Plasma Membrane Transport of 2-Ketoisocaproate by Rat Hepatocytes in Primary Culture*

Received for publication, January 26, 1983

Michael S. Kilberg‡ and Marjorie B. Gwynn
From the Department of Biochemistry and Molecular Biology, University of Florida School of Medicine, J. Hillis Miller Health Center, Gainesville, Florida 32610

The branched chain amino acids are transaminated to form KIC, KIV, and KVM from leucine, valine, and isoleucine, respectively. These 2-keto acids are then degraded by oxidative decarboxylation to generate short chain acyl-CoA derivatives which are further catabolized to form intermediates of the citric acid cycle. One of the more interesting aspects of the first two reactions is the tissue specificity of the branched chain 2-keto acid catabolism. Recently, the control of branched chain 2-keto acid metabolism by the liver has generated considerable interest, in part because of the studies by Williamson and co-workers. These investigators have demonstrated several regulatory effects of these keto acids or their metabolites on pyruvate dehydrogenase, gluconeogenesis, and ureogenesis in hepatocytes (6, 11, 12). As a result, the branched chain 2-keto acids, acting as regulators of various metabolic pathways, may play a role in maple syrup urine disease, isovaleric acidemia, and methylmalonic acidemia, three metabolic disorders in which the plasma levels of these metabolites are elevated (13-15). Considering the possible impact of the branched chain 2-keto acids on amino acid and carbohydrate metabolism in the liver, we have investigated an additional point for metabolic control of their catabolism, namely, active transport into rat hepatocytes.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats weighing between 100 and 200 g were obtained from the Division of Animal Resources, University of Florida. Radioactively labeled branched chain 2-keto acids were generated by the method of Rudiger et al. (16) and determined to be free of the precursor amino acid by thin layer chromatography. Unlabeled 2 keto acids, dexamethasone, streptozotocin, collagenase, and CHOC were purchased from Sigma. Highly purified insulin and glucagon were generous gifts from Dr. Mary Root of Lilly Laboratories.

Methods—Hepatocytes were isolated by a collagenase perfusion method as described previously (17, 18). The procedures for establishing primary cultures of normal rat hepatocytes, for maintenance of the hepatoma cell line H4-II-EC3, and for measurement of transport were the same as in our studies on amino acid uptake (18, 19). Diabetes was induced in rats by intraperitoneal injection of 8 mg per 100 g body weight of streptozotocin (20). Rats were considered to be chronically diabetic if they exhibited continual weight loss and a serum glucose level of greater than 300 mg/100 ml. Transport was divided into Na+-dependent and Na+-independent components; the CholKRP, pH 7.4 was made by replacing the corresponding Na+ salts with choline chloride and choline phosphate. For the Na+-independent transport, only the saturable component, taken as the difference between uptake in the presence or absence of an excess of unlabeled...
substrate, is shown. The estimate of the nonsaturable uptake does not take into account the reverse process which would be included in a more detailed correction (22).

The intracellular water content was determined by the 3-O-methylglucose method of Kletzien et al. (22). Prior to transport the cells were incubated for 1 h in NaKRP, pH 7.4, in order to deplete uniformly the intracellular levels of the 2-keto acids and, therefore, minimize possible trans-effects. In most cases, the experiments were repeated with 2 to 3 different preparations of cells and the standard deviations within each preparation were typically less than 10%. Kinetic data were analyzed by computer analysis and fitted to theoretical models for uptake by one or more systems (18).

RESULTS AND DISCUSSION

**Time Course of KIC Transport**—The uptake of 50 μM KIC by primary cultures of rat hepatocytes was measured over a 10-min period (Fig. 1). The saturable Na⁺-independent transport was quite rapid and attained a steady state level within the first minute. In contrast, Na⁺-dependent transport of the 2-keto acid was relatively slow and had just begun to approach a steady state after 10 min (Fig. 1). This system was concentrative, yielding an apparent distribution ratio favoring the intracellular pool, of 2 to 3. If one assumes a resting membrane potential for the hepatocyte of ~35 mV (23), the distribution ratio for Na⁺-dependent uptake of 50 μM KIC should be approximately 3 at the steady state. These results are qualitatively similar to those found for other metabolites shown to be transported into eukaryotic cells by Na⁺-dependent and Na⁺-independent processes.

**Kinetics of KIC Transport**—Fig. 2 shows the relation between velocity of total KIC uptake and the extracellular concentration. The results were subjected to computer analysis using programs which calculate and subtract the nonsaturable component prior to determining the apparent Kₐ and Vₐₘₙ values (18). For KIC transport, the data fit a theoretical model describing uptake by two saturable systems. One of these components was a low affinity process with an apparent Kₐ of about 5 mM, while the other system had a Kₐ of approximately 60 μM (Fig. 2). When these assays were repeated in a Na⁺-free medium to detect Na⁺-independent uptake alone, only the high affinity system was observed. There was good agreement between the two series of experiments with respect to the estimates for Kₐ, the computer-estimated values for the Na⁺-independent transport kinetic constants, when measured in the absence of Na⁺, were 51 μM and 1.03 nmol mg⁻¹ protein 30 s⁻¹ for Kₐ and Vₐₘₙ, respectively (compare with the values given in Fig. 2). These results serve to demonstrate the existence of at least two saturable systems for KIC transport in cultured hepatocytes.

**Inhibition of KIC Transport by 2-Keto Acids**—The uptake of KIC by isolated hepatocytes was measured in the presence or absence of individual 2-keto acids at concentrations of 0 to 5 mM. The transport of KIC was completely inhibited by unlabeled KIV and KMV and quantitatively the inhibition by each of these 2-keto acids resembled that produced by an excess of unlabeled KIC itself. The degree of inhibition for either the Na⁺-dependent or the Na⁺-independent transport process was the same whether the inhibitor under test was KIC, KIV, or KMV; the estimated Kᵢ values ranged between 0.1 and 0.5 mM (data not shown). These results suggest that all three branched chain 2-keto acids compete for the same transport systems in cultured hepatocytes. In contrast, addition of 5 mM L-leucine produced no significant inhibition of 50 μM KIC transport (the uptake rates were 102 ± 10 and 98 ± 14 pmol mg⁻¹ protein 30 s⁻¹ without or with L-leucine, respectively). The lack of an effect by amino acids, considered along with the knowledge that the radioactively labeled substrate was free of amino acid, demonstrates the specificity of these transport systems for 2-keto acids.

The uptake of KIC by isolated hepatocytes was tested for
control of hepatic branched chain 2-keto acid transport, the uptake of 50 μM KIC by cells treated previously with insulin, glucagon, dexamethasone, or the combination of the latter two hormones was measured. The hormones were used at concentrations (10^{-7} M) known to produce maximal stimulation of hepatic Na⁺-dependent neutral amino acid transport by System A (26). Insulin produced a 65% stimulation of the Na⁺-dependent system, but had no effect on the Na⁺-independent component; neither glucagon, dexamethasone, nor the combination of these two hormones had any significant effect on Na⁺-dependent or Na⁺-independent KIC transport when added to the cultured hepatocytes (Table 1).

When KIC transport was measured in hepatocytes isolated from streptozotocin-induced diabetic rats, uptake by the Na⁺-dependent system was found to be moderately, but significantly, increased (Table 1). Kinetic analysis of KIC transport in hepatocytes from normal and diabetic rats confirmed the stimulation of the Na⁺-dependent system and indicated that the effect was the result of an increase in the apparent V_{max}. Considering that streptozotocin-induced diabetes is characterized as a state of decreased plasma insulin and elevated plasma glucagon, one might view the lack of an effect on KIC transport by exogenously added glucagon as a paradox. However, recent evidence from our laboratory suggests that factors other than glucagon may be responsible for stimulation of hepatic amino acid transport by System N in the streptozotocin-treated animal (20).

Changes in KIC Transport after 2-Keto Acid Deprivation—Incubation of isolated rat hepatocytes in the absence of extracellular amino acids causes stimulation of two of the six systems for neutral amino acid transport (for a review see Ref. 27). The increased amino acid uptake, referred to as adaptive control, is considered to be the result of a derepression of a gene coding for a protein necessary for transport activity by Systems A or N. To determine if a similar mechanism might exist for regulation of 2-keto acid transport, cultured hepatocytes were incubated in the presence or absence of exogenous hormones and diabetes on the transport of KIC by cultured hepatocytes. Freshly isolated hepatocytes from normal or diabetic rats were placed in primary culture for 2 h in Waymouth’s medium containing 10% fetal calf serum. To test the effect of diabetes on KIC transport, the cells were assayed after this 2-h culture period. The donor rat from which the cells were isolated for the experiment shown here had a serum glucose value of 467 mg per 100 ml of serum. For the experiments involving hormonal stimulation, after the initial 2 h the original plating medium was changed to Waymouth’s medium lacking serum but containing the indicated hormones each at 10^{-7} M. After 3 h the uptake of 50 μM KIC was measured for 30 s at 37°C. The results are shown as the velocity, pmol·mg⁻¹ protein·30 s⁻¹, and are the averages ± S.D. of 3 to 4 determinations.

<table>
<thead>
<tr>
<th>Hormone added</th>
<th>Na⁺-dependent KIC uptake</th>
<th>Na⁺-independent KIC uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Velocity (pmol·mg⁻¹ protein·30 s⁻¹)</td>
<td>% of control</td>
</tr>
<tr>
<td>None</td>
<td>124 ± 27</td>
<td>100</td>
</tr>
<tr>
<td>Insulin</td>
<td>205 ± 22</td>
<td>165</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>133 ± 23</td>
<td>107</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>99 ± 18</td>
<td>80</td>
</tr>
<tr>
<td>Glucagon plus dexamethasone</td>
<td>155 ± 22</td>
<td>125</td>
</tr>
</tbody>
</table>

*Significant at p < 0.01.

Effect of Hormones and Diabetes on KIC Transport—Primary cultures of rat hepatocytes have been shown to respond to a variety of hormones by increasing the transport of amino acids and other metabolites. To test for possible hormonal inhibition by pyruvate. All of the Na⁺-dependent transport was pyruvate-sensitive, whereas maximal inhibition of the saturable Na⁺-independent uptake was about 60 to 65% of the total (Fig. 3). These data were analyzed with the aid of computer programs designed to describe competitive inhibition between substrates. The computer-derived apparent K⁺ values for pyruvate inhibition of Na⁺-independent and Na⁺-dependent KIC transport were 0.15 ± 0.06 and 0.27 ± 0.06 mM, respectively. It is of interest to note the Km values for pyruvate transport by isolated liver mitochondria has been reported to be about 0.2 mM (24, 25). The present results indicate that all three branched chain 2-keto acids are transported by the same systems, and the Na⁺-dependent agency also shows affinity for pyruvate.

The ability of pyruvate to inhibit completely only a portion of the Na⁺-independent KIC transport, despite a relatively low Km value, may reflect heterogeneity in this component. In this regard, CHOC has been shown to be potent inhibitor of mitochondrial pyruvate transport (24, 25). However, Patel et al. (7) have shown that CHOC does not completely inhibit the saturable transport of KIC or KIV by isolated rat liver mitochondria. Those authors postulated the presence of a branched chain 2-keto acid transport system which is distinct from the one which can accept pyruvate. Our data for Na⁺-independent KIC transport (Fig. 3) suggests that a similar situation may exist in the hepatocyte plasma membrane. Furthermore, when we tested the effect of 1 mM CHOC on the transport of 50 μM KIC by these cells, the Na⁺-dependent component was completely inhibited, whereas the Na⁺-independent transport was decreased by only 12%; the velocities were 102 ± 10 and 89 ± 3 pmol·mg⁻¹ protein·30 s⁻¹ for the Na⁺-independent uptake in the absence or presence of CHOC, respectively. Collectively, the inhibition data for Na⁺-independent 2-keto acid transport suggest the presence of a pyruvate-insensitive system in the rat hepatocyte plasma membrane.

Effect of Hormones and Diabetes on KIC Transport—Primary cultures of rat hepatocytes have been shown to respond to a variety of hormones by increasing the transport of amino acids and other metabolites. To test for possible hormonal
TABLE II

Effect of 2-keto acid starvation on KIC transport by cultured rat hepatocytes

After culturing hepatocytes for 2 h in Waymouth’s medium containing 10% fetal calf serum, the medium was changed to either Waymouth’s medium lacking serum, but containing 10 mM KIC (2-keto acid-rich medium) or to NaKRB (2-keto acid-free medium). The results were the same whether Waymouth’s medium containing 10 mM KIC or NaKRB containing 10 mM KIC was used as the 2-keto acid-rich medium. The transport of 50 μM KIC was measured for 30 s at 37 °C after incubation in the appropriate medium for 5 or 24 h. The Na+-dependent and Na+-independent uptake rates were calculated as described in the legend to Fig. 1. The data shown are the averages ± S.D. of 3 to 4 determinations.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>5 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Keto acid-rich</td>
<td>122 ± 28</td>
<td>137 ± 26</td>
</tr>
<tr>
<td>2-Keto acid-free</td>
<td>160 ± 31</td>
<td>211 ± 32</td>
</tr>
<tr>
<td>2-Keto acid-rich</td>
<td>160 ± 31</td>
<td>107 ± 23</td>
</tr>
<tr>
<td>2-Keto acid-free</td>
<td>442 ± 18*</td>
<td>114 ± 13</td>
</tr>
</tbody>
</table>

* Significant at p < 0.005.

sence of 10 mM KIC and then tested for Na+-dependent and Na+-independent transport activity. After a 5-h incubation in the absence of 2-keto acids, there was a small increase in the uptake by both systems (Table II). Maintenance of the hepatocytes for 24 h in the 2-keto acid-free medium resulted in a 3-fold stimulation of KIC transport via the Na+-dependent system, although no effect on the Na+-independent uptake was detected.

KIC Transport in Rat Hepatoma Cells—To test for changes in 2-keto acid transport after transformation, we have studied KIC transport by the rat hepatoma cell line H4-II-EC3 and have observed the following: 1) these cells contain both Na+-dependent and Na+-independent processes for KIC transport; 2) the absolute rates of transport are somewhat slower in the hepatoma cells when compared to normal rat hepatocytes; 3) the Na+-dependent KIC uptake is enhanced if the hepatoma cells are maintained for 24 h in the absence of 2-keto acid; and 4) the ability to distinguish the Na+-dependent and Na+-independent systems by means of pyruvate inhibition is more pronounced in the hepatoma cells. With respect to the latter point, the presence of 5 mM pyruvate results in little or no inhibition of Na+-independent KIC transport by the H4-II-EC3 cells, while the uptake by the Na+-dependent system is inhibited by more than 80% (data not shown).

Often the transport of nutrients into cells has been overlooked as a possible regulatory step in their overall metabolism. However, it has been demonstrated that at physiological concentrations of alanine, translocation into hepatocytes is the rate-limiting step in the metabolism of this amino acid (28, 29). It is well recognized that specific transport systems exist in the plasma membrane for most charged or hydrophilic metabolites. These systems can exhibit relatively precise structural specificity with respect to the substrates accepted, and their activity may be regulated by hormones, growth factors, and even the substrates themselves. The present communication reports the existence of such transport systems for the branched chain 2-keto acids in rat hepatocytes. The importance of these systems arises from the unique tissue distribution of the enzymes involved in the catabolism of the branched chain amino acids. Once the amino acid has been converted to the corresponding 2-keto acid by the extracellular tissues, especially muscle, the hepatocytes of the liver must remove them from the circulation before further oxidation can occur. Hence, translocation of the 2-keto acids across the hepatocyte plasma membrane actually represents the first step in the hepatic catabolism of these compounds.

REFERENCES

Downloaded from http://www.jbc.org/ by guest on October 15, 2017
Plasma membrane transport of 2-ketoisocaproate by rat hepatocytes in primary culture.
M S Kilberg and M B Gwynn


Access the most updated version of this article at http://www.jbc.org/content/258/19/11524

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/258/19/11524.full.html#ref-list-1