Effects of Insulin in Vivo and in Vitro on Amino Acid Transport into Cells from the R3230AC Mammary Adenocarcinoma and Their Relationship to Tumor Growth

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ABSTRACT

The R3230AC mammary carcinoma grows faster in diabetic rats; tumor cells from diabetic rats demonstrate increased proline transport and incorporation into proteins compared to tumor cells obtained from intact rats. Tumor cells from insulin-treated diabetic rats showed a decreased rate of proline transport and a lower amount of proline incorporation, both of which were comparable to that in cells from intact rats. The effects of insulin in vivo were time and dose related. The rate of α-aminoisobutyric acid entry, but not phenylalanine or leucine, was also higher in tumor cells from diabetic rats than from those in intact rats. Kinetic analysis of α-aminoisobutyric acid uptake indicated that α-aminoisobutyric acid was transported by two carriers. The increase in α-aminoisobutyrate uptake in the absence of insulin was due to an increase (~170%) in the Vmax of the Na+-dependent component; no difference in Kmax was observed. The Na+-independent component of α-aminoisobutyric acid uptake was not significantly increased in cells from diabetic rats. No differences were observed in 3-O-methylglucose transport, 14CO2 production from uniformly labeled glucose, or incorporation of labeled glucose into fatty acids for cells from intact or diabetic rats.

In contrast, insulin in vitro increased proline transport and its incorporation in cells from diabetic but not from intact rats. The response of cells from insulin-treated diabetic animals was blunted. These responses to insulin in vitro were shown to be dose related and occurred over the physiological range for insulin in vivo. Thus, the enhanced proline transport and incorporation seen in cells from diabetic rats may contribute to the enhanced tumor growth; administration of insulin in vivo reversed this effect on proline and reduced tumor growth. We suggest that these paradoxical effects of insulin in vivo versus those in vitro on proline transport may be due to the presence of other hormones in vivo, which modulate the effects of insulin.

INTRODUCTION

The R3230AC mammary adenocarcinoma, a transplantable tumor of Fischer rats, was classified as an autonomous, hormone-responsive neoplasm, on the basis of extensive studies with estrogens, prolactin and, more recently, insulin (4, 23). The R3230AC tumor grew faster in diabetic rats than in intact rats, but administration of pharmacological levels of insulin to intact or diabetic rats resulted in inhibition of tumor growth (4). Recent work from this laboratory has shown that cells isolated from this tumor by enzymatic dissociation bind insulin specifically and with high affinity, implying the presence of insulin receptors with properties similar to those reported for other tissues (22). Insulin-binding capacity was greater in cells from diabetic rats than in cells from intact rats; the affinity for insulin binding, however, was similar (20, 35). A passive carrier system for glucose transport was identified in these tumor cells (20, 21). Insulin in vitro was found to decrease the v of 3-O-MG transport at 4 to 6 mM 3-O-MG levels, but there was an apparent increase in the v when glucose concentration was raised to 20 mM. These findings accounted for the increase in both the Km and Vmax of 3-O-MG transport in cells from diabetic rats.

An important hormonal role for insulin is regulation of amino acid transport and/or the subsequent incorporation of amino acids into protein (1-3, 10-19, 27, 28, 33). Neutral amino acids have been shown to be transported primarily by 2 carriers, namely the A and L systems (5-7). A third transport system is limited to a narrow range of amino acids consisting of alanine, serine, and cysteine (8). Insulin has been found to stimulate uptake of those amino acids that are transported by the A system, as demonstrated by studies with the isolated rat diaphragm (33) and chick embryo heart cells (17). When AIB was used as the amino acid probe for studies with liver tissue, insulin was reported to stimulate AIB uptake by a Na+-dependent transport carrier that had somewhat different properties than the A system and hence is referred to as the "B" system (27).

With proline as the amino acid probe, previous studies from this laboratory have demonstrated the existence of a transport carrier with properties similar to those described for the A system in the Ehrlich ascites tumor cells (25). The objective of the experiments reported here was to ascertain the effect of insulin, or its lack (i.e., diabetes), in vivo on the transport of amino acids in cells from this mammary carcinoma. Experimental results are also presented on the effects of insulin in vitro. A relationship of these results to tumor growth behavior is proposed.

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2 Submitted in partial fulfillment of the requirements for the Ph.D. degree.
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MATERIALS AND METHODS

Female Fischer rats (80 to 90 g) were obtained from Charles River Breeding Laboratory (Wilmington, Mass.). The rats were offered food and water ad libitum. The R3230AC tumor was implanted s.c. in the axillary region on both sides by a sterile trochar technique as described by Hilf et al. (24).

Diabetes was induced by administration of streptozotocin into the jugular vein 2 days prior to tumor implantation (4). Streptozotocin was dissolved in 0.9% NaCl solution and rapidly adjusted to pH 4.5 with 0.025 M citric acid. Due to the instability of the drug, all injections were made within 30 min of dissolving the drug. The dose administered was 60 mg/kg. Blood glucose was determined by the glucose oxidase method (Glucostat; Worthington Biochemical Corp., Freehold, N. J.), and the urinary glucose was estimated with Clinistix (Ames Co., Elkhart, Ind.). Animals were classified as diabetics if their blood glucose levels were greater than 250 mg/100 ml and if their urine glucose level exceeded 0.5 g/100 ml. In some experiments insulin (protamine zinc insulin; Eli Lilly and Co., Indianapolis, Ind.) was administered s.c., 4 IU/day, to tumor-bearing diabetic rats for various times prior to sacrifice.

Animals were killed by cervical dislocation at 19, 20, or 21 days after tumor implantation. Tumors were excised as quickly as possible and placed in ice-cold 0.9% NaCl solution. After removal of connective and necrotic tissues, 3 g of tumor tissue were minced into 1- x 1-mm pieces on a Mcllwain tissue slicer (Brinkmann Instruments, Inc., Westbury, N. Y.). The minced tissue was incubated in 250-ml Erlenmeyer flasks with 10 ml of Hanks’ balanced salt solution (Ca²⁺- and Mg²⁺-free), containing 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) and 0.05% collagenase (type II), for 25 min at 37° in a Benco shaking water bath (80 cycles/min); cells were obtained at the end of the incubation period, the mixture was poured through a 100 mesh stainless steel strainer. The nondissociated tissue remaining on the strainer was returned to the flask with a fresh 10-ml portion of enzyme solution and incubated for an additional 25 min at 37° in a Benco shaking water bath (approximately 50 cycles/min). At the end of the incubation period, the mixture was poured through a 100 mesh stainless steel strainer. The nondissociated tissue remaining on the strainer was returned to the flask with a fresh 10-ml portion of enzyme solution and incubated for an additional 25 min; the filtrates from the first incubation mixture were discarded due to considerable contamination by RBC and cellular debris. At the end of the second hr, the incubation mixture was again strained, and the nondissociated pieces were rinsed with HEPES buffer. The filtrates and the wash mixtures were combined and centrifuged for 5 min at 80 x g. The cell pellet was then washed 3 more times with 10 volumes of buffer. Cell viability was estimated by trypan blue exclusion, and cell number was determined by the use of a hemocytometer. Cell preparations used had >90% viability (21).

Transport Studies. Transport was performed on 5 x 10⁷ cells in Falcon plastic tubes in a final volume of 1 ml of HEPES buffer, pH 7.5. The buffer consisted of 25 mM HEPES, 10 mM NaHCO₃, 130 mM NaCl, 3 mM K₂HPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 11 mM glucose, and bovine serum albumin, 1 mg/ml, with a final osmolarity of 310 mOsm. In experiments in which Na⁺ was omitted, NaCl and NaHCO₃ were replaced by choline chloride and choline bicarbonate at equimolar concentrations, thereby maintaining the same osmolarity throughout. When transport of 3-O-MG was measured, glucose was replaced by an equimolar amount of mannitol.

Initial velocity measurements were determined by adding the amino acid (labeled and unlabeled) to cells in a Benco shaking water bath (80 cycles/min); cells were obtained at 15, 30, 45, and 60 sec after addition of the labeled amino acid. Transport was terminated rapidly by adding 8 ml of ice-cold 0.9% NaCl solution to each tube followed immediately by centrifugation for 1 min at 3° (900 x g). After the supernatant was decanted, the cells were washed with another 8 ml of ice-cold 0.9% NaCl solution and centrifuged for 2.5 min (900 x g), and the supernatant was decanted. The tubes were allowed to stand (inverted) for 15 min to drain; any remaining droplets on the sides of the tubes were wiped. The cell pellet was dissolved in 2 successive 5-ml portions of aqueous counting scintillant (Amersham/Searle) and transferred to vials, and the samples were counted in a liquid scintillation counter (Isocap 300; Nuclear Chicago, Chicago, Ill.). The efficiency for counting ¹⁴C was 60% and that for ³H was 40%.

Intracellular and extracellular space measurements were determined by use of [³H]-3-O-MG and [¹⁴C]inulin in the same cell preparation. The cells were incubated under the same conditions as described above, for at least 45 min, a time when 3-O-MG has reached equilibrium; 3-O-MG was shown by Harmon and Hilf (21) to be transported into these tumor cells by facilitated diffusion reaching equilibrium at approximately 30 min at 20°. In experiments in which prolonged incubation was required, the tubes were incubated with 3-O-MG and inulin for the same period of time under study; the intracellular space thus calculated should reflect this parameter at the time when transport was measured. The tubes were centrifuged for 5 min, 100-µl aliquots of the supernatant were taken, and the remaining supernatant was decanted. The tubes were wiped and radioactivity (³H and ¹⁴C) in the pellet was counted.

The "space" measurements were calculated according to the formula:

\[
\frac{cpm \, ^3H \, (pellet)}{cpm \, ^3H \, (supernatant)} = \text{intracellular + extracellular space} \left(\mu l/5 \times 10^7 \text{ cells}\right)
\]

\[
\frac{cpm \, ^{14}C \, (pellet)}{cpm \, ^{14}C \, (supernatant)} = \text{Extracellular space} \left(\mu l/5 \times 10^7 \text{ cells}\right)
\]

\[
(1) - (2) = \text{intracellular space} \left(\mu l/5 \times 10^7 \text{ cells}\right)
\]

After the intracellular volume was obtained, the concentration of labeled amino acid taken up by the cells was calculated and a distribution ratio was calculated by the following formula:

\[
\text{Distribution ratio} = \frac{\text{Concentration of the amino acid in the intracellular space}}{\text{Concentration of the amino acid in the medium at the end of the incubation}}
\]

Uptake is reported either as distribution ratio or as µmol/min/5 x 10⁷ cells. Cells prepared as described above were found to be suitable for transport studies conducted for moderately prolonged incubation periods; cells incubated at either 37° or 4°, in the presence or absence of Na⁺,
showed no significant change in proline transport over a 4-
hr incubation period. Further, proline accumulation against a
concentration gradient was maintained. The cells dis-
played minimal changes in viability (3 to 4% change) and in
intracellular space (4.3 ± 0.13 µl/5 x 10^6 cells.)

Incorporation of Radioactivity Into Macromolecules. In-
corporation of substrates into protein by tumor cells was
estimated by incubating 10^6 cells in glass tubes containing 1
ml of HEPES buffer, pH 7.5, with either 0.1 mM proline plus
0.1 µCi ^14C-proline or 0.1 mM leucine plus 2 µCi
^3H-leucine. The tubes were placed in a shaking water bath
and incubated at 37° for various periods of time up to 4 hr.
Incorporation was terminated by adding 8 ml of ice-cold
0.9% NaCl solution to each tube, followed immediately by
centrifugation for 2 min at 3° (900 x g). After the supra-
antant was decanted, 1 ml of 10% trichloroacetic acid was
added to the pellet. The contents were mixed and then
allowed to stand for 10 min. The tubes and contents were
adjusted again and centrifuged for 5 min at 900 x g; the
resulting supernatants were removed with a Pasteur pipet.
The cell pellets were washed twice; first with 2 ml of 10% trichloroacetic acid and then with 2 ml of 0.9% NaCl solution.
One ml of hot (95°) 5% trichloroacetic acid was added to the pellet, the contents of each tube were refluxed at 105° for 30 min, and the tubes were allowed to cool, and
then centrifuged for 5 min at 900 x g. After the supernatant was discarded, the pellet was washed 3 times with 2-ml portions of 0.9% NaCl solution; the remaining pellet was
dissolved in 2 successive 5-ml portions of aqueous counting
scintillant and transferred to vials, and radioactivity was
counted. The amount of radioactivity was indicative of the
labeled amino acid incorporated into proteins.

Production of ^14CO_2 from Labeled Glucose. Quantitative
measurement of labeled CO_2 produced by the R3230AC
tumor cells was determined by incubating 10^6 cells with 5
mM D-glucose and 0.2 µCi of [U-^14C]glucose (150 to 250
mCi/mmol) in a final volume of 5.0 ml of HEPES buffer
(without glucose), pH 7.5, in a 25-ml Erlenmeyer flask. The
flasks were gassed for 30 sec with 95% O_2:5% CO_2 and then
centrifuged for 30 sec at 105° for 30 min, and the tubes were allowed to cool, and
then centrifuged for 5 min at 900 x g. After the supernatant was discarded, the pellet was washed 3 times with 2-ml portions of 0.9% NaCl solution; the remaining pellet was
dissolved in 2 successive 5-ml portions of aqueous counting
scintillant and transferred to vials, and radioactivity was
counted. The amount of radioactivity was indicative of the
labeled amino acid incorporated into proteins.

RESULTS

Effect of Insulin on Tumor Growth. Tumor growth in
diabetic rats was faster than that observed in intact rats
(Table 1), despite the fact that the total body weight of
diabetic rats was significantly reduced. The effect of dia-
etes on tumor growth becomes even more apparent when
expressed as tumor weight per 100 g body weight; this
index of tumor growth in diabetic rats was about twice that
in intact rats.

Administration of insulin to diabetic rats for various
periods of time ranging from 1 to 21 days after tumor
implantation corrected the diabetic condition as manifested by the return of blood glucose levels to normal as well as

Table 1

<table>
<thead>
<tr>
<th>Animal and therapy</th>
<th>Tumor wt (g)</th>
<th>Body wt (g)</th>
<th>Tumor wt/100 g body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (9)²</td>
<td>6.40 ± 0.42²</td>
<td>138 ± 5</td>
<td>4.66 ± 0.35</td>
</tr>
<tr>
<td>Diabetic (20)²</td>
<td>9.02 ± 0.63²</td>
<td>98 ± 3³</td>
<td>9.17 ± 0.61³</td>
</tr>
</tbody>
</table>
| Diabetic plus insu-
lin treatment for: |             |             |                        |
| 8 days (5)        | 9.73 ± 1.30 | 150 ± 7²    | 6.48 ± 1.20            |
| 21 days (5)       | 3.65 ± 0.50⁴ | 145 ± 9⁵    | 2.65 ± 0.14⁴           |

a Numbers in parentheses, number of animals per group.
b Mean ± S.E.
c Diabetic induced by streptozotocin (60 mg/kg) 2 days prior to
implantation of tumor. Insulin treatment consisted of daily s.c.
injections of 4 IU/rat.
d Significantly different (p < 0.005) from untreated diabetic
animals.
weight (by 8 days) and a relative decrease in tumor growth, was shown to be transported into these tumor cells exclusively by the A system (25).

Proline Accumulation in Tumor Cells from Intact and Diabetic Rats and Sensitivity to Metabolic Inhibitors. It was desirable to seek out a correlation between tumor growth, and specific biological responses that are affected by insulin such as substrate transport and metabolism. Since insulin has been shown to stimulate the entry of those amino acids transported by the A system, and not by the L system, the entry of proline was examined; proline was shown to be transported into these tumor cells exclusively by the A system (25).

The accumulation of proline into tumor cells from either intact or diabetic rats, as determined by incubating these cells with proline for various periods of time up to 4 hr, demonstrated differences. Cells obtained from diabetic rats accumulated proline at a faster rate and to a greater extent than cells from intact rats (Chart 1). When PCP, an inhibitor of mitochondrial ATP synthesis (32), was added to cells at the beginning of the incubation period with labeled proline, the proline accumulation in cells from diabetic or intact rats was significantly decreased (Chart 2). The level of proline accumulation in the presence of PCP, however, was still higher than that accumulated in the absence of Na*. When PCP was added to cells after 90 min or proline accumulation, cells from either intact or diabetic rats could not maintain the high intracellular proline concentrations; PCP caused a loss of accumulated proline to a level that was similar to that obtained when PCP was added at zero time. Taken together, these results indicate that the general transport characteristics of the A system were not affected by insulin such as substrate transport and metabolism. However, the rate of proline entry was altered in the absence of insulin.

AIB Entry in Tumor Cells from Intact and Diabetic Rats. To confirm the effects of diabetic on proline transport, we also measured the entry of the amino acid analog, AIB. AIB has been reported to be transported by the A system in Ehrlich ascites cells and the isolated rat diaphragm (7, 33).

We have characterized AIB transport with R3230AC tumor cells and concluded that entry of this analog occurs by both Na*-dependent and Na*-independent processes with specificities that resemble those attributed to the A and L systems, respectively (26). Therefore, the uptake of AIB (at various concentrations) was determined in the presence and absence of Na* in cells from both intact and diabetic rats. The uptake of AIB in the absence of Na* demonstrated a saturable, carrier-mediated entry (Chart 3A), and when these data were analyzed according to the Eadie-Hofstee plot they depicted a linear component (Chart 3B). At each concentration studied, the uptake of AIB in the absence of Na* was subtracted from AIB uptake in the presence of Na* (total uptake); a saturable process was then obtained (Chart 3A), which was also depicted as a linear relationship in the Eadie-Hofstee plot (Chart 3B). These results indicate that the Na*-dependent entry most probably occurs by one transport system.

The graphic analysis shown in Chart 3 indicates that the $V_{\text{max}}$ for the Na*-sensitive uptake of AIB was greater in cells from diabetic rats compared to cells from intact rats, i.e., 2035 and 1200 pmol/min/5 x 10^6 cells, respectively; no difference in $K_m$ was observed (2.13 and 2.38 mm, respectively). Furthermore, the $V_{\text{max}}$ of the Na*-independent uptake for AIB appeared to be larger in cells from diabetic rats than in those from intact rats; $V_{\text{max}}$ 740 and 534 pmol/min/5 x 10^6 cells, respectively; however, this was not a significant difference ($p > 0.05$). No difference in $K_m$ was observed; 0.42 versus 0.38 mm, respectively. Thus, the results obtained with AIB agree with those described above for proline transport; i.e., the effect of insulin in vivo is directed...
Primarily towards amino acids that are transported by the A system.

Effect of Insulin in Vivo on Proline and Leucine Transport: Time Course and Dose Response. Diabetic or intact rats were given injections of insulin, 4 IU/day, and sacrificed at various times; transport was measured in dissociated cells (Table 2). A significant decrease in proline transport was noted at 8 days in diabetic rats and by 5 days in intact rats; no change in leucine transport resulted from administration of insulin. This dose of insulin to diabetic rats returned blood glucose levels to normal by 3 days of treatment, and glucosuria was absent at this time.

The effect of dose of insulin on transport was examined in cells from diabetic rats given daily injections for 8 days. A dose-related decrease in proline transport was found, with 1 IU/day producing a significant decrease in the \( v \) for proline entry; higher doses of insulin produced further decreases (Table 2). Under the same conditions, leucine transport was unchanged. Thus, the effects of insulin in vivo on proline transport were time and dose dependent and were specific for the A system.

Effect of Insulin in Vitro on Proline Transport: Time Course. The above effects of insulin were opposite to those frequently reported for insulin on amino acid transport (3, 10, 11, 16, 33), effects that were usually examined in vitro. We therefore examined the effect of insulin, added to freshly dissociated cells (Group A), on transport of proline at times varying from 15 min to 9 hr (Table 3). No effects were seen until 5 hr of incubation; at that time and thereafter, a progressive increase in proline transport was observed.

The above experiments lasted 12 hr, and cell viability became a limiting factor. To reduce this time, we explored 2 additional protocols. In one (Group B), insulin was added once to tissues at the same time that enzymatic dissociation procedures were initiated: to the other (Group C), insulin was added as for Group B and again after the cells had been prepared. Proline transport was stimulated to a comparable degree with either protocol; further, these results were similar to those when insulin was added after cells had been prepared (Group A). For convenience, further experiments were usually conducted for 7.5 hr according to the procedure described for Group C.

Effect of Insulin on Proline Transport. In the above experiments we used \( 10^{-6} \) M insulin, a level that approximates the level of 5 \( \mu \)g/ml commonly used in tissue and organ culture systems (36, 37), as well as in studies on amino acid transport (1, 10, 12, 13, 17, 19, 27, 28, 34). To ascertain if the stimulation of proline transport was due to this unphysiological dose, a dose-response study was performed (Table 4). A small but significant increase in proline transport was observed in the presence of \( 10^{-10} \) M insulin and this response was increased as the level of insulin was increased. Similar results were obtained for insulin added to cells after enzymatic dissociation (Group A) or when the hormone was added before and after the cell dissociation procedure (Group C). The specificity of the response was confirmed by the finding that leucine transport was altered by insulin. The lower level of insulin used here is well within the physiological range and the level of \( 10^{-8} \) M insulin is comparable to that found by radioimmunoassay of plasma from rats given injections of 4 IU insulin twice daily (20).

Effect of Insulin on Leucine Incorporation into Proteins In Vitro. The ability of insulin to stimulate incorporation of leucine into trichloroacetic acid-precipitable material was
Insulin and Amino Acid Transport in Mammary Tumors

Table 2
Effect of time course and dose of insulin in vivo on transport of amino acids

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Dose of insulin (IU)</th>
<th>Intact animal</th>
<th>Diabetic animal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proline (pmol/min/5 x 10^6 cells)</td>
<td>Leucine (pmol/min/5 x 10^6 cells)</td>
<td>Proline (pmol/min/5 x 10^6 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>153 ± 10 (4)</td>
<td>1537 ± 26 (5)</td>
<td>259 ± 25 (17)</td>
</tr>
<tr>
<td></td>
<td>141 ± 21 (3)</td>
<td>1547 ± 15 (5)</td>
<td>279 ± 10 (5)</td>
</tr>
<tr>
<td></td>
<td>258 ± 9 (5)</td>
<td>1573 ± 48 (5)</td>
<td>258 ± 10 (5)</td>
</tr>
<tr>
<td></td>
<td>204 ± 7 (5)</td>
<td>1552 ± 27 (5)</td>
<td>204 ± 7 (5)</td>
</tr>
<tr>
<td></td>
<td>141 ± 10 (5)</td>
<td>1537 ± 26 (5)</td>
<td>141 ± 10 (5)</td>
</tr>
</tbody>
</table>

*Mean ± S.E.*

Numbers in parentheses, number of animals.

Significantly different (p < 0.05) from cells studied during first hr of exposure to insulin.

Table 3
Time course of effects of insulin (10^-10 M) in vitro on proline transport into cells from diabetic rats

Data are expressed as percentage of control (no insulin) examined under identical conditions. Basal level of proline transport in the absence of insulin for 74 determinations showed a mean ± S.E. of 254 ± 7 pmol/min/5 x 10^6 cells. The percentage of stimulation for the insulin response was calculated for each observation and is presented as the mean ± S.E. for each time period.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>103 ± 1(14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5-2.0</td>
<td>106 ± 2 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5-3.0</td>
<td>107 ± 2 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>114 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>119 ± 12 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>136 ± 2 (3f)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>156 ± 11 (5f)</td>
<td>172 ± 14 (4f)</td>
<td>159 ± 6 (6f)</td>
</tr>
<tr>
<td>7.0</td>
<td>176 ± 17 (3f)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>186 ± 11 (2f)</td>
<td>177 ± 6 (10f)</td>
<td>186 ± 11 (2f)</td>
</tr>
<tr>
<td>8.0</td>
<td>189 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>195 (1)</td>
<td>207 (1)</td>
<td>206 ± 22 (9f)</td>
</tr>
</tbody>
</table>

*Some results represent an average from samples examined at various time intervals within the time range given: 0 to 1 hr, 1 sample at zero time, 3 at 15 min, 3 at 30 min, 3 at 45 min, and 4 at 60 min; 1.5 to 2.0 hr, 4 samples at 1.5 hr and 3 at 2.0 hr; and 2.5 to 3.0 hr, 2 samples at 2.5 hr and 5 at 3.0 hr.

Insulin added after cells were prepared.

Insulin added during cell preparation (enzyme dissociation).

Insulin added during cell preparation steps and again, after cells were prepared.

Significantly different (p < 0.05) from cells studied during first hr of exposure to insulin (0 to 1-hr group).

studied by exposure of cells to a wide range of insulin concentrations: 10^-10 to 10^-6 M for Group A protocol experiments and 10^-11 to 10^-4 M insulin for Group C protocol experiments. A dose-response stimulation of leucine incorporation was seen (Table 5) with a small but significant effect observed at 10^-10 M insulin. As the level of insulin added was increased, the percentage of stimulation of leucine incorporated was increased; results were comparable in cells studied under both protocols. Thus, insulin in vitro was effective in stimulating leucine incorporation at hormone levels that exist in vivo.

Effect of Insulin in Vitro on Proline Transport and Incorporation into Cells from Intact, Diabetic, or Insulin-treated Diabetic Rats. Since we observed differences in transport among tumor cells from intact, diabetic, or diabetic insulin-treated rats, the response of these cells to insulin in vitro was also examined (Group C protocol). The data are shown in Chart 4. Transport of proline remained constant in cells not exposed to insulin during the times studied, and transport was highest in cells from diabetic rats; basal proline transport in cells from intact rats or from diabetic rats treated with 4 IU insulin per day for 1 week prior to sacrifice was similar. Addition of 10^-6 M insulin in vitro increased proline transport in cells from diabetic rats, the increase progressing with time. Curiously, tumor cells from intact rats showed no response to insulin in vitro, and tumor cells from diabetic rats treated with insulin in vivo demonstrated a markedly blunted response to insulin in vitro. These findings are consistent with those shown in Table 6, in which the distribution ratio for proline uptake was calculated; cells from diabetic rats demonstrated a considerable increase in the distribution ratio in response to insulin. Incorporation of proline into proteins was measured in these cells (Chart 5). All cells demonstrated a gradual time-related increase in proline incorporation (no insulin) and this was stimulated by insulin in cells from diabetic rats but not in cells from intact rats. For this parameter, insulin in vitro increased the amount of proline incorporated by cells from insulin-treated rats.

Effects of Diabetes on Other Substrates. While emphasis was directed towards transport of amino acids utilizing the A system, we wished to ascertain the specificity of the effects observed in the diabetic rat. Compared to tumor cells obtained from intact animals and studied under similar experimental conditions, diabetes produced no change in the transport of phenylalanine, leucine, or 3-O-MG, nor were there significant changes in the utilization of uniformly labeled glucose, as reflected by 14CO2 production or incorporation of label into fatty acids.

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Table 4  
Effect of dose of insulin in vitro on proline and leucine transport in cells from diabetic rats

<table>
<thead>
<tr>
<th>Level of insulin in vitro (M)</th>
<th>Transport (pmol/min/5 x 10⁶ cells)⁰</th>
<th>Group A⁵</th>
<th>Group C⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proline</td>
<td>Leucine</td>
<td>Proline</td>
</tr>
<tr>
<td>0</td>
<td>233 ± 4² (3)²</td>
<td>1480 ± 13 (3)²</td>
<td>259 ± 9 (10)²</td>
</tr>
<tr>
<td>10⁻¹⁰</td>
<td>280 ± 4 (3)²</td>
<td>1509 ± 63 (3)²</td>
<td>324 ± 8 (3)²</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>324 ± 8 (3)²</td>
<td>1466 ± 66 (3)²</td>
<td>399 ± 16 (3)²</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>426 ± 5 (3)²</td>
<td>1519 ± 67 (3)²</td>
<td>483 ± 23 (10)²</td>
</tr>
</tbody>
</table>

* Measurements made 7.5 hr after insulin was added.
* Insulin added after cells were prepared.
* Insulin added during cell dissociation steps and again after cells were prepared.
* Mean ± S.E.
* Numbers in parentheses, number of animals.
* Significantly different (p < 0.02) from cells not exposed to insulin.

Table 5  
Effect of concentration of insulin on leucine incorporation in vitro into cells from diabetic rats

Data are expressed as percentage of control (0 insulin = 100%) examined under identical conditions. The percentage stimulation was calculated for each observation and is presented as mean ± S.E. at each concentration. Numbers in parentheses represent number of tumors. Incorporation into macromolecules was measured over a 1-hr incubation period; total exposure to insulin was 7.5 hr.

<table>
<thead>
<tr>
<th>Insulin concentration (M)</th>
<th>Group A⁶</th>
<th>Group C⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 4 (3)</td>
<td>100 ± 3 (3)</td>
</tr>
<tr>
<td>10⁻¹⁰</td>
<td>127 ± 4 (3)⁶</td>
<td>120 ± 3 (3)⁶</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>129 ± 3 (3)⁶</td>
<td>148 ± 10 (3)⁶</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>145 ± 1 (3)⁶</td>
<td>146 ± 2 (3)⁶</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>178 ± 2 (3)⁶</td>
<td>187 ± 8 (3)⁶</td>
</tr>
</tbody>
</table>

* Insulin added after cells were prepared.
* Insulin added during cell preparation steps and again, after cells were prepared.
* Significantly different (p < 0.02) from cells not exposed to insulin.

DISCUSSION

As one approach to understanding of the role of insulin in growth of the R3230AC mammary tumor, experiments were conducted to examine the effects of insulin, or its lack (i.e., diabetes) in vivo, on substrate transport and metabolism. For amino acid transport, we selected: (a) proline, since we found (25) that it entered these cells exclusively by the A system; (b) AIB, a nonmetabolizable amino acid analog that has been used by others as a specific probe for the A system; and (c) phenylalanine and leucine, amino acids that enter primarily by the L system. Under the conditions used here, dissociated tumor cells from diabetic rats were capable of transporting greater amounts of proline and AIB (in the presence of Na⁺) than were cells from intact animals; no differences in the rate of entry of phenylalanine or leucine were seen. Since we also observed no differences in the transport of 3-O-MG or the metabolism of labeled glucose, we conclude that the effects of diabetes are directed towards the A system in this tumor.

This conclusion is supported by the data in which administration of insulin to tumor-bearing diabetic rats corrected the diabetic condition and decreased proline transport in a dose-related fashion to levels that were comparable to that seen in cells from intact rats. Although insulin treatment corrected the glucosuria by the second day of treatment, the effect on proline transport was not seen until some time after 3 days of therapy; interestingly, insulin treatment also reduced proline transport in tumor cells from intact rats by 5 days of therapy. Although we anticipated that insulin would affect proline transport, based on reports by others (14, 16, 33), the increase in proline transport in cells from diabetic rats and its decrease following insulin treatment of diabetic or intact rats were unexpected, since it suggested an apparent inhibitory role for insulin in vivo on proline transport in this mammary tumor. The specificity of the insulin response of the A system is suggested by the fact that phenylalanine or leucine transport was similar under various conditions of varied insulin levels; phenylalanine and leucine transport was exclusively via the L system. Furthermore, since AIB is a useful probe of the A system in other tissues (7, 33), as well as in this tumor (26), Na⁺-dependent AIB uptake in cells from diabetic rats adds support to the above results with proline.

In order to examine the effects of insulin by itself, we conducted experiments with dissociated cells exposed to insulin in vitro. Under such conditions, insulin stimulated transport of proline and incorporation into proteins in tumor cells obtained from diabetic rats. These responses were time dependent, with the increase occurring at 5 hr after exposure to insulin. Since we had chosen to use a very high level of insulin (10⁻⁶ M), assuming that lack of a response at this level would suggest problems with the in vitro system, we then examined the dose relationship of insulin to the enhanced proline uptake. The data obtained demonstrated that insulin was capable of stimulating proline transport and leucine incorporation into proteins, in direct relationship to the hormone level, and further that the stimulation was significant at levels of the hormone well within those circulating in vivo (10⁻¹⁰ to 10⁻⁸ M). Indeed, the plasma levels of insulin measured in rats treated with 4 IU insulin twice daily were 2 x 10⁻⁸ M (20), a level within the range studied in vitro. Such comparisons must be taken with caution, since it would imply that one can simply compare levels of a hormone in vitro to those in vivo.
transport processes (9, 31, 34). Studies from our laboratory effects to a reciprocal relationship between insulin and Additional studies by these investigators attribute these this proposal is the recent report by Kilberg and Neuhaus tumor growth in vivo in response to insulin therapy; en in vivo to decrease transport correlates with decreased the response of this tumor to insulin in vitro would not may play a role in the response to insulin in vivo, a role that predicts for the biological behavior of the neoplasm in vivo, ignoring the other factors present in vivo. However, the data reported here do demonstrate that proline transport was affected in a dose-related manner by insulin for in vivo versus in vitro conditions.

No simple explanation can be offered for this paradoxical action of insulin in vivo versus in vitro. The effect of insulin in vivo to decrease transport correlates with decreased tumor growth in vivo in response to insulin therapy; enhanced transport was found in tumor cells from diabetic rats in which tumor growth was accelerated. Unfortunately, the response of this tumor to insulin in vitro would not predict for the biological behavior of the neoplasm in vivo, if one assumes that transport of amino acid substrate is a rate-limiting process. It would appear that other hormones may play a role in the response to insulin in vivo, a role that would modulate or alter the response to insulin. In favor of this proposal is the recent report by Kilberg and Neuhaus (27), who demonstrated that AIB uptake into perfused liver from diabetic rats was elevated and insulin treatment (4 IU) returned AIB uptake to that seen in livers from intact rats. Additional studies by these investigators attribute these effects to a reciprocal relationship between insulin and glucagon. It has been shown earlier by others that glucocorticoids in vitro can antagonize the effects of insulin on transport processes (9, 31, 34). Studies from our laboratory have shown that estrogens can decrease insulin binding to this mammary tumor and furthermore that therapy with both insulin and estrogen was additive to cause decreased tumor growth (4, 35). We now have experimental evidence that estrogens decrease proline transport in cells from this tumor, effects that were seen both in vivo and in vitro. On this basis, we propose that the effects seen here with other hormones capable of modulating the action of insulin. Experiments are in progress to elucidate the role of other hormones in the action of insulin on transport processes although, admittedly, the results from experiments in vivo will be more difficult to interpret than those obtained in vitro.

Another aspect of the work presented here on response of tumor cells to insulin in vitro relates to the data obtained from studies of tumor cells prepared from hormonally altered hosts. It was clear that cells from diabetic rats responded to insulin in vitro with an elevation of proline transport, an increased distribution ratio, and an enhanced incorporation of label into proteins. In contrast, cells from intact rats were unresponsive to high levels of insulin in

Table 6: Effect of insulin (10⁻⁶ M) in vitro on distribution ratio for proline uptake in cells from intact, diabetic, or insulin-treated diabetic rats

<table>
<thead>
<tr>
<th>Total exposure to insulin (hr)</th>
<th>Proline uptakeb</th>
<th>Intact</th>
<th>Diabetic</th>
<th>Insulin-treated diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(hr)</td>
<td>Control</td>
<td>+Insulin</td>
<td>Control</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>5.72 ± 0.49</td>
<td>6.23 ± 0.34</td>
<td>8.91 ± 0.55</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>8.92 ± 0.69</td>
<td>8.95 ± 0.47</td>
<td>13.6 ± 1.0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>10.5 ± 0.7</td>
<td>8.93 ± 0.52</td>
<td>15.1 ± 0.91</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>10.8 ± 0.7</td>
<td>9.01 ± 1.03</td>
<td>16.3 ± 0.95</td>
</tr>
</tbody>
</table>

a Insulin (10⁻⁶ M) was added to tissues at the start of the cell preparation procedure and again after cells were prepared (Protocol Group C). The times indicated for insulin exposure are cumulative from the initial exposure to hormone (zero time). b Period of time in which cells were exposed to labeled proline (0.1 mM). c Significantly different (p < 0.05) from control (no insulin) cells.
vitro, and tumor cells from diabetic rats that have received insulin therapy prior to sacrifice demonstrated a blunted response to insulin in vitro. One possible explanation for these results may involve regulation or availability of insulin receptors. In earlier reports from our laboratory, we found that insulin binding was significantly higher in tumor cells from diabetic rats than in that seen in tumors from intact rats or diabetic animals treated with insulin (20, 35). If insulin binding and response are related, we would have expected the order of responsiveness to be in line with what we found here, namely, diabetic > insulin-treated intact. While it is not apparent why cells from intact rats showed no effect of insulin on proline transport, we have observed that \( ^4 \text{CO}_2 \) production from uniformly labeled glucose was increased by insulin in cells from intact rats (data not shown). The fact that insulin may have effects on one class of substrate in the absence of effects on other substrates has been shown for thymocytes by Goldfine et al. (13), and it suggests that hormone-induced responses may be dissociated one from another depending on time, dose, and other modulating factors.

The specificity of the response to insulin, either in vivo or in vitro, was clearly demonstrated by the results indicating that amino acids utilizing the A carrier system were affected but not those utilizing the L carrier system. The effect observed was an increase in the \( V_{\text{max}} \) for proline or AIB transport with little or no change in the \( K_{\text{m}} \) of the carrier. Results such as these have been interpreted by some (16) to suggest that the hormonal perturbation resulted in an increase in the synthesis of such carriers rather than in the affinity of the carrier for its substrate. Others have attributed an increase in \( V_{\text{max}} \) to relief of a negative feedback mechanism by the intracellular amino acid pool, which can regulate the rate of transport of preexisting membrane carriers (29). No general agreement exists in the literature regarding the mechanism of action of insulin on transport as judged by kinetic characterization of substrate entry. Depending on the tissue studied (and the experimental conditions), insulin has been reported to increase the affinity for uptake (1, 11), increase the \( V_{\text{max}} \) for uptake (11, 16, 19, 28, 29, 34), or an increase in both parameters (13, 34). Additionally, there is a report in which insulin increased amino acid incorporation into proteins without affecting transport (30) and others in which transport was altered but incorporation remained unchanged (13, 17).

Thus the results presented here are compatible with several reports and, in particular, in agreement with those in which fetal tissues (16, 19) were studied. Further experimentation is necessary to ascertain the exact mechanism for insulin action on these carriers.

ACKNOWLEDGMENTS

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ADDENDUM

The results presented here would have been less surprising had we been aware of the paper by Castles et al. (Castles, J. J., Wool, J. G., and Moyer, A. N. The Effect of Diabetes on Amino Acid Accumulation and Protein Synthesis in Isolated Rat Diaphragm. Biochim. Biophys. Acta, 100: 609-612, 1965) in which they reported that the accumulation of AIB and proline was greater in the diaphragms from diabetic rats compared to tissue taken from intact animals. It was also of interest that the response to insulin was greater in the tissues from diabetic rats, results that are similar to those reported here. No explanation was offered for the difference in sensitivity. Unlike our results, however, Castles et al. did not see an increased proline incorporation into protein, as we did in the tumor studied here. In a subsequent paper, Scharff and Wool (Scharff, R., and Wool, J. G. Effect of Diabetes on the Concentration of Amino Acids in Plasma and Heart Muscle of Rats. Biochem. J., 99: 173-178, 1966) reported that insulin deficiency produced complex alterations in the concentrations of amine acids in plasma.
and heart muscle of rats; concentrations of some amino acids were increased, others were decreased, and a small number were unchanged. They attributed the complexity of these results to diverse hormonal and metabolic changes that accompany diabetes, a proposal that is akin to our suggestion that other factors may modulate the effects of diabetes and/or insulin administration.

REFERENCES

Effects of Insulin in Vivo and in Vitro on Amino Acid Transport into Cells from the R3230AC Mammary Adenocarcinoma and Their Relationship to Tumor Growth

Paul J. Hissin and Russell Hilf


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