Hepatic uptake of γ-butyrobetaine, a precursor of carnitine biosynthesis, in rats

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Fujita M, Nakanishi T, Shibue Y, Kobayashi D, Moseley RH, Shirasaka Y, Tamai I. Hepatic uptake of γ-butyrobetaine, a precursor of carnitine biosynthesis, in rats. Am J Physiol Gastrointest Liver Physiol 297: G681–G686, 2009. First published August 13, 2009; doi:10.1152/ajpgi.00238.2009.—γ-Butyrobetaine (GBB) is a precursor in the biosynthesis of carnitine, which plays an important role in the β-oxidation of fatty acids, and is converted to carnitine by γ-butyrobetaine dioxygenase (BBD) predominantly in liver. We investigated the molecular mechanism of hepatic uptake of GBB in rat hepatocytes. Cellular localization of rat Octn2 (rOctn2;Slc22A5) was studied by Western blot analysis. Uptake of deuterated GBB (d3-GBB) was examined in HEK293 cells expressing rOctn2 (HEK293/rOctn2) and freshly isolated rat hepatocytes. d3-GBB was quantified by use of liquid chromatography-tandem mass spectrometry. Western blot analysis demonstrated an expression of OCTN2 protein in hepatic basolateral membrane but not in bile canaliculus membrane fraction. Furthermore, we found that d3-GBB was taken up by rOctn2 in an Na+-dependent manner with $K_m$ value of 13 μM. The apparent $K_m$ value for d3-GBB transport in freshly isolated rat hepatocytes was 9 μM. d3-GBB uptake by the rat hepatocytes was inhibited by γ-aminobutyric acid (GABA) to 30% of the control, whereas it was inhibited by carnitine to 62% of the control, even at 500 μM. Furthermore, d3-GBB uptake by rat hepatocytes was decreased by 45% with rat Gat2 (Slc6A13, a major liver GABA transporter) silenced by the microRNA method. Accordingly, the present study clearly demonstrates that GBB is taken up by hepatocytes for carnitine biosynthesis not only via Octn2 but also via the GABA transporter, possibly Gat2.

Octn2; transporter; hepatocytes; carnitine; γ-butyrobetaine; liver; GABA transporter

CARNITINE IS AN ESSENTIAL element in the β-oxidation of long-chain fatty acids, because it helps to carry the fatty acids as acylcarnitine esters across the mitochondrial inner membrane for energy production (12, 21). Carnitine is supplied from the diet as well as via biosynthesis from lysine and methionine (27). Previous studies revealed that cellular membrane transport of carnitine is mediated by several transporters, including OCTNs, ATB+ (SLC6A14), and CT2 (SLC22A16) (4, 17, 25). Among them, OCTN2 (SLC22A5), a Na+-dependent and high-affinity ($K_m$: 4 to 25 μM) carnitine transporter (23–25), is essential to maintain carnitine levels in serum by reabsorption of carnitine by proximal tubular cells and the uptake of carnitine into tissue (18, 23, 30), since deficiency of OCTN2 produces a shortage of carnitine in affected tissues, resulting in impairment of mitochondrial β-oxidation of fatty acid and subsequent energy production, especially during fasting or illness (18, 19).

In humans, γ-butyrobetaine dioxygenase (BBD) catalyses the conversion of γ-butyrobetaine (GBB) to carnitine in several organs, including liver, whereas the enzyme activity is found only in liver in rats. Most tissue is incapable of converting GBB into carnitine because of the lack of BBD. Since conversion of GBB to carnitine by BBD is the pathway of carnitine biosynthesis in vivo, effective delivery of GBB as a precursor of carnitine to the organs capable of producing carnitine, e.g., liver, may result in an increase in carnitine biosynthesis. Therefore, it is of pharmacological importance to clarify the mechanism of GBB uptake into the liver for the therapy of disease such as primary systemic carnitine deficiency.

OCTN2-mediated carnitine transport was strongly inhibited by acetyl carnitine and GBB, suggesting that OCTN2 transports GBB as well as carnitine (24). However, GBB administration produced an increase of carnitine in the liver of juvenile visceral steatosis (jvs) mice, in which Octn2 is dysfunctional because of mutations in the Octn2 gene (7, 24). Furthermore, GBB uptake to basolateral plasma membrane fractions prepared from rat hepatocytes was not inhibited in the presence of carnitine (1). These results suggested involvement of transporter(s) other than OCTN2/Octn2 in the hepatic uptake of GBB. Therefore, in the present study, we explored the mechanism of GBB transport into rat hepatocytes, obtaining evidence that GBB is taken up by hepatocytes via multiple transporters, including GABA-related transporters and Octn2, presumably for the biosynthesis of carnitine.

METHODS

Materials. 1-[N-methyl-3H]carnitine hydrochloride (85 Ci/mmoll) and [2,3-3H]-γ-aminobutyric acid were purchased from American Radiolabeled Chemicals (St. Louis, MO) and PerkinElmer Life and Analytical Sciences (Boston, MA), respectively. Methyl iodide (d3, 99.5%) was purchased from Cambridge Isotope Laboratories (Andover, MA). (3-Carboxypropyl)trimethylammonium chloride (GBB) and L-carnitine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from Sigma-Aldrich and Wako Pure Chemicals (Osaka, Japan).

Western blot analysis of OCTNs in liver. Canalicular membrane (CM), basolateral membrane (BM), and mixed membrane (MM) fractions were prepared from rat liver by the method previously described (13, 16). Western blot analysis was performed as described previously (10, 24). Rabbit anti-mouse Octn2 polyclonal antibody was

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raised against mouse Octn2 as described previously (24), which has been shown to cross-react with rOctn2 in Western blot analysis (10).

**Synthesis of d₃-GBB.** The deuterated GBB (d₃-GBB) was synthesized as reported by Mirzaei and Regnier (14) with minor modifications. N,N-dimethylaminobutyric acid hydrochloride (0.96 mmol), KHCO₃ (1.61 mmol), and CD₃I (3.21 mmol) were reacted in methanol for 8 h. A part of the product was purified by HPLC (model LC-20A, Shimadzu, Kyoto, Japan) on CAPCELL-PAK SCX column (150 × 20 mm ID; Shiseido, Tokyo, Japan). The isocratic elution buffer consisted of a 5 mM ammonium acetate aqueous solution, at a flow rate of 1 ml/min. Fractions containing d₃-GBB were identified by monitoring ultraviolet absorbance, combined, and lyophilized after addition of HCl. Finally, ammonium chloride was removed on an ion-exchange column (Dowex 50X8, 180–400 mesh) with 2 M HCl as reported previously (8). The structure of d₃-GBB was determined by liquid chromatography (LC)-mass spectrometry (MS) analysis with a 3200 QTRAP (Applied Biosystems, Toronto, ON, Canada) and ¹H-NMR analysis (Bruker AVANCE 300, Billerica, MA).

**Establishment of HEK293 cell line expressing rOctn2.** Plasmid DNA (pcDNA3.1, Invitrogen) containing rOctn2 cDNA (pcDNA3.1/rOctn2) was a kind gift from Dr. Yabuuchi at Genomembrane (Yokohama, Japan). HEK293 cells were transfected with pcDNA3.1/rOctn2 and then selected with 600 µg/ml G418 (Sankyo Labo Service, Hamamatsu, Japan). The resultant cell pellet was washed three times with HBSS and then resuspended in DMEM containing 5% FBS, 0.1 µM dexamethasone, and 6.25 µg/ml insulin-transferrin-selenium in addition to antibiotics. The cells were plated on collagen-coated culture plates at a density of 2 × 10⁵ cells/cm².

**d₃-GBB uptake study with HEK293/rOctn2 cells and freshly isolated rat hepatocytes.** Uptake of d₃-GBB by HEK293 cells and freshly isolated hepatocytes was examined as described previously (9, 10). Intracellular d₃-GBB was extracted from cells in 70% methanol and 30% water (vol/vol) and quantified by LC-MS/MS analysis. LC separation was carried out with an HPLC instrument (LC-20A, Shimadzu) equipped with a CAPCELL-PAK SCX column (150 × 3.0 mm ID; Shiseido) with 5 mM ammonium acetate and methanol 95:5 (vol/vol). MS was performed on a 3200 QTRAP (Applied Biosystems). Typically, the following conditions were used: source temperature 700°C, positive ion polarity with 5500 ion spray voltage. The following ion transition was obtained: d₃-GBB mass-to-charge ratio value (m/z) 149 → 87.

**RNA interference.** Kinetic parameters were obtained by fitting uptake rates to the following Michaelis-Menten equations by means of nonlinear least-squares regression analysis using KaleidaGraph (Synergy Software, Reading, PA):

\[ v = V_{\text{max}} \times S / (K_m + S) \]  
\[ v = V_{\text{max}} \times S / (K_m + S) + k_{\text{ns}} \times S \]

where \( v \), \( S \), \( K_m \), \( V_{\text{max}} \), and \( k_{\text{ns}} \) are uptake rate, substrate concentration, the Michaelis constant, maximum transport rate, and the apparent nonsaturable first-order rate constant, respectively. The statistical...
significance of differences was determined by using Student’s t-test with P < 0.05 or 0.1 as the criterion.

RESULTS

Localization of rOctn2 in rat hepatocytes. To examine the intracellular localization of Octn2 in rat hepatocytes, Western blot analysis was performed using CM, BM, and MM fractions from rat liver, as shown in Fig. 1. Anti-Octn2 antibody immunoreacted strongly with CM and weakly with MM but did not with CM, suggesting that Octn2 is functionally expressed at the basolateral membrane of rat hepatocytes.

Chemical synthesis, purification, and identification of d3-GBB. A colorless, hygroscopic product was obtained in a yield of 32.8 mg (as d3-γ-butyrobetaine chloride salt) after lyophilization. It was confirmed to be 4-(N,N-dimethyl-N-d3-methylamino)butyric acid (chloride salt) by LC-MS analysis and 1H-NMR spectroscopy. Figure 2 shows the electrospray ionization mass spectrum of the purified sample, with [M+H]+ at m/z 149 and [2M+H]+ at m/z 297. Thus the molecular weight is 148. In the 1H-NMR spectrum of the purified sample, the observed chemical shifts, relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate, were 2.10 δ [multiplet (J = 6 Hz), relative intensity 2 (CH3CH2CH2-)], 2.50 δ [triplet (J = 6 Hz), relative intensity 2 (-CH2CO2-)], 3.12 δ [singlet, relative intensity 6 (CH3)2CD3NCH2-], 3.35 δ [multiplet (J = 6 Hz), relative intensity 2 (CH3)2CD3NCH2-] (data not shown). These results confirm that the product is d3-GBB.

Characterization of d3-GBB uptake by rOctn2-expressing HEK293 and freshly isolated rat hepatocytes. Figure 3A shows the time course of the uptake of d3-GBB by HEK293/rOctn2 and HEK293/pcDNA3 cells. The d3-GBB uptake by HEK293/rOctn2 cells increased in a time-dependent manner, and intracellular accumulation of d3-GBB in 5 min was ~30-fold greater than that by HEK293/pcDNA3 cells. The initial rate of d3-GBB uptake was determined from the intracellular accumulation of d3-GBB up to 1 min, because the rOctn2-mediated uptake of d3-GBB was only linear over the first 3 min. Figure 3B shows the rOctn2-mediated uptake of d3-GBB with or without Na+. The uptake was 10-fold decreased without Na+, in agreement with the characteristic Na+ dependence of Octn2-mediated carnitine transport (20). The rOctn2-mediated d3-GBB uptake was saturable, and an Eadie-Hofstee plot showed a single straight line (Fig. 4), demonstrating the presence of a single binding site for GBB on rOctn2. The Michaelis constant and maximum uptake rate were estimated as 12.9 ± 1.8 µM and 217 ± 12.2 pmol·min⁻¹·mg⁻¹ protein, respectively.

Figure 5A shows the time course of the uptake of d3-GBB by freshly isolated rat hepatocytes at 37 and 4°C. The uptake was decreased significantly at 4°C, suggesting a contribution of a carrier-mediated transport. The initial rate of d3-GBB uptake was determined at 3 min to characterize the uptake in subsequent studies. Since uptake of d3-GBB was fivefold decreased when Na+ was replaced with N-methylglucamine (Fig. 5B) and was reduced to 62% of the control in the presence of unlabeled GBB (500 µM) (Table 1), Octn2 contributes at least in part to the hepatic uptake of d3-GBB. The initial uptake of d3-GBB was saturable, and an Eadie-Hofstee plot showed a single straight line (Fig. 6). The Michaelis constant, maximum uptake rate, and nonsaturable first-order rate constant were estimated as 9.3 ± 4.8 µM, 328.6 ± 75.6 pmol·min⁻¹·mg⁻¹ protein, and 0.85 ± 0.44 µM·min⁻¹·mg⁻¹ protein, respectively.

GBB uptake by rat hepatocytes was further characterized by measuring the inhibitory effects of various compounds on the initial uptake of d3-GBB. Unlabeled GBB (500 µM) reduced d3-GBB uptake to 16.1% of the control (Table 1). Interestingly, GABA caused a significant reduction in the uptake of d3-GBB.
Fig. 5. Time course (A) and Na\(^+\) dependence (B) of d\(_3\)-GBB uptake by freshly isolated rat hepatocytes. 

A: uptake of d\(_3\)-GBB (1 \(\mu\)M) by freshly isolated rat hepatocytes was measured at 37°C (●) or 4°C (○) and pH 7.4. B: uptake of d\(_3\)-GBB (1 \(\mu\)M) by freshly isolated rat hepatocytes was measured at 37°C and pH 7.4 for 3 min with (control) or without extracellular Na\(^+\) (−Na\(^+\); Na\(^+\) was replaced with N-methylglucamine). The uptake at 4°C was subtracted from that at 37°C to obtain the indicated values. Each value represents mean ± SE (\(n = 3\)). *\(P < 0.05\), significantly different from control.

Involvement of GABA transporter in hepatic uptake of GBB.

To determine whether GABA transporter also accepts GBB as a substrate, the inhibitory effect of GBB on \(^{[3]}\)H\(\text{GABA}\) uptake by freshly isolated rat hepatocytes was examined in the concentration range of 10 \(\mu\)M through 10 mM (Fig. 7). GBB inhibited GABA uptake in a concentration-dependent manner with an IC\(_{50}\) value of 13.3 ± 2.95 \(\mu\)M. Furthermore, to examine the contribution of GABA transporter to GBB uptake, the gene of a major rat liver GABA transporter, rGat2, was silenced by expressing shRNA to rGat2 in rat hepatocytes in primary culture. The mRNA expression of rGat2 was examined by means of semiquantitative RT-PCR (Fig. 8A), which showed a significant decrease of rGat2 mRNA expression to 45% in rat hepatocytes in which rGat2 was silenced (Fig. 8C), indicating that Gat2 might contribute to the apparent uptake of d\(_3\)-GBB in rat liver.

**DISCUSSION**

The present study has demonstrated that Octn2 and the GABA transporter rGat2 contribute to hepatic uptake of GBB, to ~30% of the control. No significant changes in d\(_3\)-GBB uptake were observed in the presence of alanine, ascorbic acid, glutamine, taurine, or taurocholic acid.

### Table 1. Inhibitory effects of various endogenous compounds and xenobiotics on d\(_3\)-GBB uptake by freshly isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration, (\mu)M</th>
<th>Relative Uptake, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100.0 ± 15.5</td>
</tr>
<tr>
<td>GBB</td>
<td>500</td>
<td>16.1 ± 3.4*</td>
</tr>
<tr>
<td>l-Carnitine</td>
<td>500</td>
<td>61.8 ± 6.5</td>
</tr>
<tr>
<td>GABA</td>
<td>500</td>
<td>29.9 ± 3.1*</td>
</tr>
<tr>
<td>Betaine</td>
<td>1,000</td>
<td>95.7 ± 3.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>5,000</td>
<td>129.3 ± 12.1</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>500</td>
<td>125.0 ± 18.3</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5,000</td>
<td>81.9 ± 13.1</td>
</tr>
<tr>
<td>Taurine</td>
<td>500</td>
<td>88.8 ± 3.6</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>500</td>
<td>98.8 ± 15.1</td>
</tr>
</tbody>
</table>

Values are means ± SE (\(n = 3\)). Uptake of deuterated \(\gamma\)-butyrobetaine (d\(_3\)-GBB; 1 \(\mu\)M) by freshly isolated rat hepatocytes was measured at 37°C and pH 7.4 for 3 min. The uptake was determined by subtracting the d\(_3\)-GBB uptake at 4°C from that at 37°C. Results are shown as percent of control uptake measured with no inhibitors. *\(P < 0.05\), significantly different from control.

Uptake of GBB by freshly isolated rat hepatocytes was Na\(^+\) dependent and seemed to be consisted of a single saturable component with a \(K_m\) value of 9.3 (Fig. 6), which is similar to the \(K_m\) value of 12.9 \(\mu\)M for GBB uptake by rOctn2 (Fig. 4). These values are also similar to the reported \(K_m\) value of 4.88 \(\mu\)M for GBB transport in rat liver plasma membrane fractions (1). Western blot analysis showed a clear localization of rOctn2 in the basolateral membrane of the hepatocytes (Fig. 1), which corresponds to the decreased hepatic uptake of carnitine in Octn2-dysfunctional jvs mice (20, 29). Therefore, rOctn2 was considered to contribute to the basolateral uptake of GBB in rat hepatocytes. However, our previous study demonstrated that Octn2-dysfunctional jvs mice exhibited an increased hepatic carnitine concentration after intravenous administration of GBB (7). This observation led us to explore a transporter, other than Octn2, that might be responsible for GBB uptake in liver.

When carnitine was used as a selective inhibitor of Octn2, uptake of GBB by the hepatocytes was not completely blocked.
even though the concentration tested was much higher (500 μM) than the $K_m$ value of Octn2-mediated carnitine transport (13 μM). This incomplete inhibition was also in agreement with the report by Berardi et al. (1) that GBB uptake was not inhibited in the presence of carnitine in the rat hepatocyte membrane fraction, suggesting a contribution of transporters other than Octn2 in the hepatic uptake of GBB.

Among compounds tested, GABA caused a significant reduction in GBB uptake by isolated rat hepatocytes (Table 1). Furthermore, GABA uptake by the hepatocytes was inhibited in a dose-dependent manner by GBB with an IC$_{50}$ of 13.3 μM (Fig. 7), which approximates the $K_m$ of GBB uptake by hepatocytes, 9.3 μM. GABA reportedly did not inhibit the transport of carnitine mediated by OCTN2 (25). In addition, we confirmed that rOctn2-mediated uptake of GBB was not inhibited even at 5,000 μM GABA (102% of control, data not shown). Therefore, the observed reduction in GBB uptake by rat hepatocytes in the presence of GABA was not explained by the inhibition of Octn2. This observation was also supported by the finding that the apparent extent of the reduction of GBB uptake (70%) by GABA was almost twofold greater than that by carnitine (38%) despite complete inhibition of Octn2-mediated carnitine transport at the same concentration (25). These results suggest that GBB and GABA share a common transport system, which is different from Octn2.

Previous studies revealed that human GABA transporters, Gat1 and Gat3, are exclusively expressed in the central nervous system, whereas Gat2 and Bgt1 are present in peripheral tissues (5). In particular, rGat2 is expressed mainly in liver and kidney (3). Accordingly, Gat2 appeared to play a major role in GBB uptake in rat liver. We further examined d$_3$-GBB uptake by rat hepatocytes with rGat2 silenced (Fig. 8). A clear reduction in d$_3$-GBB uptake corresponding to the decrease in mRNA expression of rGat2 indicates that GBB is taken up by the rat liver via rGat2 at least in part. The apparent contribution of Gat2 is approximately two-thirds, whereas that of Octn2 is approximately one-third as shown by the degree of mutual inhibition (Table 1). Moreover, the contribution of Gat2 evaluated by knockdown of GAT2 was about half, which is consistent with the inhibitory effect of GABA obtained from the inhibition study shown in Table 1.

The tissue distribution of carnitine biosynthetic enzymes in rat has been investigated by Tanphaichitr and Broquist (28). They suggested that all rat tissues produce GBB from $N^\omega$-trimethyl-lysine, after which GBB is transported to the liver for conversion into carnitine, because BBD is expressed only in the liver (2). In contrast, GABA is the major inhibitory neu-

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**Figure 7.** Inhibitory effect of GBB on $[^3H]$GABA uptake by freshly isolated rat hepatocytes. Uptake of $[^3H]$GABA (12 nM) by freshly isolated rat hepatocytes was measured at 37°C and pH 7.4 for 1 min with GBB (10 μM–10 mM). Results are shown as percent of control uptake measured without GBB. Each symbol represents means ± SE ($n=3$).

**Figure 8.** Effect of knockdown of rGat2 on uptake of d$_3$-GBB by rat hepatocytes in primary culture. Rat hepatocytes in primary culture were transfected with appropriate plasmid DNA to express short hairpin RNA (shRNA) unrelated (solid bar, control) or targeted (open bar) to rGat2. A: PCR products were electrophoresed on 2% agarose gel and visualized with ethidium bromide under ultraviolet light. B: mRNA expression of rGat2 in rat hepatocytes expressing shRNA unrelated or targeted to rGat2 was determined by semi-quantitative RT-PCR. The mRNA expression of rGat2 was normalized by mRNA expression of rat GAPDH. C: uptake of d$_3$-GBB (1 μM) by rat hepatocytes expressing shRNA unrelated or targeted to rGat2 was measured at 37°C and pH 7.4 for 1, 3, and 5 min. Each result represents mean ± SE ($n=3$ or 4). *$P<0.05$, **$P<0.01$ significantly different from control.
rotransmitter in the central nervous system and has a widespread distribution in the adult brain (5). Among GABA transporters (Gat1–3 and Bgt1) in rats, rGat2 was reportedly present only in liver (11). Therefore, Gat2 could be responsible for the physiological uptake of GBB. On the other hand, enzymes for carnitine biosynthesis are likely to present in the kidney and brain in addition to liver (22), suggesting that carnitine is synthesized in these three tissues in humans. Interestingly, Gat2 mRNA was strongly expressed in kidney and to a lesser extent in liver and brain (6). Therefore, BBD and OCTN2 are involved in GBB uptake for the efficient supply of GBB to the tissues that are capable of conversion of GBB into carnitine.

In conclusion, we introduced a new method to evaluate the transport of GBB by means of quantitation of deuterated GBB using LC-MS/MS. This analysis provided the first evidence that GBB, a precursor of carnitine biosynthesis, is transported using LC-MS/MS. This analysis provided the first evidence that GBB and OCTN2 are involved in GBB uptake for the efficient supply of GBB to the tissues that are capable of conversion of GBB into carnitine.

ACKNOWLEDGMENTS

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REFERENCES


