....	56
Materials and Methods.....	58
Results.....	59
Discussion.....	72
5. INSULIN AND GLUCOSE TRANSPORT.....	74
Introduction.....	74
Materials and Methods.....	75

Results.....	76
Discussion.....	83
6. MECHANISM OF THE ESTRADIOL-MEDIATED INCREASE IN GLUCOSE TRANSPORT.....	85
Introduction.....	85
Materials and Methods.....	89
Results.....	92
Discussion.....	99
7. SUMMARY.....	101
REFERENCES.....	105

ABSTRACT

A study of the mechanism of the estradiol-mediated increase in glucose transport in the uteri of ovariectomized rats was undertaken. An essential first step in this study was the characterization of the glucose transport process using plasma membrane vesicles.

Uterine plasma membrane preparations were obtained by centrifugation on discontinuous sucrose gradients. The specific activity of the plasma membrane marker 5'-nucleotidase was increased 10-fold while the specific activity of an endoplasmic reticulum marker glucose-6-phosphatase was increased 3-fold. D-Glucose transport into plasma membrane vesicles was inhibited by sulfhydryl reagents, phloretin, and cytochalasin B. Uptake was prevented by high osmotic pressures. The K_m of glucose transport was 12.2 ± 1.1 mM. Transport was unaffected by sodium and was energy independent.

2-Deoxyglucose transport was determined in uteri of rats grouped by stages of the reproductive cycle, i.e., diestrus 1, diestrus 2, proestrus and estrus. The rate of 2-deoxyglucose transport was highest in proestrus and lowest in diestrus 1.

The increase in glucose transport in ovariectomized rats was half-maximal at approximately 5 ng estradiol/rat and reached the maximal 2 to 3-fold response after 2 hours whether measured in whole tissue or in uterine plasma

membrane vesicles. Estrone and estriol treatment resulted in a similiar 2-fold increase in glucose transport while progesterone and dihydrotestosterone had no effect. Injec-----tion of protein synthesis inhibitors cycloheximide and emetine resulted in an increase in the basal glucose transport rate while having no effect on the estradiol-stimulated increase in glucose transport. Treatment with the transcriptional inhibitor actinomycin D resulted in a slight increase in glucose transport with no effect on the estradiol-stimulated increase in glucose transport.

Antiestrogen treatment (nafoxidine and tamoxifen) resulted in a 85-90 % decrease in the total estradiol binding sites in the cytosol but did not effect the estradiol-stimulated increase in 2-deoxyglucose transport in uterine tissue.

Insulin injection (0.2 mg/rat) resulted in a 40 % increase in 2-deoxyglucose transport in 24 h-starved rats in contrast to the 200 % increase seen with estradiol treatment. Insulin and estradiol treatment together were not additive in regard to the increase in 2-deoxyglucose transport in tissue. Estradiol treatment did not change binding of [125I]insulin to uterine plasma membranes.

Estradiol treatment resulted in a 3-fold increase in the Vmax with no apparent change in the Km for 2-deoxyglucose transport. Also, estradiol treatment did not result in an increase in the amount of glucose transporters in

uterine plasma membranes as measured by antibodies raised against the glucose transporter protein from human erythrocytes.

In summary, estradiol stimulates the rate of glucose transport in rat uterus by increasing the rate by which the transporter protein moves glucose across the plasma membrane.

LIST OF TABLES

2.1	Specific activities of membrane marker enzymes...	17
2.2	Inhibitors of glucose transport in uterine plasma membranes.....	27
3.1	Effect of estradiol on vesicular volume.....	36
3.2	Glucose transport at different stages in the reproductive cycle.....	39
3.3	Specificity of steroid hormone stimulation of glucose transport in vesicles.....	44
3.4	Effect of emetine on glucose transport.....	48
3.5	Effect of actinomycin D on glucose transport.....	50
4.1	Effect of antiestrogens on translocation of and estradiol occupancy of the cytoplasmic receptor.....	61
4.2	Effect of antiestrogens on estrogen stimulation of 2-deoxyglucose transport.....	62
4.3	Effect of estradiol on microsomal receptor number.....	70
5.1	Effect of insulin and starvation on rat serum glucose concentration.....	77
5.2	Effect on insulin on glucose transport in rat uterus.....	78
5.3	Effect of insulin and estradiol on glucose transport in rat uterus.....	80
5.4	Effect of estradiol on [125I]insulin binding to insulin receptors in rat uterine plasma membranes.....	81
6.1	Effect of various treatments on protein A binding.....	96

LIST OF FIGURES

2.1	Electron micrograph of uterine plasma membrane vesicles.....	18
2.2	Time-course of glucose transport by uterine membranes.....	19
2.3	Efflux of D-glucose from previously-loaded membrane vesicles.....	21
2.4	Effect of increasing osmolarity on glucose uptake.....	22
2.5	Fractionation of uterine membranes on a discontinuous sucrose gradient.....	23
2.6	D-glucose transport as a function of D-glucose concentration.....	25
2.7	Inhibition of D-glucose transport in membranes by cytochalasin B.....	28
2.8	Effect of cytochalasin B on the transport of 2-deoxyglucose in intact uteri.....	29
3.1	Effect of estradiol amounts on the increase in the rate of glucose transport.....	38
3.2	Time-course of the estradiol-mediated increase in glucose transport.....	41
3.3	Time-course of the estradiol-mediated increase in 2-deoxyglucose uptake in intact uterus.....	42
3.4	Time-course of glucose efflux from plasma membrane vesicles.....	43
3.5	Effect of cycloheximide on glucose transport in plasma membrane vesicles.....	45
3.6	Effect of cycloheximide on protein synthesis and 2-deoxyglucose transport.....	47
3.7	2-Deoxyglucose transport as a function of 2-deoxyglucose concentration in uterine tissue previously or not exposed to estradiol.....	51
4.1	Time-course of [3H]estradiol binding to microsomes.....	64

4.2	Saturation of [3H]estradiol binding to rat uterine microsomes.....	65
4.3	Scatchard plot derived from the data shown in Figure 4.2.....	66
4.4	Competition of [3H]estradiol binding to rat uterine microsomes with estradiol and antiestrogens.....	67
4.5	Competition of [3H]estradiol binding to rat uterine cytosol with estradiol and antiestrogens.....	69
4.6	Exchange of [3H]estradiol from microsomes with nonradioactive estradiol.....	71
5.1	Time-course of estradiol effect on plasma insulin concentration and rate of 2-deoxyglucose transport.....	82
6.1	[125I]Protein A binding with increasing amounts of human erythrocyte plasma membrane protein.....	93
6.2	Binding of [125I]protein A to increasing amounts of uterine plasma membrane protein.....	94
6.3	Western blot of SDS-solubilized plasma membrane proteins.....	98
7.1	Model of glucose transport regulation.....	104

CHAPTER 1

INTRODUCTION

Estradiol stimulates protein, RNA, and ultimately DNA synthesis in the uterus. The earliest uterine responses seen after estrogen administration occur within minutes. Some of these responses are incorporation of labelled uridine into RNA (1), histone acetylation (2), eosinophil infiltration into the uterus (3), synthesis of mRNA for induced protein (4), RNA polymerase activity (5), a decrease in arginine-rich histone content (6), an increase in glucose uptake (7), and an increase in amino acid uptake (8). Among later uterine responses to estrogen requiring 6 hours or more are generalized protein synthesis (9), and the increase in the activity of the hexose monophosphate shunt enzymes (10).

The classical model of induction of the response of the uterus to estradiol is the two-site model proposed by Gorski et al. (11) and Jensen et al. (12). This model is based on studies of the binding of radioactive estradiol to receptor in various cell fractions. According to this model, estradiol enters the cytoplasm and binds to the receptor. The receptor upon estradiol binding has a sedimentation coefficient which changes from 4S to 5S and is then translocated to the nucleus. Once in the nucleus, the receptor-estradiol complex binds to chromatin. It is

through this interaction that estradiol induces changes in cellular activity. This model, however, is inconsistent with the findings of Welshons et al. who showed that cytoplasts (cells enucleated with cytochalasin B) showed little estrogen binding activity while the nucleoplasts showed considerable estrogen binding activity (13). In addition, the studies of King and Greene, using a monoclonal antibody against the estrogen receptor and immunochemical staining, showed that specific staining was confined to the nucleus (14). These last two studies support the conclusion that the estradiol receptor resides in the nucleus and not in the cytoplasm and that the cytoplasmic receptor may be an artifact of the fractionation procedures.

Jensen and Jacobsen in 1962 showed that the uterus and vagina of rats injected with [3H]estradiol retained the label for six hours while other tissues such as kidney and blood had increased levels of hormone for approximately two hours after administration (15). This discovery suggested that a receptor molecule in these organs was specifically binding to estradiol. Noteboom and Gorski in 1965 quantitated an estrogen receptor and determined the dissociation constant (K_d) to be 0.7 nM (16). Estrogen binding was found to be localized in cytosol, nuclear, mitochondrial, and microsomal fractions. In 1979, Pietras and Szego reported the presence of an estrogen receptor on the plasma membrane (17). These results were supported by the work of

Nenci et al. using fluorescent estrogen analogs (18). However, these results are in contrast to the findings of Mueller et al. who were unable to demonstrate the presence of an estrogen receptor on the plasma membrane (19).

In addition to the estrogen receptor described above, a second estradiol receptor was first described by Best-Belsomme et al. in 1970 (20). Clark et al. determined the dissociation constant (K_d) to be 33 nM (21). This binding site (Type II) has received less attention than the high affinity site (Type I) described above. The Type II site is believed by some to be due to serum albumin or alpha-feto protein (22). More recently, the Type II site has been attributed to the presense of eosinophils in the uterus (23). The controversy surrounding the origin of the Type II site has not been settled. In addition, Clark et al. described a nuclear Type II binding site which shows a sigmoidal saturation curve (21). The precise role that each site plays in the mediation of the estradiol response is unknown. Recently, Meyers et al. found that doisyndolic-acid type estrogens bind the cytosolic receptor one to two orders of magnitude less tightly than estradiol but have uterotrophic activities close to that of estradiol (24). These results suggest that the doisyndolic acid is either bypassing the receptor step, or a different, as yet undiscovered receptor exists.

Estrogen binding to receptors can be blocked by var-

ious compounds known as antiestrogens. Terenius has divided antiestrogens into two categories depending on a limited or sustained effect in blocking estrogen action (17). The group of compounds having a sustained effect are derivatives of polycyclic phenols which include tamoxifen and derivatives of dihydronaphthalene and diphenylindene which include nafoxidine. Nafoxidine is capable of causing the translocation of the cytosolic Type I receptor to the nucleus and this event is what Katzenellenbogen and Ferguson suggest is the mechanism by which this antiestrogen blocks estradiol stimulation of uterine growth (26).

Earlier studies have indicated that estrogen injected in vivo mediates a rise in glucose transport into the uterus. Spaziani and Gutman reported, in 1965, the appearance of hexose in the uterus using in vivo permeation studies (27). Roskoski and Steiner reported a two-fold increase in the initial rate of 3-O-methylglucose uptake in vitro 2 hours after in vivo estrogen administration (28). In 1968, Smith and Gorski reported a detectable rise in the rate of deoxyglucose uptake into uteri in vitro as early as 30 minutes after in vivo estrogen administration (7).

Glucose transport regulation has been studied in detail in a number of other systems. The ability of Ehrlich ascites tumor cells to take up glucose increases during the course of tumor development. Using D-glucose inhibitable cytochalasin B binding, Chan et al. demonstrated

that the increase in glucose transport can be explained by an increase in the number of glucose transporters in the plasma membrane and that changes in the turnover and affinity for substrate of the glucose transporter need not be invoked (29). Another system where glucose transport regulation has been investigated is in chicken embryo cells infected by Rous sarcoma virus. Salter et al. showed that the increase in glucose transport following viral infection is due to an increase in the number of glucose transporters as measured by antibody raised against human erythrocyte glucose transporter (30). A third system used to study the mechanism of the increase in glucose transport is insulin-stimulated glucose transport in rat adipocytes. Wardzala et al. demonstrated, using D-glucose inhibitable cytochalasin B binding, that the increase in glucose transport in fat cells following insulin administration is due to an increase in the number of transporters in the plasma membrane (31). In addition, Cushman and Wardzala showed that the source of the additional transporter is the microsomal fraction (32).

In addition to the translocation of preformed glucose transporter proteins to the plasma membrane as a mechanism to increase glucose transport into the cell, other mechanisms are possible to explain estradiol action on the uterus. For example, estradiol may induce an increase in the affinity of the transporter for glucose or it may induce

changes in the composition of the plasma membrane which result in an increase in the intrinsic velocity of the transport process. Neither of these possibilities require an increase in the number of transporters in the plasma membrane. An additional possibility is the translocation of newly synthesized glucose transporters to the plasma membrane. These newly synthesized proteins could conceivably be a different transporter protein than the one already present in the plasma membrane.

The transducing mechanisms that convert hormonal signals to an increase in glucose transport via the translocation of the transporter protein from the microsomal membranes to the plasma membrane are unknown. In the case of Rous sarcoma virus infection of chicken embryo fibroblasts, a number of effects including a fall in cAMP concentration (34) and an increase in phosphatidyl inositides (35) occur temporally with an increase in glucose transport. These events are possible candidates in the, as yet undetermined, transducing mechanism.

The mechanism that transduces the insulin-receptor binding signal to the translocation of transporter proteins is also unknown. Several candidates have been proposed as elements in the transducing mechanism, including cAMP, cGMP, calcium, and intracellular pH (36,37,38). Reduction in cAMP levels has correlated well with insulin action (39,40), while the increase in cGMP levels in fat cells

was not specific for insulin and its actions (41). Changes in intracellular calcium concentration may be involved in some insulin action (42) but its precise role has not yet been established. In conclusion, none of the putative regulators appear to be the sole mediator of insulin action.

The goal of this research was to elucidate the mechanism by which estradiol stimulates the increase in glucose transport into the uterus. In order to study these mechanisms, two different model systems have been employed. These systems are plasma membrane-enriched membrane vesicles and a whole organ in vitro system. Plasma membrane-enriched vesicles were generated in order to characterize and quantitate the glucose transporter protein from the uterus while the whole organ system was used to answer questions more readily obtained with tissue uptake studies than with vesicles. Among the questions examined are the role of estrogen receptors and the role of insulin in the increase in glucose transport. Mechanistic studies include determining if translocation of newly synthesized or preformed transporters occurs, if the affinity of the transporter for glucose increases, and if the intrinsic velocity of transport increases. Other mechanistic studies involve identifying the system that transduces the estradiol signal to an increase in glucose transport. Possible transducing systems include cAMP dependent and

calcium dependent systems.

Plasma membrane vesicles have been generated from a number of cell types in order to characterize the glucose transporter protein. Using plasma membrane vesicles, the glucose transporter protein has been characterized in rat adipocytes (43), human placenta (44), calf thymus (45), and in rat skeletal muscle (46). Among the characteristics of the glucose transporter protein determined using vesicles are specific, bidirectional transport of D-glucose, sensitivity of the transporter to sulfhydryl reagents, and affinity of binding between the transporter protein and D-glucose. Furthermore, in this system one can analyze dose-response relationships and the time course of the response to estradiol administration. One advantage of this model is that one can directly measure glucose transport.

Injection of estradiol in vivo followed by removal of the uterus after an appropriate time period and incubation of the whole uterus with glucose analogs such as 3-O-methylglucose and 2-deoxyglucose is a system utilized by Roskoski and Steiner (28) and Smith and Gorski (29). Using this model, the transporter protein cannot be quantitated nor can, in the case of 2-deoxyglucose, transport be directly measured. Using 2-deoxyglucose, transport and phosphorylation is measured. The validity of this model rests on the assumption that transport and not phosphory-

lation is rate-limiting. This assumption is based, among other considerations, on the fact that glucose transport is hormonally regulated; i.e., only a rate-limiting step would be under hormonal regulation. In addition, Smith and Gorski showed that the increase in glucose transport occurred well before any increase in hexokinase activity (7). One advantage of this model over vesicles is that nonspecific uptake is slow while in the vesicle system, nonspecific uptake is much higher than specific transport which complicates data collection. In addition to transport studies, the plasma membrane vesicle model allows the quantitation of the transporter protein. There are two techniques used to quantitate the transporter protein. The first technique employs labeled cytochalasin B as a ligand (47). In this procedure, the specific binding is the difference in binding between cytochalasin B and cytochalasin B plus high amounts of D-glucose. The second technique employs polysera raised against the purified human red blood cell glucose transporter (48). In this procedure, quantitation can be achieved via an ELISA procedure or a Spot immunochemistry technique.

CHAPTER 2

CHARACTERIZATION OF THE GLUCOSE TRANSPORTER

Introduction

Some of the effects of estradiol on the uterus of ovariectomized rats include increased amino acid (8,49-51) glucose (7,28,52) and RNA precursor (53) uptake as well as increased vascular permeability (54). Uterine cell surface glycoproteins are altered as determined by changes in lectin binding (55,56). All of these effects were observed to occur within 4 h after exposure to estradiol. These phenomena appear to involve actions at the plasma membrane.

Of particular interest to me is the stimulation in glucose transport. Whether this effect occurs through an increase in the intrinsic activity of the existing transport protein or through changes in the amount of transport protein in the plasma membrane is not known. It has been demonstrated that cycloheximide, an inhibitor of protein synthesis, can block the stimulation in glucose transport by estradiol, as measured by the accumulation of 2-deoxyglucose-6-phosphate in uterus in organ culture (7). There is increased protein synthetic activity during this early time period although the synthesis of significant quantities of protein does not begin until about 4-6 h after estradiol exposure (9,57). In order to investigate the

mechanism of the regulation of glucose transport in uterus by steroid hormones, characterization of glucose transport in uterine tissue is an essential first step.

In this chapter I describe certain properties of the glucose transport system in uterine plasma membranes. The presence of a glucose transport protein in the plasma membrane vesicles was indicated by stereospecific uptake of D-glucose and its inhibition by classic glucose transport inhibitors, e.g., cytochalasin B, phloretin, and sulfhydryl reagents.

Materials and Methods

Materials

L-[1-¹⁴C]glucose (47 mCi/mmol) and [4-³H]cytochalasin B (10.3 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. D-[U-¹⁴C]mannitol (210 mCi/mmol), D-[1-¹⁴C]glucose-6-phosphate (225 mCi/mmol) and D-[1-³H]glucose (25 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. Nonradioactive cytochalasin B, phloretin, and N-ethyl maleimide were purchased from Sigma Chemical Co., St. Louis, Mo. Sucrose was obtained from Schwarz/Mann, Inc., Spring Valley, N.Y. Liquid scintillation counting solution, BetaPhase, was obtained from WestChem, Inc., San Diego, Calif. Other reagents were obtained at the highest purity available.

Buffers

Buffer 1 consisted of 0.01 M Tris-HCl, pH 8.1, 0.1 mM EDTA and 4 mM sodium azide. Buffer 2 was Buffer 1 which also contained 0.25 M sucrose. Buffer 3 was Buffer 1 which also contained 1 mM magnesium chloride and 1 mM calcium chloride. Stop Solution was buffer 3 which also contained 1 mM mercuric chloride.

Membrane Preparation

All procedures were performed at 0-5 degrees Centigrade. Uterine membranes were prepared from intact Sprague-Dawley-derived rats (160-180 g) obtained from Small Animal Supply Co., Omaha, Neb. The rats were maintained on a 12 h dark/light cycle at 25 degrees Centigrade and were fed ad libitum. The uteri (4-16) were excised and placed in ice-cold Buffer 2. The uteri from animals in random stages of the estrous cycle were stripped of fat, cut into small pieces with a razor blade and homogenized, 2 uteri at a time, in a 10 ml glass-teflon homogenizer using 20-25 strokes. The pestle was polished with emery cloth to increase the clearance with the glass from approximately 0.15 mm to 0.35 mm. Homogenization with a Polytron homogenizer equipped with a PT-10 generator at full speed twice for 10 s each gave results comparable to the glass-teflon homogenizer. The homogenate was centrifuged at 2 000 x g for 5 min. (Centrifugal forces were

estimated from the bottom of the tube.) The supernatant solution was saved and the pellet was washed by resuspending it in 4 ml of the same buffer and centrifuging again at 2 000 x g for 5 min. The supernatant solutions were combined and centrifuged at 210 000 x g for 45 min. The pellet was resuspended in 2 ml of Buffer 2 and applied to the surface of a discontinuous gradient consisting of 2 ml of 1.1 M sucrose in Buffer 1 and 0.5 M sucrose in the same buffer. After centrifugation at 41 000 rpm (286 000 x g) in an SW-41 swinging bucket rotor for 90 min, the turbid material at the interface was removed with a pipet, diluted with an equal volume of Buffer 1, and centrifuged at 210 000 x g for 45 min. The pellet was resuspended in Buffer 3 to give a protein concentration of approximately 3 mg/ml.

Transport Assays

Stock ethanol solutions of D-[3H]glucose and L-[14C]-glucose were blown to dryness in a stream of nitrogen and then resuspended in Buffer 3 containing 0.1 mM D- and L-glucose each unless otherwise indicated. The specific activities were 4.65 dpm/fmol and 2.37 dpm/fmol, respectively. Five microliters of this solution were incubated with 10 to 15 microliters of the membrane suspension (approximately 50 micrograms protein/assay) in a total volume of 25 microliters for an appropriate time period at

20 degrees Centigrade. Uptake was terminated by the addition of 3 ml of ice-cold Stop Solution. The mixtures were filtered through 0.65 μ m Millipore filters (DAWP) and washed three times with 3 ml of ice-cold Stop Solution. The moist filters were dissolved in 1.0 ml methyl cello-solve during 10 min at room temperature. To this solution was added 14 ml of BetaPhase scintillation solution for the determination of radioactivity. Uptake is defined as the retention of any solute by membranes. D-glucose transport is defined as D-glucose uptake minus L-glucose uptake. Stereospecific transport was only 20 % of the total amount of sugar taken up. D-[14C]mannitol could be used in place of L-[14C]glucose with identical results. Cold stop solution stopped both specific uptake and efflux. D-glucose transport rates were measured during a 7 s incubation.

Enzyme Determinations

The enzymes, 5'-nucleotidase and glucose-6-phosphatase, were measured as described by Mueller et al. (64) by measuring the rate of release of phosphate from 5'-AMP and glucose-6-phosphate (58); ouabain-sensitive sodium/potassium ATP'ase was measured by the method of Bers (59); phosphodiesterase was measured by the method of Kelley et al. (60); and cytochrome c oxidase was measured by the method of Stocco and Hutson (61). Protein was determined

according to Bradford (62) using a commercially available protein assay reagent (obtained from Bio-Rad Laboratories, Richmond, Calif.) with Fraction V bovine albumin (Sigma Chemical, Co.) used as the standard.

Electron Microscopy

Membrane pellets were fixed with 2.5 % glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.3, for a minimum of 2 h. The pellets were then post-fixed for 1 h in 2 % osmium tetroxide, pH 7.0, dehydrated through a graded series of ethanol solutions, and embedded in an Epon epoxy resin. Sections were cut on a Sorvall MT-2B ultramicrotome, stained in uranyl acetate and lead citrate, and examined in a Hitachi H-600 electron microscope at 75 kV.

Curve Fitting

Analysis of kinetics experiments was accomplished by an iterative nonlinear curve-fitting procedure performed on-site with an IBM 370 computer using a program originating from SAS Institute, Box 8000, Cary, N.C., 27511. The results are expressed as estimates of the kinetic parameters $\pm$ the asymptotic standard errors.

Results

Uterine plasma membranes were prepared by centrifugation on a discontinuous sucrose gradient consisting of 0.5

M sucrose layered over 1.1 M sucrose. Plasma membrane markers were located mainly at the interface between the two solutions. A typical purification summary is shown in Table 2.1. This procedure is similar to the procedures of Kidwai et al. (63) and Mueller et al. (64). Enzyme markers determined in several preparations were: 5'-nucleotidase, ouabain-sensitive ATP'ase, and phosphodiesterase (plasma membrane); glucose-6-phosphatase (endoplasmic reticulum); and cytochrome c oxidase (mitochondria). The ATP'ase and 5'nucleotidase marker enzymes were purified about 10-fold while phosphodiesterase was purified only about 3-fold. The increase in specific activity of cytochrome c oxidase indicated some contamination with mitochondria membranes.

Electron micrographs (Figure 2.1) of these preparations showed mainly closed vesicles of varying size apparently from plasma membranes. Other recognizable structures such as intact mitochondria or contractile elements were not observed. In other photographs, it could be seen that the pelleted membranes were homogeneous from the top to the bottom of the pellet. The intravesicular volume of 1.8 microliters/mg protein was determined from uptake which was allowed to proceed for 90 min.

These preparations were capable of stereospecific transport, as expected for a carrier-mediated process. Shown in Figure 2.2 is a typical time-course of the trans-

Table 2.1

Specific activities of membrane marker enzymes

Enzyme	Preparation	
	Homogenate	Membranes
5'-nucleotidase	5.3 $\pm$.6(6)	49 $\pm$ 11(6)
phosphodiesterase	0.32 $\pm$.08(2)	1.0 $\pm$ 0.3(2)
ouabain-sensitive ATP'ase	6.24 (1)	57.8 (1)
glucose-6-phosphatase	1.6 $\pm$.8(2)	5.7 $\pm$ 1.7(2)
cytochrome c oxidase	0.49 $\pm$.17(2)	0.81 $\pm$.28(2)
(protein, mg/uterus)	15.6 $\pm$ 2.0(6)	0.39 $\pm$.15(6)

Specific activities are expressed as micromoles of product formed/h per mg protein except for cytochrome c oxidase, which is expressed as the change in absorbance at 550 nm/min per mg protein. Values are expressed as the mean $\pm$ SD (number of preparations). Membranes were collected at the interface between 0.5 M sucrose and 1.1 M sucrose.

Figure 2.1. Electron micrograph of uterine plasma membranes. Electron micrograph was prepared as described in the Materials and Methods section. The bar represents 500 nm.

•

Figure 2.2. Time-course of glucose transport by uterine membranes. Glucose transport was measured as described in the Materials and Methods section.

port of D-glucose. It is assumed that L-glucose uptake represents nonspecific uptake due to leakiness of the vesicular membrane while D-glucose uptake represents both nonspecific uptake and specific uptake mediated by a transport protein. Therefore the difference between the two curves (Figure 2.2) represents stereospecific, carrier-mediated, transport of D-glucose. By the end of a 90-min incubation, complete equilibration had been reached. The half-time of D-glucose transport was 20 ± 4 s. Membrane vesicles prepared from ovariectomized rats had similar transport properties.

These preparations were also capable of transporting glucose stereospecifically in both directions. Vesicles were loaded with both D- and L-glucose during a 90 min incubation and then diluted with buffer and filtered at various times after dilution. It can be seen in Figure 2.3 that the loss of D-glucose was more rapid than the loss of L-glucose having a half-time of 4-8 s.

Membranes prepared by these procedures behave according to the van't Hoff relationship in that the uptake of D-glucose was markedly reduced by increasing the osmolality of the medium. The line shown in Figure 2.4 extrapolated to zero indicating that there was no detectable binding of glucose to the membranes.

Shown in Figure 2.5 is the distribution of membrane markers on a discontinuous sucrose gradient which was

Figure 2.3. Efflux of D-glucose from previously-loaded membrane vesicles. Reaction mixtures identical to those described for the measurement of glucose uptake were allowed to incubate at 20 degrees Centigrade for 90 min, allowing D- and L-glucose to equilibrate in the vesicles. Three ml of Buffer 3 was added and the incubation was continued for the indicated time and filtered and washed 3 times with cold Stop Solution. Radioactivity was determined as described. D-glucose efflux is defined as pmol L-glucose retained by the filter minus pmol D-glucose retained by the filter. Values are positive indicating that efflux of D-glucose was more rapid than the efflux of L-glucose. Error bars represent the standard deviation of 3 separate determinations.

Figure 2.4. Effect of increasing osmolarity on glucose uptake. Medium osmolarity was adjusted with D-mannitol in Buffer 3. The line was fitted by linear regression with $r > 0.99$.

Figure 2.5. Fractionation of uterine membranes on a discontinuous sucrose gradient. Crude membranes were layered over a sucrose gradient and centrifuged at 41 000 rpm (286 000 x g) in a SW-41 swinging bucket rotor for 1 1/2 h. The gradient consisted of layers of 1.3 ml of each of 9 solutions of Buffer 1 which had sucrose concentrations ranging from 0.5 M to 1.3 M in increments of 0.1 M. In some experiments, no membranes were obtained at the interface between 0.5 M and 0.6 M. Turbid material at each interface was collected, diluted with Buffer 1 and centrifuged at 210 000 x g for 45 min. Pellets were resuspended in Buffer 3 and assayed as indicated. Specific activities of 5'-nucleotidase (open circles), glucose-6-phosphatase (closed circles) and D-glucose transport (open triangles) were measured.

prepared from a series of sucrose solutions in Buffer 1 ranging from 0.5 M at the top of the centrifuge tube to 1.33 M at the bottom of the tube. It can be seen that there was significant D-glucose transport into membranes associated with the same regions of the gradient in which the plasma membrane marker, 5'-nucleotidase, was found with little D-glucose transport into membranes associated with the endoplasmic reticulum marker. Thus for purposes of this study, discontinuous gradients were constructed which maximized yield of vesicles which had D-glucose uptake properties. By selecting slightly different sucrose solutions for the gradient, e.g., 0.6 M and 0.9 M, a 15-fold increase in the specific activity of 5'-nucleotidase was obtained with only a 2-fold increase in the specific activity of glucose-6-phosphatase. With this gradient there was an overall 4-fold increase in the specific activity of phosphodiesterase and an overall slight decrease in the specific activity of cytochrome c oxidase (not shown). In this case there was an additional 40 % decrease in the yield of plasma membranes. Longer centrifugation times did not improve the yield or purity of plasma membranes. Fifteen-fold purification is typical of such preparations (65).

D-glucose uptake was found to be saturable having a K_m value of 12.2 ± 1.1 mM, as shown in Figure 2.6.

Stereospecific uptake was inhibited by several known

Figure 2.6. D-glucose transport as a function of D-glucose concentration. The rate of D-glucose transport was measured as described in the Materials and Methods section. A K_m value of 12.2 ± 1.1 mM was determined computer fit as described.

inhibitors of glucose transport (Table 2.2) including phloretin (66), cytochalasin B (67) and by sulfhydryl reagents such as mercuric ion and N-ethyl maleimide (68). The inhibitions are underestimated due to the long incubation time.

The inhibition by cytochalasin B suggested the possibility of measuring the amount of transport protein from [3H]-cytochalasin B binding studies. As seen in Figures 2.7 and 2.8, the concentration of cytochalasin B required for 50 % inhibition of D-glucose transport by plasma membrane vesicles and uterine tissue was 1-3 micromolar. This value is about 10-fold higher than that usually measured in plasma membranes from other tissues (71). Measurement of the K_d of cytochalasin B binding which could be displaced by D-glucose was unsuccessful because of high amounts of nonspecifically bound cytochalasin B, even in the presence of cytochalasin E, which did not inhibit D-glucose transport. The data indicated that the value of the K_d was larger than 1 micromolar.

Discussion

Using the enzyme markers 5'-nucleotidase and glucose-6-phosphatase in conjunction with a series of discontinuous sucrose gradients (Fig. 2.5), I was able to determine the gradient shelf from which to collect membranes in such a way as to maximize yield of plasma membranes versus purity

Table 2.2

Inhibitors of glucose transport in
uterine plasma membranes

Additions	Concentration, (mM)	% Control
none	-----	(100)
N-ethyl maleimide	15	15
phloretin	0.5	20
cytochalasin B	0.005	21
mercuric ion	1.0	25

Inhibitors were added to the membranes 15 min before addition of radiolabel. The complete reaction mixture was incubated for 2 min prior to filtration.

Figure 2.7. Inhibition of D-glucose transport in membranes by cytochalasin B. D-Glucose was measured after a 7 s incubation as described.

Figure 2.8. Effect of cytochalasin B on the transport of 2-deoxyglucose in intact uteri. One quarter uteri were incubated with $[3H]$ 2-deoxyglucose for 30 min. After incubation, uteri were ground in 5 % TCA and 2-deoxyglucose-6-phosphate was collected on anion exchange columns, eluted with 1 N HCl and assayed by liquid scintillation counting. The open square represents the effect of cytochalasin E on 2-deoxyglucose transport.

(absence of endoplasmic reticulum). The separation of plasma membranes from endoplasmic reticulum is important especially for studies attempting to quantitate the glucose transporter protein in plasma membranes subsequent to perturbation of the rate of glucose transport by estradiol and other factors. Endoplasmic reticulum contains glucose transporter protein and therefore presents a potential source of contamination.

Glucose transport into uterus occurs through a system of facilitated diffusion, since it is stereospecific (Fig. 2.2), saturable (Fig. 2.6), and displays bidirectional transport (Fig. 2.3). Phloretin, cytochalasin B and sulfhydryl reagents inhibit glucose transport into plasma membranes (Table 2.2), indicating the presence of a carrier-mediated transport process. These data indicate that the glucose transport system in uterus is similar to the systems isolated from muscle (45), adipocytes (69), erythrocytes (70), etc.

CHAPTER 3

CHARACTERIZATION OF ESTRADIOL STIMULATION OF GLUCOSE TRANSPORT

Introduction

Estradiol stimulates glucose transport in the uterus (7,28,52) and the maximum stimulation of transport by estradiol has been reported to occur by 2 h (28). In this chapter, the time-course and dose-response relationship of estradiol to glucose transport was determined. The time required for estradiol to stimulate the maximal increase in glucose transport will be compared with the time required by other hormones, providing information useful in determining the mechanism of estradiol action. The dose-response relationship of estradiol to glucose transport will provide information relating to physiological versus pharmacological action, thus providing information relating to the physiological significance of this system.

As a further criterion for physiological significance, the estradiol-mediated increase in glucose transport should be steroid specific. Consequently, the effect of other steroid hormones on glucose transport will be reported in this chapter.

If the estradiol relationship to glucose transport is physiologically significant, then the rate of glucose transport in rat uterus should reflect the plasma concen-

tration of estradiol. Therefore, the relationship between glucose transport in the uterus and plasma concentration of estradiol will be reported.

Estradiol could increase glucose transport by a number of mechanisms including through increased synthesis of transporter proteins. To investigate this possibility, the effect of the protein synthesis inhibitors cycloheximide and emetine on estradiol stimulation of glucose transport was determined.

Actinomycin D, an inhibitor of RNA synthesis, has been shown to block the estradiol-mediated increase in glucose oxidation (72). In order to determine the role of RNA synthesis on glucose transport, actinomycin D was administered in vivo before estradiol administration.

The effect of estradiol on the kinetic parameters K_m and V_{max} of glucose transport was determined. These results will provide information relating to the mechanism by which glucose transport is increased.

Materials and Methods

Materials

D-[1- ^{14}C]glucose-6-phosphate (225 mCi/mmol) and L-[4,5- 3H]leucine (58 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. D-2-[G- 3H]deoxyglucose (8.3 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. Cycloheximide, emetine, estradiol, estriol,

estrone, progesterone, dihydrotestosterone, and cytochalasins B and E were purchased from Sigma Chemical Co., St. Louis, Mo. Other reagents were obtained at the highest purity available.

Buffers

Buffers for assays with vesicles are the same as described in Chapter 2.

Membrane Preparation

Plasma membrane vesicles were prepared as described in Chapter 2.

Transport Assays

Transport assays with vesicles are as described in Chapter 2.

Glucose transport in intact uterus was estimated by measuring the amount of 2-deoxyglucose-6-phosphate formed during incubation of uterine tissue with 2 ml of 0.1 mM D-2-[3H]deoxyglucose, specific activity 1.76 dpm/pmol, in 0.15 M NaCl and 0.02 M potassium phosphate buffer at pH 7.0. Cytochalasin B was added to certain incubations as indicated. Tissue was removed, rinsed twice with the incubation buffer without 2-deoxyglucose and homogenized with 2 ml of 5 % trichloroacetic acid with a Polytron homogenizer. Approximately 3000 dpm of D-[14C]glucose-6-phosphate were added just before homogenization as a re-

covery indicator. The mixture was centrifuged at 15 000 x g for 15 min. The supernatant solution was extracted 3 times with ethyl ether (to remove trichloroacetic acid). The aqueous layer was then passed through a 0.7 x 1.2 cm column containing AG 1-X2, 200-400 mesh (BioRad), which had been prepared by washing with several ml of 1 N HCl, followed by several ml of water. The sample was followed by 3 1-ml aliquots of water. Radioactive 2-deoxyglucose-6-phosphate was eluted with 2 1-ml aliquots of 1 N HCl. The eluates were combined and added to 15 ml of BetaPhase scintillation fluid. Tritium dpm values were corrected to 100 % recovery of D-[¹⁴C]glucose-6-phosphate.

Cycling of Intact Rats

The estrus cycle stages of individual rats were determined for a week or more. Vaginal cell samples were obtained with a eye dropper and the estrus cycle stages of the animals were determined according to the major cell type or ratio of two major cell types present in the samples. Cell types were cornified (estrus), lymphocytes along with less numbers of nucleated cells (diestrus 1), nucleated cells along with less numbers of lymphocytes (diestrus 2), and nucleated cells (proestrus).

Injections In Vivo

Intraluminal injections were as follows: Surgery was performed on ovariectomized rats under ether anesthesia.

A vertical incision was made on the dorsal side of the rat through the skin. Blunt-edged tweezers were used to pull the uterine fat pad through the opening. The uterine horn end is located in this fat pad. The uterine horn end was held steady by holding the fat pad and a 10 microliter phosphate-buffered saline solution containing the appropriate compound was injected via a 26 gauge needle into the lumen.

Intraperitoneal injections were performed as follows: Compounds (estradiol, estrone, cycloheximide, etc.) were injected via a 26 gauge needle into the peritoneal cavity. Compounds were dissolved in 0.155 M NaCl.

Results

Uterine plasma membranes from control and estradiol-treated rats were prepared by centrifugation on a discontinuous sucrose gradient consisting of 0.5 M and 1.0 M sucrose. Since differences in the intravesicular volumes between vesicles from control and estradiol-treated rats would complicate data interpretation, the intravesicular volume was determined in vesicles with and without prior estradiol treatment (Table 3.1). Since no difference in intravesicular volume/mg protein was noted, I believe that the differences in glucose transport in the vesicles could be attributed to differences in either the activity

Table 3.1

Effect of estradiol on vesicular volume

Hours After Estradiol Administration	Vesicular Volume (microliters/mg protein)
0	1.84 $\pm$ 0.07
2	1.89 $\pm$ 0.06
4	1.89 $\pm$ 0.07
6	1.86 $\pm$ 0.01

D- and L-glucose were incubated with vesicles at 20 degrees Centigrade for 90 min, allowing equilibration of the label to occur between the buffer and the vesicles. After 90 min, the vesicles were collected on Millipore filters (HAWP) and the radioactivity was determined via liquid scintillation counting. Volumes of the vesicles were determined from the original cpm/microliter of the incubation buffer. Values are expressed as the mean $\pm$ standard deviation where n = 7.

of the transporter or an increase in the number of transporters.

Shown in Figure 3.1 is the effect of increasing amounts of estradiol administered in vivo on the rate of glucose transport in plasma membrane vesicles. An increase in the rate of glucose transport over controls is first seen at 1 ng and a near maximal transport rate at 1 000 ng estradiol.

It is important to measure phosphorylation of 2-deoxyglucose in the uterus during the time when incorporation is linear. It was found that the incorporation of phosphate into 2-deoxyglucose is linear up to 30 min (data not shown). All assays involving 2-deoxyglucose were incubated for 20 or 30 min.

In order to relate the estradiol-mediated increase in glucose transport in ovariectomized rats to the normal in vivo situation, the rates of 2-deoxyglucose transport were determined in the whole tissue isolated from rats in various phases of the reproductive cycle (Table 3.2). The rate of 2-deoxyglucose transport into the uterus was highest at proestrus. The rate of transport decreased in estrus and diestrus 1. In diestrus 2 the rate of 2-deoxyglucose transport began to increase again, this rise apparently preceding the high rate seen in proestrus.

The maximal increase in the rate of glucose transport is seen between 1 and 2 h after estradiol ad-

Figure 3.1. Effect of estradiol amounts on the increase in the rate of glucose transport. Estradiol was injected intraperitoneally into ovariectomized rats. Three hours later rats were killed and the uteri were removed and homogenized. Plasma membranes were collected on a 0.5/1.0 M sucrose interface. Plasma membrane vesicles (0.05 mg) were incubated with label for 30 s. The vesicles were collected on Millipore filters and radioactivity was determined by liquid scintillation counting.

Table 3.2

Glucose transport at different stages in
the reproductive cycle

Stage	2-Deoxyglucose Transport (pmol/30 min/mg DNA)
Diestrus 1	3700 $\pm$ 660
Diestrus 2	5240 $\pm$ 810
Proestrus	7910 $\pm$ 202
Estrus	5480 $\pm$ 380

Uterine tissue (1/4 uterus per assay) was incubated with labeled 2-deoxyglucose (0.1mM) for 30 min at 37 degrees Centigrade. After 30 min, the tissue was homogenized in 5 % TCA and the 2-deoxyglucose-6-phosphate was recovered from anion exchange columns and counted by liquid scintillation counting. The results are expressed as the mean $\pm$ standard deviation where n = 4.

ministration as measured in plasma membrane vesicles (Figure 3.2). The maximal response is stable up to 4 h after estradiol administration. Measuring 2-deoxyglucose phosphorylation in uterine tissue from starved rats, a maximal response is not detected until 3 h after estradiol administration (Figure 3.3). The reason for the delay in the increase in glucose transport is not known. However, the discrepancy may be related to the starved state of the animals. Animals were starved in the course of insulin studies (see Chapter 5).

If the increase in the rate of glucose influx into vesicles is increased as a result of estradiol administration, it was reasoned that the rate of efflux should be affected as well. In Figure 3.4 it is shown that the rate of efflux from vesicles isolated from rats treated with estradiol was higher than from vesicles isolated from control rats.

Steroid specificity in the stimulation of glucose transport was examined using progesterone, dihydrotestosterone, estriol, and estrone (Table 3.3). Progesterone and dihydrotestosterone had no effect on the rate of glucose transport into vesicles, while estriol and estrone were as effective as estradiol in stimulating the rate of glucose transport.

In Figure 3.5 it is shown that cycloheximide administration has no effect on estradiol's ability to stimulate

Figure 3.2. Time-course of the estradiol-mediated increase in glucose transport in plasma membrane vesicles. Ten micrograms of estradiol was injected intraperitoneally into ovariectomized rats. Plasma membrane vesicles were prepared and incubated with radiolabel. After 30 s incubation, vesicles were collected on Millipore filters and radioactivity was determined by liquid scintillation counting.

Figure 3.3. Time-course of the estradiol-mediated increase in 2-deoxyglucose uptake in intact uterus. Ten micrograms of estradiol was injected intraperitoneally into ovariectomized, 24 h-starved rats. Whole uteri were incubated with 0.1 mM [3H]2-deoxyglucose for 20 min. [3H]2-deoxyglucose-6-phosphate was collected on anion exchange columns and eluted with 1 N HCl. Radioactivity was determined by liquid scintillation counting.

Figure 3.4. Time-course of glucose efflux from plasma membrane vesicles. Membranes were preloaded with D-glucose and D-mannitol for 90 min, allowing equilibration of both compounds to occur. Three ml of buffer (20 degrees Centigrade) was added and the incubation was continued for the indicated time. Vesicles were collected on Millipore filters and radioactivity was determined by liquid scintillation counting. Open circles represent glucose transport in uteri from rats treated with estradiol. Closed circles represent glucose transport in uteri from untreated rats.

Table 3.3

Specificity of steroid hormone stimulation of
glucose transport in vesicles

Treatment	Glucose Transport (pmol/min/mg protein)
none	10.9 $\pm$ 1.6
progesterone	11.1 $\pm$ 0.9
dihydrotestosterone	11.7 $\pm$ 0.8
estrone	19.6 $\pm$ 3.1
estradiol	17.8 $\pm$ 1.5
estriol	19.3 $\pm$ 1.9

Plasma membrane vesicles (55 micrograms) were incubated with radiolabel for 30 min. The vesicles were collected on HAWP Millipore filters. The filters were dissolved in methylcellosolve and the entrapped radiolabel was assayed by liquid scintillation counting. The values for each treatment are expressed as the mean $\pm$ standard deviation where n = 3.

Figure 3.5. Effect of cycloheximide on glucose transport in plasma membrane vesicles. Varying amounts of cycloheximide were injected intraperitoneally. After 3 h, the rats were killed and plasma membranes were obtained. Vesicles (0.05 mg protein) were incubated with glucose for 30 s and the membranes were collected on Millipore filters (HAWP). Radioactivity on the filters was determined by liquid scintillation counting.

rates of glucose transport at any concentration while higher amounts of cycloheximide results in an increase in the rate of glucose transport in vesicles isolated from animals not treated with estradiol. Similar results can be seen with 2-deoxyglucose transport in tissue (Figure 3.6). The effect of cycloheximide on protein synthesis is seen in Figure 3.6. This is a lower estimate of protein synthesis inhibition since leucine incorporation was assayed 3 h after cycloheximide administration.

It is possible that cycloheximide may increase the basal rate of glucose transport by nonspecific effects not related to the inhibition of protein synthesis. Therefore, the effect of emetine on glucose transport was investigated. It can be seen in Table 3.4 that the basal rate of glucose transport was increased with emetine and that the rate of glucose transport with estradiol was unchanged by emetine. At 12.5 mg emetine/rat, protein synthesis was inhibited by more than 90 % (889 ± 592 cpm for control leucine incorporation vs 37 ± 12 cpm for emetine-treated leucine incorporation; $n = 3$ for both groups). Leucine incorporation was determined 3 h after emetine administration.

In order to determine the role of RNA synthesis in basal and estradiol-induced glucose transport, 20 micrograms of actinomycin D was injected into the left uterine horn. The amount of actinomycin D injected has

Figure 3.6. Effect of cycloheximide on protein synthesis and 2-deoxyglucose transport. Varying amounts of cycloheximide were injected subcutaneously. Leucine was injected into the lumen of the uterus. For leucine incorporation, the uteri were ground in 5 % TCA and the radioactivity in the NaOH-dissolved pellet was determined by liquid scintillation counting. For 2-deoxyglucose transport, uteri were removed and incubated in 0.1 mM $[3H]2$ -deoxyglucose. After 30 min, the uteri were ground in 75 % isopropyl alcohol and the $[3H]2$ -deoxyglucose-6-phosphate was collected on anion exchange columns, eluted with 1 N HCl and assayed by liquid scintillation counting.

Table 3.4

Effect of emetine on glucose transport

Treatment	Glucose Transport (fmol/7 sec/mg protein)
none	455 $\pm$ 242
estradiol	1067 $\pm$ 57
emetine	1366 $\pm$ 275
estradiol + emetine	1391 $\pm$ 153

Plasma membrane vesicles (68 micrograms) were incubated with radiolabel for 7 sec. Vesicles were trapped on Millipore filters (600 nm). The filters were dissolved in methlycellosolve and radioactivity was determined by liquid scintillation counting. Values for each treatment is expressed as the mean $\pm$ standard deviation where n = 3.

been previously shown to inhibit RNA synthesis (73). Thirty minutes after actinomycin D administration, estradiol (10 micrograms) was injected intraperitoneally. After 4 h 30 min, animals were killed and the uteruses removed and cut into halves. In Table 3.5 it can be seen that actinomycin D raised the basal rate of glucose transport but did not affect the estradiol-mediated increase in glucose transport. These results suggest that the putative inhibitor of glucose transport requires continual RNA synthesis in addition to continual protein synthesis. Again, as stated for the protein synthesis inhibitors, no clear interpretation of the data is possible in this in vivo system.

The kinetic parameters K_m and V_{max} were determined by measuring uptake and phosphorylation of 2-deoxyglucose into whole uterine tissue (Figure 3.7). The V_{max} of the influx of 2-deoxyglucose into the tissue from control animals was 30.0 ± 4.2 nmol 2-deoxyglucose/20 min/microgram DNA while the V_{max} of the influx into the tissue from estradiol-treated rats was 90.7 ± 7.5 nmol 2-deoxyglucose/20 min/microgram DNA. 2-Deoxyglucose influx was saturable in both control and estradiol-treated tissues. The K_m value for the controls was 4.6 ± 2.1 mM while the K_m value for the experimental group was 3.4 ± 1.0 mM.

Table 3.5

Effect of actinomycin D on glucose transport

Treatment	2-Deoxyglucose Transport (cpm/30 min/half uterus)
none	2225 $\pm$ 234
estradiol	3649 $\pm$ 597
actinomycin D	2699 $\pm$ 336
actinomycin D + estradiol	3485 $\pm$ 652

Neumann-Keul's test for significance employed:

none vs actinomycin D: $p < 0.05$

actinomycin D vs actinomycin D + estradiol: $p < 0.01$

estradiol vs actinomycin D + estradiol: not significant.

The values for each treatment is expressed as the mean $\pm$ standard deviation where $n = 12$.

Figure 3.7. 2-Deoxyglucose transport as a function of 2-deoxyglucose concentration in uterine tissue previously or not exposed to estradiol. 2-Deoxyglucose transport was measured by incubating intact uteri with 0.1 mM $[3H]$ 2-deoxyglucose for 20 min. After 20 min, uteri previously treated with estradiol in vivo (open circles) or control uteri (closed circles) were ground in 5 % TCA and $[3H]$ 2-deoxyglucose-6-phosphate was collected on anion exchange columns, eluted with 1 N HCl and assayed by liquid scintillation counting.

Discussion

The relationship between estradiol and glucose transport has been shown to be physiologically relevant since rates of glucose transport in uteruses from rats grouped in stages of the estrus cycle corresponded to plasma estradiol concentrations for each stage as reported in the literature (72). For example, the highest rate of glucose transport is seen in uteruses from rats in proestrus which has the highest concentration of plasma estradiol (approx. 150 pM) and the lowest rate of glucose transport is seen in uteruses from rats in diestrus 1 which has the lowest concentration of plasma estradiol (approx. 40 pM).

The amount of estradiol (10 ng) required to elicit approximately 50 % of the increase in glucose transport (Figure 3.1) is reasonably close to the K_d of binding of estradiol to the receptor (0.1-1.0 nM). That is, with the assumption that a 250 g rat has a volume of 100 ml and that the injected estradiol distributes evenly throughout the animal without enzymic alteration, then the serum concentration of 10 ng estradiol would be approximately 0.4 nM, a value near the K_d for the binding of estradiol to the receptor and near the value of the concentration of estradiol in blood during proestrus. Since the amount of estradiol used to elicit a 50 % increase in the rate of glucose transport is near physiological concentrations, then it is reasonable to assume that the response to

estradiol in this system is physiological and not pharmacological.

A physiological system under specific hormonal control should not be affected by hormones having different regulatory functions. Glucose transport rates are increased by estrogens but not by progesterone or dihydrotestosterone. These data confirm the specificity and therefore the physiological relevance of the estradiol-mediated increase in glucose transport in rat uterus.

With the physiological significance established, I have attempted to determine if certain mechanistic features of the estradiol stimulation of glucose transport compare with other well-studied systems which regulate glucose transport.

The time-course of the increase in the rate of glucose transport (Figure 3.2) is similar to the time-courses of epidermal growth factor (74) and tumor growth factor (75) in that 30 min or more is required to reach a maximal rate in glucose transport. In contrast, the insulin-induced increase in the rate of glucose transport requires only 10-15 min to achieve the maximal rate. The mechanism by which insulin increases glucose transport in adipocytes is at least partly through the translocation of preformed transporters from the endoplasmic reticulum to the plasma membrane (32). Epidermal growth factor and

tumor growth factor are believed to induce an increase in glucose transport via the modulation of protein kinase activity (75). Other, specific features of the mechanism by which estradiol increases the rate of glucose transport in rat uterus is discussed in Chapter 6.

To determine if estradiol increases glucose transport in the uterus by initiating specific protein synthesis, varying amounts of the protein synthesis inhibitors cycloheximide and emetine were injected into rats prior to estradiol administration. The data show that inhibition of protein synthesis results in an increase in the basal rate of glucose transport, suggesting that the basal rate is under control of an inhibitor protein having a relatively short half-life. In other words, once the synthesis of the putative inhibitor is blocked, the rate of glucose transport increases. In regard to the effect of cycloheximide on the estradiol-induced increase in glucose transport, the results are inconclusive due to the inability to adequately manipulate the whole animal model.

In order to determine if estradiol increases glucose transport through a process requiring RNA synthesis, the effect of actinomycin D on basal rates of glucose transport and on the estradiol-induced increase in the rate of glucose transport was examined. Actinomycin D, like cycloheximide, raised the basal rate of glucose transport. Furthermore, actinomycin D did not prevent the increase in

the rate of glucose transport by estradiol. These results suggest that the basal rate of glucose transport is under negative control and that the putative glucose transport inhibitor turns over rapidly, thus requiring continual mRNA synthesis. In addition, estradiol does not increase the rate of glucose transport through a process requiring mRNA transcription since estradiol stimulated the rate of glucose transport in the presence of actinomycin D.

Various mechanisms operating at the plasma membrane can be postulated to explain how estradiol mediates the increase in the rate of glucose transport. One approach to provide information on the mechanism is seen in the determination of the kinetic parameters of glucose transport. While a small decrease was seen in the K_m of glucose transport, the difference in V_{max} is sufficient to explain the increase in glucose transport. The increase in V_{max} of glucose transport can be explained by either an increase in the number of transporter proteins in the plasma membrane or by an increase in the intrinsic rate of the glucose transport process. Data resolving these two mechanisms can be found in Chapter 6.

CHAPTER 4

GLUCOSE TRANSPORT AND ESTROGEN RECEPTORS

Introduction

The generally accepted mechanism of steroid hormone action was first proposed by Jensen (12). This model involves the diffusion of steroid through the plasma membrane into the cytoplasm where it binds to a soluble receptor. The existence of a soluble cytoplasmic receptor has recently been questioned by Welshons et al. (13) and King and Greene (14) who show data that suggest that this steroid receptor may reside in the nucleus. Although the existence of a cytoplasmic receptor is under question, it is agreed that the binding of the estradiol-receptor complex to the chromatin initiates changes in mRNA transcription.

In addition to the type I receptor mentioned above, Best-Belsomme et al. first described a second estrogen binding protein (20). Clark et al. determined the K_d of binding of this protein to estradiol to be 33 nM (21), which is larger than the K_d of binding for the Type I receptor (0.8 nM). This estrogen binding protein (Type II) is believed by some to be artifactual contamination (22,23). Clark et al. suggest that the Type II sites may be extracellular binding proteins that help to accumulate estrogens. It is also possible that Type II sites are a precursor form of the Type I site and as such represent a

cytoplasmic reservoir of Type I sites (21). The Type II site also differs from the Type I site in that it is not found in the nucleus. Finally, two types of nuclear estrogen binding sites (Type I and Type II) have been characterized. The nuclear Type I site is believed to be identical to the cytosol Type I receptor. After estradiol administration, the nuclear Type I increases corresponding to a depletion in the cytosol Type I site. The nuclear Type I site is believed to be involved in the regulation of specific mRNA transcription while the function of the Type II nuclear binding site is unknown. The nuclear Type II site is believed to be different than the cytosol Type II site. This site has been reported to display a sigmoidal binding curve with estradiol.

In this chapter, the effect of Type I receptor occupancy and translocation (or nuclear retention of receptors during homogenization) by antiestrogens on the ability of estradiol to stimulate glucose transport was examined. These experiments will determine: (i) if the cytoplasmic location of the receptor is necessary for estradiol to increase glucose transport, and (ii) what effect prior blocking of the estradiol binding site with antiestrogens will have on the ability of estradiol to increase glucose transport. In addition, the relative affinity of binding of estradiol, nafoxidine, and tamoxifen to the microsomal receptor was investigated. The relative binding of

estradiol and the antiestrogens were done in light of a report by Parikh et al. (76) who showed that tamoxifen has approximately 1500-fold less affinity for a microsomal binding site than estradiol. This microsomal binding site is a potential mediator of estradiol stimulation of glucose transport.

Materials and Methods

Materials

D-2[G-3H]Deoxyglucose (8.3 Ci/mmol) was purchased from New England Nuclear, Boston, Massachusetts. [6,7-3H]estradiol was purchased from Amersham Corporation, Arlington Heights, Illinois. Nafoxidine, tamoxifen and estradiol were purchased from Sigma Chemical Co., St. Louis, Missouri. Other reagents were obtained at the highest purity available.

Buffers

Buffer 1 consisted of 10 mM Tris, pH 7.5 and 0.1 mM EDTA. Buffer 2 consisted of 10 mM Tris, pH 7.5, 150 mM KCl, 2.5 mM magnesium chloride and 250 mM sucrose.

Estradiol Receptor Preparation

Cytosolic receptor supernatant was prepared by grinding tissue in Buffer 1 and centrifuging the homogenate at 50 K (210 000 x g) for 90 min. Microsomal membranes were prepared by homogenizing the tissue in Buffer 2 and cen-

trifuging 10 K (12 000 x g) for 15 min followed by centrifuging at 50 K (210 000 x g) for 90 min. The resulting membranes were resuspended in Buffer 2.

Estradiol-Receptor Binding Assay

Estrogen binding to receptor was accomplished through the dextran-coated charcoal method. For total receptor assay, cytosol (usually 0.2 uterus) in centrifuge tubes was incubated with 1 microliter of 1000 nM [3H]estradiol with or without 1 microliter 100-fold excess nonradioactive estradiol. After 1.5 h incubation at 30 degrees Centigrade, tubes were put on ice for 20 min. Then, 0.02 ml dextran-charcoal (0.5 % dextran, 5 % charcoal) was added for 2 min. The tubes were spun in a Fisher microfuge for 10 min (approximately 12 000 x g). One hundred sixty five microliters of supernatant solution was mixed with 10 ml of Betaphase counting fluid and radioactivity was determined by liquid scintillation counting. Unfilled receptor sites were assayed using the above procedure except all incubations were done at zero degrees Centigrade.

Results

In vivo treatment of tamoxifen or nafoxidine is known to result in occupation of estradiol binding sites and in either translocation of the cytosol receptor to the nucleus or nuclear retention of the receptor during homo-

genization (26). To determine if the occupation of the cytoplasmic Type I estradiol binding sites by these compounds would inhibit the estradiol-mediated increase in glucose transport in the uterus, the antiestrogens were injected into rats prior to estradiol administration. In addition, this experiment was designed to determine if the presence of the receptor in the cytoplasm is required for the induction in the rate of glucose transport. In Table 4.1 it is shown that 1 mg of either antiestrogen results in an 85-90 % decrease in total binding sites in the cytosol. In spite of the dramatic decrease in these binding sites, the increase in glucose transport as a result of estradiol administration is not inhibited by nafoxidine (Table 4.2). In addition, the increase in glucose transport occurs although 72 % of the remaining receptors in the cytoplasm are filled by nafoxidine.

The fact that a 90 % decrease in cytoplasmic Type I sites by tamoxifen did not result in a decrease in the estradiol-mediated increase in glucose transport and the fact that tamoxifen had a 1500-fold less affinity for the microsomal receptor than estradiol in calf uterus (76) suggested the possible role of the microsomal estradiol receptor as the site mediating the increase in glucose transport. Demonstrating the existence of a microsomal receptor having a 1500-fold less affinity for the antiestrogens than estradiol would provide a receptor candi-

Table 4.1

Effect of antiestrogens on translocation of and estradiol occupancy of the cytoplasmic receptor

Treatment	Total sites	Unfilled sites
	cpm/uterus	
none	17 670	18 980
nafoxidine	1 953	547
tamoxifen	2 847	53

One mg of tamoxifen and nafoxidine was injected subcutaneously into ovariectomized rats 16 h before killing. Cytosol (0.2-0.45 mg protein) was incubated with 1 micromolar [3H]estradiol with and without a 100-fold excess of nonradioactive estradiol. The difference of radioactivity between binding with and without excess nonradioactive estradiol is reported above. Total sites refers to specific binding of [3H]estradiol at 30 degrees Centigrade while Unfilled sites refers to specific binding of [3H]estradiol at zero degrees Centigrade. The variation within assay was < 5 %.

Table 4.2

Effect of antiestrogens on estrogen stimulation
of 2-deoxyglucose transport

Treatment	2-Deoxyglucose Transport % Control
none	100 $\pm$ 12
estradiol	217 $\pm$ 30
nafoxidine	143 $\pm$ 17
estradiol + nafoxidine	194 $\pm$ 19

One mg of nafoxidine was injected subcutaneously into ovariectomized rats 13 h before estradiol administration (0.010 mg). 2-Deoxyglucose transport was assayed as previously described. The values for each treatment is expressed as the mean $\pm$ standard deviation where n = 2.

date that could mediate the increase in glucose transport in the presence of antiestrogens.

Before assaying the relative affinity of tamoxifen and estradiol to the microsomal receptor, the estradiol-microsomal receptor binding conditions were characterized. In Figure 4.1 the time-course of estradiol binding to the microsomal receptor at 22 degrees Centigrade is shown to be essentially complete between 1 and 2 h.

At a low concentration range of estradiol (0.1-5 nM), rat uterine microsomal membranes exhibited one estradiol binding site having a K_d of approximately 0.5 nM which is similar to the cytosolic K_d (data not shown). In order to determine if site(s) with less affinity for estradiol were present, high concentrations of estradiol were utilized. Figure 4.2 shows binding to rat uterine microsomes as a function of high estradiol concentrations at 22 degrees Centigrade. At least two microsomal estradiol binding sites are evident in the Scatchard plot seen in Figure 4.3. The different binding sites could represent a mixture of functional estradiol receptors and nascent receptors being formed on polysomes.

Having characterized the binding of estradiol to various cellular receptors, the relative affinity of estradiol and the antiestrogens were determined in cytosol and microsomal membranes. In Figure 4.4 it can be seen that tamoxifen and nafoxidine show similar abilities to

Figure 4.1. Time-course of [3H]estradiol binding to microsomes. One hundred microgram quantities of rat uterine membranes were incubated with varying amounts of [3H]estradiol with or without a 100-fold excess of non-radioactive estradiol. The difference between total and nonspecific binding is plotted above.

Figure 4.2. Saturation of $[^3\text{H}]$ estradiol binding to rat uterine microsomes. One hundred fifteen microgram quantities of microsomal membranes were incubated with varying amounts of $[^3\text{H}]$ estradiol with or without a 100-fold excess of nonradioactive estradiol. Both total and nonspecific binding is plotted above.

Figure 4.3. Scatchard plot derived from the data shown in Figure 4.2.

Figure 4.4. Competition of [3H]estradiol binding to rat uterine microsomes with estradiol and antiestrogens. [3H]Estradiol (0.5 nM) and estradiol (open squares), nafoxidine (closed circles), or tamoxifen (open circles) were incubated simultaneously with membranes (0.11 mg) for 4 h at 22 degrees Centigrade. The 100 % binding corresponds to 530 cpm.

displace low amounts of [3H]estradiol from the microsomal receptor which is approximately 200-fold less than estradiol. Tamoxifen and nafoxidine have similar affinities to the cytoplasmic receptor which is approximately 100-fold less than the affinity of estradiol to the cytoplasmic receptor (Figure 4.5). The affinities of estradiol, tamoxifen and nafoxidine for the cytoplasmic and the high affinity form of the microsomal receptor are similar. This finding, in conjunction with the finding that the dissociation constants are the same between the cytoplasmic and the high affinity microsomal receptor suggests that these receptors may be the same protein.

The microsomal receptor does not translocate into the nucleus after estradiol administration (Table 4.3). Therefore, it was considered possible that binding of estradiol to the microsomal receptor could have local effects resulting in the translocation of microsomal-bound glucose transporters to the plasma membrane. To test this hypothesis, an attempt to correlate the degree of estradiol binding to the microsomal receptor and the increase in glucose transport was attempted. Unfortunately, this experiment turned out to be unfeasible since receptor-bound [3H]estradiol readily exchanged with nonradioactive estradiol at zero degrees Centigrade (Fig. 4.6). Therefore, significant amounts of estradiol bound to the microsomal receptor would be lost during the homogenization

Figure 4.5. Competition of [3H]estradiol binding to rat uterine cytosol with estradiol and antiestrogens. [3H]estradiol (0.05 nM) and estradiol (open squares), tamoxifen (open circles), or nafoxidine (closed circles) were incubated simultaneously with cytosol (0.075 mg protein) for 4 h at 22 degrees Centigrade. The 100 % binding refers to 657 cpm.

Table 4.3
Effect of estradiol on microsomal
receptor number

Treatment	Specific Estradiol Binding cpm/uterus
none	1666 $\pm$ 186
estradiol	2253 $\pm$ 421

Values between control and experimental are not significantly different at $p > 0.1$. Estradiol binding was determined by incubation of 0.22 mg microsomal protein with 100 nM [3H]estradiol with or without 100-fold excess diethylstilbestrol (estradiol is not soluble in water at 0.01 mM). The values for each treatment is expressed as the mean $\pm$ standard deviation where $n = 3$.

Figure 4.6. Exchange of [3H]estradiol from microsomes with nonradioactive estradiol. [3H]Estradiol was incubated with microsomes (0.080 mg protein) for 1 h 15 min at 22 degrees Centigrade in 0.1 ml buffer. At the end of the incubation time, 1 ml ice-cold buffer (excess nonradioactive estradiol) was added. Aliquots at various times were removed and radioactivity was determined. The 100 % binding refers to 3200 cpm.

procedure. Furthermore, accurate measurement of occupied sites would not be possible since exchange occurs at zero degrees Centigrade.

Discussion

Estradiol stimulates glucose transport maximally in rat uterus even in the presence of nafoxidine at concentrations that occupy 75-86 % of the estradiol receptor. This statement is derived from the following argument: One milligram nafoxidine and 1 microgram of estradiol injected into ovariectomized rats results in a 1000-fold excess of nafoxidine to estradiol. These amounts of nafoxidine and estradiol are each in excess of the value of the K_d for each compound, thus predicting saturation of the estradiol receptor. Since estradiol has a 100 to 200-fold higher affinity for the receptor than nafoxidine and using the relationship, $K_e/K_n = [\text{estradiol}]/[\text{nafoxidine}] \times [\text{N-R}]/[\text{E-R}]$ where K_e = dissociation constant for estradiol binding, K_n = dissociation constant for nafoxidine binding, $[\]$ = molar concentration of estradiol, nafoxidine, N-R (nafoxidine-receptor complex), and E-R (estradiol-receptor complex), it can be calculated that estradiol bound approximately 14-25 % of the receptor. The result of this calculation is supported by the fact that the 72 % of the cytosolic receptors are bound in animals treated with nafoxidine.

These results open the possibility of "spare" receptors similar to that seen with peptide hormone receptors located in the plasma membrane, e.g., insulin receptors (77). Furthermore, these results are similar to that of Anderson et al. (78) who showed that less than 50 % of nuclear receptor binding by estradiol was sufficient to effect a maximum increase in glucose oxidation in rat uteri.

The lack of a 1-to-1 correspondence in estradiol receptor occupancy to glucose transport rates and glucose oxidation also raises the possibility that a receptor, different from the classical receptor, exists having these properties: (i) low affinity for nafoxidine, (ii) higher affinity for estradiol than the classical estradiol receptor, and (iii) high estradiol dissociation rate. The latter property would prevent it from measurement by the assay used for the estradiol receptor; i.e., the dextran-coated charcoal method.

CHAPTER 5

INSULIN AND GLUCOSE TRANSPORT

Introduction

One of the major actions of insulin is to promote glucose metabolism. An early event (within minutes) of insulin action is the increase in the rate of glucose transport (79). A number of laboratories have shown that insulin increases glucose transport by increasing the number of functional transporters in the plasma membrane without affecting the K_m of glucose binding to the transporter (80,81). Recently, Whitesell and Abumrad reported that insulin results predominantly in an increased affinity of glucose for the transporter in the adipocyte (82), although an increase in the V_{max} is also reported.

Since insulin is known to stimulate the rate of glucose transport in various tissues, experiments were designed to demonstrate whether or not insulin stimulated the rate of glucose transport in the uterus and whether insulin and estradiol shared common pathways. In addition, experiments were done to determine whether or not estradiol affected changes in the binding of insulin to the insulin receptor. Since estradiol has been shown to induce luteinizing hormone receptors in rat ovaries (83), the possibility exists that estradiol increases glucose

transport in the uterus by increasing the number of insulin receptors in rat uterine plasma membranes.

Materials and Methods

Materials

[125I] porcine insulin (100 mCi/mg) was purchased from ICN Pharmaceuticals, Inc., Irvine, California. The insulin radioimmunoassay kit was purchased from Amersham Corporation, Arlington Heights, Illinois. The glucose assay kit was purchased from Sigma Chemical Co., St. Louis, Missouri. All other reagents were purchased as previously described.

Plasma Insulin Assay

Blood was removed from the axillary vein of anesthetized rats (ovariectomized) using heparin-coated pasteur pipettes and put in centrifuge tubes at zero degrees Centigrade. Blood samples were spun and the plasma was removed from the pelleted cells. Aliquots of plasma were assayed immediately for insulin using antibody raised against human insulin. After the appropriate steps, insulin-[125I]antiinsulin-antiIgG complexes were pelleted in a clinical centrifuge for 30 min at 4 degrees Centigrade. The supernatant was decanted and the pellets were counted in a Beckman gamma counter. Units of plasma insulin were determined by using a standard curve generated from known amounts of insulin.

Serum Glucose Assay

Blood was drained from decapitated rats into centrifuge tubes. The blood was allowed to clot overnight and then the samples were centrifuged. Aliquots of serum were assayed for glucose using a colorimetric assay kit purchased from Sigma. The assay employed the enzymes glucose oxidase and peroxidase to generate a brown color.

Results

To determine if insulin increases the rate of glucose transport in the uterus, fed and 24 hour-starved rats were injected subcutaneously with 0.2 mg insulin. Two hours later, the rats were killed. In Table 5.1 it is shown that insulin reduced the serum glucose concentration in both fed and starved rats. This experiment was done in order to demonstrate that insulin was present in sufficient amounts in the body in order to affect physiological processes. It can be seen in Table 5.2 that insulin increased the rate of 2-deoxyglucose transport 45 % in starved but not fed rats. To my knowledge, this is the first demonstration of an increase in the rate of glucose transport in the uterus as a consequence of insulin administration. Why insulin increases glucose transport in the uteruses in starved but not in fed rats is not known.

In order to determine if estradiol and insulin mediate the increase in glucose transport via the same or

Table 5.1

Effect of insulin and starvation on rat
serum glucose concentration

Treatment	Serum glucose concentration (mM)
Fed	2.00 $\pm$ 0.20
Fed + insulin	0.15 $\pm$ 0.17
Starved	0.58 $\pm$ 0.03
Starved + insulin	0.05 $\pm$ 0.03

Animals were starved overnight. Results are expressed as the mean with the standard deviation. The value for each treatment is expressed as the mean $\pm$ standard deviation where $n = 2$.

Table 5.2

Effect of insulin on glucose transport
in rat uterus

Treatment	2-Deoxyglucose Transport nmol/20 min/uterus	
	Fed	Starved
none	2.15 $\pm$ 0.61	1.05 $\pm$ 0.11*
insulin	2.00 $\pm$ 0.32	1.66 $\pm$ 0.22*

* The increase in 2-deoxyglucose transport is significantly higher in the uteruses of insulin-treated animals vs the control animals with $p < 0.01$. The value of the transport rates are expressed as the mean $\pm$ standard deviation where $n = 4$.

different mechanisms, insulin and estradiol were injected either alone or together in starved rats. In Table 5.3 it can be seen that increases in glucose transport is not additive in the presence of both compounds.

In order to determine if estradiol administration results in changes in the binding of insulin to its receptor, plasma membrane vesicles from control and estradiol-treated rats were incubated with 1.0 nM labelled insulin with or without excess nonradioactive insulin and binding was assayed (Table 5.4). No difference in insulin binding was seen after estradiol treatment. Assuming that 1.0 nM estradiol is under the maximal insulin binding to the receptor, then one can deduce that estradiol does not effect either the number of receptors or the K_d of binding of insulin to the receptor.

To determine if estradiol affected the plasma insulin concentration, the concentration of plasma insulin was determined in 24-hour starved rats at various time points after estradiol administration (Fig. 5.1). One hour after estradiol treatment, a statistically significant rise in plasma insulin concentration was seen. The concentration of insulin appeared to remain elevated for at least the next 3 hours.

Table 5.3

Effect of insulin and estradiol on glucose
transport in rat uterus

Treatment	2-Deoxyglucose Transport nmol/20 min/uterus
none	2.98 $\pm$ 0.48
insulin	4.08 $\pm$ 0.12
estradiol	5.98 $\pm$ 0.60
estradiol + insulin	6.14 $\pm$ 0.64

Insulin versus estradiol, $p < 0.001$

Insulin versus estradiol + insulin, $p < 0.001$

Estradiol versus estradiol + insulin, $p > 0.7$

The value of each treatment is expressed as the mean $\pm$ standard deviation where $n = 4$. 2-Deoxyglucose transport was determined as previously described.

Table 5.4

Effect of estradiol on [125I]insulin binding to insulin receptors in rat uterine plasma membranes

Treatment	[125I]Insulin Bound fmol/mg protein
none	35.8 $\pm$ 3.3
estradiol	36.3 $\pm$ 5.3

Rats were killed 3 hours after the rats were estradiol injection plasma membranes prepared. Plasma membranes were collected from a 0.5/1.0 M sucrose shelf and incubated for 30 min at 22 degrees Centigrade with 1.0 nM labelled insulin with or without excess nonradioactive insulin. Membranes were collected on cellulose acetate filters and counted in a Beckman gamma counter.

Figure 5.1. Time-course of estradiol effect on plasma insulin concentration and rate of 2-deoxyglucose transport. 2-Deoxyglucose transport was measured as described previously. Plasma insulin was measured via radioimmunoassay. The mean values are plotted with the standard error of the mean. The numbers next to the insulin values represent the numbers of rats and each assay was measured in duplicate. The difference between the zero time point and the 1 h time point is statistically significant with $p < 0.005$.

Discussion

The data presented in this chapter demonstrate that estradiol and insulin share certain but not all features in the mechanism that increases glucose transport in the uterus.

Insulin administration increases glucose transport in rat uterus in starved rats but not in fed rats, indicating that, in fed rats, the glucose transport rate in the uterus is already maximally stimulated. Estradiol, on the other hand, stimulates glucose transport in uteri from either fed or starved rats, indicating that a signal mechanism exists for estradiol which is not used by insulin.

Estradiol and insulin, however, share some portion of a common pathway resulting in the increase in the rate of glucose transport since the simultaneous treatment of rats with both compounds does not result in an additive response. In order for this interpretation to be valid, at least one of the compounds must be at a concentration high enough to elicit maximal rates in glucose transport. In regards to estradiol, 0.010 mg of the compound was injected--an amount previously shown to elicit the maximal increase in the rate of glucose transport. The amount of insulin used (0.2 mg) was sufficient to lower serum glucose concentration by an order of magnitude. Since the amount of estradiol used gave the maximal response and

since the amount of insulin used was probably near the maximal response, the conclusion from this experiment is that estradiol and insulin increase the rate of glucose transport via common features of a pathway.

Although estradiol administration appears to result in a small increase of plasma insulin concentration, insulin is not the mediator of the estradiol stimulation of glucose transport for a number of reasons. First, insulin treatment alone only results in a 45 % increase in glucose transport while estradiol treatment causes a 100 % increase. Second, insulin administration does not result in an increase in glucose transport in fed animals while estradiol has a full effect on glucose transport. Third, the time course of the increase in glucose transport (hours) is not consistent with the time course of the increase in glucose transport seen with insulin (minutes).

CHAPTER 6

MECHANISM OF THE ESTRADIOL-MEDIATED INCREASE IN GLUCOSE TRANSPORT

Introduction

Glucose transport is hormonally regulated in a number of cells including adipocytes, muscle cells, fibroblasts, (84) and uterine cells (50). The regulation of the rate of glucose transport could be accomplished by: (i) increasing the intrinsic activity of the transporter protein by lipid changes in the plasma membrane, (ii) increasing the number of transporter proteins, (iii) increasing the affinity of the transporter protein for glucose, or (iv) any combination of the above.

Compounds shown to be involved in the stimulation of the rate of glucose transport include: (i) hormones (84, 85, 50) such as insulin, testosterone, and estradiol, (ii) growth factors (75) such as platelet-derived growth factor, and epidermal growth factor, and (iii) mitogens (87, 88) such as phytohemagglutinin and concanavalin A. Furthermore, other factors such as tumor viruses (86), chemical carcinogens (86), and temperature have been shown to affect the rate of glucose transport in vitro (89).

The effect of insulin on the increase in glucose transport has been well-studied (84). A number of laboratories have provided evidence that glucose transport

rates are increased as a result of the translocation of transporter proteins from intracellular sites to the plasma membrane. Initially, kinetic data were used to argue for the translocation hypothesis. In these studies, the kinetic parameters K_m and V_{max} were compared between control and insulin-stimulated cells by using the glucose analogs 2-deoxyglucose or 3-O-methylglucose. In these studies, the V_{max} was shown to increase without a significant change in the K_m for glucose transport (81,80).

Another interpretation of these results is that changes in the intrinsic activity of the transporter as a results of insulin treatment occurs. Olefsky (90), studying 2-deoxyglucose transport in rat adipocytes, showed a 3-fold increase in the V_{max} of transport without a change in the K_m value. In addition, when the V_{max} of basal and insulin-stimulated transport was measured as a function of temperature, parallel Arrhenius plots were obtained yielding equal activation energies, implying that insulin increases the V_{max} by increasing the number of transporters rather than enhancing the intrinsic activity of the transporter.

In addition to kinetic studies, assays involving D-glucose-inhibitable cytochalasin B binding and binding of antiglucose transporter antibodies to the glucose transporter have been used to show increases in the number of transporters in the plasma membrane subsequent to insulin

administration. Karnieli et al., using specific cytochalasin B binding, showed a 5-fold increase in the number of transporters in the plasma membrane with a roughly corresponding (3-fold) decrease in the number of transporters in low density microsomal fractions (91). Using antibody raised against purified human red blood cell glucose transporter, Wheeler et al. (92) showed that insulin treatment resulted in an increase in antibody binding to rat adipocyte plasma membrane preparations. In addition, the results showed a decrease in antibody binding in microsomal membranes subsequent to insulin treatment.

On the other hand, Whitesell and Abumrad (82), using adipocytes reported that insulin increases glucose and 3-O-methylglucose transport rates by a 2 to 3-fold increase in the V_{max} of transport and a 10-fold decrease in the K_m value. The K_m for glucose transport changed from 75 mM to 8 mM. The authors previously reported no change in the K_m between control and insulin-treated adipocytes (93), but the rates called "basal" in those studies were significantly higher than those subsequently reported by other workers (94), suggesting that the cell preparations were partially activated by experimental conditions. For example, it is known that low temperatures have an insulin-like effect on the basal rate of glucose transport (95); i.e., the basal rate of 3-O-methylglucose is higher at 25 degrees centigrade than it is at 37 degrees. In

addition recent data obtained by Whitesell et al. (94,98) indicate that the basal rate of glucose transport is altered through K_m changes in response to metabolic factors.

A decrease in the K_m for glucose transport as a result of insulin treatment has also been shown to occur in the heart (99), in skeletal muscle (100,101) and in epididymal fat tissue (102).

The insulin-induced decrease in K_m for glucose transport may reflect activation of the transporter via a phosphorylation event. This concept is indirectly supported by the observation that glucose transport is stimulated in muscle cells by the diester 12-O-tetradecanoyl phorbol 13-acetate, a compound known to activate protein kinase C (103). Protein kinase C is an enzyme implicated in the regulation of cell growth. Another possible mechanism for transport stimulation is modification in membrane fluidity (104,105) which could enhance the function of the transporter.

In this chapter, data are provided, using polyserum raised against the human glucose transporter, that indicate no change in the number of transporter proteins in the plasma membrane after a number of experimental manipulations. The experimental manipulations include starvation and insulin, estradiol, and cycloheximide treatments. These results can be explained by postulating either a

change in the K_m value or in the intrinsic activity of the transporter. In Chapter 3, I reported no significant change in the K_m of glucose transport subsequent to estradiol treatment. However, the report by Whitesell and Abumrad (82) opens the possibility that the K_m value of the basal lowered to the value of the stimulated tissue as a consequence of removing the uterus from the rat. These studies should be repeated taking in account temperature and nutritional factors.

Materials and Methods

Materials

[125I]Protein A (70-100 Ci/g) was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. Peroxidase-linked goat antirabbit IgG and diaminobenzidine were purchased from Sigma Chemical Co., St. Louis, Mo. Nitrocellulose filters were purchased from Amersham Corporation, Arlington Heights, Illinois and from Millipore Corp., Bedford, Mass. Antiglucoase transporter antiserum was obtained from Dr. Peter Hinkle, Cornell University, Ithaca, New York. Other reagents were purchased from the usual sources at the highest purity available. The protein transblotting apparatus was purchased from Hoefer Scientific Instruments, San Francisco, California.

Buffers

Buffer 1 consisted of 20 mM Tris, pH 7.4. Buffer 2

sisted of phosphate-buffered saline (20 mM potassium phosphate, pH 7.4 and 0.155 M NaCl). Buffer 3 was Buffer 2 with 0.05 % Tween.

Membrane Preparation

Plasma membranes were isolated from a 0.5/1.0 M sucrose shelf as previously described (see Chapter 2). Membranes were taken up in Buffer 1 and protein concentration was determined by the Bradford method (62). Plasma membranes were dissolved by adding 10 % SDS, giving a final concentration of 1 % SDS.

Radioimmunoassay of Transporter Protein

Fifteen microliters of antigen in Buffer 1 with 1 % SDS were spotted on 13 mm dia. Millipore filters (HATF) in Falcon 24-well tissue culture plates. Typically 500 - 5000 ng uterine protein and 60 - 500 ng human erythrocyte ghost protein were spotted per filter. After 15 min, the filters were covered with 1 ml of Buffer 2 containing 2 % Carnation milk. Forty five minutes later, the solution was removed and the filters were rinsed once quickly with 1 ml Buffer 3 and then washed with 1 ml Buffer 3 two times for 5 min. Polyserum (39 micrograms protein) was added to 0.5 ml of Buffer 3 and the reaction was allowed to proceed for 5 h. The solution was removed and the filter was again washed quickly with 1 ml Buffer 3 followed by 3-1 ml washes of 5 min. One microliter [125 I]protein A (0.2

mCi/ml) was added to 1 ml Buffer 3. Twenty five minutes later, the solution was removed and the filters were washed as previously described. The filters were then dissolved in 0.5 ml methylcellosolve and to the resulting solution was added 7.5 ml Betaphase scintillation fluid. All steps were performed at room temperature and with constant agitation.

Western Blotting

Protein samples were separated by SDS-polyacryamide electrophoresis according to the method of Laemmli (106). The protein was then transferred to nitrocellulose paper using a Hoefer transblotting system. Transfer was allowed to proceed for 1 - 3 h in Towbin buffer (107) at 1.2 amperes current. After transfer of protein was accomplished, the filter was washed in 50 ml of Buffer 2 for 5 min in a plastic container followed by incubation of the filter with 50 ml of Buffer 2 containing 2 % Carnation milk for 45 min. The filter was then washed quickly with 50 ml of Buffer 3 followed by a 5 min wash with Buffer 3. Seventy five microliters of antiglucose polyserum (0.293 mg protein) was added to 50 ml Buffer 3 and binding was allowed to proceed for 3 h. The solution was removed and the filter was washed quickly with 1 ml of Buffer 3 followed by 3 5-min washes with 50 ml of the same buffer. Fifty microliters of goat antirabbit antibody linked with

peroxidase was added to 50 ml Buffer 3 (1/1000 dilution of antibody) and binding was allowed to proceed for 45 min. The filter was washed quickly once with Buffer 2 followed by 3 5-min washes. The filter was then immersed in 50 ml phosphate-buffered saline containing 0.5 mg/ml diaminobenzidine and 0.006 % hydrogen peroxide. Color development was usually allowed to proceed for approximately 30 min to 1 h.

Results

To produce meaningful results about the effects of various treatments on changes in the number of glucose transporters in the plasma membrane, it was first necessary to determine the linearity of the immunoassay with antigen. In Figure 6.1 it can be seen that two to thirty nanograms of human red blood cell (RBC) plasma membrane protein blotted onto nitrocellulose paper produced a linear increase in the amount of [¹²⁵I]protein A binding ($r > 0.99$).

Using a range of uterine plasma membrane protein from 0.5 to 4 micrograms, it was shown in Figure 6.2 that the binding of protein A was proportional to the amount of membrane protein added. Furthermore, it can be seen that plasma membranes derived from ovariectomized rats pretreated for 3 h with 10 micrograms of estradiol exhibited no difference antigen amounts as seen by protein A

Figure 6.1. $[^{125}\text{I}]$ Protein A binding with increasing amounts of human erythrocyte plasma membrane protein. The binding of labeled protein A was linear up to 30 ng membrane protein ($r > 0.99$). Immunoassay was performed as described under Material and Methods.

Figure 6.2. Binding of $[^{125}\text{I}]$ protein A to increasing amounts of uterine plasma membrane protein. Closed circles refers to uterine membrane protein from ovariectomized rats and the open circles refers to uterine membrane protein from ovariectomized rats injected with 10 micrograms of estradiol 3 h before removal of uteri. Immunoassay was performed as described under Materials and Methods.

binding.

In Table 6.1 it can be seen that neither starvation, starvation + insulin, nor cycloheximide produce changes in protein A binding, even though all of these manipulations are known to change the rate of glucose transport (see Chapters 4,5).

These results suggest that the differences in glucose transport rate from the above manipulations are not due to an increase in the number of transporter proteins in the plasma membrane but a change in either the K_m of transport or a change in the intrinsic activity of the transporter. Since kinetic data (Chapter 3) show no significant change in the K_m of glucose transport after estradiol treatment, the simplest explanation is that estradiol increases glucose transport rate in rat uterus by increasing the intrinsic rate of the glucose transport process.

Another interpretation of the data is the possibility that transporter proteins not reactive with the antibody are inserted into the plasma membrane as a consequence of estradiol and other treatments. Finally, the possibility exists that the antibody is reacting to proteins in the plasma membrane other than the glucose transporter and that the number of the transporter proteins in fact did change during these treatments but was not detected.

In order to address the last point, a western blot of the plasma membrane proteins from human RBC and rat uterus

Table 6.1

Effect of various treatments on
protein A binding

	Treatment	% Control
Part 1	none	100 $\pm$ 7
	starved	88 $\pm$ 7
	starved + insulin	94 $\pm$ 27
Part 2	none	100 $\pm$ 3
	cycloheximide	106 $\pm$ 16

Differences are not significant between any of the treatments and controls. In part 1, the binding of protein A/ng membrane protein is seen. One hundred percent represents 2.68 cpm/ng protein (930 ng protein/assay). In part 2, the binding of protein A/ng protein is seen. One hundred percent represents 1.57 cpm/ng protein (2 270 ng protein/assay). All experiments were repeated at least 3 times.

was produced (Figure 6.3). Lane A shows the protein reactive to the polyserum from the human RBC. Two major bands are evident: one band has a molecular weight of approximately 20 000 daltons and the other of approximately 40 - 45 000 daltons. The latter band has the size and appearance of what would be expected for the glucose transporter protein. That is, the size of the protein reactive with the polyserum is consistent with the reported size of the human RBC transporter and the spread of the band, characteristic of glycoproteins, is consistent with previously published results of approx. 45 000 daltons (91). The smaller protein is suspected to be globin chains. Lane B shows proteins reactive to the polyserum from rat uterus plasma membranes. The protein in the major band appears to have a molecular weight of 50 000 daltons and has the characteristic band spread of a glycoprotein. These results show that the polyserum is reacting with a protein having the same size and banding characteristics reported for the glucose transporter. However, the possibility exists that the antibody is reactive to a glycoprotein having similar characteristics of the glucose transporter protein.

One way to show conclusively that the polyserum is reacting with the transporter protein would be to purify a protein with this antibody and show that this purified protein is capable of stereospecifically transporting

Figure 6.3. Western blot of SDS-solubilized plasma membrane proteins. Visualized in lane a are human RBC proteins and in lane b are proteins from rat uterus. The membrane proteins were incubated with polyserum raised against purified human RBC glucose transporter protein. Antibody binding was visualized using a peroxidase-linked second antibody in conjunction with diaminobenzidine.

glucose into liposomes. Another way would be to use these antibodies to block glucose transport in plasma membrane vesicles.

Finally, it should be noted Drs. Hinkle and Sogin, the source of the polyserum, have shown that polyserum binding assays give amounts of transporters/mg membrane protein consistent with the amounts of transporters/mg membrane protein generated by D-glucose inhibitable cytochalasin B binding assays (108). Furthermore, they report that this polyserum inhibited specific glucose transport 50 % when glucose transporters are reconstituted in liposomes.

Discussion

The immunoassay used to measure amounts of rat uterine glucose transporter protein in plasma membranes meets the requirements of sensitivity, linearity and specificity. Using this assay, I have determined that estradiol does not increase the rate of glucose transport in rat uterus by causing an increase in the number of transporter proteins in the plasma membrane. In conjunction with the fact that estradiol increases the V_{max} without a significant change in the K_m of glucose transport, I conclude that estradiol increases the rate of glucose transport in rat uterus by increasing the intrinsic rate of the transport process. The rate of the

transport process could be effected in a number of ways, including: (1) phosphorylation-dephosphorylation of the transporter protein, (2) local membrane lipid modifications, and (3) involvement of a regulatory protein that interacts with the glucose transporter protein.

Phosphorylation-dephosphorylation events may cause conformation shifts in the transporter protein, allowing greater mobility of the transporter protein which would result in a faster shuttling of glucose across the plasma membrane. Greater mobility of the transporter protein could also result from increases in fluidity in the lipids surrounding the transporter protein. Finally, the interaction of a regulatory protein with the transporter protein could restrict mobility of transport action. In this example of negative control, the regulatory protein could be released from the transporter protein as a consequence of phosphorylation-dephosphorylation of the transporter protein as stated above or by phosphorylation-dephosphorylation of the regulatory protein itself.

.

CHAPTER 7

SUMMARY

A study of the mechanism of the estradiol-stimulated increase of glucose transport in uteri from ovariectomized rats was undertaken. Two systems employed to study glucose transport rates were: (i) D-glucose transport into uterine plasma membrane vesicles and (ii) 2-deoxyglucose transport and phosphorylation in uterine tissue in vitro.

Glucose transport in the rat uterus is stereospecific, energy and sodium independent and is inhibited by classic inhibitors of glucose transport, e.g., phloretin, mercuric ion, and cytochalasin B. In addition, it was shown for the first time that the estradiol-stimulated increase in glucose transport persists in isolated plasma membrane vesicles, suggesting a relatively permanent membrane effect by estradiol.

Estradiol, estrone and estriol administration increase the rate of glucose transport in rat uteri while dihydrotestosterone and progesterone have no effect. In addition, 2-deoxyglucose transport studies in the uteri of rats grouped in various stages of the reproductive cycle showed that the rate of glucose transport corresponded to the serum concentration of estradiol at each stage of the cycle. These results provide evidence for the physiological relevance of the study of the estradiol-

mediated increase in glucose transport in rat uterus.

Insulin treatment also stimulates glucose transport in the uterus in starved rats but not in fed rats. The increase in glucose transport with estradiol and insulin together is not additive, suggesting common pathways in the stimulatory process.

The V_{max} of glucose transport was increased in the uteri from rats pretreated with estradiol with no significant change in the K_m value, suggesting the increase in glucose transport is due to either an increase in the intrinsic activity or an increase in the number of transporters in the plasma membrane. Direct determination of the relative amounts of glucose transporters in uterine plasma membranes was accomplished with antiserum raised against the human glucose transporter. Estradiol treatment did not effect the number of glucose transporter proteins in rat uterine plasma membranes. In addition, treatments known to change the rate of glucose transport including starvation and in vivo insulin and cycloheximide administration did not change the number of glucose transporters in the plasma membrane as assayed with this antiserum.

The simplest explanation of the data is that estradiol mediates the increase in glucose transport in the uterus by changing the intrinsic activity of the transport process. The rate of the transport process could be

effected by (i) phosphorylation-dephosphorylation of the transporter protein, (ii) local membrane lipid modifications, and (iii) a regulatory protein interacting with the glucose transporter. Figure 7.1 depicts a simplified model of the regulation of glucose transport in rat uterus.

Figure 7.1. Model of glucose transport regulation. The model depicts continual production of a protein (inhibitor) that depresses the rate of glucose transport. Estradiol induces glucose transport by either direct effects on the glucose transporter complex or by depressing the production of the glucose transport inhibitor (see page 99 for further discussion). The plus sign (+) refers to stimulation of glucose transport while the minus signs (-) refer to points of possible estradiol-receptor depression of inhibitor production. E represents estradiol, R represents the receptor, and E-R represents the estradiol-receptor complex.

REFERENCES

1. Means, A.R., and Hamilton, T.H. Proc. Natl. Acad. Sci. USA 56: 1594 (1966).
2. Libby, P.R. Biochem. J. 130: 663 (1972).
3. Tchernitchin, A., Roorijck, J., Tchernitchin, X., Vandenhende, J., and Galand, P., Nature (London) 248: 142 (1974).
4. DeAngelo, A.B., and Gorski, J. Proc. Natl. Acad. Sci. USA 66: 693 (1970).
5. Hamilton, T.H., Widnell, C.C., and Tata, J.R. Biochim. Biophys. Acta 108: 168 (1965).
6. Barker, K.L. Biochemistry 10: 284 (1971).
7. Smith, D.E., and Gorski, J. J. Biol. Chem. 243: 4169 (1968).
8. Noall, M.W., and Allen, W.M. J. Biol. Chem. 236: 2987 (1961).
9. O'Malley, B.W., and Means, A.W. Science 183: 610 (1974).
10. Smith, D.E., and Gorski, J. Exp. Biol. Med. 124: 747 (1967).
11. Gorski, J., Toft, D.O., Shyamala, G., Smith, D., and Notides, A. Rec. Prog. Horm. Res. 24: 45 (1968).
12. Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W., and De Sombre, E.R. Proc. Natl. Acad. Sci. USA 59: 632 (1968).
13. Welshons, W.V., Lieberman, M.E., and Gorski, J. Nature 307: 747 (1984).
14. King, W.J., and Greene, J.L. Nature 307: 745 (1984).
15. Jensen, E.V., and Jacobsen, H.I. Recent Prog. Hormone Res. 18: 387 (1962).
16. Noteboom, W.D., and Gorski, J. Arch. Biochem. Biophys. 111: 559 (1965).
17. Pietras, R.J., and Szego, C.M. J. Steroid Biochem. 11: 1471 (1979).

18. Nence, I., Marchetti, E., Margola, A., and Fabris, G. J. Steriod Biochem. 14: 1139 (1981).
19. Mueller, R.E., Johnston, T.C., and Wotiz, H.H. J. Biol. Chem. 254: 7895 (1979).
20. Best-Belsomme, M., Fries, J., and Erdos, T. Eur. J. Biochem. 17: 425 (1970).
21. Clark, J.H., Hardin, J.W., Eriksson, H., Upchurch, S., and Peck, E.J. Jr. "Ontogeny of Receptors and Reproductive Hormone Action", edited by T.H. Hamilton, J.H. Clark, and W.A. Sadler, p 65. Raven Press, New York 1979.
22. Michel, G., Jung, I., Baulieu, E.E., Aussel, C., and Chiel, J. Steroids 24: 437 (1974).
23. Lyttle, C.R., Medlock, K.L., and Sheehan, D.M. J. Biol. Chem. 259: 2697 (1984).
24. Meyers, C.Y., Kolb, V.M., and Dandliker W.B. Res. Commun. Chem. Pathol. Pharmacol. 35: 165 (1982).
25. Terenius, L. Acta Endocrinologica 64: 47 (1970).
26. Katzenellenbogen, B.S., and Ferguson, E.R. Endocrinology 97: 1 (1975).
27. Spaziani, E., and Gutman, A. Endocrinology 76: 470 (1965).
28. Roskoski, R., and Steiner, D.F. Biochim. Biophys. Acta 135: 717 (1967).
29. Chan, T.W., Fung, K.P., Choy, Y.M., and Lee, C.Y. Arch. Biochem. Biophys. 225: 458 (1983).
30. Salter, D.W., Baldwin, S.A., Lienhard, G.E., and Weber, M.J. Proc. Natl. Acad. Sci. USA 79: 1540 (1982).
31. Wardzala, L.J., Cushman, S.W., and Salans, L.B. J. Biol. Chem. 253: 8002 (1978).
32. Cushman, S.W., and Wardzala, L.J. J. Biol. Chem. 255: 4758 (1980).
33. Osten, J., Bader, J., Johnson, G.S., and Pastan I. J. Biol. Chem. 247: 1632 (1972).

34. Sugimoto, Y., Whitman, M., Cantley, L.C., and Ericksons, R.L. *Proc. Natl. Acad. Sci. USA* 81: 2117 (1984).
35. Czech, M.P. *Annu. Rev. Biochem.* 46: 359 (1977).
36. Denton, R.M., Brownsey, R.W., and Belsham, G.J. *Diabetologia* 21: 347 (1981).
37. Kahn, C.R. *Trends Biochem. Sci.* 4: N263 (1979).
38. Pilkis, J.J., Claus, T.H., and Johnson, R.A. *J. Biol. Chem.* 250: 6328 (1975).
39. Kashiwagi, A., Huecksteadt, T.P., Foley, J.E. *J. Biol. Chem.* 258: 13685 (1983).
40. Fain, J.N., and Butcher, F.R. *J. Cyclic Nucleotide Res.* 2: 71 (1976).
41. Clausen, T. *Curr. Top. Membr. Transp.* 6: 169 (1975).
42. Ludvigsen, C., and Jarrett, L. *J. Biol. Chem.* 254: 1444 (1979).
43. Bisonette, J.M., Black, J.A., Wickham, W.K., and Acott, K.M. *J. Membrane Biol.* 58: 75 (1981).
44. Schraw, W.P., and Regen, D.M. *Biochim. Biophys. Acta* 649: 726 (1981).
45. Klip, A., and Walker, D. *Arch. Biochem. Biophys.* 221: 175 (1983).
46. Smith, D.E., and Gorski, J. *Life Sci.* 6: 1263 (1967).
47. Jung, C.Y., and Rampal, A.L. *J. Biol. Chem.* 252(15): 5456 (1977).
48. Sogin, D.C., and Hinkle, P.C. *Proc. Natl. Acad. Sci. USA* 77: 5725 (1980).
49. Riggs, T.R., Pan, M.W. and Feng, H.W. *Biochim. Biophys. Acta* 150: 92 (1968).
50. Roskoski, R., and Steiner, D.F. *Biochim. Biophys. Acta* 135: 727 (1967).
51. Walters, M.R., Lawrence, A.L., and Hazelwood, R.L. *Biol. Reprod.* 12: 383 (1975).

52. Walters, M.R., Lawrence, A.L., and Hazelwood, R.L. *Endocrinology* 108: 1915 (1981).
53. Means, A.R., and Hamilton, T.H. *Proc. Nat. Acad. Sci.* 56: 686 (1966).
54. Peterson, R.P., and Spaziani, E. *Endocrinology* 89: 1280 (1971).
55. Pietras, R.J., and Szego, C.M. *Endocrinology* 97: 1445 (1975).
56. Murphy, C.R., and Rogers, A.W. *Cell Biophysics* 3: 305 (1981).
57. Noteboom, W., and Gorski, J. *Proc. Nat. Acad. Sci.* 50: 250 (1963).
58. Aronson, N.N., and Touster, O. *Meth. Enzymol.* 31: 90 (1974).
59. Bers, D.M. *Biochim. Biophys. Acta* 555: 131 (1979).
60. Kelley, S.J., Dardinger, D.E., and Butler, L.G. *Biochemistry* 14: 4983 (1975).
61. Stocco, D.M., and Hutson, J.C. *Cancer Res.* 40: 1486 (1980).
62. Bradford, M. *Anal. Biochem.* 72: 248 (1976).
63. Kidwai, A.M., Radcliffe, M.A., and Daniel, E.E. *Biochim. Biophys. Acta* 233: 538 (1971).
64. Mueller, R.E., Johnston, T.C., and Wotiz, H.W. *J. Biol. Chem.* 254: 7895 (1979).
65. Neville, Jr., D.M. In *Methods in Membrane Biology* (Korn, E.D., ed.), Vol. 3, pp. 1-49, Plenum Press, New York.
66. LeFevre, P.G. *Synp. Soc. Exp. Biol.* 8: 118 (1954).
67. Lin, S., and Spudich, J.A. *J. Biol. Chem.* 249: 5778 (1974).
68. LeFevre, P.G. *J. Gen. Physiol.* 31: 505 (1948).
69. Illiano, G., and Cuatrecasas, P. *J. Biol. Chem.* 246: 2472 (1971).

70. Baldwin, J.M., Gorga, J.C., and Lienhard, G.E. J. Biol. Chem. 256: 3685 (1981).
71. Plagemann, P.G.W., Graff, J.C., and Wohlhueter, R.M. J. Biol. Chem. 252: 4191 (1977).
72. Smith, M.S., Freeman, M.E., and Neill, J.D. Endocrinology 96: 219 (1975).
73. Moulton, B.C., and Barker, K.L. Endocrinology 91: 491 (1972).
74. Barnes, D., and Colowick, S.P. J. Cell. Physiol. 89: 633 (1975).
75. Inman, W.H., and Colowick, S.P. Proc. Natl. Acad. Sci. USA 82: 1346 (1985).
76. Parikh, I., Anderson, W.L., and Neame, P. J. Biol. Chem. 255: 10266 (1980).
77. Gammeltoft, S. Physiol. Rev. 64: 1321 (1984).
78. Anderson, J.N., Peck, E.J., Jr., and Clark, J.H. Endocrinology 92: 1488 (1973).
79. Kahn, C.R. Ann. Rev. Med. 36: 429 (1985).
80. Cushman, S.W., and Wardzala, L.J. J. Biol. Chem. 255: 4758 (1980).
81. Suzuki, K., and Kono, T. Proc. Natl. Acad. Sci. USA 77: 2542 (1980).
82. Whitesell, R.R., and Abumrad, N.A. J. Biol. Chem. 260: 2894 (1985).
83. Louvet, J-P., Harman, S.M., Schreiber, J.R., and Ross, G.T. Endocrinology 99: 758 (1976).
84. Klip, A. Life Sciences 31: 2537 (1982).
85. Mills, T.M., and Spaziani, E. Biochem. Biophys. Acta 150: 435 (1968).
86. Hatanaka, M., and Hanafusa, H. Virology 41: 647 (1970).
87. Osihiro, Y., DiPaolo, J.A. J. Cell. Physiol. 83: 193 (1974).

88. Peters, J.H., and Hansen, P. Eur. J. Biochem. 19: 509 (1971).
89. Katzen, H.M., Soderman, D.D., and Green, B.G. Biochem. Biophys. Res. Commun. 98: 410 (1981).
90. Chang, K., and Cuatrecasas, P. J. Biol. Chem 249: 3171 (1974).
91. Olefsky, J.M. Biochem. J. 172: 137 (1978).
92. Wardzala, L.J., Cushman, S.W., and Salans, L.B. J. Biol. Chem. 253: 8002 (1978).
93. Wheeler, T.J., Simpson, K.A., Sogin, D.C., Hinkle, P.C., and Cushman, S.W. Biochem. Biophys. Res. Comm. 105: 89 (1982).
94. Whitesell, R.R., and Gliemann, J. J. Biol. Chem. 254: 5276 (1979).
95. Vega, F.V., Key, R.J., Jordan, J.E., and Kono, T. Arch. Biochem. Biophys. 203: 167 (1980).
96. Ezaki, O., and Kono, T. J. Biol. Chem. 257: 14306 (1982).
97. Abumrad, N.A., Whitesell, R.R., Briscoe, P., and Park, C.R. Fed. Proc. 43: 1573 (1984).
98. Whitesell, R.R., and Lynn, W.S. Fed. Proc. 38: 914 (1979).
99. Cheung, J.Y., Conover, C., Regen, D.M., Whitfield, C.F., and Morgan, H.E. Am. J. Physiol. 234: E70 (1978).
100. Field, R. A., and Cori, C.F. (1963) in Perspectives in Biology (Cori, C.F., Foglia, V.V., Leloir, L.F., and Ochoa, S., eds) pp162-173, American Elsevier Publishing Co., New York.
101. Narahara, H.T., Oezand, P., and Cori, C.F. J. Biol. Chem. 235: 3370 (1960).
102. Crofford, O. B., and Renold, A.E. J. Biol. Chem. 240: 3237 (1965).
103. Farese, R.V., Standaert, M.L., Barnes, D.E., Davis, J.S., and Pollet, R.J. Endocrinology 116: 2650 (1985).

104. Yuli, I., Wilbrandt, W., and Shinitzky, M. Biochem. 20: 4250 (1981).
105. Yuli, I., Incerpi, S., Luly, P., and Shinitzky, M. Experientia 38: 1114 (1982).
106. Laemmli, U.K. Nature (London) 227: 680 (1970).
107. Towbin, H., Staehelin, T., and Gordon, J. Proc. Natl. Acad. Sci. USA 76: 4350 (1979).
108. Sogin, D.C., and Hinkle, P.C. Proc. Natl. Acad. Sci. USA 77: 5725 (1980).