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INHIBITORY ACTION OF ISOVALERYL-L-CARNITINE ON PROTEOLYSIS IN PERFUSED RAT LIVER

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SUMMARY: Isovaleryl-l-carnitine inhibits the proteolysis induced by amino acid deprivation in the perfused rat liver to an extent equivalent, or, below 0.4 mM, even greater than that previously found for l-leucine (Ref.1). Also the typical concentration-response curve previously found for leucine (Ref.1) is mimicked by isoveleryl-l-carnitine. The maximum inhibition (approximately 50% of the control) occurred for both l-leucine and isovaleryl-l-carnitine above 0.8 mM. Only at these high concentrations also l-carnitine and isobutyryl-l-carnitine exhibit a significant, albeit lower, degree of inhibition. The possible mechanism of this proteolysis inhibition is discussed.

There is a good reason to consider that the free amino acids present in liver may be the primary regulators of proteolysis in rat tissue (for a review see 1). Following their depletion in perfused rat liver, for instance, there is a big increase in the rate of proteolysis (2), which is strongly inhibited by addition to the perfusate of a mixture containing all proteic amino acids at concentration as down to half their normal plasma levels (2). However a few amino acids inhibit proteolysis to the same extent as the complete mixture (3). Among these, leucine is easily the most effective, suppressing the deprivation response by about 50% at 4 times its normal plasma concentration (3). An analogous effect is obtained with α-ketoisocaproate, (1) the transamination product of leucine. This observation raises the question whether other leucine catabolites could also be inhibitory. One of these is isovaleryl-CoA formed from α-ketoisocaproate by oxidative decar-

Abbreviations: IVC, isovaleryl-l-carnitine.

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boxylation. This substance cannot itself cross cell membranes, but its carnitine analogue can do so.

In the present paper we report that IVC, from 0.1 to 1 mM does indeed inhibit the proteolysis induced by amino acids deprivation in perfused liver and to an extent equivalent, or even greater, than that found with leucine.

METHODS AND MATERIALS

Liver perfusion: liver donors for the perfusion experiments were male wistar rats maintained on standard laboratory chow and water ad libitum weighting 130-140 g. All animals were anaesthetized by intraperitoneal injection of ketamina (16 mg/100 gr) and heparinized (1000 U).
Livers were perfused by the **in situ** technique (4). Perfusion was started in the single pass mode (not recirculating) and continued for 40 min. At the end of the single pass phase, the flow of the perfusate was switched to a second stage cyclic perfusion reservoir containing 50 ml of the basic medium and cycloheximide 18 µM. Following 30 sec. washout, the medium was recirculated for 15 min. All experiments were performed at a flow rate of 11 ml/min. and at a temperature of 37°C.
Perfusion medium: the basic perfusion medium consisted of Krebs-Ringer bicarbonate buffer with 4% bovine plasma albumin (fraction V, Sigma Chemical Co.) and 10 mM glucose, gassed with 95% O_2 - 5% CO_2. After the addition of the substances to be tasted the albumin buffer solution was passed through 0.45 µs Millipore filter, and the pH adjusted to 7.4 in the presence of 95% O_2 - 5% CO_2.
Protein degradation: proteolysis was determined from the rate of valine release in second stage cyclic perfusion in the presence of cycloheximide according to the procedure described by Khairallah and Mortimore (5). Four 0.5 ml perfusate samples were taken between 5 and 15 min, and stored at -25°C. Total free valine accumulation was computed taking as total volume of distribution the perfusate volume plus liver water (6). Proteolytic rates then were calculated by the least square regression. Values were expressed as nmoles of valine min^{-1}/g liver wet weight.
Analytical procedures: determination of valine was obtained by HPLC analysis of samples previously deproteinizated in ice cold PCA (6% final concentration) and derivatized with dansyl chloride (Sigma Chemical Co.),using the method described by Taphui (7). To improve the accuracy of quantitative analysis, l-norleucine (66 µM final) was added as an internal standard to all samples. The cromatographic apparatus consisted of a Beckman System Gold, a column 250 x 4 mm Bio-sil ODS-5S protected with a guard column 30 x 4.6 mm ODS-5S (Bio-rad Laboratories, Richmond, CA.),a Waters 712 Wisp autosampler, and a Perkin Elmer LS 5 Spectrofluorimeter set at 340 - 520 nm as the excitation and emission wavelengths. Dansylated valine was separated by a gradient at a flow rate of 0.8 ml/min for the first 20 min, 1 ml/min between 20 and 40 min, and 0.8 ml/min for the last 5 min . The mobile phase consisted of two eluants: 75/25 (v/v) water/methanol containing 1 vol% glacial acetic acid and 0.03 vol% triethylamine (eluant A) and 50/50 (v/v) acetonitrile/methanol containing 3 vol% glacial acetic acid and 0.03 vol% triethylamine (eluant B). The gradient conditions were: initial, 33% of B then arised to 50% in 15 min.,

isocratic conditions for 5 min and eventually an increase to 95% of B in 5 min. hold for 10 min. The initial conditions were restored in 1 min. and 15 min. of requilibration were used before a new injection. The analysis was performed at room temperature.

RESULTS

The rates of hepatic proteolysis in the presence of either IVC or an equimolar amount of l-leucine are shown in Fig. 1.

The two compounds inhibit proteolysis to almost the same extent throughout the concentration range used. They share also an anomalous dose-response curve: inhibition increases with increasing concentration down to 0.2 mM, but above this value it declines; above 0.4 mM the proteolysis inhibition increases once more. The only difference found was the significantly higher degree of inhibition afforded by IVC in the range around 0.2 mM. The maximum inhibition (approximately 50% of the control) occurred for both l-leucine and IVC at a concentration above 0.8 mM.

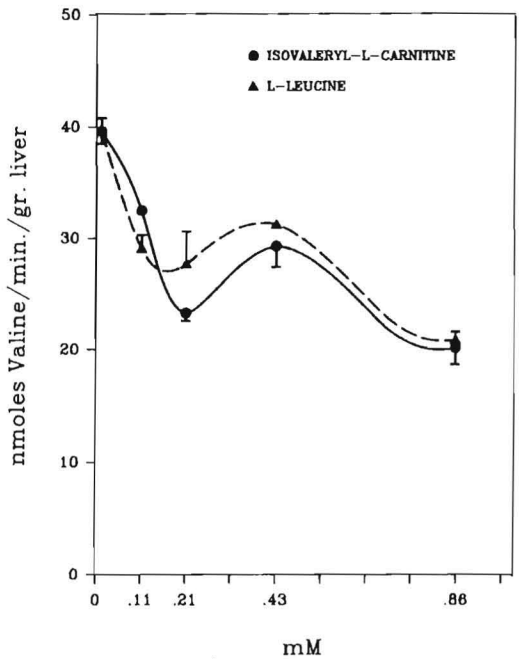

Fig. 1. Comparison of the effect of graded concentrations of l-leucine and isovaleryl-l-carnitine on perfused liver proteolysis.
Rat livers were perfused in the single-pass mode for 40 min. in the presence of different concentration of l-leucine or isovaleryl-l-carnitine. Protein breakdown was determined from the release of valine in the second-stage cycloheximide perfusion (see Methods). Values are ± S.E. of 4 to 14 determination.

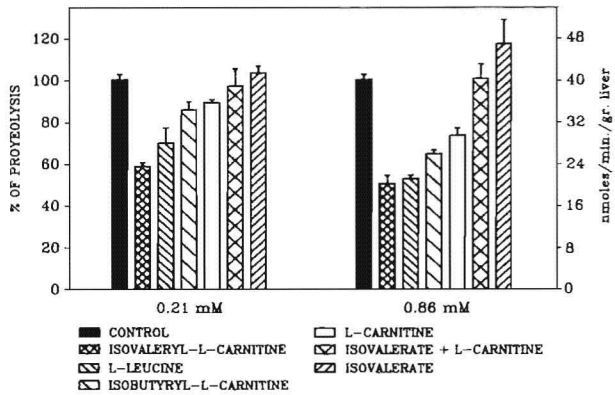

Fig. 2. Inhibition of proteolysis by l-leucine, isovaleryl-l-carnitine and related compounds.
The two chosen concentrations (0.21 and 0.86 mM) are significant (see Fig. 1) for the evaluation of the concentration dependence of the tested compounds. See Fig. 1 for the experimental conditions. Values are ± S.E. of 4 to 14 determinations.

We have compared the inhibition degree at 0.21 and 0.86 mM of some compounds metabolically related to IVC (Fig. 2). At 0.21 mM only IVC and l-leucine inhibited significantly but at 0.86 mM both isobutyryl-l-carnitine and l-carnitine also did so, though to a lesser extent than IVC or l-leucine.

The inhibition by high concentration of l-carnitine was suppressed when isovalerate was added together to perfusate. Note that isovalerate added to the perfusate in the absence of l-carnitine stimulated protein breakdown.

The lack of inhibition of isobutyryl-l-carnitine at 0.21 mM is in line with the observation that the amino acid from which it is derived, i.e. valine, is devoid of inhibitory activity (3) as also is l-isoleucine.

DISCUSSION

Our results show that in perfused liver IVC inhibits proteolysis with a concentration profile very similar to that already reported for l-leucine (1). Unlike α-ketoisocaproate, which is inactive at low concentrations (3), IVC is more active than leucine in the range around 0.21 mM. The problem as to whether α-ketoisocaproate exerts its action as such, or upon transamination into leucine has not been unequivocally solved. On surer ground the inhibition of proteolysis by IVC is a process that certainly does not involve leucine, since the oxidative decarboxylation of α-ketoisocaproate to isovaleryl CoA is known to be irreversible.

Since both l-leucine and α-ketoisocaproate catabolise to IVC, their common inhibitory properties could be due to formation in each case of the later. One possibility is that the isovaleryl group might be the signal recognizable by a receptor regulating proteolysis. The fact that α-ketoisocaproate mimics the inhibitory effect of l-leucine only in the upper concentration range, and is much less active, (or even inactive) in the physiological range (around 0.1 mM), might be because α-ketoisocaproate has a lower accessibility than either leucine or IVC to this putative receptor. On the other hand, IVC - the most active compound at low concentration - is rapidly taken up by perfused liver(8). The peculiar profile of the concentration-response curve of both leucine and IVC can not be explained by the present results. Conceivably, as already outlined by Mortimore (1), it may be accounted for by the complexity of the cellular proteolytic system (lysosomal and non lysosomal) and the putative multeplicity of the regulatory sites.

The inhibitory action of IVC seems remarkably specific, since neither isobutyryl-l-carnitine nor the other IVC related compounds tested exhibited much effect (Fig.2).

Isobutyryl-l-carnitine and l-carnitine, which inhibit only at high concentration, could do so after prior conversion to IVC. Thus Hokland (8) has found that in the perfused live l-carnitine consistently produces IVC. Likewise the inhibitory effect of isobutyryl-l-carnitine at a high concentration could be due to the release from it of free carnitine via the reaction catalysed by carnitine: CoA acetyl transferase. On the other hand owing to the very limited leucine transamination and the rate of α-ketoisocaproate decarboxilation in the liver (1), the amount of isovaleryl-carnitine generated from leucine should be rather small. The effect of carnitine itself at high concentration could perhaps be due to an interaction of this dipolar compound with lisosomal membrane. The abolition of carnitine inhibition by lipophilic isovalerate could be due to its prior binding to the membrane countractiving carnitine interaction.

Whether and to what extent IVC acts as a physiological regulator in vivo is an open question which can only be answered when the concentration of IVC in liver has been determined under conditions of modified proteolysis. On the basis of the

data presently available (9) the IVC concentration in normal liver is lower than that which one could aspect to be reached in perfused liver in our experiments. However under certain conditions, for instance during physical exercise (10), the level of IVC in blood may increase considerably. Since in muscle, unlike in liver, leucine transamination is very rapid (11), the IVC formed there could be transported to the liver for further metabolism.

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