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INSULIN BINDING AND GLUCOSE TRANSPORT IN THE R3230AC MAMMARY ADENOCARCINOMA

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Cells dissociated from the R3230AC mammary adenocarcinoma from intact and diabetic rats were examined for insulin binding and glucose transport. The K_d for insulin binding, $\sim 10^{-10}$ M, was similar in all tumors studied. However, the apparent number of receptor sites per cell increased in cells from diabetic rats. Kinetic analysis of 3-0-methyl glucose (3-OMG) entry showed both diffusional and passive carrier characteristics. Insulin (4×10^{-9} M) in vitro did not affect diffusional entry, whereas the hormone altered the passive carrier system, as reflected by an increase in K_m and V_{max}. Insulin decreased initial velocity of glucose transport at 4–6 mM glucose levels but increased initial velocity of glucose transport at 20 mM glucose. An explanation of the role of insulin on tumor growth in vivo from effects on glucose transport in vitro is proposed.

INTRODUCTION

The R3230AC mammary adenocarcinoma of the rat has been shown previously to be a hormone-responsive, autonomous tumor with regard to estrogen, prolactin, and insulin (1, 2). In the diabetic animal, the tumor demonstrated slightly increased growth rates compared with the intact animal and administration of pharmacological doses of insulin resulted in inhibition of tumor growth (2). To better define the effect of insulin on this tumor, studies involving determination of insulin binding and glucose transport were carried out on isolated tumor cell suspensions from animals in which the insulin levels had been modified.

MATERIALS AND METHODS

Female Fischer rats (80–90 g), obtained from Charles River Breeding Laboratory, Wilmington, Mass., were individually housed and offered food and water ad libitum. The R3230AC tumor was transplanted by a sterile trochar technique as described by Hilf et al. (3). Animals were sacrificed by cervical dislocation at least 3 weeks after tumor transplantation.

Diabetes was induced by intravenous injection of streptozotocin 1 wk prior to tumor implantation (2); diabetes was confirmed by blood glucose levels > 250 mg/100 ml and urinary glucose exceeding 0.5 mg/100 ml. At necropsy, serum insulin levels were $< 2 \times 10^{-10}$ M as determined by radioimmunoassay (4).

In some experiments, tumor-bearing animals received insulin (Isophane insulin, E. R. Squibb, New Brunswick, N.J.) by subcutaneous injection 16 hr (2 IU) and 0.5 hr (2 IU) prior to sacrifice.

Isolated cell suspensions were obtained according to the procedure of Pitelka et al. (5) using 0.05% collagenase and 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, Mo.). Cells obtained after a second incubation with enzymes were washed four times in Medium 199 (with or without glucose). Cell viability was monitored by trypan blue exclusion; preparations used had > 85% viability. Cell number was estimated by the use of a hemocytometer.

The insulin binding assay was carried out at 20°C in a total volume of 1 ml Medium 199 with glucose (5–6 mM), utilizing 1×10^6 cells, 0.1% bovine serum albumin (Sigma), and insulin, either radioactively labeled alone (20–50 μCi/μg) or with unlabeled insulin. Plastic tubes were employed to decrease adsorption of insulin. After the appropriate incubation, the samples were centrifuged (900 × g) for 1 min, the supernatant was decanted, the cell pellet was resuspended in 1 ml buffer, and the suspension was rapidly filtered through an insulin assay filter (Amersham-Searle Corp., Arlington Heights, Ill.), which had been previously soaked in 10^{-5} M insulin and rinsed with 1 ml 10^{-5} M insulin. The filter was washed twice with 5 ml of buffer. Air-dried filters were counted in a liquid scintillation counter, ^{131}I counting efficiency was 72%.

Glucose transport was measured at 20°C with 3-0-methyl-(^{14}C)-D-glucose (3-OMG) (57 mCi/mmole, Amersham-Searle). Five million cells were suspended in Medium 199 (without glucose) with 0.1% BSA; ^{14}C-3-OMG plus unlabeled 3-OMG was added to give a final volume of 1 ml. Mannitol was added to maintain uniform osmolarity of 60 mM. Transport was halted by addition of 10 ml of ice-cold 0.9% NaCl followed by centrifugation. The cells were rapidly washed once with 10 ml 0.9% NaCl at 0°C and centrifuged; the cell pellet was resuspended in 200 μl 5% TCA. After 15 min at room temperature, the cell pellet was obtained by centrifugation. A portion of the supernatant was placed in 1 ml of a mixture containing Beckman Bio-Solv and toluene (1:2), 10 ml toluene-Omnifluor was added, and ^{14}C was counted with an efficiency of 80%.

RESULTS

At 10^{-9} M [^{131}I] insulin, specific binding was directly related to cell count. Labeled insulin was used alone (total binding) or in the presence of a 1,000-fold excess of cold insulin (estimate of nonspecific binding). The difference between total binding and nonspecific binding was considered to be the specific binding of insulin to the cell. Washing experiments were conducted to decrease nonspecific ^{131}I-insulin binding to the filter. The results of these experiments indicated that one or two successive washings of the cells yielded specific binding that represented 25–60% of the total insulin bound. For convenience, one washing was routinely employed.

The time course of insulin binding demonstrated that specific binding reached a plateau by 60 min at 20°C when 10^{-9} M insulin was used (Fig. 1).

The kinetics of insulin binding to tumor cells from intact, intact pretreated with insulin, and diabetic animals were examined (Fig. 2). At insulin concentrations as low as 10^{-11} M, there were differences in the apparent specific insulin binding to tumor cells dissociated from animals exposed to different hormonal environments. Tumor cells from diabetic animals exhibited greater specific insulin binding than cells from intact

animals or intact animals pretreated with insulin. These data were analyzed using a program for simple linear regression to determine the best fitting straight line; the slope and intercept were obtained from the above analysis. Scatchard analysis (Fig. 3) of these data demonstrated a family of curves with the same dissociation constant, K_d (no significant difference by analysis of covariance, P = 0.91); however, the estimates of the number of receptor sites per cell appeared to be different at insulin concentrations between 10^{-11} M and 10^{-10} M. These are summarized in Table I.

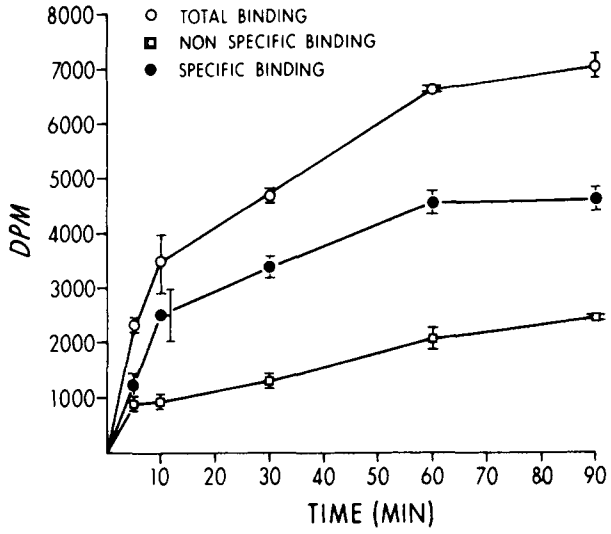

Fig. 1. Time course of insulin binding. At 10^{-9} M [^{131}I] insulin, specific binding reached a plateau by 60 min at 20°C. Each point is the average of three assays; vertical line is ± SEM.

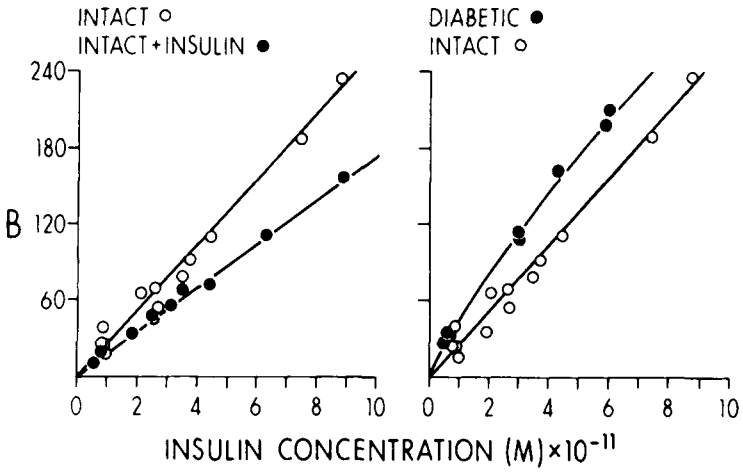

Fig. 2. Effect of concentration on binding. An increase in [^{131}I] insulin binding to tumor cells from the diabetic animal was seen compared to tumor cells from intact animals and tumor cells from intact animals pretreated with insulin. B = molecules of insulin bound per cell.

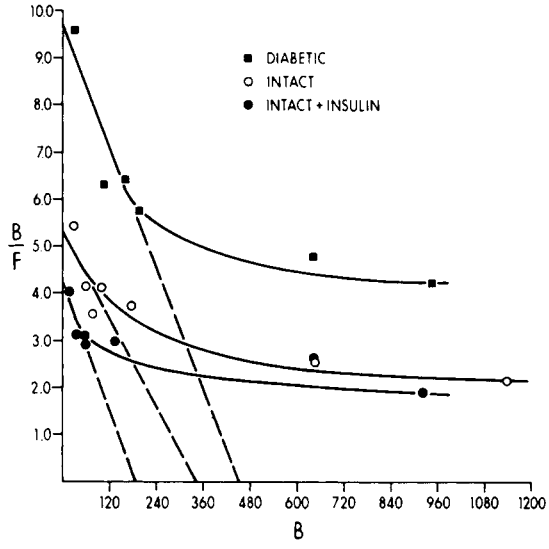

Fig. 3. Scatchard analysis of data from three experiments of insulin binding. Intercept on abscissa (determined by least square analysis of data obtained over a range of 10^{-11} to 10^{-10} M insulin) was used to calculate number of molecules of insulin bound per cell. B = molecules of insulin bound per cell, F = concentration of insulin in the assay (M). B/F × 10^{12}.

TABLE I. Levels of Insulin and Glucose in Tumor-Bearing Rats

	Intact	Intact + Insulin	Diabetic
Tumor growth (% control)	100	56	140
Serum insulin (M)[1]	2×10^{-9}	2×10^{-8}	10^{-10}
Serum glucose (mg/100 ml)	105	114	434
(mM)	5.8	6.3	24

Characteristics of Insulin Binding in Tumor Cells

	Intact	Intact + Insulin	Diabetic
Insulin Binding			
K_d (M)	0.9×10^{-10}	1×10^{-10}	1×10^{-10}
Sites/cell	340	190	450

3-0-Methyl Glucose Transport in Tumor Cells

	Intact			Diabetic	
Insulin in vitro (M)	0	4×10^{-9} [1]	10^{-6}	0	10^{-6}
3-OMG Transport					
K_m (mM)	5.6	8.8	8.9	4.9	12.0
V_{max}[2]	1.9	2.3	2.2	1.7	2.4
v_i (at 5 mM)[2]	0.90	0.84	0.81	0.84	0.70

[1] Determined by radioimmunoassay for insulin.
[2] (μmoles/min × 10^6 cells) × 10^{-4}.

Glucose transport was examined under the same conditions employed for insulin binding. Initial velocity determinations were made at 15, 30, 45, and 60 sec using ^{14}C-3-OMG. The transport system was examined by competing 1 mM 3-OMG with phloridzin (200 μM), phloretin (200 μM), and D-glucose (10 mM) (Fig. 4). The inhibition of 3-OMG transport by glucose indicated that both substances utilize the same carrier system. Phloretin completely inhibited passive-mediated transport. This effect was observed when phloretin was added either 10 min prior to or simultaneously with the 3-OMG. Cells incubated with phloretin were used routinely in order to estimate diffusional entry of glucose into tumor cells (Fig. 5). The slight inhibition observed with phloridzin was attributed to a 5% impurity of phloretin.

The inhibitory effects of osmolarity on glucose transport were observed above 10 mM glucose. Mannitol, which does not compete with 3-OMG for passive transport, was added in appropriate amounts to maintain the osmolarity at 60 mM.

Kinetic analysis of the passive carrier was carried out on tumor cells from intact and diabetic animals (Fig. 6A, B). In the absence of insulin in vitro, the K_m and V_{max} were similar. The addition of insulin (4×10^{-9} M – 10^{-6} M) in vitro caused an increase in K_m, which was significant in cells from intact rats ($P = 0.05$); an even greater increase in K_m occurred in cells from diabetic rats ($P = 0.004$), as calculated from analysis of covariance. The estimated V_{max} also appeared to be increased in the presence of insulin. These results are summarized in Table I.

DISCUSSION

The data obtained with the R3230AC tumor cells indicate that the K_d for insulin binding was similar in cells from all groups studied. The number of insulin receptor sites per cell showed an apparent increase in tumors from diabetic rats. This may be explained

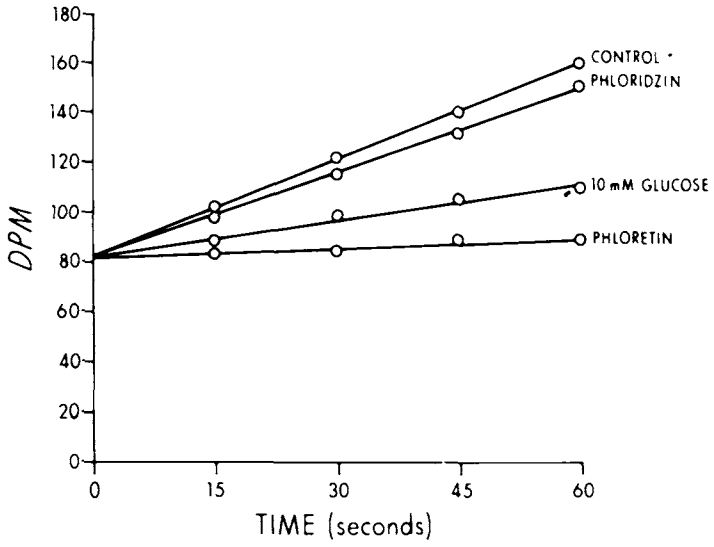

Fig. 4. Effect of competitors on 3-0-Methyl glucose transport. Each point is the average of duplicate samples.

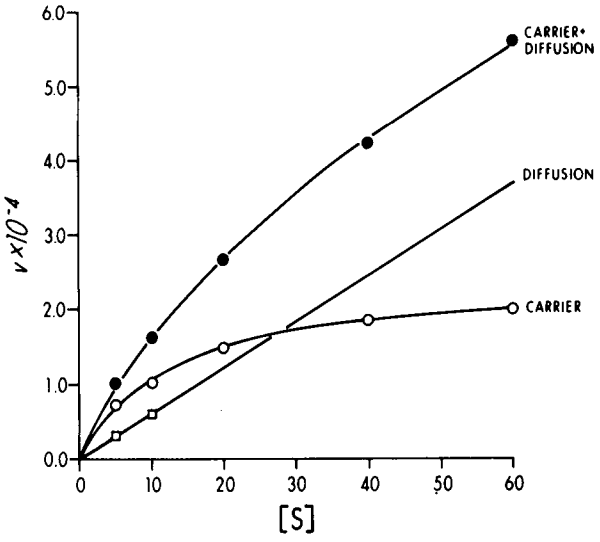

Fig. 5. Kinetics of 3-0-methyl glucose transport. Initial velocity obtained at 20°C. The diffusional component was estimated by the addition of 200 μM phloretin to 5 and 10 mM 3-0-methyl glucose samples. The difference between total transport (carrier + diffusion) and diffusional transport gave the carrier-mediated transport. v = initial velocity (μmoles/min × 10^6 cells). S = concentration of 3-0-methyl glucose (mM).

by: (a) a lack of endogenous insulin interfering with the estimation of receptor sites; (b) a synthesis of new receptor sites in the diabetic animal; or (c) a combination of both of these factors. Divergence from linearity shown in the Scatchard analysis suggests the presence of more than one class of receptor. However, DeMeyts et al. (6) have proposed that negative cooperativity, rather than multiple classes of receptors, might explain this lack of linearity. Preliminary experiments, designed to explore the existence of negative cooperativity in the tumor cells, gave results as predicted by the model proposed by DeMeyts et al. (6).

Kinetic analysis of 3-OMG transport suggested both diffusional and carrier transport. Glucose and phloretin competed with 3-OMG transport indicating the presence of a passive transport carrier for glucose. Diffusional transport was not altered by the addition of insulin in vitro. However, the passive carrier system was altered as reflected by a significant increase in K_m and an apparent increase in V_{max} at physiological levels of insulin (4×10^{-10} M). Insulin decreased the initial velocity of glucose transport in vitro at 4–6 mM glucose levels, but appeared to increase the v_i of glucose transport at 20 mM glucose.

On the basis of the earlier reported growth patterns of the R3230AC tumor in intact and diabetic rats (2), we interpret the results of the experiments on glucose transport presented here as: (a) inhibition of tumor growth resulting from exogenous administration of insulin (2 IU insulin/day per rat which produced $\sim 10^{-8}$ M levels in serum) could be due to inhibition of glucose transport, i.e. the decrease in v_i observed here by insulin in vitro; (b) the increased growth of the tumor in the diabetic rat may be primarily due to increased glucose uptake by diffusion (glucose levels in diabetic rats approximate 20 mM); and (c) the further increase in tumor growth in diabetic rats given small amounts of insulin (0.5 IU) may have resulted from both increased diffusional transport and an increase in

both V_{max} and v_i of the passive carrier, which was observed at > 20 mM glucose in vitro.

Studies are in progress to elucidate the effect of insulin on the R3230AC tumor with regard to further definition of glucose transport, amino acid transport, and glucose metabolism.

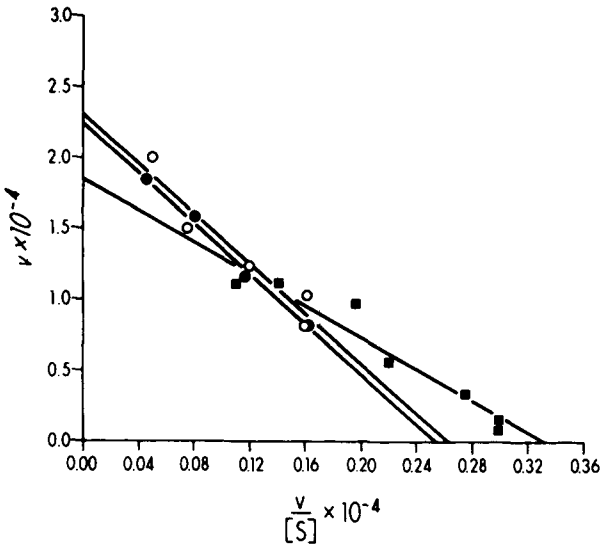

Fig. 6A. Data plotted according to Eadie-Hofstee (by linear regression analysis for best fitting line) showing the effect of insulin in vitro on the 3-0-methyl glucose carrier in tumor cells from intact rats. v = Initial velocity (μmoles/min x 10^6 cells), S = concentration of 3-0-methyl glucose (mM). Solid square, no insulin; open circle, 10^{-6} M insulin; solid circle, 4×10^{-9} M insulin.

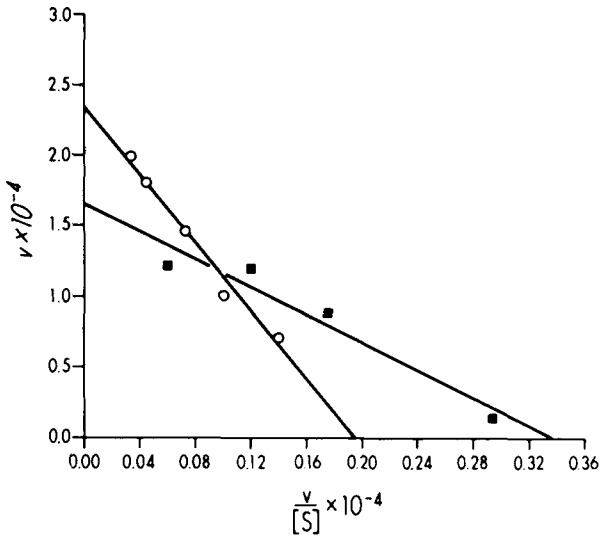

Fig. 6B. Data plotted according to Eadie-Hofstee showing the effect of insulin in vitro on the 3-0-methyl glucose carrier in tumor cells from diabetic rats. v = Initial velocity (μmoles/min x 10^6 cells), S = concentration of 3-0-methyl glucose (mM). Solid square, no insulin; open circle, 10^{-6} M insulin.

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