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SHORT COMMUNICATION

EFFECT OF AMINO ACID DEPRIVATION ON L-ALANINE UPTAKE THROUGH THE ASC SYSTEM IN FRESHLY ISOLATED TROUT HEPATOCYTES

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Fasting in mammals and other vertebrates induces an increase in the ability of liver to extract from blood gluconeogenic substrates, such as plasma amino acids, which can also be used as an energy source (Cowey *et al.* 1977; Newsholme and Leech, 1983). In mammalian hepatocytes, food deprivation induces the appearance of a high-affinity component for short-chain amino acid transport, with the properties of system A, while there are no changes in the activities of a low-affinity system (the ASC system), system L (Fehlmann *et al.* 1979) or glutamine uptake (Hayes and McGivan, 1982). 'A' is the abbreviation for a Na^+-dependent carrier which has L-alanine and other short-chain neutral amino acids as preferred substrates. Its tolerance to *N*-methylated analogues differentiates it from the ASC system. 'L' is the abbreviation for a Na^+-independent carrier which has L-leucine as preferred substrate. '*asc*' is the abbreviation for a carrier similar to the 'ASC' carrier with respect to preferred substrates, but it is Na^+-independent.

The ASC system is a widely distributed, short-chain neutral amino acid carrier that transports alanine, serine, cysteine and threonine in a Na^+-dependent mode, although the scope of its substrates is now thought to be wider than when it was first described in Erlich ascites tumour cells (Christensen *et al.* 1967). Characteristically, it does not accept *N*-methylated amino acid derivatives (allowing easy distinction from the A system).

In a previous study, we demonstrated that L-alanine is taken up by isolated trout hepatocytes by means of two systems, one of them showing the properties of an ASC system, the other resembling an *asc* system (Canals *et al.* 1992). Although system A is absent from fish hepatocytes (Ballatori and Boyer, 1988; Canals *et al.* 1992), we have shown that fasting induces an increase in the rate of uptake of L-alanine and in its rate of transformation to glucose (Canals *et al.* 1992). The present study extends previous information and demonstrates that, in trout hepatocytes, the ASC system is affected by amino acid deprivation *in vitro*.

Brown trout (*Salmo trutta*) (250–400g) were obtained from a fish farm (Departament Medi Ambient, Generalitat de Catalunya) in the Pyrenees (Pont de Suert, Spain) and were

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maintained in an open-water circuit, directly connected with the river Noguera at the farm facilities (low-salinity water, pH8.0). Fasting animals were deprived of food for 30 days before experiments were performed. Control animals were fed Ewos pellets (20g/100 g bodymass). Experiments were carried out between January and April, 1992.

All chemicals were from Sigma Chemical Co. (USA), except L-[3-^{3}H]alanine which was obtained from New England Nuclear (Germany).

Hepatocytes from trout anaesthetized in $NaHCO_3$-buffered MS222 were obtained essentially as described by French *et al.* (1981), except that hyaluronidase was omitted and 5mmol l^{-1} instead of 25mmol l^{-1} $NaHCO_3$ was used, because of the difference in the gas mixture used to equilibrate all solutions (99.5% O_2:0.5% CO_2). The final pH was adjusted to 7.5. Osmolality was determined and adjusted with NaCl to 305mosmol kg^{-1} with a micro-osmometer (model 3MO, Advanced Instruments, Inc. USA). Cell viability was routinely assessed by means of the exclusion of Trypan Blue and more than 95 % viability was obtained. Cell integrity over time was measured by lactate dehydrogenase (LDH) release and less than 5% of initial intracellular LDH was found in the medium after 6h of incubation. Following isolation, cells were finally suspended in Hanks' solution containing 2% fatty-acid-free bovine serum albumin. In some instances, K^+ was used instead of Na^+ as the main extracellular cation. No loss of cell viability was observed under these conditions.

For uptake experiments, both cells and solutions were pre-equilibrated at 15˚C before uptake was started by mixing (1:1, v/v) the hepatocyte suspension with L-[^{3}H]alanine (0.3 μCi ml^{-1} cells) to obtain the desired concentrations and a final 'hepatocrit' of about 5 %. Incubations were performed in a shaking bath at 15˚C, using the indicated gas mixture as atmosphere. The uptake was stopped by diluting with Hanks' solution. Cells were rinsed with Hanks' solution and centrifuged (1000 ***g***, 8min at 4˚C) three times before finally lysing with 0.1% Triton X-100. The resulting solution was counted in a well-type liquid scintillation counter (Packard, Great Britain). 2mmol l^{-1} amino-oxyacetic acid (AOA; a transaminase inhibitor) was used throughout uptake experiments to inhibit alanine metabolism. The DNA content of hepatocytes was measured as described by Buckley and Bulow (1987).

The concentration dependence of L-alanine uptake by hepatocytes from fed or fasted trout was measured (Fig. 1). As reported previously (Canals *et al.* 1992), the rate of incorporation into cells from fasted animals was higher than that into cells from fed trout. The value of V_{max} obtained for the Na^+-dependent system (total uptake minus Na^+-independent uptake) in cells from fed animals was $0.9\times10^3\pm0.1\times10^3$ nmol mg^{-1} DNA $10min^{-1}$, whereas from fasted animals it was $2.1\times10^3\pm0.2\times10^3$ nmol mg^{-1} DNA $10min^{-1}$ (these values differ significantly, $P<0.01$). K_m values varied from 3.5±0.3mmol l^{-1} in cells from fed animals to 5.5±0.04mmol l^{-1} in cells from fasted trout (these values differ significantly, $P<0.001$). Kinetic variables for the Na^+-independent uptake were as follows: V_{max} values were $0.57\times10^3\pm0.04\times10^3$ and $1.7\times10^3\pm0.07\times10^3$ nmol mg^{-1} DNA $10min^{-1}$ in cells from fed and fasted trout, respectively ($P<0.01$); K_m was 8.3±0.4mmol l^{-1} for cells from fed animals and 8.8±0.5mmol l^{-1} for hepatocytes from fasted trout. Both K_m and V_{max} of the Na^+-dependent system differ from those previously published. A strong seasonal effect has been observed

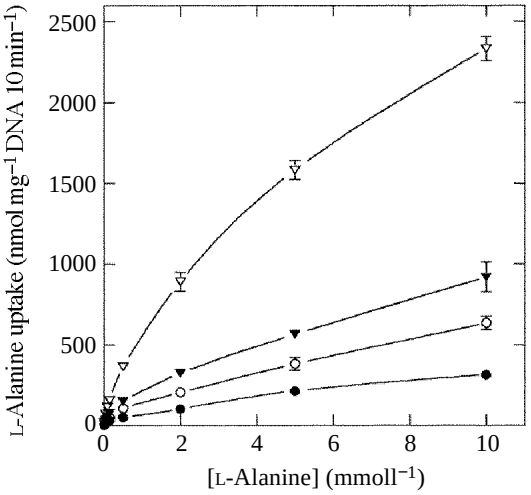

Fig. 1. Concentration dependence of L-alanine uptake by hepatocytes from control (circles) or fasted (triangles) trout. Initial rates were measured in the presence of buffers containing either sodium or potassium. Cells were incubated for 10min before the uptake was stopped, as described in the text. Open symbols, total uptake; filled symbols, Na^+-independent uptake. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean. Kinetic constants for both Na^+-dependent and Na^+-independent L-alanine uptake are given in the text.

in the Na^+-dependent uptake of L-alanine by these cells, which will deserve further analysis.

Because the K_m of the Na^+-dependent system in cells from fasted trout was different from that in cells from fed trout, the inhibitory action of several amino acids on this uptake of L-alanine was tested. The Na^+-dependent uptake was inhibited most by alanine, serine and cysteine, and less inhibition was caused by all other amino acids tested, except α-(methylamino)isobutyric acid, which had no effect (data not shown). These results were similar to those obtained when hepatocytes from fed animals were used (Canals *et al.* 1992). Moreover, the pH dependence of the uptake of 150 μmol l^{-1} L-alanine by cells from fasted animals was the same as that found in hepatocytes from non-fasted animals (data not shown). All this indicates that fasting induces an increase in the activity of systems already present in trout hepatocytes.

Among the different factors that may be involved in the increase in the rate of uptake induced by fasting is the stimulating effect that amino acid deprivation can exert on the activity of amino acid carriers. Two mechanisms have been suggested to explain how amino acid deprivation can induce an increase in the activity of the carrier: release from *trans*-inhibition and adaptive regulation (Guidotti *et al.* 1978; Shotwell *et al.* 1983; Englesberg and Moffet, 1986; Saier *et al.* 1988; Cheeseman, 1991). Both mechanisms are involved in the regulation of system A activity. *Trans*-inhibition can be defined as the decrease in the rate of uptake of an amino acid through a determinate carrier caused by the presence of this amino acid or some other amino acid transported by the same carrier on the other side of the membrane. Thus, an incubation carried out in the absence of external

amino acid should provoke a depletion of the intracellular amino acid pool, inducing an increase in the ability of cells to take up substrates through this system. This process is fast and independent of protein synthesis. Adaptive regulation implies an increase in the initial rate of amino acid transport dependent on protein synthesis, due to a depletion of external amino acids, inducing an enhancement of carrier expression (Englesberg and Moffet, 1986).

The ASC system from mammalian cells is insensitive to amino acid deprivation *in vitro* (Christensen and Kilberg, 1987; Saier *et al.* 1988) and to starvation of the donor animal (Fehlmann *et al.* 1979; Hayes and McGivan, 1982). From our results, a different picture emerges in trout hepatocytes, because the rate of L-alanine uptake through the Na^+-dependent system increased during 6h of incubation without amino acid, while the activity of the Na^+-independent system remained unaltered (Fig. 2). Because this effect was suppressed by 1mmol l^{-1} cycloheximide (a protein synthesis inhibitor) (Fig. 3), and the ASC system is not subjected to *trans*-inhibition, but to *trans*-stimulation, in cells of higher vertebrates (Gazzola *et al.* 1980; Barker and Ellory, 1990) and fish (M. A. Gallardo and J. Sánchez, unpublished data), it may be concluded that this system is subject to adaptive regulation in trout hepatocytes.

This substrate deprivation specifically stimulated the activity of the Na^+-dependent system, but had no effect on the activity of the Na^+-independent system. Thus, the increase observed in its activity (see Fig. 1) must be related to another factor, such as hormones. We tested the influence of insulin on the uptake of L-alanine by trout hepatocytes and only the activity of the ASC system was increased, while the activity of the *asc* system remained unaltered (P. Canals, M. A. Gallardo and J. Sánchez, in preparation).

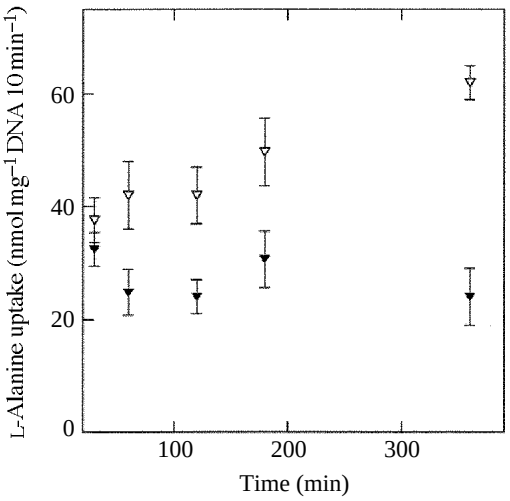

Fig. 2. Effect of amino acid deprivation on the rate of uptake of L-alanine by Na^+-dependent (△) and Na^+-independent (▲) systems by hepatocytes from fasting trout. Cells were incubated in media without L-alanine and, at the selected times, labelled L-alanine (150 μmol l^{-1} final concentration) was added. The uptake was followed for 10min. Results are the mean of three individual experiments and bars show the standard deviation.

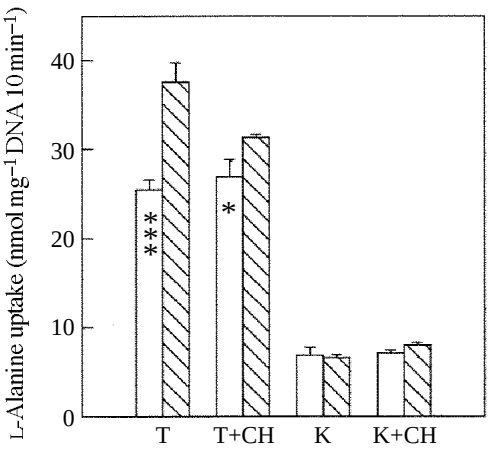

Fig. 3. Effect of 1mmol l^{-1} cycloheximide on the rate of uptake of L-alanine by hepatocytes from fasted trout. Cells were incubated for 1h (open columns) or 6h (hatched columns) in the absence of L-alanine, with or without cycloheximide (CH). At these times, labelled L-alanine (150 μmol l^{-1} final concentration) was added and the uptake was followed for 10min. T refers to the uptake in the presence of Na^+ as the main external cation, while K refers to the Na^+-independent uptake. Results are the mean of three individual experiments ± the standard deviation. Statistical significance between uptake rates at 1 and 6h (*P<0.05; ***P<0.001).

Adaptive regulation has been demonstrated *in vitro*; it remains to be determined whether it plays an important role in modifying cell behaviour as environmental conditions change *in vivo*. It is not clear whether an absolute deprivation of an amino acid may be paralleled by a drop in its plasma concentration, as occurs during fasting. However, in a previous study (Canals *et al.* 1992) we showed that there is an inverse relationship between the rate of uptake of L-alanine by trout hepatocytes and its plasma concentration. As indicated there, a chemical factor varying in parallel with this plasma concentration could be responsible for the changes observed in the rate of incorporation of L-alanine. However, the results presented here point to the involvement of adaptive regulation in supplying increasing amounts of gluconeogenic or energy substrates by stimulation of a Na^+-dependent carrier. This does not exclude hormonal involvement *in vivo*, and it should be considered that the effect observed is clearer in cells from fasting animals than in those from fed fish. In any case, these results open an interesting door to the study of how the properties of amino acid carriers have changed during the course of vertebrate evolution.

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