Down-regulation by Extracellular ATP of Rat Hepatocyte Organic Anion Transport Is Mediated by Serine Phosphorylation of Oatp1*

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Joseph S. Glavy‡, Suet M. Wu§, Pi Jun Wang§, George A. Orr‡, and Allan W. Wolkoff‡

From the ‡Department of Molecular Pharmacology and §Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461

Recent studies implicate a role in hepatocyte organic anion transport of a plasma membrane protein that has been termed oatp1 (organic anion transport protein 1). Little is known regarding mechanisms by which its transport activity is modulated in vivo. In previous studies (Campbell, C. G., Spray, D. C., and Wolkoff, A. W. (1993) J. Biol. Chem. 268, 15399–15404), we demonstrated that hepatocyte uptake of sulfobromophthalein was down-regulated by extracellular ATP. We have now found that extracellular ATP reduces the V_max for transport of sulfobromophthalein by rat hepatocytes; K_m remains unaltered. Reduced transport also results from incubation of hepatocytes with the phosphatase inhibitors okadaic acid and calyculin A. Immunoprecipitation of biotinylated cell surface proteins indicates that oatp1 remains on the cell surface after exposure of cells to ATP or phosphatase inhibitor, suggesting that loss of transport activity is not caused by transporter internalization. Exposure of [35S]-loaded hepatocytes to extracellular ATP results in serine phosphorylation of oatp1 with the appearance of a single major tryptic phosphopeptide; oatp1 from control cells is not phosphorylated. This phosphopeptide comigrates with one of four phosphopeptides resulting from incubation of cells with okadaic acid. These studies indicate that the phosphorylation state of oatp1 must be an important consideration when assessing alterations of its functional expression in pathobiological states.

Transport of various organic anions, including sulfobromophthalein (BSF), 1 is an important function of the hepatocyte (1, 2). Recent studies have implicated a role in this process of a protein that has been termed oatp1 (organic anion transport protein 1) (3, 4). Oatp1 is the first member of a newly described unique family of transport proteins (5–10). Computer modeling suggests that oatp1 and the other members of the family are hydrophobic and have 12 transmembrane domains (2). These proteins are highly conserved but differ in substrate specificities and tissue distributions. Expression of oatp1 is limited to the basolateral plasma membrane of the hepatocyte (11–13) and the apical plasma membranes of the S3 segment of the proximal tubular epithelial cell (11) and the choroid plexus epithelial cell (12, 13). Other studies indicate that oatp1 is an electroneutral anion exchanger in which uptake of a compound such as BSF is accompanied by efflux of a counter-ion such as HCO_3^- (14) or GSH (15). Although oatp1 is under strong developmental regulation (13), little is known regarding mechanisms by which its activity may be modulated in vivo.

In previous studies, we demonstrated that hepatocyte uptake of BSF was down-regulated rapidly and specifically by extracellular ATP (16). In particular, it appeared that this effect was caused by the tetra-anion ATP^-4. Characteristics of nucleotide specificity suggested that ATP was interacting with a purinergic receptor that was similar to the P2Z (now P2X_7) receptor (17). This receptor has been described in macrophages and other cells as forming a channel that facilitates anion permeability (18, 19). That ATP^-4 reduces organic anion permeability in hepatocytes suggests that it acts by a different mechanism in these cells. Purinergic receptors in the P2Y class are G protein-coupled, and several have been described with nucleotide specificities similar to that described previously for inhibition of organic anion transport by hepatocytes (20–22). The mechanism(s) by which activation of a purinergic receptor might influence oatp1 function is unknown. Candidates include phosphorylation of the transporter with subsequent reduction of activity or internalization from the cell surface. The purpose of the present study was to examine these mechanistic possibilities.

**EXPERIMENTAL PROCEDURES**

**Cells**

Preparation of Isolated Rat Hepatocytes—Hepatocytes were isolated from 200–250-g male Harlan Sprague Dawley rats (Tacoma Farms, Germantown, NY) after perfusion of the liver with collagenase type I ( Worthington) (13). All animals used in this study received humane care in compliance with the institution’s guidelines. Viability of isolated hepatocytes was >90% as judged by trypan blue exclusion.

Culture of Isolated Rat Hepatocytes—In some experiments, hepatocytes were cultured overnight as described previously (13, 23). In brief, freshly isolated hepatocytes were suspended in Waymouth 752/1 medium (Life Technologies, Inc.) containing 5% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 1.7 mM additional CaCl_2, 5 μg/ml bovine insulin (Sigma), 100 units/ml penicillin (Life Technologies, Inc.), 0.1 mg/ml streptomycin (Life Technologies, Inc.), and 25 mM HEPEs, pH 7.2. Approximately 1.5 × 10^6 cells in 3 ml of medium were placed in 60-mm Primaria culture plates (Becton Dickinson, Franklin Lakes, NJ) and cultured in a 5% CO_2 atmosphere at 37 °C. After 2 h, the medium was changed, and cells were cultured overnight for approximately 18 h.

HeLa Cells Stably Transfected with Oatp1—HeLa cells (ATCC) stably transfected with pMPE-4-oatp1 plasmid were cultured and grown in selective medium as described previously (24). The pMPE-4-oatp1 plasmid was constructed so that expression of oatp1 was under the control of a metallothionein promoter (24). For induction of oatp1, cells were cultured for 24 h in medium containing 100 μM ZnSO_4 and then 24 h following an additional 50 μM (total of 150 μM) ZnSO_4 (24).

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Preparation of Antibody to Oatp1

A peptide containing the amino-terminal 14 amino acids of the derived oatp1 sequence was synthesized with a cysteine residue at the carboxyl terminus to be used as a linker. Synthesis was performed on an Applied Biosystems 430A peptide synthesizer using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry. The peptide structure was verified by amino acid analysis and by electrospray ionization mass spectrometry using a PE Sciex API-III instrument. These procedures were performed in the Laboratory of Macromolecular Analysis at the Albert Einstein College of Medicine. This peptide is unique to oatp1, and the sequence is distinct from protein sequences of the other known members of the oatp family of proteins. This cysteine-terminating peptide was linked to maleimide-activated keyhole limpet hemocyanin (Pierce) according to the manufacturer’s directions. Rabbits were immunized with this keyhole limpet hemocyanin-linked peptide by Covance Research products, Inc. (Denver, PA). Specificity of the resulting anti-serum was tested by immunoblot as described previously (11).

Chemicals

Oakoic acid was obtained from Alexis Biochemicals (San Diego, CA). Calcyolin A, Nα-benzoyl-l-arginine methyl ester (BAME), soybean trypsin inhibitor, Nα-p-tosyl-l-arginine methyl ester (TAME), phenylmethylsulfonyl fluoride, leupeptin, ATP, BSP, and 1-1tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin were from Sigma. 32P-BSP was prepared as described previously (24). 32P-A as a carrier-free aqueous orthophosphate solution (370 MBq/ml) was from Amersham Pharmacia Biotech.

32P Labeling of Cells

10 million isolated rat hepatocytes were washed three times with 1 ml of phosphate-free Eagle’s minimum essential medium (Sigma) supplemented with 25 mM HEPES, pH 7.2. Cells were suspended with gentle rotation in 1 ml of this medium at 37 °C for 30 min. After three washes, cells were resuspended in 1 ml of phosphate-free minimum essential medium, 25 mM HEPES, pH 7.2, and 100 μl (1 mM) of 32P-P was added. Cells were rotated gently at 37 °C for approximately 1 h. Cell viability remained unchanged during these incubations. In some studies, after this incubation period ATP (final concentration 5 mM) or okadaic acid (final concentration 0.6 μM) was added to the medium for an additional 10 min. Cells were then quickly pelleted and washed three times in 1 ml of phosphate-buffered saline containing 20 μg/ml BAME, 20 μg/ml TAME, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 5 mM EDTA, 1 mM EGTA, 2 mm phenylmethylsulfonyl fluoride, and 1 μM okadaic acid at 4 °C. The cell pellets were then processed for immunoprecipitation.

Immunoprecipitation of Oatp1

1 ml of lysis buffer consisting of 150 mM NaCl, 0.1% BSA, 1% Triton X-100, 20 mM octyl glucoside, 20 μg/ml BAME, 20 μg/ml TAME, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 5 mM EGTA, 1 mM EDTA, 2 mm phenylmethylsulfonyl fluoride, and 1 μM okadaic acid, and 10 mM Tris, pH 7.4, was added to each cell pellet and vortexed vigorously. The tubes were allowed to sit on ice for 5 min and then centrifuged at 16,000 × g for 10 min at 4 °C in a table top centrifuge (Hermle Z230M, Hermle GmbH & Co., Gosheim, Germany). The supernatant and 10 μl of nonimmune rabbit serum were placed in a tube containing 40 μl of protein A/G agarose beads (Pierce) which had been prewashed twice with ice-cold lysis buffer. This mixture was rotated gently at 4 °C for 30 min and centrifuged to remove nonspecifically adsorbed material. A 10-μl aliquot of anti-oatp1 was added to the supernatant and rotated at 4 °C overnight. 40 μl of protein A/G agarose beads was then added, and tubes were rotated at 4 °C for 30 min. After centrifugation, supernatants were removed by aspiration, and the beads were washed five times at 4 °C with 1 ml of buffer consisting of 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 10 mM Tris, pH 7.2. The samples were subsequently washed with 1 ml of Tris-buffered saline, pH 7.2, and subjected to 10% SDS-PAGE in the absence of reduction. After electrophoresis, the samples were transferred onto NitroBind nitrocellulose transfer membrane (Micron Separations, Inc., Westboro, MA). Radioautography was performed utilizing BioMax MS film (Kodak) with an intensifying screen at −70 °C for 1–4 days.

Phosphopeptide Mapping

Tryptic phosphopeptide analysis was performed by a modification of methods described previously (25–27). 32P-Labeled bands corresponding to the location of immunoprecipitated oatp1 were excised from the nitrocellulose membrane and prepared for tryptic digestion as described previously (25, 26). After drying, digested samples were resuspended in 7 μl of pH 1.9 buffer consisting of a mixture of 88% formic acid, glacial acetic acid, and twice deionized water (50:156:1.794, v/v/v). Samples were applied onto a 100-μm cellulose thin layer chromatography plate (Merck). The plates were electrophoresed in the first dimension at 650 volts in pH 1.9 buffer for 1 h in a Hunter thin layer peptide mapping system. HTLC-7000 (C.B.S. Scientific Co., Inc., Del Mar, CA). After blow drying, plates were rotated 90° and chromatographed in a solution consisting of isobutyric acid, n-butyl alcohol, pyridine, glacial acetic acid, and twice deionized water (125:3:8.9:6.5:5.8:55.8, v/v/v). After drying, plates were exposed to BioMax MS film at −70 °C with an intensifying screen for 7–10 days.

Phosphoamino Acid Analysis

Tryptic peptides were prepared as above. 50 μl of 6 N HCl was added to the dried hydrolysate and then heated at 110 °C for 70 min. The sample was dried, resuspended in pH 1.9 buffer, and subjected to two-dimensional electrophoresis as described previously (26). Migration of unlabeled amino acid standards was revealed by subsequent exposure of the plate to ninhydrin.

Biotinylation of Cell Surface Oatp1

Cell surface biotinylation was performed as a modification of a method described previously (28). Overnight cultured rat hepatocytes were washed three times with 1.5 ml of buffer A, consisting of 135 mM NaCl, 1.2 mM MgCl2, 0.81 mM MgSO4, 27.8 mM glucose, 2.5 mM CaCl2, and 25 mM HEPES, pH 7.2. Cells were then incubated at 37 °C in 1 ml of this buffer for 15 min. ATP (5 mM final concentration) or okadaic acid (0.6 μM final concentration) was then added, and incubation was continued for 10 min. Cells were then washed three times at 4 °C with phosphate-buffered saline, pH 8.0. They were then incubated for 80 min at 4 °C with 0.5 mg/ml sulfoconjugated avidin (Pierce), a membrane-impermeant biotinylation reagent. After biotinylation, cells were washed three times with phosphate-buffered saline, pH 8.0, and then processed for immunoprecipitation of oatp1, as described above.

To ensure that cells remained intact during this procedure and that internal protein was not biotinylated, immunoprecipitation was also performed using an antibody to a common determinant of the Yb rat GST subunit, kindly provided by Dr. Irving Listowsky (29). This protein is abundant in rat hepatocyte cytosol. That Yb could be biotinylated in disrupted cells was confirmed by harvesting cells in phosphate-buffered saline, homogenizing them by 20 strokes in a tight Dounce, and performing the biotinylation procedure on the supernatant following table top centrifugation for 10 min at 4 °C. Immunoprecipitates were resolved on a 10% PAGE, and biotinylated oatp1 (detected by ECL (Amersham Pharmacia Biotech) following transfer to nitrocellulose and probed with horseradish peroxidase-conjugated avidin (Pierce). Densitometry of immunoblot bands was performed using an Ultrascan XL densitometer (Amersham Pharmacia Biotech). 35S-BSP Transport Studies

Transport of 35S-BSP was quantified in cultured rat hepatocytes as described previously (13, 23). In brief, cells were washed three times with 1.5 ml of buffer A. They were then incubated for 15 min at 37 °C in 1 ml of buffer A containing 0.1% BSA. After this period, cells were incubated for 5 min at 37 °C in 1 ml of buffer A with or without 5 mM ATP, 0.6 μM okadaic acid, or 50 mM calyculin A. 1 μM 35S-BSP (approximately 5000 cpm) was added, and incubation was continued for an additional 5 min. The solution was then aspirated rapidly, and cells were washed five times at 4 °C with 1.5 ml of buffer A. The third wash contained 5% BSA and was allowed to stand for 5 min. Cells were harvested, and radioactivity was determined. Cell protein was determined in replicate plates by the BCA assay (Pierce) according to the manufacturer’s instructions using BSA as standard. In studies of saturation of BSP uptake, varied concentrations of BSP were used, keeping the ratio of BSA to BSP constant as described previously (23). K0 and Vmax were quantified by non-linear least squares fit of the data (SigmaPlot v. 4.0, Jandel Corporation, San Rafael, CA) to Equation 1.

\[ V = \frac{V_{\text{max}} \times [\text{BSP}]}{K_0 + [\text{BSP}]} + b \times [\text{BSP}] \]  

In this equation, b is a constant representing nonspecific association of ligand with cells. Similar studies were performed in oatp1-transfected HeLa cells, except that BSA was not included in the incubation of cells with 35S-BSP (24).
RESULTS

Influence of ATP and Okadaic Acid on 35S-BSP Uptake by Cultured Rat Hepatocytes—In these studies, overnight cultured rat hepatocytes were incubated for 5 min at 37 °C in 0.1% BSA containing 1 μM 35S-BSP in the presence and absence of 0.6 μM okadaic acid, 5 mM ATP, or a mixture of both okadaic acid and ATP. As seen in Fig. 1A, incubation with the phosphatase inhibitor (okadaic acid) alone reduced 35S-BSP uptake by approximately 35% (p < 0.001). ATP inclusion in the medium resulted in an approximately 77% reduction in 35S-BSP uptake (p < 0.001). Inclusion of both ATP and okadaic acid in the medium reduced uptake by 85%, a significantly greater reduction than was seen with either agent alone (p < 0.001). It was possible that addition of okadaic acid and ATP reduced 35S-BSP uptake because of competition for uptake. However, as seen in Fig. 1B, these agents had no effect (p > 0.91) on 35S-BSP uptake by HeLa cells that had been stably transfected with oatp1.

Fig. 1. Influence of okadaic acid (OA) and ATP on uptake of 35S-BSP by rat hepatocytes and oatp1-transfected HeLa cells. Overnight cultured rat hepatocytes (panel A) or oatp1-transfected HeLa cells (panel B) were prepared and cultured as described under “Experimental Procedures.” Cells were preincubated in the presence or absence of 0.6 μM okadaic acid for 10 min at 37 °C. Uptake of 35S-BSP (1 μM) was then determined over 5 min at 37 °C with or without the addition of 5 mM ATP as described under “Experimental Procedures.” The uptake of 35S-BSP in untreated (control) hepatocytes was 2.0 ± 0.52 pmol/min/mg of protein (mean ± S.E., n = 3 independent studies, each performed in triplicate). Uptake of 35S-BSP in untreated (control) oatp1-expressing HeLa cells was 52.5 ± 7.4 pmol/min/mg of protein (mean ± S.E., n = 3 independent studies, each performed in triplicate).

* p < 0.05 compared with control. ** p < 0.05 compared with ATP alone.

Fig. 2. Extracellular ATP reduces V_max for 35S-BSP uptake by rat hepatocytes. Overnight cultured rat hepatocytes were incubated for 5 min at 37 °C in increasing concentrations of 35S-BSP in the presence of a 15-fold molar excess of BSA. Panel A, total uptake was quantified under control conditions (●) and in the presence of 5 mM ATP (■). Computer fit to a single component Michaelis-Menten equation was performed to obtain the saturable (— — —) and nonsaturable (—) components for studies performed in the absence (panel B) and presence (panel C) of 5 mM ATP. Note the change in ordinate scale in panel C. In these representative studies of three independent studies that were performed, KM was 0.096 μM (control) versus 0.062 (ATP); V_max was 2.50 pmol min⁻¹ mg⁻¹ of protein⁻¹ (control) versus 0.61 (ATP); b was 0.7 pmol min⁻¹ mg⁻¹ of protein⁻¹ (control) versus 0.20 (ATP).

As seen in Fig. 2A, the initial uptake of 35S-BSP was reduced over a range of BSP concentrations. Non-linear analysis of these data revealed saturable and nonsaturable components (Fig. 2, B and C). There was no effect of ATP treatment on KM (0.0699 μM ± 0.009, control versus 0.0601 μM ± 0.018, ATP.
Oatp1 Phosphorylation Modulates Transport Activity

Transport of organic anions is a fundamental function of the hepatocyte (1, 2). Over the past few years, several proteins that mediate hepatocyte organic anion transport have been cloned based upon their functional expression (5–10). These proteins include oatp1, an 80-kDa glycoprotein that is present on the basolateral (sinusoidal) plasma membrane of the adult rat hepatocyte (2, 4, 11, 13). Oatp1 is best modeled as a 12-transmembrane domain protein and is the first member of a unique family of plasma membrane transporters of varied tissue and substrate specificities. Studies that examined functional expression of oatp1 in the presence and absence of specific antisense oligonucleotides suggested that this protein mediates a substantial proportion of hepatocyte transport of the synthetic organic anion, BSP (32). When this compound is administered intravenously, it binds tightly to albumin in the circulation from which it is cleared rapidly (1). Previous studies showed that BSP was also extracted efficiently from its albumin carrier by rat hepatocytes in culture (23, 33). These characteristics of

in the immunoprecipitate. When disrupted cells were biotinylated, biotinylated Y_b was detected readily after immunoprecipitation (data not shown).

**Extracellular ATP Stimulates Oatp1 Phosphorylation**—The studies presented above suggest the possibility that ATP, when added to the cell medium, interacts with a purinergic receptor that stimulates phosphorylation of oatp1 through a signal transduction mechanism. In support of this hypothesis is the finding that okadaic acid and calyculin A, structurally distinct phosphatase inhibitors (31), are equally effective in inhibiting 35S-BSP transport (Fig. 4). These results suggest that the phosphorylation state of oatp1 represents the net balance between kinase and phosphatase activities. As seen in Fig. 5a, under control conditions, no oatp1-derived tryptic phosphopeptides are seen. After a 10-min exposure of cells to extracellular ATP, a single major tryptic phosphopeptide appears (Fig. 5b). Phosphoamino acid analysis revealed exclusive serine phosphorylation (data not shown). After exposure of cells to okadaic acid, four distinct tryptic phosphopeptides are seen (Fig. 5c). One of these phosphopeptides comigrates with the phosphopeptide that results from ATP treatment (Fig. 5d). Exclusive serine phosphorylation of oatp1 was also observed in okadaic acid-treated hepatocytes (data not shown). In contrast to results in rat hepatocytes, oatp1 did not become phosphorylated after ATP treatment of HeLa cells that had been stably transfected with the oatp1 expression vector (data not shown).

**DISCUSSION**

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receptors responds to both ATP and ADP and produces free Ca\(^{2+}\) transients of short duration. The other responds to ATP only and produces Ca\(^{2+}\) transients of longer duration. Whether this latter receptor type regulates organic anion transport may be speculated, but direct evidence is lacking. Molecular characterization of these proteins in the liver has not as yet been established.

The present study provides evidence that oatp1 undergoes serine phosphorylation in response to extracellular ATP. A number of other plasma membrane transporters that undergo phosphorylation have been described. The Na\(^+/\)H\(^+\) exchanger is phosphorylated on a serine residue by protein kinase C, and this reduces its activity by altering its conformational state (30, 31). The present study indicates that similar to the Na\(^+/\)H\(^+\) exchanger, when oatp1 is phosphorylated, it loses transport activity but does not leave the cell surface. Retention on the cell surface was demonstrated by quantitative biotinylation of oatp1 with a membrane-impermeant reagent. Even though this transporter remains on the cell surface, \(V_{\text{max}}\) for transport is reduced by more than 60% (Fig. 2). This reduction in transport activity correlates with appearance of a single major tryptic peptide that becomes phosphorylated in response to extracellular ATP. The location of this peptide in the protein and the mechanism by which phosphorylation alters its transport function are not as yet known.

As noted above, recent studies have shown that oatp1 is an anion exchanger (14, 15). It is possible that addition of a negatively charged phosphate group to the inner domain of the protein prevents this exchange from occurring. However, numerous alternative possibilities exist, and structure-function relationships of oatp1 remain to be elucidated. It is of interest that HeLa cells that have been stably transfected with oatp1 show no phosphorylation of this protein in response to extracellular ATP or okadaic acid. Likewise, in these oatp1-expressing cells, BSP uptake is not affected by these agents. It is likely that they lack either the purinergic receptor or the kinase that mediates this response to ATP.

The physiologic consequences of ATP-mediated down-regulation of organic anion transport are unknown. It is possible that in states in which there is liver injury, ATP is released locally and reduces the ability of hepatocytes to extract organic anions from the blood. This could be protective by keeping cells from being overloaded with potentially toxic anionic compounds, or it could exacerbate effects of already compromised liver function. It is clear that the phosphorylation state of the transporter must be considered when assessing alterations of its functional expression in pathobiological states.

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