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Molecular and Functional Identification of Organic Anion Transporter Isoforms In Cultured Bovine Mammary Epithelial Cells (BME-UV)

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Abstract

Mammary epithelial cells express a diversity of membrane transporters including members of organic cation and organic anion (OAT) transporter subfamilies. Four mammal OAT isoforms have been identified: OAT-1, OAT-2, OAT-3, and OAT-4. The pharmacological significance of OAT isoforms has been emphasized because of their role in the movement of a wide variety of substrates across epithelial barriers. The present study identified (molecularly and functionally) bOAT isoforms in BME-UV cells. mRNA expression levels of all tested transporters in BME-UV cells were less than expression levels of the corresponding transporters in bovine kidney. Directionality in the flux of *P*-aminohippuric acid and acetylsalicylate, compounds known to interact with OAT-1 and OAT-2, respectively, across BME-UV monolayers was not observed at the concentrations used in this study. Directionality was, however, observed in the flux of estrone sulfate. Adding probenecid, penicillin G or non-radiolabeled estrone sulfate to the apical donor compartment significantly increased the apical to basolateral flux of estrone sulfate across the BME-UV monolayer. These results suggest that BME-UV cells express an organic anion transport system, making it a potentially useful model to study the role of this transport system in the mammary epithelial barrier.

Keywords

Mammary epithelium; organic anion transporter; Pharmacokinetic; BME-UV cell; RT-PCR

INTRODUCTION

Numerous compounds, such as drugs, environmental pollutants, and metabolites of both exogenous and endogenous origins, exist as organic anions at physiological pH (Miyazaki et al., 2004). These compounds are handled by the organic anion transporter (OAT)1 system expressed in different epithelial cells, including mammary epithelial cells (Alcorn et al., 2002; Sekine et al., 2006; Al-Bataineh et al., 2009; Gilchrist and Alcorn, 2009). The OAT

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¹Organic anion transporter

subfamily, encoded by the *SLC22A*, is a group of the organic ion transporter family, which includes organic cation transporter (OCT)², OCT novel type/carnitine, and OAT subfamilies (Tamai et al., 1997; Sekine et al., 1998b; Sekine et al., 1998a; Tamai et al., 1998; Koepsell et al., 1999).

The pharmacological significance of OAT isoforms has been emphasized because of their role in the movement of a wide variety of substrates across the epithelial barrier including clinically important compounds such as loop and thiazide diuretics, nonsteroidal anti-inflammatory drugs, antiviral agents, environmental pollutants (e.g., the anionic herbicide 2,4-dichlorophenoxyacetic acid), as well as sulfonamide and β -lactam antibiotics, (Sekine et al., 2000; Sweet et al., 2001).

Several mammal OAT isoforms have been identified, but four of them were well characterized: OAT-1 (Sekine et al., 1997), OAT-2 (Sekine et al., 1998b), OAT-3 (Kusuhara et al., 1999), and OAT-4 (Cha et al., 2000). These OAT subfamily members differ in substrate selectivity and localization. For instance, OAT-1, OAT-2, and OAT-3 are involved in the uptake of various endogenous and exogenous substances via the basolateral membrane, whereas OAT-4 is involved in either the uptake or the secretion of different substances via the apical membrane (Takeda et al., 2001; Miyazaki et al., 2004; Sekine et al., 2006; Giacomini et al., 2010). On the other hand, the differences and similarities in substrate affinities make it possible to functionally map transporters that are involved in transepithelial processes across the epithelial monolayer.

Knowledge of transporter expression in the mammary epithelium and their apical or basolateral directionality is a necessary first step in drug transport studies. In the human, mRNA coding for different members from organic ion transporter family have been isolated from mammary epithelial cells either by being purified from pooled fresh breast milk samples (Alcorn et al., 2002) or their mRNA have been detected in human mammary epithelial cell culture (Kimura et al., 2006). Several *in vivo* studies have also suggested that organic anions (N⁴-acetylated para-aminohippurate, N⁴-acetylated sulfanilamide and nitrofurantoin) are actively transported across the bovine, caprine, and rat mammary epithelium respectively into milk. This is because their observed Milk/Plasma ratio was 3 to 30 times higher than the predicted value which calculated based on the pK_a of the drug and the pH of both milk and plasma (Rasmussen, 1969a, b; Kari et al., 1997).

Information about mammary transport mechanisms are still relatively limited compared with our knowledge of transport systems in other epithelia. Different cell culture *in vitro* models have been established to study drug flux across mammary epithelial cells (Toddywalla et al., 1997; Kimura et al., 2006). It is, however, important that the chosen cell culture model displays some of the morphological and functional properties that are representative of the corresponding *in vivo* cell layers were established (Wilson, 1990). The study described in this manuscript is part of a long-term project to develop an *in vitro* model to study the xenobiotic excretion into milk using an immortalized bovine mammary epithelial cell line (BME-UV) 6. This cell line was selected as a model for the blood-milk barrier due to its ability to express transport proteins like P-glycoprotein (Al-Bataineh et al., 2010), establish a cell polarity with well-formed tight junctions between adjacent cells, and exhibit a transepithelial electrical resistance (Schmidt et al., 2001; Quesnell et al., 2007). The specific aim of this study was to identify molecularly and functionally bOAT isoforms in BME-UV cells cultured under non-hormonally treated conditions and serves as a baseline for future comparison with native tissues.

²Organic cation transporter

⁶Bovine mammary epithelial cell line

MATERIALS AND METHODS

Expression of genes encoding for the different bOAT isoforms in cultured BME-UV cells were determined using real time-PCR technique (RT-PCR)⁸. Transport studies using compounds known to interact with the OAT isoforms were conducted to determine whether the transporters were functional and significantly influenced the flux of substances across the epithelial barrier.

Cell Culture

The BME-UV cells were cultured under conditions similar to those described previously (Schmidt et al., 2001; Quesnell et al., 2007). Briefly, the BME-UV cells were grown to 65-75% confluency in 25 cm² plastic culture flasks (Corning, Inc., Corning, NY). Then, dissociated and dispersed cells were seeded on permeable polyester inserts, (Transwell, Corning, Inc.) and fed with asymmetrical media. Typical bovine medium (TBM)⁹, which contains little lactose and has concentrations of electrolytes that closely mirror serum, bathed the basolateral aspect (facing the lower compartment, which represents blood circulation) of the cells throughout all experiments. The apical aspect (facing the upper compartment, which represents milk) was exposed to apical bovine medium (APM)¹⁰ of low electrolyte-high lactose composition that resembles the ionic composition of milk. Composition of TBM and APM were reported previously (Schmidt et al., 2001; Quesnell et al., 2007). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Media on both the apical and basolateral aspects of the cells were refreshed daily. Cells were maintained in culture on permeable supports for 2 weeks to form a confluent, polarized and electrically tight monolayer prior to all experiments.

Identification of bOAT transporters in BME-UV cells

RT-PCR—Expression levels of bOAT isoforms (bOAT-1, bOAT-2, bOAT-3, and bOAT-4) in BME-UV cells were determined and compared with expression levels of the corresponding transporters in bovine kidney.

RNA was isolated from confluent monolayers of BME-UV cells and bovine kidney lysates using RNeasy Mini kit (QIAGEN, Foster City, California) according to the manufacturer's directions. Primer sequences and amplicon size (base pairs) for each transporter gene and GAPDH gene were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/>), a web-based primer design program, and are listed in Table 1. The expression of target bOAT isoforms in mammary epithelial cells was determined by RT-PCR (iCycler, Bio-Rad Laboratories, Hercules, CA) with the use of a commercial kit (Quantitect SYBR Green RT-PCR kit and reagents, Qiagen). Reactions were performed in a final volume of 25µL containing 100 ng of template RNA. This experiment was repeated five times (i.e. RNA isolated in 5 different occasions) with RT-PCR conducted on separate days. RT-PCR assay was conducted in the manner where samples of bovine kidney, no-template reaction and no reverse transcriptase were included in the assay. Relative gene expression for OATs in BME-UV cells to the corresponding gene expression in bovine kidney was calculated using 2^{-ΔΔCT} method after normalized the expression of each gene in both bovine kidney and BME-UV cells to the house keeping gene (GAPDH) according to Livak and Schmittgen (2001).

⁸Real Time-PCR

⁹Typical bovine medium

¹⁰Apical bovine medium

Transport studies—To study the function of bOAT members in the BME-UV monolayer, we compared the flux of different anionic compounds known to interact with OAT isoforms from apical-to-basolateral (Ap-to-BL)¹¹ side and vice versa in the presence and absence of other compounds known to interact with different drug transporters including bOAT. Test compounds were prepared in TBM or APM together with mannitol (Mnt)¹². Because Mnt is a hydrophilic and relatively small molecule, mol. wt. 182, it was used as a marker for paracellular movement to monitor the integrity of the mammary epithelial monolayer (Kimura et al., 2006).

Flow-Through Diffusion Cell System—Confluent monolayers of BME-UV cells on permeable supports were mounted as a barrier in a flow-through diffusion cell system (PermeGear, Bethlehem, PA). The system has automated receptor fluid collection and a high precision multichannel dispenser (Ismatec SA, Switzerland). The flow rate of receptor fluid through the receiver compartment of each diffusion chamber was controlled at 4 ml/h. Temperature was maintained at 37°C by circulating water through the diffusion chambers' holders. Donor compartments of diffusion chambers were capped directly after adding test solutions. Sequential samples were collected from the receiver side at regular time intervals (15 min) over a period of 120 min. Samples of either [¹⁴C]- or [³H]-radiolabeled test compounds were assayed by dual labeled β-scintillation counting with quench correction (Beckman LS6500, Beckman Coulter, Inc., CA). Automatic calculation of disintegrations per minute (DPM)¹³ was used for each sample.

bOAT-1—The flux of 2.0 μM *P*-aminohippuric acid ([¹⁴C]-Pah, specific activity: 52.7 mCi/mmol, American Radiolabeled Chemicals, MO), a compound known to interact with OAT-1 (Sekine et al., 2006), together with 0.025 μM [³H]-Mnt (specific activity: 20 Ci/mmol, American Radiolabeled Chemicals, MO) was studied across the BME-UV monolayer from Ap-to-BL side and vice versa in the presence and absence of probenecid (1 mM, Invitrogen Corporation, CA), a compound known to interact with OAT (Lash et al., 2007), Verapamil (50 μM, Sigma-Aldrich, Inc., MO), a compound known to interact with P-glycoprotein (Kim, 2002), and EsS (100 μM, Sigma-Aldrich, Inc., MO), a compound known to interact with OAT-3 (Dantzler and Wright, 2003). These compounds were added separately into the donor compartment together with Pah at the start of each experiment.

bOAT-2—The flux of acetylsalicylate ([¹⁴C]-Sal, specific activity: 55 mCi/mmol, American Radiolabeled Chemicals, MO), a compound known to interact with OAT-2 (Miyazaki et al., 2004; Sekine et al., 2006) was studied across the BME-UV monolayer from Ap-to-BL side and vice versa in the presence and absence of probenecid (1 mM). The transport experiment was initiated by adding 1 ml of test solution containing 1.0 μM [¹⁴C]-Sal together with 0.025 μM [³H]-Mnt into the donor compartment.

bOAT-4—The flux of estrone sulfate ([³H]-EsS, specific activity: 50 Ci/mmol, American Radiolabeled Chemicals, MO), a compound that has been reported to interact with OAT-4 with high affinity (Takeda et al., 2002; Sai and Tsuji, 2004; Sekine et al., 2006), was studied across the BME-UV monolayer in both directions (Ap-to-BL and BL-to-Ap). The transport experiment was initiated by adding 1 mL of test solution containing 0.02 μM [³H]-EsS together with 2 μM [¹⁴C]-Mnt (specific activity: 55 mCi/mmol, American Radiolabeled Chemicals, MO) to the donor compartment. Moreover, the Ap-to-BL flux of [³H]-EsS across the BME-UV monolayer was determined in the presence and absence of 1 mM probenecid, 100 μM penicillin G (PenG, Sigma-Aldrich, Inc., MO)¹⁴, and 20 μM of non-

¹¹Apical-to-basolateral

¹²Mannitol

¹³Disintegrations per minute

radiolabeled EsS. These compounds were added separately into the donor compartment together with EsS.

Data Analysis

The flux ($\mu\text{mol}/\text{cm}^2$ per hour) of tested compounds was calculated based on the amounts of tested compound recovered in the receiver compartment per unit time and then divided by mannitol flux at each measured time point to account for changes in overall epithelial integrity. Then, it was normalized by the effective diffusion area (cm^2) of the cell culture insert.

Statistical Analysis

All statistical analyses were performed using the commercially available software SIGMASTAT (Systat Software Inc., San Jose, CA). Group comparisons were made by analysis of variance (ANOVA)¹⁵. When the ANOVA showed significant differences among treatments, the Holm-Sidak posttest was performed to determine the specific pairs of treatments between which statistically significant differences occurred. The level of significance for all tests was set at $P < 0.05$.

RESULTS

mRNA expression of bOAT isoforms in BME-UV cells

Expression levels of all tested transporters in BME-UV cells were less than expression levels of the corresponding transporters in bovine kidney by at least 2 orders of magnitude (table 2). The relative expression of bOAT-4 in BME-UV cells to that in bovine kidney was the highest among tested bOAT isoforms (bOAT-4: $3.1 \pm 0.7 \times 10^{-2}$ fold; $n = 5$).

Transport studies of organic anion compounds across BME-UV monolayers

The Mnt-normalized flux of Pah across the BME-UV monolayer in the Ap-to-BL direction was not significantly different from the Pah flux in the opposite direction (BL-to-Ap) at the concentration used in the current study (Fig. 1). The intracellular amount of PAH was $5.5 \pm 0.5 \times 10^{-4}$ μg . Adding probenecid or verapamil together with Pah to the apical donor compartment had no distinguishable effect on the Ap-to-BL flux of Pah. The same results were observed in the Pah flux in the opposite direction (BL-to-Ap) after adding probenecid or EsS together with Pah to the basolateral donor compartment.

Directionality in the Mnt-normalized flux of Sal across the BME-UV monolayer was not observed at the concentration used in the current study (Fig. 2). Furthermore, adding probenecid together with Sal to the donor compartment had no distinguishable effect on the Sal flux in either direction.

The Mnt-normalized flux of EsS across the BME-UV monolayer from BL-to-Ap direction was more than 2 times the flux in the opposite direction (Fig. 3). The Ap-to-BL flux of EsS across the BME-UV monolayer was significantly increased by adding probenecid together with EsS to the apical donor compartment (Fig. 4). Adding PenG also increased the Ap-to-BL flux of EsS but to a lesser degree than when probenecid was added. The maximum increase in the Ap-to-BL flux of EsS was observed after adding non-radiolabeled EsS together with [^3H]-EsS to the donor compartment.

¹⁴Penicillin G

¹⁵Analysis of variance

The amount of EsS that crossed BME-UV monolayers in the presence of probenecid, PenG or non-radiolabeled EsS was significantly higher than the amount of EsS that crossed the BME-UV monolayer when [³H]-EsS was added alone to the apical donor compartment. The highest amount value ($5.4 \pm 0.1 \times 10^{-3}$ nmol) was when non-radiolabeled EsS was added together with [³H]-EsS to the donor compartment, which was double the amount value of EsS that crossed BME-UV monolayer when [³H]-EsS was added alone. Furthermore, the detected intracellular content of EsS, when probenecid, PenG or non-radiolabeled EsS were added together with [³H]-EsS to the apical donor compartment, was higher than when EsS was added alone. The recovery of EsS from the entire system at the conclusion of experiment was 93 ± 2 %.

DISCUSSION

The expression levels of all tested transporters in BME-UV cells were less than the expression levels of the corresponding transporters in bovine kidney. The fluxes of either Pah or Sal across the BME-UV monolayer are similar in both directions (Ap-to-BL or BL-to-Ap) at the concentrations used in this study. Directionality was, however, observed in the flux of EsS across the BME-UV monolayer, this could be attributed to the involvement of the organic anion transport system in the molecules' passage across BME-UV cells.

The Bronaugh-type flow-through diffusion cell system that was used in our study has numerous advantages. The system allows for automatic sampling of receptor fluid to accurately monitor the absorption/time profile. Multiple replicates of treatment conditions could be obtained cost-effectively. Most importantly, receptor fluid flow maintained apparent infinite sink conditions in the receptor fluid throughout the experiment, which reduces the effect of test compound molecules diffusing back from the basolateral to the apical side or vice versa, thereby isolating directional processes of drug movement across the epithelial barrier (Bronaugh and Stewart, 1985).

EsS is a substrate for both OAT-3 and OAT-4. Both of these transporters were found to be expressed in BME-UV cells, with bOAT-4 having the highest relative expression compared to the kidney, where it plays an important role in the excretion of anionic substances. In a previous study (Al-Bataineh et al., 2009), we elucidated the involvement of a carrier-mediated transport system in the passage of EsS across the BME-UV monolayer in the BL-to-Ap direction that was inhibited by probenecid and became saturated at higher concentration.

The movement of EsS from apical to basolateral side may involve more than one transport system as demonstrated by the differences in the increase in the EsS flux when probenecid and non-radiolabeled EsS were added. Probenecid is a compound known to interact with OAT, while non-radiolabeled EsS may interact with bOAT as well as other transporters including BCRP and this may explain the higher EsS flux after adding non-radiolabeled EsS than after adding probenecid to the donor compartment. Probenecid, PenG and non-radiolabeled-EsS increased the Ap-to-BL flux of [³H]-EsS by either competing with [³H]-EsS or inhibiting directly the transporters that involved in EsS efflux. The concentration of 0.02 μ M EsS was selected because it is well below the K_m value of EsS when it is up taken by OAT-4 that expressed in *Xenopus laevis oocytes* ($K_m = 1 \mu$ M) (Cha et al., 2000). Using multiple concentrations of inhibitor would be an important goal for future studies to help clarifying this process.

Pah and Sal were added to the donor compartment at concentrations of 2 μ M and 1 μ M, respectively, which are well below the K_m values of both compounds ($K_m = 14.3 \mu$ M, and 89 μ M, respectively) as determined by uptake into OAT expressing *Xenopus laevis oocytes*

(Sekine et al., 1997; Sekine et al., 1998b). The absence of directionality in the flux of either test compounds may be explained by low expression of the transporters for these compounds in BME-UV cells. This conclusion is further supported by the unchanged flux of Pah and Sal when other anionic compounds (i.e. probenecid) known to interact with OAT were added to the donor compartment. The observed flux of both Pah and Sal across the BME-UV monolayer is therefore most likely attributed to saturated transporters-mediated processes with the possible addition of some passive diffusion. Studies with a range of concentrations would be needed to confirm this hypothesis.

Results from the transport studies reported in this manuscript are consistent with low observed mRNA expression of bOAT in BME-UV cells as well as with observations reported from other species. OAT-1 and OAT-2 were not detected in human mammary epithelial cells purified from pooled fresh breast milk samples (Alcorn et al., 2002), or lactating rat mammary tissue (Gerk et al., 2001b). These authors did not detect OAT-3 and OAT-4 either, although we did detect relatively higher bOAT-4 expression. The most likely explanation for this disagreement is experimental method. We used cell culture (higher amount of mRNA), while tissue and collected cells in milk samples may have yielded less mRNA and was therefore less sensitive. Another reason is that the OAT expression is species or lactation stage-dependent (Alcorn et al., 2002; Buist and Klaassen, 2004; Gilchrist and Alcorn, 2009).

The flux of different anionic compounds known to interact with OAT in our *in vitro* cell culture model (BME-UV) is consistent with other observations reported in lactating cows and other species such as goat, mouse and rat. Earlier *in vivo* studies in lactating cows and female rats showed that experimental and theoretical (calculated on the basis of pH partition concept) milk-to-plasma ultra-filtrate ratios were in close agreement for Pah and Sal and not altered by drug concentrations or probenecid (Miller et al., 1967; Gerk et al., 2001a). Thus, a passive diffusion process may govern the movement of both Pah and Sal from plasma to milk.

The milk concentration of endogenous EsS and EsS infused close-arterially into the mammary gland in bovine and a non-pregnant goat, respectively was significantly greater than that in plasma, indicating that EsS was actively transferred from blood to milk against a concentration gradient (i.e. EsS cross mammary epithelial barrier by a carrier mediated process) (Heap et al., 1983, 1984).

In summary, the results from the current study indicate that EsS flux across BME-UV cells is transporter-mediated. The transporter that most likely plays a role in this flux is bOAT-4, due to its relatively higher expression compared to other members of this transporter family. bOAT-3 and other transporters such as BCRB may also play a role in EsS flux across the BME-UV cells. The expression of organic anion transport system in mammary epithelial cells has important implications for understanding the barrier function of the mammary epithelium and provides insight into the role of bOAT system in the accumulation and/or removal of anionic molecules from milk and/or plasma. The potential effect of hormones on the transporters expression in BME-UV cells remains unknown and further studies should be performed.

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ABBREVIATION LIST

OAT	Organic anion transporter
bOAT	Bovine Organic anion transporter
OCT	Organic cation transporter
Pah	<i>P</i> -aminohippuric acid
EsS	Estrone sulfate
Sal	Acetylsalicylate
BME-UV	Bovine mammary epithelial cell line
RT-PCR	Real Time-PCR
TBM	Typical bovine medium
APM	Apical bovine medium
Ap-to-BL	Apical-to-basolateral
Mnt	Mannitol
DPM	Disintegrations per minute
PenG	Penicillin G
ANOVA	Analysis of variance

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Figure 1. Pah (2.0 μ M) flux across the BME-UV monolayer from apical to basolateral side (Ap) and from basolateral to apical side (BL) in the presence and absence of probenecid (+Prob), verapamil (+Verp), and estrone sulfate (+EsS). Data represent mean $\pm$ SE of three replicates. No directionality observed in the mannitol-normalized flux of *P*-aminohippuric acid (Pah) across the BME-UV monolayer.

Figure 2.

Sal (1.0 μM) flux across the BME-UV monolayer from the apical to the basolateral side (Ap) and from the basolateral to the apical side (BL) in the presence and absence of probenecid (+Prob). Data represent mean ± SE of three replicates. No directionality observed in the mannitol-normalized flux of Acetylsalicylate (Sal) across the BME-UV monolayer.

Figure 3. Mannitol-normalized flux of 0.02 μM [^3H]-EsS across the BME-UV monolayer from apical to basolateral side (Ap), and from the basolateral to the apical side (BL). Data represent mean $\pm$ SE of three replicates. BL-to-Ap flux of EsS across the BME-UV monolayer was significantly higher than EsS flux in the opposite direction (Ap-to-BL) ($P < 0.05$).

Figure 4. Mannitol-normalized flux of 0.02 μM [^3H]-EsS across the BME-UV monolayer from apical to basolateral side (Ap) in the presence and absence of probenecid (+Prob), penicillin G (+PenG), and non-radiolabeled EsS (+EsS). Data represent mean $\pm$ SE of three replicates. Probenecid, penicillin G, and non-radiolabeled estrone sulfate (EsS) significantly ($P < 0.05$) increased [^3H]-EsS flux across the BME-UV monolayer.

Table 1

Primer sequences and amplicon size (base pairs) for each transporter gene and GAPDH gene.

Transporter gene	Forward primer	Reversed primer	Amplicon size
<i>bOAT-1</i>	GGTGTACCCCAATGATCC	CCACGTCTTGCACTGTGTCT	218
<i>bOAT-2</i>	CCCTGAACAAAGTAGCTGCC	CACCGACAGATAGACCAGCA	239
<i>bOAT-3</i>	ATTTGATGACCCTGAGGACG	GTGTCTCAGGCAGGAAGAGG	270
<i>bOAT-4</i>	CACCTCCACCATTGTGACTG	TGTAACCCTAGCCCCAGATG	194
<i>bGAPDH</i>	AAGATTGTCAGCAATGCC	ACAGACACGTTGGGAG	297

Table 2

mRNA transporter expression in BME-UV cells relative to the corresponding transporter mRNA expression in bovine kidney. Data represent mean $\pm$ SE of five replicates.

	Transporter			
	OAT-1	OAT-2	OAT-3	OAT-4
mRNA expression ¹	9.2E-04	33E-04	7E-04	310E-04
SEM ²	4.5E-04	14E-04	2E-04	70E-04

¹ Expression of mRNA transporter in BME-UV cells relative to mRNA expression in bovine kidney.

² SEM: standard error of the mean.