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Control of Amino Acid Transport in the Mammary Gland of the Pregnant Mouse

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The regulation of the uptake of the amino acid analog α-aminoisobutyric acid was studied in diced mammary glands from pregnant mice. Stimulation of uptake by insulin was not prevented by inhibitors of protein synthesis; protein synthesis inhibitors decreased uptake by 20%; this response occurred more promptly in insulin-treated tissues. Elimination of extracellular amino acids led to a substantial increase in transport which was not abolished by inhibitors of protein synthesis. These results indicate that insulin does not increase amino acid transport in this system by altering synthesis and degradation of transport protein. They are consistent with a model in which the activity of the existing amino acid transport protein is subject to negative feedback regulation from the intracellular amino acid pool.

Key words: amino acid transport, mammary gland, cell proliferation, feedback regulation

Increases in amino acid transport have been observed as an early event following the stimulation of cell proliferation in many systems (1–4). Sander and Pardee (5) and Topper et al. (6) found cell-cycle-related changes in amino acid transport. Recent studies by Oxender and co-workers (7) demonstrated reciprocal changes in the activity of the A and L systems for neutral amino acid transport when Balb/3T3 cells reach confluency (see also 8, 9). Neither the mechanism nor the significance of these changes is well-understood, although both Holley (10) and Pardee (11) have proposed that alterations in the membrane transport of small molecules may play a role in the control of cell proliferation.

Oka, Topper, and their co-workers (12–14) demonstrated that at least 1 round of cell division ensues within 24 h when explants of mammary glands from pregnant mice are treated with insulin in vitro. A significant increase in the transport of nonmetabolizable amino acid analog, α-aminoisobutyric acid (AIB), occurred within 4 h of insulin treatment (13, 14). We undertook the present study of the mechanism of the insulin stimulation of AIB transport in mammary gland in vitro with the hope of gaining further insight into the mechanisms by which amino acid transport is controlled in proliferating tissue. Our results indicate that AIB transport is regulated in response to changes in the intracellular amino acid pool by a mechanism which does not involve alterations in the synthesis and degradation of amino acid transport protein. A preliminary report of this work has appeared (15).

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MATERIALS AND METHODS

Solutions

Unless otherwise indicated all incubations were carried out in TC199 medium modified to increase the buffer capacity by addition of 10 ml of a solution containing 50 mM HEPES buffer, 1.2 mM KCl, 26.4 mM NaCl, 4.9 mM $CaCl_2$, and 0.7 mM KH_2PO_4 to 25 ml of reconstituted TC199. Solid glucose and bovine serum albumin were added to concentrations of 27 mM and 2%, respectively, and the pH adjusted to 7.4 with NaOH. For the amino acid-free medium we used a modified Ringer bicarbonate containing 104 mM NaCl, 5 mM $NaHCO_3$, 6 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, 20 mM HEPES, 2 mg/100 ml phenol red and 2% bovine serum albumin with a final pH of 7.4. Insulin (bovine) was obtained from Eli Lilly (Iletin®). Isotopes were obtained from New England Nuclear Corporation. All chemicals were reagent grade. Budget-solv (RPI) was used for all scintillation counting.

Incubation

All experiments were carried out on tissues acutely isolated from the mammary glands of pregnant Balb-C mice. Our breeding colony was originally started with mice obtained from the colony maintained at the American Medical Center in Denver. For all reported experiments, the mammary gland from a single 16–18-day pregnant mouse was dissected free from excess connective tissue and lymph nodes, diced into 1 mm^2 pieces with crossed razor blades and preincubated in TC199 medium, modified as described above, for 1 h at 37°C to stabilize the tissue. The tissue was then further incubated in the presence or absence of insulin and protein synthesis inhibitors for 0.5–6 h. To measure the amino acid transport capacity 100 mg of tissue was placed in 1 ml of solution containing 0.5 mM α-aminoisobutyric acid (AIB) and 0.5 μCi/ml [^{14}C] AIB for 10 minutes. Experiments to be reported elsewhere indicate that this time of incubation allows estimation of the initial rate of AIB entry into the tissue. Experiments using dissociated alveoli gave quantitatively similar results, suggesting that AIB transport into diced mammary tissue represents transport by the alveolar cells. Methods for determining [^{14}C]alanine incorporation into lipids as well as [^{14}C]tryptophane into protein are given in the legends.

By using relatively large volumes of medium for preincubation (30 ml for 1 g of tissue), pH changes attributable to the high lactate production by this tissue were avoided. When present, insulin was used at 0.4 μU/ml and cycloheximide at 0.5 mg/ml. The sucrose space of the diced tissue averaged 25%, a value used to correct the data for extracellular amino acid.

RESULTS

Figure 1 shows the effects of insulin and protein synthesis inhibitors on AIB uptake in diced mammary glands from pregnant mice. Note that in the control tissue the rate of AIB uptake remains constant over a 6-h period. After 3 h of preincubation insulin had a marked stimulatory effect on the initial rate of AIB uptake. A concentration of cycloheximide sufficient to inhibit protein synthesis by 90% (data not shown) depressed AIB uptake by 20% after 2 h of preincubation. These effects, although quantitatively small, were quite consistent, including the single step decrease of AIB uptake to a new steady level in the presence of cycloheximide. When insulin and cycloheximide were added together, AIB uptake did not differ significantly from the control. This experiment did not

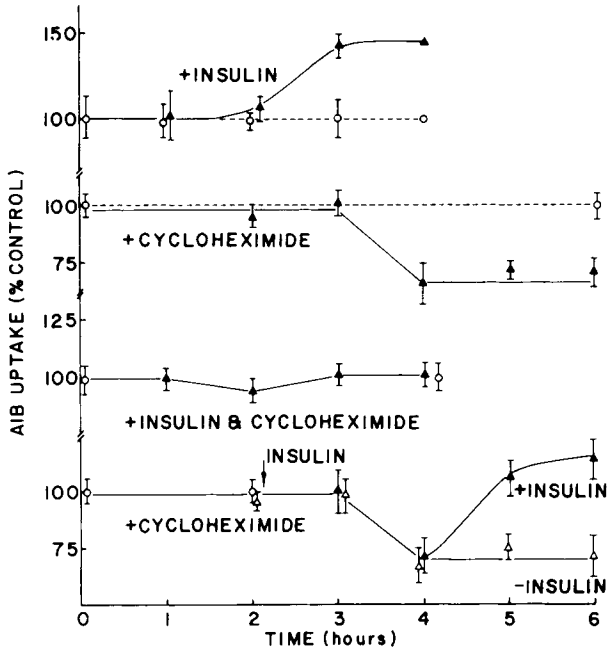

Fig. 1. The effect of insulin, cycloheximide, insulin plus cycloheximide, and cycloheximide followed by insulin on AIB uptake by diced mammary gland from a pregnant mouse. Tissues were preincubated with the indicated agent for varying periods of time (abscissa), followed by incubation with [^{14}C]AIB and 0.5 mM AIB for 10 min. After blotting and weighing, the radioactive amino acid was extracted with 5% trichloroacetic acid and counted in a liquid β-scintillation counter. Here and in Figs. 2–4 all points are the average of quadruplicate determinations. The distance between bars represents 2 standard errors of the means. Control shown as (○) with a dotted line.

allow us to decide whether cycloheximide inhibited the insulin action or whether the effects of the 2 agents canceled each other out. To clarify this problem, insulin was added to cycloheximide-treated tissues (Fig. 1, bottom) after 2 h of preincubation. Two hours after insulin addition, there was an increase in AIB uptake similar to that seen in the absence of cycloheximide. The finding that the effect of insulin on AIB uptake is not inhibited by inhibitors of protein synthesis suggests that insulin does not act by stimulating the synthesis of transport protein.

We next considered the possibility that insulin prevents amino acid carrier degradation in mammary tissue as has been reported for muscle (16). In Fig. 2, we show the results of the addition of cycloheximide to insulin-stimulated tissue. If insulin were acting by inhibiting carrier degradation, we would expect no effect or a delayed effect of cycloheximide in the presence of insulin. Instead, cycloheximide addition is followed by a prompt decrease in AIB uptake. During the same period cycloheximide had no effect on AIB uptake by the control tissue. Therefore insulin does not appear to act by decreasing carrier degradation in mammary tissue.

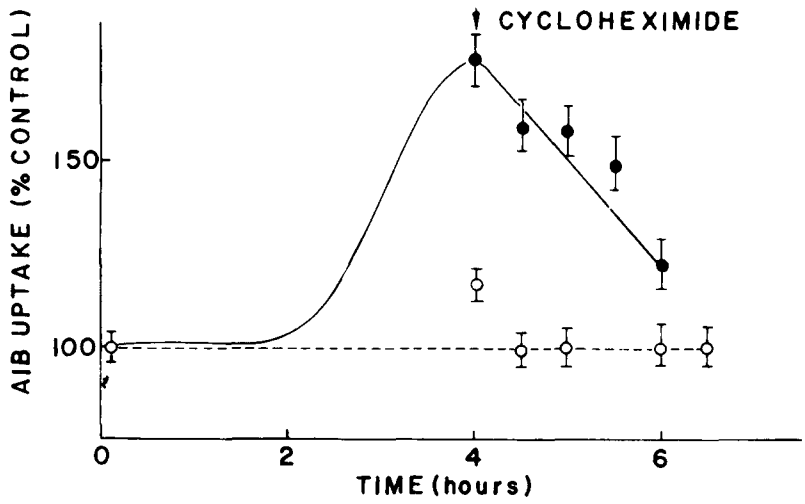

Fig. 2. The effect of cycloheximide on AIB uptake in insulin-treated tissue. Tissues were preincubated in the presence (•) and absence (○) of insulin for the time shown on the abscissa after which AIB transport was determined as detailed in the legend to Fig. 1. Cycloheximide was added to both tissues at 4 h.

Finally we tested the hypothesis that insulin alters the size of the intracellular amino acid pool, regulating amino acid transport through a negative feedback mechanism. If this hypothesis is correct, it should be possible to demonstrate that insulin increases amino acid utilization. To do this we measured alanine incorporation into lipid and tryptophane incorporation into protein as a function of preincubation time in the presence of insulin. Figure 3 shows that both functions are markedly increased over controls, indicating that insulin does increase the rate of cellular processes which utilize intracellular amino acids.

If insulin works through alteration in amino acid pool size, other treatments which increase or decrease these pools should have predictable effects on amino acid transport. The effect of the protein synthesis inhibitor cycloheximide, has already been shown on Fig. 1. The observed decrease in AIB uptake is the result one would expect from decreased amino acid utilization. Puromycin was also studied and found to have similar effects. Figure 2 illustrates the effect of cycloheximide on insulin stimulated tissue; here the decrease in AIB uptake occurs more rapidly than in nonstimulated tissue. This finding suggests that, when the flow of amino acids through the pool was increased in response to insulin, blockage of protein synthesis led to a rapid build-up of the intracellular amino acid concentration necessary to decrease amino acid uptake.

Removal of all amino acids from the bathing medium should decrease intracellular amino acid pools. The results of such an experiment are shown on Fig. 4. When the tissue is removed from TC199 and placed in a Ringer solution containing no amino acids there is a prompt increase in AIB uptake which begins to level off at 2 h. This increase cannot simply be the result of removing competing amino acids from the medium since the extracellular space in this tissue equilibrates within 5 min (experiment not shown). A

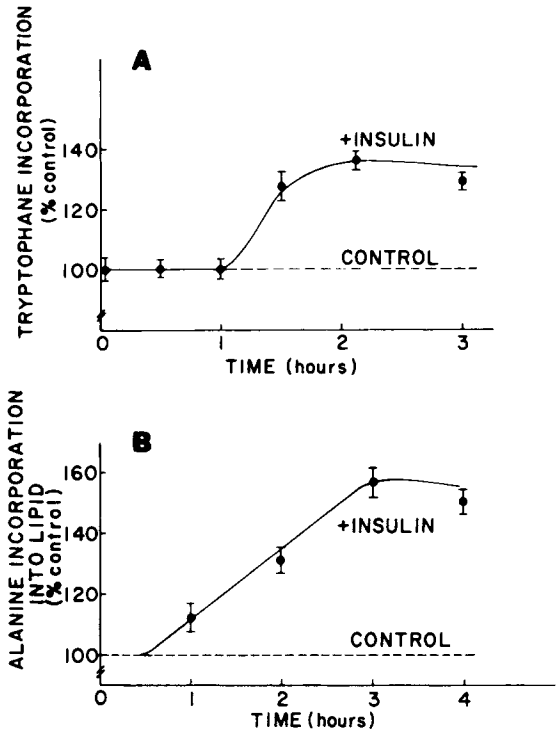

Fig. 3. The effect of insulin on amino acid utilization by diced mammary gland from a pregnant mouse. A) the effect of insulin on tryptophane incorporation into protein. After preincubating, the tissue was incubated in the presence of 5 μCi/ml [^{14}C] tryptophane for 10 min. After blotting and weighing, free radioactivity was extracted with 3 changes of trichloroacetic acid and the protein dissolved in 1 N NaOH and counted. No precipitation of protein occurred during the counting interval using this technique. B) The effect of insulin on alanine incorporation into lipid. After preincubation with or without insulin for the time indicated on the abscissa, the tissues were incubated in the presence of [^{14}C] alanine for 30 min. After blotting and weighing, the tissues were placed in a glass scintillation vial with 10 ml of a toluene based fluid and shaken at room temperature overnight to extract the lipid soluble components. Prior to counting, 1 ml of water was added to the vials to dissolve the water soluble radioactivity and partition it away from the fluor. A separate experiment shows that no more than 11% of the water soluble activity is counted by this technique.

similar but quantitatively smaller result is seen in the presence of cycloheximide (Fig. 4), again suggesting that synthesis of new protein is not involved in the response to decreased amino acid supply.

DISCUSSION

In studies to be reported elsewhere on diced mammary glands from pregnant mice, we found that insulin increases V_{max} without altering the K_m of transport. From the studies reported here, this alteration in transport capacity is related neither to synthesis of

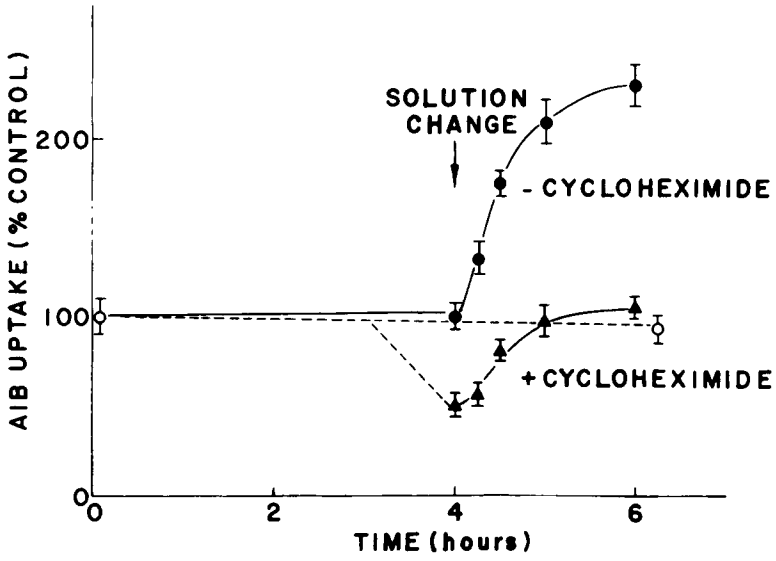

Fig. 4. The effect of removal of external amino acids on AIB uptake by diced mammary gland from a pregnant mouse. Tissues were incubated in TC199 with (▲) or without (●) cycloheximide for 2 h after which the solution was changed to Ringer bicarbonate. AIB uptake was assayed as described in the legend to Fig. 1.

new carrier protein nor to alteration of the rate of carrier degradation. However, the effects on AIB uptake of insulin, protein synthesis inhibitors, and removal of external amino acids are all consistent with the model for regulation of amino acid transport shown on Fig. 5. In this model, negative feedback from the intracellular amino acid pool regulates the rate of transport by preexisting membrane transport elements.

This conclusion differs from that reached by workers studying regulation of amino acid transport in other tissues. For example, Guidotti and his co-workers (16) concluded that insulin acted on the A system of muscle both by protecting membrane sites from degradation and by increasing the rate of synthesis of transport protein (see also 17). A substantial increase in amino acid transport when tissues are preincubated in amino acid-free media has been noted in uterus (18), newborn rat kidney cortex (19), placenta (20, 21), and a variety of avian and mammalian tissues (22, 23). This response to amino acid starvation, termed "adaptive regulation" (19, 22), often appears to be abolished by cycloheximide or puromycin (18, 19, 21, 22). This observation suggested to several workers that synthesis of new transport protein is involved (18, 19, 22). In this regard the results of an early study on yeast by Grensen et al. are instructive (24). These workers observed an increase in arginine uptake during nitrogen starvation and a decrease in arginine uptake in the presence of cycloheximide; because cycloheximide had no effect in nitrogen-starved yeast, they concluded that the alterations in the transport of the amino acid were the result of changes in the level of free intracellular amino acids rather than alterations in the

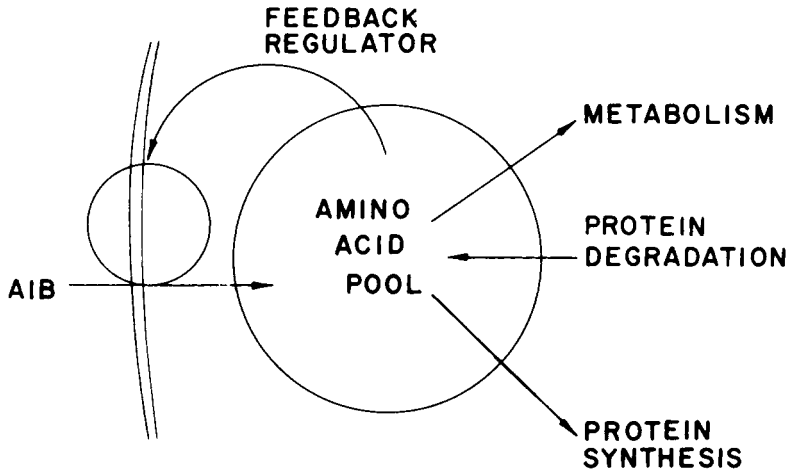

Fig. 5. Model for regulation of amino acid transport in diced mammary gland. For explanation, see text.

amount of transport protein. They suggested that the transinhibition of amino acid transport by intracellular amino acids first proposed by Ring and Heinz (25) might constitute a type of feedback control in this system. Neither the experiments of Guidotti and co-workers (22, 23) nor those of Reynolds et al. (19) on adaptive regulation were designed in such a way as to eliminate feedback effects of intracellular amino acids. The studies of Smith (22) suggest that pool size does play a role in placental tissue.

Recently Oxender and co-workers (7) measured the actual amino acid pools in Balb/3T3 mouse cells under depressed growth conditions. They found that a 2- to 3-fold increase in the amino acid pools was accompanied by a decrease in transport by the A system. These results again suggest a central role for the amino acid pool in the regulation of amino acid transport.

The mechanism of the apparent local feedback regulation in mammary tissue is not yet clear. It could involve direct transinhibition of the type suggested by Ring and Heinz (25). Changes in the membrane potential which alter the electrochemical gradient for Na^+, the apparent driving force for transport by the A system (26, 27), could be involved. A more complex feedback loop involving metabolites of the amino acids in question cannot at this time be ruled out. Although there is no evidence for transcriptional or translational control in the present study, it is important to be aware that regulation of the synthesis of transport protein could be superimposed on local control mechanisms. For example, Pall (28) has observed both transcriptional and local control of methionine transport in yeast. Further understanding of the regulation of amino acid transport in eukaryotes will only come from a detailed correlation of amino acid pools with transport activity under a variety of conditions.

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