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Modulation of succinate transport in Hep G2 cell line by PKC

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Abstract

The cellular uptake of the tricarboxylic acid cycle (TCA) intermediates is very important for cellular metabolism. However, the transport pathways for these intermediates in liver cells are not well characterized. We have examined the transport of succinate and citrate in the human hepatoma cell line Hep G2 and found that it exhibited a higher rate of succinate compared to citrate transport, which was sodium dependent. Comparison of the transport properties of Hep G2 to that of human retinal pigment epithelial (HRPE) cells transfected with human sodium dicarboxylate transporters, hNaDC-1, hNaDC-3, and hNaCT indicated that Hep G2 cells express a combination of hNaDC-3 and hNaCT. Short period activation of protein kinase C (PKC) by phorbol 12-myristate, 13-acetate (PMA) and α-adrenergic receptor agonist, phenylephrine (PE), downregulated sodium-dependent succinate transport presumably via hNaDC-3. The inhibition by PMA was partially prevented by cytochalasin D, suggesting that PKC reduces the hNaDC-3 activity, at least in part, by increased endocytosis. In contrast, activation of PKA by both forskolin and epidermal growth factor (EGF) had no effect on succinate transport. Our results suggest that Hep G2 cells provide a useful model for studies of di- and tricarboxylate regulation of human liver.

Keywords: Succinate; Citrate; Hep G2; Dicarboxylate; Protein kinase C; Protein kinase A

1. Introduction

Numerous studies have reported that the cellular uptake of the tricarboxylic acid cycle (TCA) intermediates, di- and tricarboxylates, is very important for cellular metabolism. They are not only intermediates in the TCA cycle for biological energy production, but also are a source of cytosolic acetyl CoA for the synthesis of fatty acids, isoprenoids, cholesterol, and for the elongation of fatty acids [1–3]. The mammalian liver is of physiologic importance for the synthesis of fatty acids, isoprenoids, and cholesterol as well as glucose homeostasis, biochemical pathways in which TCA cycle intermediates have critical roles. These intermediates are mainly transported into the cells by sodium-dicarboxylate cotransporters (NaDCs) and the transport processes are classified into the low- and high-affinity transporters based on substrate affinity. The low-affinity sodium dicarboxylate transporter-1 (NaDC-1) prefers succinate as a substrate with a Km value of 0.9 mM and is primarily expressed in the brush border membrane of rabbit and human renal proximal tubule and intestine [4–6]. The high-affinity sodium dicarboxylate transporter-3 (NaDC-3) is found widely expressed in placenta, brain, liver, kidney, and pancreas of human and rat, with a Km value for succinate in the range of 20–102 μM [7–9]. Recently, a sodium citrate transporter (NaCT) has been cloned from rat brain [1] and was found to accept citrate as the most preferred substrate.

The intermediates of TCA cycle have been reported to be transported in the liver cells by both sodium-dependent and -independent transport systems [10–16]. Studies using isolated, perfused rat liver have shown that hepatic uptake of dicarboxylates, such as α-ketoglutarate and malate, is localized in perivenous hepatocytes [12–14]. Purified plasma membrane vesicles from rat liver contain an electrogenic sodium-dependent transport system for succinate and other dicarboxylates that closely resembles the high-affinity NaDC-3 transporter from the renal basolateral membrane [11,15,16]. Sodium-independent transport of α-ketoglutarate has also been reported in basolateral membrane from rat liver [10]. At present, citrate transport in liver cells has not been characterized.

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The human hepatoma Hep G2 cells are differentiated cells that display morphological and functional features of normal hepatocyte cells, including synthesis of major plasma proteins and expression of organic solute transporters [17–19]. These cells provide a useful model system with which to study the regulation of transporters for TCA cycle intermediates. The purpose of the present study was to examine the transport pathways for both succinate and citrate in Hep G2 cell line. The characteristics of succinate and citrate transport found in Hep G2 cells were compared with those of human retinal pigment epithelial (HRPE) cells transiently transfected with human sodium dicarboxylate transporters, hNaDC-1, hNaDC-3, and hNaCT. The main finding is that the transport of succinate and citrate in Hep G2 cells is mediated by a combination of two members of the SLC13 gene family, the human high-affinity sodium dicarboxylate cotransporter, hNaDC-3, and the human sodium citrate cotransporter, hNaCT. The characteristics of succinate and citrate transport found in Hep G2 were consistent with those of succinate and citrate transport described for the hNaDC-3, and hNaCT expressed in HRPE cells. RT-PCR analysis verified the presence of hNaDC-3 and hNaCT transcripts in Hep G2 cells. Furthermore, kinetic analysis in Hep G2 cells revealed a high-affinity succinate transport consistent with hNaDC-3. Finally, the regulation of sodium-dependent succinate transport by PKC and PKA were evaluated. Our experimental data clearly indicated that the Hep G2 cells represent an excellent cell model system for further studies on the regulatory mechanisms of endogenous di- and tricarboxylate transporters.

2. Methods

2.1. Culture of Hep G2 cell line

Hep G2 cells, a human hepatoblastoma carcinoma cell line (The American Type Culture Collection, Manassas, VA) were cultured in DMEM (Hyclone) containing 10% heat-inactivated fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Hyclone). Cell monolayers were maintained in a humidified incubator at 37 °C and 5% CO_2. Hep G2 cells were plated in 24-well cell culture plates (Corning Inc.) at a density of 3.0×10^5 cells/well until they reached confluence, approximately within 4 days.

2.2. Functional expression in HRPE cells

Human retinal pigment epithelial (HRPE) cells (Coriell Institute) were transiently transfected with plasmids containing cDNA coding for hNaDC-1 [6,20], hNaDC-3 [9], and hNaCT [2] in pcDNA3.1 vector. They all are kindly provided by Professor Ana M. Pajor (University of Texas Medical Branch, Galveston, Texas). HRPE cells were cultured in Modified Eagle's medium (Gibco/BRL) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. Prior to transfection, cells were plated at 1.2×10^5 cells/well in 24-well plastic plates. When cell monolayers reached 50–80% confluence, approximately 24 h after plating, cells in each well were transfected with 1.8 μg of FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN) and 0.6 μg of recombinant plasmid DNA. The cells were incubated for 48 h before measurement of transport activity. Background counts in control cells transfected with pcDNA 3.1 vector were subtracted from counts in experimental cDNA-transfected cells.

2.3. Transport assay

Transport of radiolabeled [^{14}C]-succinate (specific activity 44 Ci/mol, Moravek Biochemicals, Inc.) and [^{14}C]-citrate (specific activity 107 Ci/mol, Amersham Biosciences) were measured at a final concentration of 10 μM. The sodium-dependent transport buffer contained (in mM): NaCl (120), KCl (5), $MgSO_4$ (1.2), $CaCl_2$ (1.2), D-glucose (5), and HEPES (25), pH adjusted to 7.4 with 1 M Tris. In sodium-free transport buffer, NaCl was substituted by 120 mM choline chloride. Uptake experiments were performed at 37 °C. Cell monolayers were first washed twice with prewarmed sodium-free transport buffer to remove the medium. The transport reaction was started by the addition of 0.20 ml of transport buffer containing [^{14}C]-substrate and the cells were incubated for 10 min. The transport buffer containing [^{14}C]-substrate was then aspirated off to terminate the uptake and the cells were washed four times with 1 ml of transport buffer without the radiolabeled substrate. Cell monolayers were then solubilized with 0.20 ml of 20% sodium dodecyl sulfate (SDS) and 0.20 ml of the supernatant were transferred to scintillation vials for liquid scintillation counting.

The transport of [^{14}C]-succinate and [^{14}C]-citrate in transfected HRPE cells were measured essentially as described for Hep G2 cells. Uptake into HRPE cells transfected with vector alone was subtracted from cDNA-containing plasmid transfected cells. Before adding radioactive uptake solution, HRPE cells were preincubated for 15 min at room temperature with sodium-containing transport buffer, which help to reduce background counts obtained in these transfected cells.

2.4. RT-PCR

Hep G2 cells were cultured in T-75 plastic flasks (Corning Incorporated, NY) and resuspended using 0.25% trypsin-EDTA. Total RNA isolation was then performed using RNeasy Mini kit (QIAGEN) according to manufacturer's instructions. Reverse transcription and PCR were carried out using one-step RT-PCR kit (QIAGEN). The specific primers (BSU Bioservice unit, NSTDA, Thailand) for hNaDC-1, hNaDC-3, and hNaCT are shown in Table 1. The following reaction cycle was used for the RT-PCR : 50 °C for 30 min, 95 °C for 15 min, 40–45 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 60 s, then followed by a 10-min final extension at 72 °C. The quality of the cDNA were assessed by amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (BSU Bioservice unit, NSTDA, Thailand), using the primers 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′ and 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′. PCR products were separated by electrophoresis in 2% agarose gels and visualized by staining with ethidium bromide.

2.5. Na/K-ATPase activity assay

The protocol for cell permeabilization by alamethicin and assay of Na/K-ATPase activity followed the methods of Woolcock and Specht, with some modifications [21]. Briefly, Hep G2 cells were grown in 75 cm^2 tissue culture flasks. After they reached confluence, cells were detached by incubating with 0.25% trypsin-EDTA for 3 min. After centrifugation at 250×*g* for 5 min at 25 °C, cells were then resuspended in warm buffer containing (in mM): HEPES (20) pH 7.4, NaCl (132), KCl (5), $MgCl_2$ (1.5), $CaCl_2$ (0.55), glucose (24.5), and 0.2 mg/ml alamethicin [22] and incubated at 37 °C for 20 min. For phorbol 12-myristate, 13-acetate (PMA) treated group, 100 nM PMA dissolved in DMSO were added to cell suspension and preincubated at 37 °C for 20 min prior to incubating with alamethicin for 20 min. Cells were then cooled briefly in ice prior to Na/K-ATPase activity assay.

Table 1
Oligonucleotides used in RT-PCR reactions

Gene (predicted size, bp)	Genbank accession number	Sequence (sense and antisense strand)
hNaDC-1 (581)	U26209	5′-CCC TTA ATC CTG TTC CCT ATG A-3′ 5′-TGG GGG AAG AGC GAG TTG A-3′
hNaDC-3 (750)	AF154121	5′-CAC CGC CTC CAC TGC CAT GAT GC-3′ 5′-GAC GGG AAG AAG AAC AAG ATG GTG-3′
hNaCT (647)	Y151833	5′-GGA GCT GCC AGG GAG TCA AGT G-3′ 5′-GGA GGG GGA TAA AAT GGA GTT TTC-3′