Effect of Insulin on Amino Acid Transport in Isolated Rat Hepatocytes

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Summary. The effects of insulin on amino acid transport were studied in freshly prepared suspensions of isolated hepatocytes from adult rats. Insulin stimulated the active transport of α -aminoisobutyric acid by increasing the influx. The onset of the insulin effect was delayed by thirty to sixty min. Insulin increased the $V_{\max}$ of transport by about 60% without affecting the K_m . Cycloheximide and actinomycin D inhibited hormonal action by 60 to 80%. Only the "A" system of transport was affected by insulin. Half-maximal stimulation of transport was observed with insulin at 2 to 3 nmol/l, a concentration which also occupies about 50% of insulin-specific binding sites at steady state. Insulin did not antagonize the stimulatory effect of glucagon on amino acid transport.

Key words: Amino acid transport, isolated hepatocytes, liver, insulin action, insulin binding.

The hormonal regulation of amino acid transport is of particular physiological importance in eukaryotic cells. Thus, insulin has been shown to enhance the accumulation of amino acids in skeletal and cardiac muscle, bone, lymphoid cells, mammary epithelial cells and hepatoma culture cells (see Ref. 1 for review). Insulin has also been reported to stimulate the uptake of α -aminoisobutyric acid by the isolated perfused rat liver [2] and more recently to induce amino acid transport in freshly isolated rat hepatocytes [3] and in primary cultures of adult rat liver parenchymal cells [4]. We have previously characterized the transport systems that are operative for neutral amino acids in freshly isolated hepatocytes from the adult rat [5]. We have also demonstrated

that glucagon [6, 7], catecholamines [8] and glucocorticoids [9] stimulate amino acid transport in the hepatocyte by specifically increasing the activity of the A system of transport.

The present studies were designed to investigate the effect of insulin on amino acid transport in isolated hepatocytes in more detail. Several aspects of insulin effect on transport have been analyzed and the relationship between insulin binding and insulin action on amino acid transport has been examined.

Materials and Methods

Isolated hepatocytes were prepared from 6 to 8 week-old male Wistar rats following the procedure previously described [10]. The animals were maintained in a constant temperature (23°C) animal room with a fixed 12h light-12h dark cycle (the light period starting at 8 a.m.), and had free access to laboratory chow containing 21% protein, 4% fat and 51% carbohydrate until the time of sacrifice (9–11 a.m.). Freshly prepared hepatocytes have been shown to retain well preserved ultrastructure [10], metabolic capabilities [10] and active transport systems for amino acids [5]. The intracellular levels of ATP (15 to 20 nmol/ 10^6 cells) and K^+ (130 to 150 mmol/l of cell water) remained stable for periods of 3h at 37°C following cell isolation. All experiments were carried out at 37°C in Krebs-Ringer bicarbonate buffer containing bovine serum albumin fraction V (10 mg/ml), gentamycin (50 μ g/ml) and bacitracin (1 mg/ml), and gassed with O₂:CO₂ (95:5, V/V). Cells at about 1.5×10^6 /ml were incubated with various agents for the times indicated; they were then washed twice with fresh buffer before the addition of a mixture of labelled and unlabelled amino acid as indicated in the legends to figures. The analytical procedure employed to measure transport and the mode of expression of the results have previously been described [5, 6].

Studies of insulin binding to hepatocytes were performed under the same experimental conditions of buffer, temperature, and cell concentration as those used in transport experiments. 125 I-insulin (specific activity $\approx 300 \mu$ Ci/ μ g) was iodinated as described elsewhere [11] and exhibited receptor-binding ability that was similar to that of mono 125 I-insulin [12] obtained after DEAE-cellulose chromatography (data not shown). The specific binding of 125 I-insulin (0.5 ng/ml, 0.08 nmol/l) was determined after 20

Fig. 1. Effect of insulin on the time course of AIB uptake. Suspensions of hepatocytes were preincubated for 2h in the absence (basal) or presence of insulin. The uptake of 2mmol/l [¹⁴C]AIB was then measured at the times indicated. The distribution ratio was calculated by dividing the intracellular concentration of AIB by the extracellular concentration; intracellular water ($\approx 4 \mu\text{l}/10^6$ cells) was determined in the same cell preparation. Each point is the mean $\pm$ SEM of four determinations

Fig. 2. Effect of duration of cell pre-exposure to insulin on AIB influx. Suspensions of hepatocytes were incubated in the absence or presence of insulin. At the times indicated, initial rates of transport were measured by incubating aliquot samples with 2 mmol/l [¹⁴C]AIB for 15 min. Each value is the mean $\pm$ SEM of four determinations. Time 0 on figure represents a 15 min exposure of hepatocytes to insulin since the hormone was present during the transport assay

min incubation at 37°C, a period which achieves apparent steady state of binding at this temperature [13]. At the end of incubation 300 μl -samples were rapidly transferred to plastic tubes containing chilled buffer. Cells were sedimented by centrifugation and washed once with chilled buffer. Non-specific binding was estimated by measuring cell-bound radioactivity in the presence of a large excess (50 to 100 $\mu\text{g}/\text{ml}$) of unlabelled insulin. The non-specific component of binding did not exceed 10 to 15% of total binding.

Glucose was assayed in the incubation medium by the glucose oxidase method [14].

Porcine monocomponent insulin and glucagon were gifts from J. Schlichtkrull (Novo Research Institute, Copenhagen, Denmark) and G. Jouve (Novo Paris, France). α -Aminoisobutyric acid

Fig. 3. Persistence of insulin effect after insulin withdrawal. Hepatocytes were incubated in the absence or presence of 100 nmol/l insulin. After 15 min of cell exposure to the hormone, hepatocytes were isolated by centrifugation, washed twice with buffer, and resuspended in insulin-containing medium ("insulin present continuously") or insulin-free medium ("insulin present for the first 15 min") for the times indicated. The influx of [¹⁴C]AIB (2 mmol/l) was then measured over 15 min. Each point is the mean of four determinations

(AIB), aminocyclopentane carboxylic acid (cycloleucine), cycloheximide, gentamycin and bacitracin were purchased from Sigma. Actinomycin D and the glucose oxidase kit were from Boehringer Co.

The amino acid analogue α -(methylamino)-isobutyric acid (N-methyl AIB) was a gift from H. N. Christensen (Ann Arbor, Michigan); α -amino [1-¹⁴C]isobutyric acid and L-aminocyclopentane [1-¹⁴C]carboxylic acid were purchased from the Radiochemical Centre (Amersham, England).

Results

Effect of Insulin on the Time Course of AIB Uptake

Insulin at 100nmol/l increased both the initial rate and the steady state level of AIB transport in isolated hepatocytes (Fig. 1). In cells previously exposed to insulin, the distribution ratio was increased by 50 to 60%. The extent of increase observed at each time point was similar, suggesting that the hormone effect was to enhance the influx without altering the efflux. The fractional efflux of AIB from cells exposed to insulin was indeed indistinguishable from that observed with control cells (not shown).

Dependence of Stimulation of AIB Transport on the Duration of Cell Exposure to Insulin

The stimulatory effect of insulin on AIB uptake did not occur instantaneously. When cells were incubated with or without insulin for various lengths of

Fig. 4. Effect of insulin on kinetic parameters of AIB transport. Hepatocytes were preincubated for 2 h in a sodium-containing medium and in the absence (Δ — Δ and $\circ$ — $\circ$) or presence of insulin ($\blacktriangle$ — $\blacktriangle$ and $\bullet$ — $\bullet$). Initial rates of transport were then measured for the external AIB concentrations indicated. Sodium was replaced by choline when a sodium-free medium was required during transport assay. Closed symbols represent insulin-treated cells ($\blacktriangle$ — $\blacktriangle$: sodium present during transport assay; $\bullet$ — $\bullet$: sodium absent during transport assay); open symbols represent control cells (Δ — Δ : sodium present during transport assay; $\circ$ — $\circ$: sodium absent during transport assay). The upper and lower dotted lines represent the saturable components of transport. This was calculated for insulin-treated and control cells, respectively, by subtracting from total the transport measured in the absence of sodium, ($[\text{Na}^+] = 0$). Each point is the mean $\pm$ SEM of four determinations

time prior to the addition of ^{14}C AIB, the hormone effect became significant only after 30 min of preincubation, to reach a maximum by 2 h (Fig. 2). The continuous presence of insulin was, however, not required since the stimulatory effect was of similar degree in hepatocytes permanently exposed to the hormone and in cells exposed to insulin for only the first 15 min of the preincubation period (Fig. 3).

Effect of Inhibitors of RNA and Protein Synthesis on Basal and Insulin-Stimulated AIB Transport

Actinomycin D at 1 $\mu\text{g}/\text{ml}$ (0.8 $\mu\text{mol}/\text{l}$) and cycloheximide at 0.1 mmol/l inhibited the insulin stimulation of transport by 60 to 80%, without significantly altering the basal (i.e., non-insulin-stimulated) uptake of AIB (Table 1). Under the same conditions, actinomycin D inhibited RNA synthesis and protein synthesis by 95% and 40%, respectively, and cycloheximide inhibited protein synthesis by 90% (not presented).

Effect of Insulin on the Kinetic Parameters of AIB Transport

Insulin did not influence the non-saturable, sodium-independent component of AIB transport (Fig. 4). The V_{max} of the saturable, sodium-dependent component of transport ($\approx 130 \text{ nmol}/10^6 \text{ cells. 15 min}$) was increased by insulin ($\approx 210 \text{ nmol}/10^6 \text{ cells. 15 min}$), whereas the apparent K_m remained essentially

Table 1. Effect of cycloheximide and actinomycin D on basal and insulin-stimulated AIB influx

Inhibitor	Insulin		
	0	1 nmol/l	100 nmol/l
AIB influx: nmol/ 10^6 cells. 15 min			
None	18.5 $\pm$ 0.6	27.5 $\pm$ 0.3	33.0 $\pm$ 0.6
Cycloheximide 0.1 mmol/l	16.5 $\pm$ 0.4	17.7 $\pm$ 0.3	21.8 $\pm$ 0.3
Actinomycin D 1 $\mu\text{g}/\text{ml}$	16.0 $\pm$ 0.6	19.3 $\pm$ 0.4	21.4 $\pm$ 0.6

Hepatocytes were incubated for 30 min in the absence or presence of cycloheximide or actinomycin D prior to addition of insulin. Incubations were continued for 2 h. ^{14}C AIB influx (2 mmol/l) was then measured. Each value is the mean $\pm$ SEM of four determinations

unaltered ($\approx 20 \text{ mmol}/\text{l}$) (Fig. 4).

Effect of Insulin on Transport Systems

The saturable, sodium-dependent transport of AIB in the hepatocyte occurs through the A ("Alanine" preferring) and ASC ("Alanine, Serine, Cysteine") systems [5]. When the influx of AIB was measured in a sodium-containing medium and in the presence of an excess of N-methyl AIB, conditions under which the entry of AIB through the A system is largely

Fig. 5. Effect of insulin on amino acid transport systems. Cells were preincubated for 2 h without (basal) or with insulin (50 nmol/l), in a sodium-containing medium. After washing twice, cells were resuspended in buffer without or with sodium and without or with insulin. AIB (0.25 mmol/l) influx was then measured in the absence or presence of 10 mmol/l N-methyl-AIB. Cycloleucine (0.2 mmol/l) influx was measured in sodium-free medium or in sodium-containing medium in the presence of 30 mmol/l AIB. Each value represents the mean $\pm$ SEM of six determinations

Fig. 6. Dose response curves for insulin binding and insulin stimulation of AIB transport. Hepatocytes were preincubated for 2 h in the presence of varying concentrations of insulin before the addition of 2 mmol/l [¹⁴C]AIB. Amino acid influx (●—●) was then measured after 15 min. The results are expressed as percent of maximal insulin effect obtained at 100 nmol/l and represent the mean $\pm$ SEM of four separate experiments. The binding of insulin to hepatocytes (using ¹²⁵I-insulin and increasing concentrations of unlabelled insulin) was measured at steady state after 20 min incubation under conditions (buffer, temperature, cell concentration) similar to those employed in transport experiments. Binding (▲—▲) is expressed as percent of maximal binding taken as the amount of hormone specifically bound at 10 nmol/l insulin, a concentration at which the specific binding of insulin is virtually saturated under the experimental conditions employed. Each point represents the mean $\pm$ SEM of three separate experiments

inhibited, the stimulatory effect of insulin was no longer observed; insulin was also without effect on the sodium-independent entry (physical diffusion) of AIB, measured in a choline medium (Fig. 5). Thus, insulin specifically increases the activity of transport system A.

The saturable transport of cycloleucine in hepatocytes occurs through the L ("Leucine" preferring) system (sodium-independent) for about 80% and through the A and ASC systems for 20% [5]. Two conditions allow for the measurement of cycloleucine transport through system L: the saturation of systems A and ASC by an excess of AIB, or the use of a sodium-free (choline) medium. Under both conditions, insulin did not significantly affect the transport of cycloleucine (Fig. 5), therefore excluding an effect of the hormone on the L system of transport.

Dose Response of Insulin Stimulation of AIB Influx; Comparison with Insulin Binding

The stimulatory effect of insulin on AIB transport could be detected with hormone concentrations as low as 0.1 nmol/l (0.6 ng/ml or 15 μ U/ml); the apparent half-maximal stimulation (Effective Dose 50, ED₅₀) occurred at 2 to 3 nmol/l insulin, and maximal effect was achieved with 50 to 100 nmol/l (Fig. 6). When the binding of insulin to hepatocytes was measured at steady state (after 20 min at 37°C), the apparent value for half-maximal receptor occupancy was 1 to 2 nmol/l insulin (Fig. 6). Therefore, the apparent ED₅₀ for insulin-stimulated AIB transport and the apparent insulin concentration for half-maximal receptor occupancy appear to be very similar. Despite the different time dependence of both processes, such comparison between insulin binding at steady state and subsequent effect on amino acid transport seems to be warranted by the observation that the continuous presence of insulin is not required (beyond 15 min of cell exposure to the hormone) for the subsequent generation of stimulation of amino acid transport (Fig. 3). The dose dependence relationships for insulin binding and subsequent effect on amino acid transport could have been affected by the degradation of insulin in the medium during its exposure to hepatocytes; however, under the conditions used (after 15 min incubation at 37°C in the presence of bacitracin), the degradation did not exceed 20% with insulin at 100 nmol/l and 26% with insulin at 0.1 nmol/l.

Combined Effects of Insulin and Glucagon on Glycogenolysis and AIB Transport

Insulin largely inhibited the glucagon-stimulated glycogenolysis in isolated hepatocytes, without signifi-

cantly affecting the basal rate of glycogenolysis (Fig. 7, left). Since there was a good quantitative agreement between the disappearance of cell glycogen and the release of glucose into the medium (unpublished observations), it is likely that gluconeogenesis contributed very little of the glucose output. In the same cell suspension, both insulin and glucagon enhanced AIB transport; in contrast to their action on glycogenolysis, the two hormones exerted no antagonistic effect on the transport of AIB measured after 2 hours of cell exposure to the hormones (Fig. 7, right) used in combination at maximally effective concentrations (10 nmol/l glucagon and 100 nmol/l insulin).

Discussion

The present study has provided evidence that insulin, at concentrations within the physiological values in hepatic portal blood, stimulates directly the concentrative uptake of AIB in suspensions of freshly isolated hepatocytes from the adult rat. These observations confirm and extend previous reports on insulin effect on AIB uptake in the isolated perfused rat liver [2], in primary cultures of adult rat liver parenchymal cells [4], and in isolated hepatocytes [3].

The use of isolated hepatocytes has made it possible to analyze the effects of insulin in kinetic and quantitative terms. The stimulatory effect of insulin on amino acid transport in hepatocytes shares many characteristics with that elicited by other hormones. Thus, as previously observed with glucagon [6, 7], catecholamines [8] and glucocorticoids [9], the stimulatory effect of insulin was restricted to system A, the sodium-dependent, active and concentrative transport system for neutral amino acids [1, 5]. This is in keeping with the observation that the A mediation of amino acid transport is the sole system subject to regulation (both hormonal and non-hormonal) in a variety of cells [1], and stresses the physiological importance of this system. As also observed with glucagon [6, 7], catecholamines [8] and glucocorticoids [9], the stimulation of amino acid transport by insulin was dependent on new protein synthesis, since it was largely suppressed under conditions where protein synthesis was virtually abolished (cycloheximide) or substantially depressed (actinomycin D). Although it is clear that new protein synthesis is implicated in the effect of insulin on amino acid transport in hepatocytes, attempts to discriminate between a possible effect of insulin at the transcriptional level and its effect at the translational level [15] have not been conclusive (not presented). Along with the lag period observed (about 30 min) before

Fig. 7. Effect of insulin and glucagon on glycogenolysis and AIB transport. Hepatocytes were preincubated in the absence or presence of each hormone used alone or combined. Glycogenolysis was evaluated by measuring the glucose release in the incubation medium after 15 min. The influx of [¹⁴C]AIB (2 mmol/l) was measured over 15 min in cells previously exposed to hormones for 2 h. Each bar is the mean $\pm$ SEM of four determinations

the onset of insulin action, and with the increase in the V_{max} of transport elicited by the hormone, the results suggest that insulin enhances amino acid transport by inducing the new synthesis of carrier proteins. This is similar to the characteristics of insulin's effect on amino acid transport in primary cultures of hepatocytes [4] and in a number of other tissue and cell types [1].

Comparisons between the binding of insulin to its receptors and subsequent biological effects of the hormone have generally revealed complex relationships [16]. In the present study, such comparison has been further complicated by the fact that insulin binding and effect on amino acid transport appear to be rather distant processes, both temporally and sequentially, since a number of events (e. g., new protein synthesis) supervene between hormone binding and expression of biological effect. It was observed however that, after hepatocytes had been exposed to insulin for 15 min at 37° C (a time sufficient to achieve steady state binding of the hormone), the continuous presence of insulin in the incubation medium was no longer required to observe the subsequent stimulation of transport (Fig. 3). Also, under these conditions (resuspension of hepatocytes in insulin-free medium following exposure to ¹²⁵I-insulin for 20 min at 37° C), 70% and 80% of cell-associated radioactivity is released from hepatocytes after 60 and 120 min, respectively (unpublished results). It thus appears that, once insulin has bound to receptors, a signal is generated which does not depend on the continuous presence of the hormone in the extra-

cellular medium or on a cellular site accessible to dissociation. It therefore seems valid to compare the dose-response relationships of insulin binding at steady state and subsequent effect on amino acid transport. Such comparison has revealed that the stimulation of amino acid transport by insulin bears some proportional relationship to the occupancy of receptor sites by the hormone throughout the whole range of dose-response curves. Similar results have been reported in comparing insulin binding and effect on amino acid transport in rat thymocytes [17, 18]. This is in contrast to the relationship between insulin binding and effects in rat adipocytes [19–21] and in mouse skeletal muscle [22]; in these systems, maximal insulin effects on glucose transport and metabolism are achieved when only a fraction of total receptors is occupied by the hormone [19–22]. Whether this apparent discrepancy in insulin action implicates the generation of different signal(s) and/or represents a distinctive feature of the long term “trophic” (and possible growth-promoting) effects of the hormone, as opposed to its rapid metabolic effects, is unknown at present.

Finally, the physiological implications of these findings deserve mention. Since insulin and glucagon usually exert opposite actions on liver metabolism, it is remarkable that both promote amino acid transport in the liver (Fig. 7). The rise in both insulin and glucagon secretion following a protein meal or amino acid administration is also a rather unique physiological situation where the secretions of A and B cells change in the same direction. Thus, the teleology of the rise in glucagon secretion in response to protein ingestion, i. e., to prevent hypoglycaemia as a consequence of aminogenic insulin secretion [23] appears to have a corollary in the concerted action of both hormones to increase amino acid transport in the liver. That glucagon exerts a faster effect than insulin on this variable [7] may be consistent with the need for a rapid activation of gluconeogenesis (a concomitant of the acute metabolic effects of insulin on glucose storage in the liver and utilization at the periphery), thus permitting subsequent stimulation by insulin of anticatabolic and — or — anabolic processes through sustained entry of amino acids in the liver.

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