SHORT COMMUNICATION

SUPPLEMENTARY INFORMATION

The importance of incorporating OCT2 plasma membrane expression and membrane potential in IVIVE of metformin renal secretory clearance

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Drug Metabolism and Disposition

Materials and Methods

Chemicals and Reagents

Synthetic signature peptides for OCT2 were obtained from New England Peptides (Boston, MA). The corresponding stable isotope labeled (SIL) peptides for OCT2 and Na^+-K^+ ATPase, total protein quantification bicinchoninic acid assay (BCA) kit, dithiothreitol (DTT), iodoacetamide (IAA), mass spectrometry grade trypsin, DMEM (Dulbecco's Modified Eagle Medium) high glucose medium (Gibco, Life Technologies), DPBS (Dulbecco's Phosphate-Buffered Saline), Krebs-Ringer-HEPES (KRH) buffer, and Pierce cell surface protein isolation kit were obtained from Thermo Scientific (Rockford, IL). Pierce cell surface protein isolation kit contains sulfosuccinimidyl-2-(biotinamido) ethyl-1, 3-dithiopropionate (sulfo-NHS-SS-biotin), quenching solution (100 mM glycine), lysis buffer, neutravidin agarose gel, wash buffer, column accessory pack, DTT, phosphate buffer and Tris buffer. Metformin [biguanido-^{14}C] hydrochloride (98 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). ScintiVerse BD Cocktail liquid scintillant and HPLC-grade acetonitrile were purchased from Fischer Scientific (Fair Lawn, NJ). Cimetidine, metformin (hydrochloride salt) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were analytical grade.

Human kidney cortex and transporter-expressing cell lines

Transplant quality human kidney cortices (for demographics see Supplementary Table 2S in Prasad et al., 2016) were obtained from Newcastle University, England (n = 5, randomly selected). OCT2-expressing HEK293 and MDCKII cells were created as described previously (Yin et al., 2015; Yin et al., 2016).

Measurement of OCT2 transporter uptake activity in HEK293 and MDCKII cells

Transport studies were conducted in OCT2-expressing MDCKII and HEK293 cells with [^{14}C]-metformin with or without 1mM cimetidine (OCT2 inhibitor). For transport assays, OCT2-expressing MDCKII and HEK293 cells were grown in 24-well poly-D-lysine coated plates, at a density of 75,000 cells per well with 1 mL of high glucose DMEM medium (changed daily). At day 3, cells were washed with 1mL/well DPBS buffer and incubated at 37 °C with KRH buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM HEPES) containing 5.5 µM [^{14}C]-metformin with or without 1mM cimetidine (OCT2 inhibitor). After 2 minutes (when uptake is linear), the uptake (in triplicate) was terminated by washing the cells three times with ice-cold KRH buffer (1 mL each). Then, the cells were lysed with 0.5 mL 1 M NaOH at 37 °C for 2 hours and neutralized with 0.5 mL 1 M HCl. 30 µL of this lysate solution were used for total protein estimation using the BCA method and 200 µL were used to analyze total radioactivity by Tri-Carb Liquid Scintillation Counters

(PerkinElmer). OCT2-mediated uptake was determined by the difference in the metformin uptake in the presence/absence of 1 mM cimetidine. The *in-vitro* OCT2-mediated clearance of metformin was calculated as the ratio of OCT2-mediated uptake and metformin concentration in the media.

LC-MS/MS quantification of total OCT2 expression in human kidney cortex as well as total and plasma membrane OCT2 expression in HEK293 and MDCKII cells

Plasma membrane isolation from OCT2-expressing HEK293 and MDCKII cells: Plasma membranes from OCT2-expressing HEK293 and MDCKII cells were isolated in 3 and 2 independent experiments respectively, using a biotinylation methodology optimized in our laboratory (Kumar et al., 2017). Briefly, HEK293 and MDCKII cells were grown in 75 cm^2 flasks till 80-100% confluency and incubated with 10 mL of 0.78 mg/mL of sulfo-NHS-SS-biotin at 37 °C for one hour to biotinylate extracellular free primary amines (lysine or arginine) or N-terminal primary amines of extracellular peptide residues in the plasma membrane. After cell lysis, biotinylated plasma membranes were isolated using neutravidin resin columns.

Determination of percent plasma membrane expression of OCT2 in OCT2-expressing cells: The expression of OCT2, Na^+-K^+ ATPase, and calreticulin in the lysate, the non-biotinylated and the biotinylated fractions was quantified using

LC-MS/MS (see below). Stable labeled internal standards of OCT2 and Na^+-K^+ ATPase unique peptides were used to determine the area ratio (analyte:SIL ratio) of the respective peaks in the samples obtained from the biotinylation experiment. For calreticulin, the stable labeled internal standard was not available and therefore the stable labeled internal standard of Na^+-K^+ ATPase was used to estimate the calreticulin peak area ratio (Kumar et al., 2017).

OCT2 total protein quantification in kidney cortex: Sample preparation and quantitative proteomics were performed as described previously (Prasad et al., 2016). Total protein content (wt/wt) in kidney cortex homogenate was quantified by BCA assay. OCT2 expression was determined in triplicate for each kidney cortex homogenate. Briefly, 20 µL of 2.0 mg/mL (or lower concentration) of the human kidney cortex or transporter-expressing cell line or samples obtained through biotinylation experiments was treated with 15 µL of ammonium bicarbonate buffer (50 mM, pH 7.8), 18 µL of 3% sodium deoxycholate (w/v), 6 µL DTT (100 mM), and 10 µL of human albumin (10 mg/mL). Proteins present in sample was denatured and disulfide bond between cysteine residues was reduced by incubation at 95 °C for 5 minutes, followed by addition of 6 µL of IAA (200 mM; an alkylating agent) and treatment with ice-cold methanol (0.5 mL), chloroform (0.2 mL), and water (0.45 mL) and centrifugation. Lastly, the protein sample was digested with 20 µL of trypsin followed by centrifugation at 5000 g for 5 minutes at 4 °C, and 5 µL of the supernatant was introduced into the LC-

MS/MS system. The calibration standards were prepared by spiking peptide standards into the extraction buffer II of the membrane protein extraction kit. Samples were analyzed as described before by Kumar et al., 2017. Briefly, AB Sciex 6500 triple-quadrupole mass spectrometer (Sciex, Framingham, MA, U.S.A.) coupled to the Water Acquity UPLC system (Waters Corporation, Milford, MA) was operated in electrospray positive ionization mode for liquid chromatography-tandem mass spectroscopy (LC-MS/ MS) analysis of the signature peptides (Supplemental Table 1). The transitions from doubly charged parent ion to singly charged product ions for the analyte peptides and their respective SIL peptides were monitored (Supplemental Table 1). The chromatographic separation and resolution were obtained on an Acquity UPLC HSS T3 Column, (1.8 µm, 2.1 mm X 100 mm) with a 0.2 µm inlet frits (Waters, Milford, MA, USA). Mobile phases (0.3 mL/min) consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program for UPLC method for determination of OCT2 in human kidney cortex and OCT2-expressing HEK293 and MDCKII cells was: 0–2 minutes: 3% B; 2–20 minutes: 3–45% B; 20–23 minutes: 45–90% B; 23–24 minutes: 90% B; 24–24.5 minutes: 90–3%B; 24.5–28 minutes: 3–3%B including washing and re-equilibration for 3.5 minutes. Seven calibration standards ranging from 0.14 to 70.4 fmol (on the column) were used to quantify OCT2.

Supplemental Table 1. MRM parameters used for quantification of OCT2 and marker proteins.

Protein	Surrogate Peptide	Parent ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (V)
OCT2	LNPSFLDLVR	587.3	946.5, 228.1	50	28
	LNPSFLDLV***R***	592.3	956.5, 228.1		
Na^+-K^+ ATPase	AAVPDAVGK	414.2	586.3	50	18
	AAVPDAVG***K***	418.2	594.3		
Calreticulin (MDCKII)	FYALSAR	414.2	517.3	50	19
Calreticulin (HEK293)	EQFLDGDGWTSR	705.8	893.4, 778.4	50	33

Italicized and bolded letters indicate stable labeled amino acid residues. Two different surrogate peptides of calreticulin (FYALSAR in CHO and MDCKII cells and EQFLDGDGWTSR in HEK293 cells) were used because of their species-dependent specificity.

Supplemental Table 2. Renal clearance (CL_r) of metformin in healthy human subjects

Metformin Dose	(CL_r) ($L*hr^{-1}$)	$CL_{r,sec}$ (mL/min)	Number of healthy subjects	Reference
1850 mg, PO	46.0±4.2	643±71	12	(Hibma et al., 2016)
850 mg, PO	42.3±12.9	585±215	12	(Li et al., 2016)
2000 mg, PO	32.3±6.7	418.3±112.2	18	(Devineni et al., 2015)
500 mg, PO	36.4±6.5	486.0±108	24	(Klamerus et al., 2014)
1000 mg, PO	38.2±8.5	516.7±141.7	27	(Shin et al., 2014)
1000 mg, PO	36.1±8.4	481.7±140.2	14	(Johansson et al., 2014)
500 mg, PO	33.0±8.5	430.0±141.7	43	(Oefelein et al., 2013)
250 mg, PO	23.7±1.9	275.0±31	8	(Kusuhara et al., 2011)
850 mg, PO	26.5±6.5	340±97	14	(Chen et al., 2009)
850 mg, PO	31.5±3.4	405.0±57.2	9	(Sambol et al., 1996)
1700 mg, PO	27.3±2.3	335.0±38.9	9	
2550 mg, PO	27.7±2.3	341.7±37.8	9	
927±12 mg, IV	20.1±2.8	215.0±46	5	(Sirtori et al., 1978)
250 mg, PO	31.6±9.9	407±165	7	(Somogyi et al., 1987)
250 mg, IV	32.6±3.7	408.5±68.3	4	(Tucker et al., 1981)
500 mg, PO	31.5±7.5	389.5±104.8	4	
1500 mg, PO	31.1±16.7	383.5±247.9	4	
500 mg, IV	27.2±4.9	333.7±81.5	3	(Pentikainen et al., 1979)
500 mg, PO	26.6±3.0	323.6±50.7	5	
CL_r values listed above are mean ± SD, PO, Oral; IV, intravenous. $CL_{filtration}$ (if not stated in the cited manuscript) was assumed to be 120 mL/min, the geometric mean $CL_{r,sec}$ of metformin was calculated as 415 mL/min.				

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