Insulin Regulation of Amino Acid Transport in Mesenchymal Cells from Avian and Mammalian Tissues

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Insulin regulation of amino acid transport across the cell membrane was studied in a variety of mesenchymal cells directly isolated from avian and mammalian tissues or collected from confluent cultures. Transport activity of the principal systems of mediation in the presence and absence of insulin was evaluated by measuring the uptake of representative amino acids under conditions approaching initial entry rates. Insulin enhanced the transport rate of substrate amino acids from the A system (α -aminoisobutyric acid, L-proline, glycine, L-alanine and L-serine) in fibroblasts and osteoblasts from chick-embryo tissues, in mesenchymal cells (fibroblasts and smooth muscle cells) from immature rat uterus, in thymic lymphocytes from young rats and in chick-embryo fibroblasts from confluent secondary cultures. In these tissues, the uptake of amino acid substrates of transport systems L and Ly^+ (L-leucine, L-phenylalanine, L-lysine) was not affected by the presence of the hormone. No insulin control of amino acid transport was detected in chick-embryo chondroblasts and rat peritoneal macrophages. These observations identify the occurrence of hormonal regulatory patterns of amino acid transport for different mesenchymal cell types and indicate that these properties emerge early during cell differentiation.

Many features of amino acid transport across the cell membrane, including the existence of distinctive systems of mediation with specific reactivity to substrates, ion- and energy-dependence, sensitivity to pH and metabolic inhibitors, capacity for exchange diffusion, trans-stimulation, trans-inhibition etc., have been elucidated in the past few years (Christensen, 1969, 1973, 1975; Christensen *et al.*, 1973, 1974). Moreover, regulatory mechanisms that continuously adapt the efficiency of the transport process to the actual needs of the intracellular machinery have been found to operate in a number of mesenchymal and epithelial tissues from avian and mammalian origin (Guidotti, 1972; Guidotti *et al.*, 1974*a,b*, 1975). Among these mechanisms, hormonal regulations are of major physiological importance in highly integrated organisms (Riggs, 1970). Insulin, in particular, enhances the accumulation of several amino acids in such tissues as skeletal (Akedo & Christensen, 1962; Manchester & Wool, 1963; Elsas *et al.*, 1968; Narahara & Holloszy, 1974) and cardiac muscle (Guidotti *et al.*, 1968; Manchester *et al.*, 1971), bone (Hahn *et al.*, 1971), lymphoid cells (Goldfine *et al.*, 1972), mammary epithelial cells (Friedberg *et al.*, 1970) and hepatoma culture cells (Risser & Gelehrter, 1973).

In a model muscle preparation (chick-embryo heart cells) insulin control is restricted to amino acid substrates of transport system A (Guidotti *et al.*,

1974*a*). Evidence that the hormone acts predominantly or entirely on the A system in rat uterus and diaphragm has been reported by Riggs and co-workers (Riggs & McKirahan, 1973; Mohri *et al.*, 1974).

The latter agency is also the sole transport system that exhibits adaptive regulation (Gazzola *et al.*, 1973; Guidotti *et al.*, 1975).

To extend these observations and to define these aspects of the hormonal control we studied insulin regulation of amino acid transport in a variety of mesenchymal cells from avian and mammalian sources. The correct evaluation of the hormonal effect required an experimental procedure capable of minimizing the interference of adaptive control (cf. Guidotti *et al.*, 1975). The results of the experiments to be reported identify the occurrence of specific regulatory patterns of amino acid transport for different mesenchymal tissues and indicate that these properties emerge early during cell differentiation.

Experimental

Experimental design

The activity of the principal systems of mediation for amino acid transport in animal tissues and cells in culture can be evaluated by measuring the uptake of representative substrates under conditions approaching initial entry rates (Guidotti *et al.*, 1971;

Manchester *et al.*, 1971; Gazzola *et al.*, 1972). Insulin has been shown to affect the activity of the A transport system by modulating the rate of synthesis and degradation of specific transport proteins (Guidotti *et al.*, 1974a), a time-requiring process. During the incubation period needed to render the effect of insulin large enough to be compatible with an accurate determination of it, several biological preparations placed in a defined amino acid-free medium undergo changes in their transport activity for the various systems of mediation (Guidotti *et al.*, 1975). In particular, the activity of transport system A increases with time as a result of an adaptive mechanism acting at the transcription level (Franchi-Gazzola *et al.*, 1973). Therefore a correct measurement of the hormonal effect requires an experimental procedure in which control and insulin-treated tissues are incubated under the same conditions (except for the presence of the hormone) for the same length of time. Preliminary experiments showed that an incubation period of 2 h was sufficient to detect a definite hormonal effect in insulin-sensitive cells. Under the conditions selected for incubation (see next paragraph), all the biological preparations under study (fibroblasts, chondroblasts and osteoblasts from avian embryonic tissues; fibroblasts, smooth muscle cells, thymocytes and macrophages of mammalian origin) were viable for several hours, as assessed by measurements of oxygen consumption and incorporation of labelled amino acids into protein (Guidotti *et al.*, 1969, 1975).

Materials

The sources for most of the materials and reagents used are listed in preceding papers (Guidotti *et al.*, 1974a,b, 1975).

Methods

The biological models included mesenchymal cells isolated from chick-embryo tissues (fibroblasts from corneas, chondroblasts from cartilaginous pelvic bones, osteoblasts from membrane bone calvaria), chick-embryo fibroblasts from secondary confluent cultures and mesenchymal cells of mammalian origin (fibroblasts and smooth muscle cells from immature rat uterus, thymocytes from rat thymus glands and peritoneal rat macrophages). Handling of tissues and preparation of cell suspensions have been described in detail previously (Guidotti *et al.*, 1969, 1971, 1975). Chick embryo fibroblasts for cell culturing were obtained from 11-day-old embryos by the method of Rubin (1973). Secondary confluent cultures were placed in suspension by a 30 min incubation at 37°C in Ca^{2+} - and Mg^{2+} -free Krebs-Ringer bicarbonate buffer (Guidotti *et al.*, 1969) contain-

ing collagenase (1 mg/ml) and gentle scraping with a rubber 'policeman'.

Incubations (2 h) were carried out in silicone-treated glass vessels at 37.5°C under continuous mild stirring (Guidotti *et al.*, 1969). The incubation medium was Krebs-Ringer bicarbonate buffer, pH 7.4, containing 8 mM-glucose, in an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5). When present, insulin was added at a concentration of 5 $\mu\text{g}/\text{ml}$ of medium.

Initial rates of amino acid uptake were measured by transferring samples of cell suspension into flasks containing Krebs-Ringer bicarbonate buffer supplemented with the labelled amino acid under study (0.1 mM, final concn.) and incubating the flask at 37.5°C for 5 min. The means for determining intracellular accumulation of the tracer amino acid and for evaluating the proper corrections to be introduced were as described by Guidotti *et al.* (1969, 1971, 1975).

Results

The effects of insulin on the transport of amino acids in mesenchymal cells of avian origin incubated in Krebs-Ringer bicarbonate buffer are shown in Tables 1 and 2. In fibroblasts (from corneal tissue and from secondary confluent cultures) and in osteoblasts (from calvaria) the hormone accelerated the uptake of amino acids (α -aminoisobutyric acid, L-proline, glycine, L-alanine and L-serine) taken up, primarily or appreciably, by the A transport system (Oxender & Christensen, 1963; Christensen, 1969; Gazzola *et al.*, 1972, 1973). It was ineffective on the transport rate of L-leucine, L-phenylalanine and L-lysine, which are preferential substrates of transport systems L and Ly^+ . Evidence that identifies the A transport system as being insulin-sensitive in myoblasts from chick-embryo hearts has been provided (Guidotti *et al.*, 1974). In chondroblasts (from cartilaginous pelvic bones) the hormone did not change the transport rate of any amino acid studied.

An acceleration by insulin for the transport of α -aminoisobutyric acid, L-proline, glycine, L-alanine and L-serine (preferential amino acid substrates of the A transport system) has been observed with mesenchymal cells from immature rat uterus (Table 3), i.e., with a cell population represented mainly by fibroblasts and smooth muscle cells (Guidotti *et al.*, 1975) of mammalian origin.

In rat thymic lymphocytes, the entry rate of all the substrate amino acids tested (α -aminoisobutyric acid, L-proline, glycine, L-alanine and L-serine) of the A transport system was enhanced by insulin, whereas the uptake of L-leucine, L-phenylalanine and L-lysine (taken up by systems L and Ly^+) was not (Table 4). Results from Table 4 also indicate that insulin does not affect the transport rate of any amino acid studied in rat peritoneal macrophages.

Table 1. *Effect of insulin on amino acid uptake by isolated fibroblasts from chick-embryo corneal tissue and from secondary confluent cultures*

Cell suspensions (from 9-day-old chick-embryo corneas and from secondary confluent cultures of chick-embryo fibroblasts) were incubated for 2 h in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 8 mm-glucose in the absence and in the presence (5 µg/ml) of insulin. Incubation was at 37.5°C in an atmosphere of O₂+CO₂ (95:5). Amino acid uptake was measured by transferring samples of cell suspensions (2×10⁶–4×10⁶ cells) into flasks containing Krebs-Ringer bicarbonate supplemented with 8 mm-glucose and the ¹⁴C-labelled amino acid under study (0.1 mm, final concn.) and incubating for 5 min at 37.5°C. The values are means±S.E.M. of six to nine separate determinations.

Amino acid added	Amino acid uptake (nmol/min per ml of cell water)					
	Corneal fibroblasts			Cultured fibroblasts		
	No insulin	Insulin	Difference (%)	No insulin	Insulin	Difference (%)
α-Aminoisobutyric acid	211±20	262±13*	+24	154±4	186±5*	+21
L-Proline	313±26	433±36*	+38	206±23	262±22*	+27
Glycine	101±7	144±12*	+43	—	—	—
L-Alanine	378±18	477±20*	+26	360±41	422±36*	+17
L-Serine	314±18	397±19*	+26	—	—	—
L-Leucine	90±11	78±7	-13	42±1	41±1	-2
L-Phenylalanine	49±5	48±5	-2	40±1	37±1	-8
L-Lysine	87±12	89±12	+2	92±2	92±2	0

* Significant difference ($P < 0.01$) versus 'No insulin'.

Table 2. *Effect of insulin on amino acid uptake by isolated chick-embryo chondroblasts and osteoblasts*

Cell suspensions were obtained from cartilaginous pelvic bones (chondroblasts) and from membrane bone calvaria (osteoblasts) of 11-day-old chick embryos. Incubations and amino acid uptake measurements (on samples of 3×10⁶ cells) were as described in Table 1. The values are means±S.E.M. of six separate determinations.

Amino acid added	Amino acid uptake (nmol/min per ml of cell water)					
	Chondroblasts			Osteoblasts		
	No insulin	Insulin	Difference (%)	No insulin	Insulin	Difference (%)
α-Aminoisobutyric acid	262±28	249±35	-5	190±21	293±31*	+54
L-Proline	366±29	373±33	+2	357±22	490±29*	+37
Glycine	131±15	129±12	-2	117±12	192±22*	+64
L-Alanine	571±41	596±34	+4	430±35	537±34*	+25
L-Serine	361±33	347±32	-4	403±37	654±43*	+62
L-Leucine	53±8	51±7	-4	52±5	52±5	0
L-Phenylalanine	24±2	24±2	0	41±3	41±3	0
L-Lysine	57±8	55±8	-4	82±8	83±9	+1

* Significant difference ($P < 0.01$) versus 'No insulin'.

Discussion

Our previous investigations (Guidotti *et al.*, 1974a,b) indicated that the A system for the transport of neutral amino acids is the sole such agency that undergoes control of its activity by insulin in chick-embryo heart cells. The results in the present paper show that insulin also accelerates the transport of substrate amino acids of the A system in fibroblasts either obtained directly from tissues (chick-embryo corneas),

or collected from cell cultures (Table 1), in osteoblasts from chick-embryo calvaria (Table 2) and in thymic lymphocytes from young rats (Table 4). Indirect evidence for this effect in fibroblasts and smooth muscle cells can be found in studies by Mohri *et al.* (1974) with immature rat uterus. Indeed, a cell population obtained from this tissue (mainly consisting of fibroblasts and smooth muscle cells) responds to insulin with an increased uptake of amino

Table 3. *Effect of insulin on amino acid uptake by cells isolated from immature rat uterus*

Cell suspensions (fibroblasts and smooth muscle cells) were obtained from uteri of immature female rats (wt. 50–70 g). Incubations and amino acid uptake measurements (on samples of 3.5×10^6 cells) were as described in Table 1. The values are means $\pm$ S.E.M. of five separate determinations.

Amino acid added	Amino acid uptake (nmol/min per ml of cell water)		
	No insulin	Insulin	Difference (%)
α -Aminoisobutyric acid	83 $\pm$ 8	163 $\pm$ 12*	+96
L-Proline	98 $\pm$ 10	127 $\pm$ 10†	+30
Glycine	42 $\pm$ 6	77 $\pm$ 10*	+83
L-Alanine	290 $\pm$ 21	497 $\pm$ 34*	+71
L-Serine	197 $\pm$ 16	257 $\pm$ 27*	+30
L-Leucine	29 $\pm$ 4	27 $\pm$ 4	–7
L-Phenylalanine	24 $\pm$ 4	22 $\pm$ 4	–8
L-Lysine	57 $\pm$ 9	54 $\pm$ 7	–5

* Significant difference ($P < 0.01$) versus 'No insulin'.

† Significant difference ($P < 0.05$) versus 'No insulin'.

Table 4. *Effect of insulin on amino acid uptake by isolated rat thymocytes and rat peritoneal macrophages*

Thymocyte suspensions were obtained from thymus glands of 13–19-day-old rats. Macrophage suspensions were collected from peritoneal exudates of paraffin-oil-injected adult rats. Incubations and amino acid uptake measurements (on samples of 1.5×10^7 cells for thymocytes and 4×10^6 cells for macrophages) were as described in Table 1. The values are means $\pm$ S.E.M. of ten (thymocytes) and six (macrophages) separate determinations.

Amino acid added	Amino acid uptake (nmol/min per ml of cell water)					
	Thymocytes			Macrophages		
	No insulin	Insulin	Difference (%)	No insulin	Insulin	Difference (%)
α -Aminoisobutyric acid	37 $\pm$ 3	57 $\pm$ 4*	+54	34 $\pm$ 3	32 $\pm$ 4	–6
L-Proline	25 $\pm$ 2	39 $\pm$ 3*	+56	32 $\pm$ 4	32 $\pm$ 3	0
Glycine	11 $\pm$ 1	18 $\pm$ 2*	+64	22 $\pm$ 2	20 $\pm$ 2	–9
L-Alanine	52 $\pm$ 6	66 $\pm$ 5*	+27	173 $\pm$ 10	175 $\pm$ 11	+1
L-Serine	41 $\pm$ 5	64 $\pm$ 4*	+56	97 $\pm$ 7	91 $\pm$ 8	–6
L-Leucine	8 $\pm$ 1	8 $\pm$ 1	0	23 $\pm$ 3	24 $\pm$ 3	+4
L-Phenylalanine	8 $\pm$ 1	8 $\pm$ 1	0	39 $\pm$ 4	38 $\pm$ 4	–3
L-Lysine	56 $\pm$ 4	53 $\pm$ 4	–5	46 $\pm$ 4	46 $\pm$ 7	0

* Significant difference ($P < 0.01$) versus 'No insulin'.

acid substrates of transport system A (Table 3). A stimulation by insulin of the transport of α -aminoisobutyric acid (a typical substrate of the A system in several animal tissues) in human fibroblasts from confluent-monolayer cultures (Hollenberg & Cuatrecasas, 1975) and in aggregates of cultured cardiac cells from chicken embryos (Elsas *et al.*, 1975) have been reported.

No insulin stimulation of amino acid uptake has been observed with such mesenchymal cells as chick-embryo chondroblasts (Table 2) and rat peritoneal macrophages (Table 4). The fact that insulin-sensitive osteoblasts (from membrane bone calvaria)

and insulin-insensitive chondroblasts (from cartilaginous pelvic bones) have been obtained at a corresponding early stage of embryological development (11-day-old embryos, see Table 2) indicates that insulin regulation of amino acid transport is a property of the cell that emerges rather precociously during differentiation. Whether the appearance of this control mechanism depends on the assembly of functional insulin receptors at the cell membrane during ontogeny or on differentiation of specialized intracellular functions remains to be elucidated. In chick-embryo heart a stimulation by insulin of amino acid transport is already operative at a 5-day stage of embryological

development (Guidotti *et al.*, 1968), whereas a saturable transport system for glucose appears and becomes sensitive to insulin between day 7 and 9 of development (Guidotti *et al.*, 1966). In foetal bone cells (from calvaria), which exhibit a definite hormonal acceleration of amino acid transport, no insulin-sensitive systems for cellular uptake of sugars have been detected (Hahn *et al.*, 1971). When the stimulatory effects of insulin on amino acid transport in thymic lymphocytes and macrophages are confronted, one finds that hormonal regulation is effective in the former and absent from the latter cells (Table 4), whereas recent results point to monocytes (phagocytic cells germane to macrophages) rather than to lymphocytes (including T lymphocytes) as the insulin receptor-bearing cells in peripheral blood (Schwartz *et al.*, 1975). These discrepancies require the introduction of an arbitrary distinction between effects of insulin (or of postulated mediators formed at the cell membrane after the interaction of the hormone with specific receptors) on differentiated intracellular functions and existence of insulin receptors, defined as sites to which the hormone binds specifically with high affinity (Cuatrecasas, 1974). The high concentrations of insulin used in the present and other studies (Akedo & Christensen, 1962; Manchester & Wool, 1963; Elsas *et al.*, 1968, 1975; Guidotti *et al.*, 1968; Hahn *et al.*, 1971; Goldfine *et al.*, 1972; Risser & Gelehrter, 1973; Riggs & McKirahan, 1973; Mohri *et al.*, 1974) to investigate the action of the hormone on amino acid transport in cells and tissues *in vitro* might in fact cause effects by non-receptor interactions with the cell surface. With chick-embryo hearts, a definite stimulation of α -aminoisobutyric acid uptake has been observed at insulin concentrations close to the physiological range (0.10 nM), but the biological effect increased with the concentration of the hormone above 0.1 μ M (Guidotti *et al.*, 1968). Therefore caution must be exercised in 'receptor' interpretation of biological activities when high concentrations of the hormone are used.

One consistent finding in our study requires further consideration. In fibroblasts (Table 1), osteoblasts (Table 2) and myoblasts (Guidotti *et al.*, 1974a,b), the group of neutral amino acids that responds to insulin with an accelerated uptake rate also undergoes adaptive regulation (Guidotti *et al.*, 1975). The transport system involved, formally identified as the A system in chick-embryo heart cells (Gazzola *et al.*, 1973; Guidotti *et al.*, 1974a), appears to correspond to the same A system as a whole in fibroblasts, osteoblasts and possibly in smooth muscle cells (cf. Guidotti *et al.*, 1975, for adaptive regulation). Adaptive control in thymic lymphocytes, however, is restricted to some amino acids (namely α -aminoisobutyric acid and L-proline) among those that are considered typical substrates of the A transport system (Guidotti *et al.*, 1975). In these cells, the transport rate of L-

alanine and L-serine does not increase under derepressive conditions; conversely, all substrates of the A transport system (including L-alanine and L-serine) respond to insulin with enhanced uptake. This result suggests that L-alanine and L-serine are preferential substrates of different mediations in lymphoid cells. If this were the case, insulin might shift the reactivity of these amino acids toward transport system A in thymocytes.

The study of the regulation of transport in cells of the higher animals is still at a fairly primitive stage of investigation. However, there is now convincing evidence that various control mechanisms for molecular transport find expression at the cell membrane (Franchi-Gazzola *et al.*, 1973; Guidotti *et al.*, 1974a,b, 1975; Elbrink & Bihler, 1975). The recognition of these hormonal and adaptive control mechanisms and their characterization by occurrence, target system, site and mode of action, magnitude of effect and specific properties provide a basis for a functional discrimination of the cell types after differentiation, and may offer the means to deal successfully with problems of tissue histogenesis in eukaryotes.

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