Amino Acids (2015) 47:693–705
DOI 10.1007/s00726-014-1889-6

ORIGINAL ARTICLE

Human intestine luminal ACE2 and amino acid transporter expression increased by ACE-inhibitors

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Received: 2 September 2014 / Accepted: 3 December 2014 / Published online: 23 December 2014

Abstract Sodium-dependent neutral amino acid transporter B^0AT1 (SLC6A19) and imino acid (proline) transporter SIT1 (SLC6A20) are expressed at the luminal membrane of small intestine enterocytes and proximal tubule kidney cells where they exert key functions for amino acid (re)absorption as documented by their role in Hartnup disorder and iminoglycinuria, respectively. Expression of B^0AT1 was shown in rodent intestine to depend on the presence of the carboxypeptidase angiotensin-converting enzyme 2 (ACE2). This enzyme belongs to the renin-angiotensin system and its expression is induced by treatment with ACE-inhibitors (ACEIs) or angiotensin II AT_1 receptor blockers (ARBs) in many rodent tissues. We show here in the *Xenopus laevis* oocyte expression system that human ACE2 also functionally interacts with SIT1. To investigate in human intestine the potential effect of ACEIs or ARBs on ACE2, we analysed intestinal biopsies taken during routine gastroduodenoscopy and ileocolonoscopy from 46 patients of which 9 were under ACEI and 13 ARB treatment. Analysis of transcript expression by real-time PCR and of proteins by immunofluorescence showed a co-localization of SIT1 and B^0AT1 with ACE2 in the brush-border membrane of human small intestine enterocytes and a distinct axial expression pattern of the tested gene products along the intestine. Patients treated with ACEIs displayed in comparison with untreated controls increased intestinal mRNA levels of ACE2, peptide transporter PEPT1 (SLC15A1) and AA transporters B^0AT1 and PAT1 (SLC36A1). This study unravels in human intestine the localization and distribution of intestinal transporters involved in amino acid absorption and suggests that ACEIs impact on their expression.

Keywords B^0AT1 · SIT1 · ACE2 · Intestine

Abbreviations

B^0AT1	Sodium-dependent broad substrate selectivity neutral amino acid transporter 1
SIT1	Sytem imino transporter 1
ACE2	Angiotensin-converting enzyme inhibitors

Introduction

Protein digestion refers to the hydrolysis of dietary proteins by stomach and pancreas proteases and by brush-border membrane-bound peptidases into absorbable units, namely tri-, dipeptides and amino acids (AA) (Daniel 2004). Transepithelial absorption across enterocytes involves then sequential transport across the luminal brush-border and basolateral membranes. The luminal step is mediated by the peptide transporter PEPT1 and various AA transporters (Broer et al. 2011; Broer and Palacin 2011; Chillaron et al. 2010; Daniel 2004). From enterocytes, amino acids are then released into the extracellular space by another set of AA transporters located in the basolateral membrane (Daniel and Kottra 2004).

The major small intestine luminal transporter for neutral AAs B^0AT1 (broad neutral AA transporter 1, SLC6A19) is also expressed in kidney proximal tubule and its defect

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was shown to cause Hartnup disorder (Kleta et al. 2004; Seow et al. 2004). Intestinal B^0AT1 expression and function depends on the presence of the accessory protein ACE2 (angiotensin-converting enzyme 2) (Camargo et al. 2009; Kowalczuk et al. 2008). This membrane-anchored monocarboxypeptidase is a structural homologue of ACE and is expressed in various tissues, including heart, kidney, testes, lung and intestine, where it negatively regulates the renin-angiotensin system by degrading angiotensin I and II into Ang-(1-9) and Ang-(1-7), respectively (Kuba et al. 2010). At the brush-border membrane of small intestine enterocytes, ACE2 displays a high expression level and is suggested to participate to peptide digestion (Fairweather et al. 2012; Kowalczuk et al. 2008). The catalytic domain of ACE2 is fused to a membrane anchor domain that shows high structural similarity with the renal protein collectrin (TMEM27) (Kuba et al. 2010) which interestingly functions as B^0AT1 associated protein in kidney proximal tubule (Danilczyk et al. 2006).

The sodium-dependent imino transporter 1 (SIT1, SLC6A20) is a high-affinity luminal L-proline (Pro) transporter, expressed—among other tissues—in small intestine enterocytes and proximal kidney tubule cells (Romeo et al. 2006; Takanaga et al. 2005). Mutation of its gene has been suggested to cause—in combination with polymorphisms of other proline and glycine transporters—the metabolic disorder Iminoglycinuria (Broer et al. 2008). SIT1 (SLC6A20) is structurally closely related to B^0AT1 (SLC6A19) but an analogous functional interaction with ACE2 and/or collectrin has not been demonstrated, although suggested by the observation that mice lacking the ACE2-related protein collectrin display reduced proximal tubule SIT1 expression and significant prolinuria (Danilczyk et al. 2006). Similarly, *ACE2* knock-out mice show decreased intestinal Pro absorption; however, effects on SIT1 protein expression could not be tested due to lack of a specific antibody (Singer et al. 2012).

Drugs interfering with the renin-angiotensin system (RAS), such as ACEIs and ARBs have become first-line medications to treat arterial hypertension (Werner et al. 2010). Interestingly, both types of drugs have been shown in rodents to increase the expression of ACE2 mRNA in different organs and tissues, including heart, kidney and the aorta (Chappel and Ferrario 2006; Ferrario and Varagic 2010; Igase et al. 2005). However, it is not known, whether ACEIs or ARBs also affect the expression of small intestine ACE2, which is involved—as a carboxypeptidase—in protein digestion and—by interacting with B^0AT1 and potentially SIT1—in AA absorption. Additionally, the knowledge about the axial distribution of AA- and peptide transporters along the intestine is sparse and mainly originates from animal studies (Dave et al. 2004; Terada et al. 2005).

In this study we (a) addressed the question whether human ACE2 can interact functionally with the proline transporter SIT1 using the *Xenopus laevis* oocyte expression system and (b) whether it co-localises with SIT1 as with B^0AT1 at the luminal surface of human intestinal mucosa. Furthermore (c), we analysed the axial distribution of ACE, ACE2, amino acid- and peptide transporters in human intestine and (d) assessed whether ACEIs and ARBs potentially impact on ACE2 and AA transporter expression in the intestine of human patients.

Materials and methods

Cloning and cRNA synthesis of transporters for expression in *X. laevis* oocytes

Human SIT1 cDNA was amplified from human total RNA (Takara Clontech, Mountain View, CA, USA). The PCR product was ligated into a plasmid using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA), and excised with *Eco*RI before inserting into the expression vector pBluescript-KSM. For the insertion of the Flag tag, SIT1 was excised with *Eco*RV and *Spe*I, ligated to Flag tag containing vector (FastBac-Flag, kindly provide by Thierry Hennet) and cut with *Sfo*I and *Spe*I. The fusion protein of SIT1 with N-terminal Flag tag was returned to the expression vector using *Eco*RV and *Spe*I. SIT1 and SIT1-Flag constructs were linearized respectively with *Xba*I and *Sac*I for the synthesis of cRNA using MEGAscript T3 Transcription Kit (life technology, Carlsbad, CA, USA). Human ACE2 cRNA was prepared as previously described (Camargo et al. 2009).

Transport Studies in *X. laevis* oocytes

Transport studies using radiolabeled amino acid tracers were performed as described previously (Meier et al. 2002). Briefly, after cRNA injection (SIT1 alone: 5 ng, SIT1 and ACE2: 5 and 20 ng, SIT1 and collectrin: 5 and 5.4 ng) oocytes were incubated 2–3 days in ND96 solution at 16 °C. Thereafter, 6–10 oocytes per condition were washed 3–4 times at room temperature (RT) with uptake buffer (pH 7.4, 10 mM HEPES, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) and pre-incubated at 25 °C for 2 min. The uptake solution containing radiolabeled amino acids (2 µCi of radiolabeled tracer per group of oocytes) was added for 10 min and uptake was ended by washing oocytes 4 times with uptake buffer at 4 °C. Oocytes were then dissolved separately in SDS (2 %) for 60 min. Finally, 3 ml of scintillation solution (Emulsifier-Safe TM) was added to each oocyte and radioactivity was determined using a scintillation counter (TRI-CARB 2900TR, Packard Instrument Co., Meriden, CT, USA). Data were expressed as pmol/h × oocyte, and values obtained for non-injected oocytes were subtracted. Kinetic

Table 1 Patients' data

	Control[a]	ACEI[b]	ARB[c]	ACEI vs. control	ARB vs. control
Age (years)	51.5 ± 2.7	64.4 ± 3.2	65.2 ± 2.0	**	**
Gender (m/f)	13/11	4/5	4/9		
BMI (kg/m^2)	25.2 ± 0.75	26.4 ± 1.9	26.5 ± 1.5	ns	ns
MAP (mmHg)	87.9 ± 2.5	87.1 ± 3.3	90.3 ± 3.9	ns	ns
ACE activity (U/l)	35.2 ± 3.4	14.8 ± 1.7	33.0 ± 4.4	**	ns
Renin (mU/l)	12.2 ± 1.8	33.1 ± 9.1	613 ± 392	ns	ns
Aldosterone (ng/l)	102 ± 14.2	69.6 ± 9.5	148 ± 32.9	ns	ns
Angiotensin I (ng/ml)	0.49 ± 0.083	0.43 ± 0.077	0.59 ± 0.17	ns	ns
Angiotensin II (pg/ml)	11.3 ± 3.4	9.8 ± 3.7	65.0 ± 40.1	ns	ns

BMI body mass index, *MAP* mean arterial pressure

** $p < 0.01$; ns not significant ($p > 0.05$) (ANOVA with post hoc Dunnett's test)

[a] Patients without RAS-active medication

[b] Patients treated with ACE-inhibitors

[c] Patients treated with angiotensin II AT_1 receptor blockers

experiments were performed with five different amino acid concentrations ranging from 10 μM to 1 mM.

Study population

A total number of 46 (21 male and 25 female) patients was included in the current study, with 9 (20 %) patients treated with ACE-Inhibitors, 13 (28 %) patients treated with AT_1-receptor blockers and 24 control patients (Table 1). Medical supply was independent from the present study or hereby obtained results. 24 (52 %) control patients were not on medication that affected the renin-angiotensin system. All patients were examined at one single institution. Patients underwent either gastroduodenoscopy only ($n = 34$), a combined gastroduodenoscopy and ileocolonoscopy ($n = 10$) or ileocolonoscopy only ($n = 2$) as part of a routine medical checkup. During this procedure, mucosal biopsies were taken at four different parts of the gastrointestinal tract: Duodenum parts II (descending) and III (inferior/horizontal), terminal ileum and ascending colon. Blood and urine samples were collected from all patients in order to quantify amino acid levels using high performance liquid chromatography (HPLC) measurements. Laboratory values of the RAS (ACE-activity, renin, aldosterone, angiotensin I and II; measured at the Institute of Clinical Chemistry, University Hospital of Zurich), as well as different physiologic parameters, such as body mass index (BMI), mean arterial blood pressure [MAP = (systolic blood pressure + 2 × diastolic blood pressure)/3] and heart rate were assessed. The age of included patients ranged from 18 to 80 years and patients BMI was between 18 and 35 kg/m^2. Patients with severe pathologies of the gastrointestinal tract, such as coeliac disease, Crohn's disease and ulcerative colitis, as well as patients with carcinomas, kidney- or hepatic insufficiency, bleeding disorders, infectious diseases, oral anticoagulation, drug- or alcohol abuse or mental retardation, were excluded from the present study.

Mean age of treated patients (65 years) was higher than of non-treated controls (52 years). BMI, mean arterial pressure, angiotensin I and II and renin plasma levels were not different between groups. Plasma ACE-activity was significantly lower in patients treated with ACEIs when compared to non-treated controls (Table 1).

Intestinal biopsies

Mucosal biopsies were taken at four different intestinal localizations (duodenum part II, part III, terminal ileum and ascending colon). After removal, tissue specimens were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction or cryosection was performed.

Immunofluorescence

Cryosection

Harvested biopsies and PFA fixed oocytes were embedded in optical coherence tomography (OCT) cryostat medium (Medite Medizinaltechnik AG, Switzerland) and put into tubes containing liquid propane. Tubes were transferred into liquid nitrogen for quick freezing. Obtained blocks were stored at −80 °C until further processing. A cryotome, (Leica CM 1850 Cryostat, Switzerland) was used to produce 5 μm (tissue specimens) and 9 μm sections (oocyte specimens) that were immediately transferred onto

polylysine slides (O. Kindler & CO GmBH, Germany). The slides were stored at −20 °C until later processing.

Immunostaining

Immunostaining of frozen tissue sections was performed as described previously (Ramadan et al. 2006). Briefly, samples were defrosted at RT for 5 min in a wet chamber. For fixation, slides were immersed in Methanol (70 %) for 90 s. After fixation, slides were washed (1 × 15 min, 2 × 5 min) with phosphate buffered saline (PBS, 0.1 M) (137.0 mM NaCl, 2.7 mM KCl, 12.0 mM $HPO_4^{2-}/H_2PO_4^-$). To reduce unspecific antibody binding, tissues were kept in 2 % bovine serum albumin (BSA) diluted in PBS (0.1 M) for 1 h at RT. The following primary antibodies were applied to the section samples for 1 h (tissue specimens) and 3 h (oocytes) at RT: 1. Affinity purified mouse anti human polyclonal SIT1 (Abnova, Taipei, Taiwan), 2. Affinity purified goat anti human polyclonal ACE2 (R&D Systems, Minneapolis, USA), 3. Affinity purified rabbit anti human polyclonal B^0AT1 (Pineda, Berlin, Germany), 4. Affinity purified rabbit anti-flag antibody (Sigma-Aldrich, Switzerland). Antibodies were diluted 1:100 in PBS (0.1 M) enriched with 2 % BSA and 0.04 % Triton X-100. Thereafter, tissue sections were rinsed again in PBS (0.1 M) (3 × 5 min) and oocyte sections in PBS (0.2 M) (2 × 5 min) and PBS (0.1 M) (1 × 5 min) before incubation with the secondary antibodies (Alexa Fluor® 594 donkey anti-mouse IgG, dilution 1:500, Alexa Fluor® 488 donkey anti-mouse IgG, dilution 1:500, Alexa Fluor® 594 donkey anti-goat IgG, dilution 1:500, Alexa Fluor® 488 donkey anti-goat IgG, dilution 1:500, Alexa Fluor® 594 donkey anti-rabbit IgG, dilution 1:500, Alexa Fluor® 488 donkey anti-rabbit IgG, dilution 1:500) and 4′, 6′-diamidino-2-phenyl-indole (DAPI, Merck, NJ) (0.1 mg/ml) (diluted 1:5,000) for 1 h at RT (tissue specimens) or Alexa Fluor® 488 donkey anti-rabbit IgG, dilution 1:1,000 and Texas red Phalloidin (diluted 1:1,000) for 45 min at RT (oocytes specimens). Tissue sections were washed with PBS (0.1 M) (3 × 5 min) and oocyte sections with PBS (0.2 M) (2 × 5 min) and PBS (0.1 M) (1 × 5 min) before mounting with DAKO fluorescence mounting media (DakoCytomation, Baar, Switzerland). Sections were examined using a Nikon Eclipse TE300/200 inverted microscope fitted with a DS-5M Standard charge-coupled device camera. Pictures were captured with NIS-Elements software (Nikon Instruments Inc, Melville, NY, USA) and processed using Adobe Photoshop software. Incubation of sections with secondary antibodies only did not result in a detectable signal (data shown for oocytes).

Western blot analysis

Western blotting using oocyte protein lysates was performed as described previously (Ramadan et al. 2006). Oocytes were homogenised in 10 ul of lysis buffer per oocyte (250 mM sucrose, 0.5 mM EDTA, 5 mM Tris–HCl, pH 6.9, 1 mM PMSF, and 10 ul/ml protease inhibitor cocktail, Sigma) by passing 10× through a 25 G needle. The solution was centrifuged at 100×*g* for 10 min, and the supernatant was transferred into a new tube. The supernatant was used for SDS–PAGE analysis on a 8 % gel. The gel was loaded with the equivalent of 0.5 oocyte per lane. Proteins were transferred subsequently electrophoretically to a PVDF-membrane (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 2 % Top BLOCK™ (Juro, Lucerne, Switzerland) in Tris-buffered saline/0.1 % Triton X-100 (Sigma-Aldrich, Switzerland), the blots were incubated with the rabbit anti-FLAG antibody (dilution 1:1,000), followed by horseradish peroxidase-conjugated (HRP) anti-rabbit antibody (1:5,000; Promega, Madison, WI, USA) for 1 h at room temperature. To compare the loading, the membrane was stained with mouse anti-tubulin (Sigma, 1:5,000) and alkaline phosphatase (AP)-conjugated anti-mouse antibody. Antibody binding was detected with the Luminata Classico Western HRP substrate (Merck Millipore, Billerica, MA, USA) or CDP-Star AP substrate (Roche, Basel, Switzerland). For chemiluminescence detection a Fuji LAS 4000 camera (Fujifilm Life Science, Minato, Japan) was used.

Intestinal gene expression

RNA extraction

RNA extraction was performed by disrupting tissue in 350 µl RLT-Beta-Mercapto-Ethanol buffer (10 µl β-ME/1 ml RLT-buffer) with MagNALyser Green Beads (Roche, Switzerland) for 30 s at 6,000 rpm using a Precellys® 24 tissue homogenizer (Bertin Technologies, France). The solution was centrifuged at 10,000×*g* for 5 min at 10 °C. The supernatants were directly used for RNA isolation with the QiagenRNeasy mini kit (QIAGEN, Switzerland), which was employed according to the manufacturer's instructions.

RNA concentrations were determined using the NanoDrop 1000 Spectrophotometer (Witec AG, Switzerland) at 260 nm wavelength. Beside RNA concentrations, purity of the extractions was assessed.

Reverse transcription

For reverse transcription, Applied Biosystems Taq Man RT-PCR reagents and the Biometra T Gradient Thermocycler (Biolabo Scientific Instruments SA, Switzerland) were used. Final concentrations in the reaction mix were: RT buffer (1×), $MgCl_2$ (5 mM), random hexamers (2.5 µM), deoxyNTP mix (500 µM each), RNAse inhibitor (0.4 U/

µl), multiscribe reverse trancriptase enzyme (1.25 U/µl), RNA template (33 ng/µl) and RNAse free water. All reactions were executed with negative controls (RT-) using the same protocol without adding the multiscribe reverse trancriptase enzyme to the reaction mix. Until further analysis, samples were stored at −20 °C.

Primers and probes

Primers and probes were designed according to a previous report (Nishimura and Naito 2005) or using the software Primer Express 3.0 (Applied Biosystems, Switzerland). Self-designed primers were chosen to generate amplified fragments of 70–140 base pair length spanning intron–exon boundaries to avoid contaminating genomic DNA. Primer specificity of all primers was tested using mRNA from human intestine and/or human kidney and resulted in a single product of expected length (data not shown). Probes were labelled with the fluorescent reporter dye FAM at the 5′ end and the quencher dye TAMRA at the 3′ end (Microsynth AG, Switzerland).

Real-time PCR

Quantitative real time PCR (qRT-PCR) was performed as described previously (Dave et al. 2004; Ramadan et al. 2006). Briefly, a 20 µl PCR reaction volume was prepared using cDNA (1 µl), TaqMan Universal PCR master mix (10 µl) (Applied Biosystems AG, Switzerland), Primers (0.8 µl each), Probe (0.4 µl) and DEPC-Water (7 µl). Final concentrations in the reaction volume were: Primers (1 µM each) and Probe (0.1 µM). Reactions were run in 96-well optical reaction plates using the 7500 Fast Real-Time System Thermocycler (Applied Biosystems AG, Switzerland). 45 thermal cycles were set at 95 °C (10 min), 60 °C (15 s) and 72 °C (1 min). For analysing data, an individual threshold was set for each gene in the linear range of the amplicon curves. All reactions were run in triplicates and mean values were used for further processing. Negative controls (RT-) were run for each gene. If maximal cycle difference within triplicates was ≥1 cycle or if the difference between the mean value of the triplicates and the negative control was ≤5 cycles, obtained results were discarded and not used for further analysis. Abundance of target mRNA was calculated relative to a Villin mRNA, which is commonly used as reference gene for epithelial content in human intestinal samples (Meier et al. 2007). In order to verify non-varying Villin mRNA expression along the human digestive tract, target and Villin mRNA was calculated relative to a second reference gene (HPRT, encoding the Hypoxanthine phosphoribosyltransferase) (data not shown) (Wehkamp et al. 2004). Relative gene expression values were determined using the ΔCt method (relative expression $= 2^{-\Delta Ct}$, ΔCt = average Ct value of target − average Ct value of reference).

Comparative promoter analysis

The proximal promoter regions of human ACE2, B^0AT1 and PEPT1 genes were retrieved using the software Gene2Promoter (Genomatix). The proximal promoter regions used were generally defined as 500 nucleotides upstream and 100 nucleotides downstream from the transcription start site (TSS). TSSs were automatically assigned to genes based on 5′ cap site databases integrated into promoter identification programs (Eldorado, Genomatix). Promoter sequences of relevant transcripts and in a second analysis of relevant transcripts plus CompGene promoters (promoters with no transcript listed) were included in the analysis. Genomatix FrameWorker database was used to identify new TFBS modules (Cartharius et al. 2005). A module is defined as a set of two or more TFBSs with a specific order, strand orientation, and distance range between the individual TFBSs. A threshold of 100 % genes containing the module, a distance of 5–200 bp and an intersite variability of 15 bp or less were permitted between TFBSs. The software ModelInspector (Genomatix) was used with the default settings to identify the previously characterised TF binding modules in additional promoter regions of the Genomatix Human Promoter Database (Version Eldorado 12-2012) (Frech et al. 1997).

Statistical analysis

For data representation and statistical analysis, the statistical software Graphpad Prism 5 (GraphPad Software, San Diego, CA, USA) and *R*, an open-source language and environment for statistical computing (http://www.R-project.org/) were used. Error bars correspond to standard error of the mean (SEM). *p* values of 0.05 or less were considered statistically significant.

Ethics

Human experiments

Written informed consent was obtained from each patient. The study was conducted according to Good clinical practice guidelines and was approved by the local ethics committee (reference number: EK-1744).

Animal experiments

Animal experiments (i.e., removal of oocytes from *Xenopus laevis* frogs) were performed according to the Swiss Animal Welfare Laws and as approved by the local Veterinary Authority (Kantonales Veterinäramt Zurich).

Results

Functional interaction of human L-proline transporter SIT1 with ACE2 and collectrin in *X. laevis* oocytes

To test whether human ACE2 and human collectrin functionally interact with SIT1—as shown for B^0AT1 (Kowalczuk et al. 2008)—we co-expressed these proteins in *Xenopus laevis* oocytes and assayed their transport function using L-proline as substrate. Pro uptake rate was increased more than twofold, when ACE2 or collectrin was co-expressed with SIT1, whereas no significant Pro uptake was observed when ACE2 or collectrin RNA was injected alone (Fig. 1a). To test whether these two accessory proteins modulate SIT1 transport kinetics differentially, the concentration-dependence of L-proline uptake was measured. The apparent affinity of SIT1 alone ($K_{0.5}$ 67.3 μM) was similar in oocytes co-expressing ACE2 ($K_{0.5}$ 50.4 μM) or collectrin ($K_{0.5}$:60.0 μM), respectively, as shown by the $K_{0.5}$ derived from Lineweaver–Burk analysis. In contrast, the maximal transport capacity (V_{max}) was more than twofold higher in oocytes co-injected with ACE2 or collectrin, respectively (Fig. 1b).

Fig. 1 Functional interaction of amino acid transporter SIT1 with accessory proteins ACE2 and collectrin expressed in *X. laevis* oocytes. **a** L-proline (100 μM) uptake. *X. laevis* oocytes were injected with human SIT1 (*white bar*), human SIT1 plus human ACE2 (*dark grey bar*), human SIT1 plus human collectrin (*black bar*), human ACE2 (*third last bar*; *light grey*), or human collectrin (*second last bar*; *light grey*) cRNA. The last *bar* (*light grey*) represents non-injected oocytes. L-proline transport was determined 2–3 days after injection. Each *bar* represents the mean transport rate ± SEM. (n = total of 40 oocytes analysed in four independent experiments). $p < 0.05$; Bonferroni-corrected p values for the indicated comparisons were calculated using a mixed model including the experiment number as random factor to take into account the grouping structure of the data caused by the data acquisition in four independent experiments. **b** Lineweaver–Burk plot showing concentration-dependence of L-proline uptake. The half maximal uptake rate $K_{0.5}$ and maximal transport capacity V_{max} of L-proline by human SIT1 was assessed in the absence (SIT1; *circles*; *continuous line*) or presence of human ACE2 (SIT1+ACE2; *squares*; *dotted line*) or human collectrin (SIT1+collectrin; *diamonds*; *dashed line*). Uptake rates using 3–4 different L-proline concentrations, ranging from 10 to 1000 μM, were determined 2–3 days after injection. Each data point represents the mean transport rate ± SEM. (n = total of 24 oocytes from four independent experiments). $K_{0.5}$ was similar in all three groups ($K_{0.5}$ SIT1: 67.3 (95 % confidence interval (CI) 41.0–137.9) μM, $K_{0.5}$ SIT1+ACE2: 50.4 (CI 29.5–104.6) μM, $K_{0.5}$ SIT1+collectrin: 60.0 (CI 26.5–296.7) μM. Maximal transport capacity (V_{max}) was about twofold higher in oocytes co-injected with ACE2 or collectrin, respectively (V_{max} SIT1: 35.5 (CI 24.2–66.1) pmol/h × oocyte, V_{max} SIT1+ACE2: 74.0 (CI 50.9–136.0) pmol/h × oocyte, V_{max} SIT1+collectrin: 81.5 (CI 46.3–341.4) pmol/h × oocyte

Increased oocyte plasma membrane expression of SIT1 transporter upon co-expression with ACE2

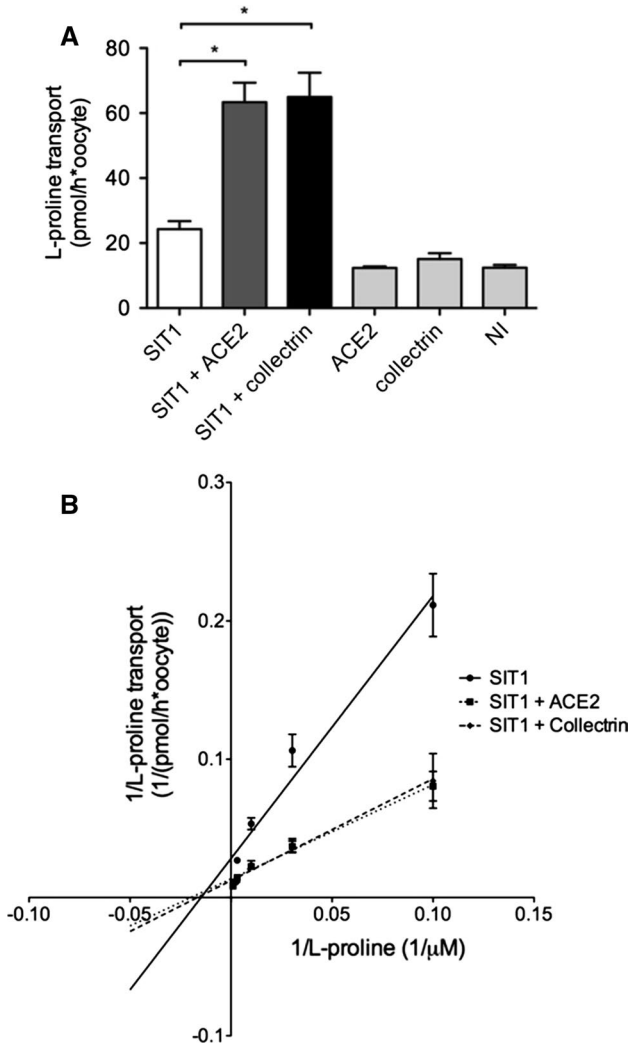
To test the impact of ACE2 co-expression on amount and localization of human SIT1, a human SIT1-Flag construct was expressed in *X. laevis* oocytes and visualised by immunohistochemistry (Fig. 2a) and Western blotting of membrane lysates (Fig. 2b). When co-expressed with human ACE2, human SIT1 transporter (green) produced a clearly stronger signal at the plasma membrane (Fig. 2a, lower panel) where it colocalized with actin (phalloidin, red) that labels the surface membrane folds and microvilli. Western blot analysis performed with 3 different batches of oocytes expressing SIT1 (S) or SIT1 and ACE2 (SA) confirmed the increased SIT1 expression level in the presence of the accessory protein ACE2 (Fig. 2b). That the Flag tag inserted at the N-terminus of SIT1 did not alter its transport function was verified in all oocyte batches used for Western blot and immunofluorescence (data not shown). Thus, as previously reported for the related transporter B^0AT1, the co-expression of ACE2 also increased cell surface expression and transport function of SIT1.

Localization of amino acid transporters B^0AT1 and SIT1 and of ACE2 in human small intestine

The distribution of the AA transporters B^0AT1 (Fig. 3a; green) and SIT1 (Fig. 3d; green) and of ACE2 (Fig. 3b, e; red) along the villi (V) and in the crypts (C) was visualised by immunohistochemistry. The two transporters co-localised with ACE2 along the brush-border membrane of duodenum (Fig. 3c, f) and terminal ileum (not shown) enterocytes on villi. The signal of AA transporter B^0AT1 (Fig. 3a) appeared to be stronger towards the tips of the villi and weaker in the crypts, whereas no clear statement about the expression along the crypt-to-villus axis of SIT1 and ACE2, respectively, may be made based on the present pictures. Some cells lining the small intestine lumen showed a diffuse labelling with all antibodies and represent presumably goblet cells that are non-specifically stained. Whereas in the colon B^0AT1 was not detected by immunofluorescence, ACE2 and SIT1 were labelled within colonic crypts where they co-localised to the apical membrane of epithelial cells (data not shown).

Axial expression of AA- and peptide transporters as well as of ACE and ACE2 mRNAs along human intestine

Using mucosal biopsies taken from 10 patients referred for combined gastroduodenoscopy and ileocolonoscopy, real-time PCR was performed to measure the relative abundance of SLC transporter mRNAs in the epithelial layer of duodenum parts II and III, terminal ileum and ascending colon. As internal standard, the transcript of the small intestine enterocyte housekeeping gene Villin was used (Meier et al. 2007). No statistical tests were applied to the transporters' distribution: terms like higher, lower and equal only qualitatively



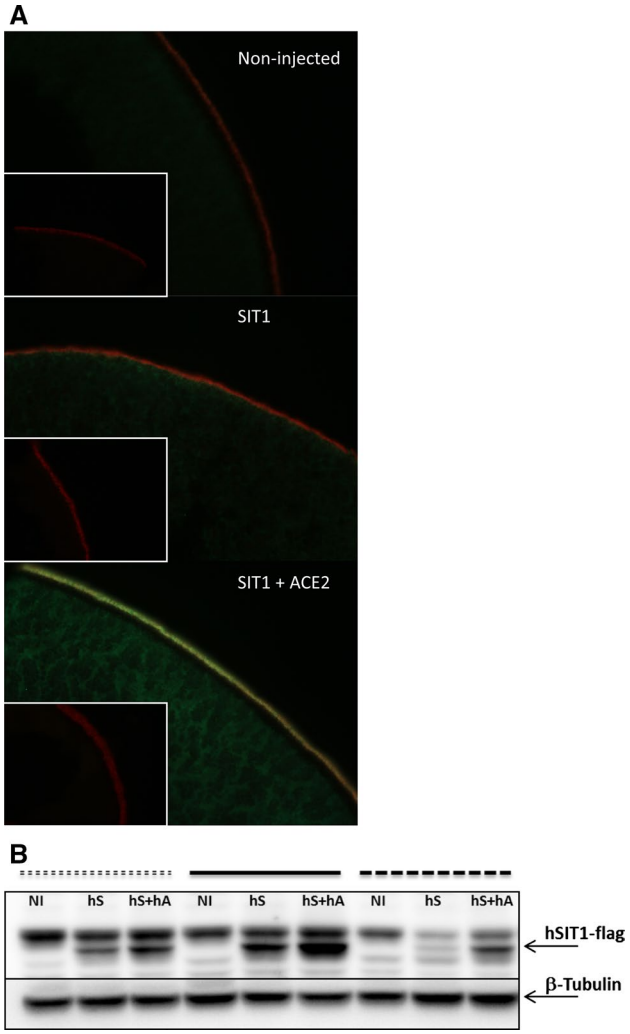


Fig. 2 Human ACE2 increases oocyte membrane human SIT1 expression. **a** Three representative oocytes; without injection (Non-injected; upper picture), injected with human SIT1 alone (SIT1; middle picture), with human SIT1 and human ACE2 (SIT1+ACE2 lower picture) were stained for human SIT1 expression at the oocytes surface (anti-flag, *green*) or with membrane marker (actin phalloidin, *red*). *White boxes* in the lower left quadrant of the pictures show negative controls without addition of the primary (anti-flag) antibody. **b** The expression level of labelled proteins (hSIT1-flag) was analysed by Western blot. Membranes of non-injected oocytes (NI), oocytes expressing human SIT1-flag construct (S) and SIT1-flag+human ACE2 (hS+hA) from three different batches of oocytes were analysed. The loading of oocyte membranes was controlled using a β-Tubulin antibody. Each band represents lysates made from 9 to 33 oocytes. The three *different lines* (*dotted*, *continuous* and *dashed*) indicate different batches of oocytes used

describe the data and do not refer to statistical significant differences. These measurements revealed distinct axial gene expression patterns (Fig. 4): (a) All tested transporter mRNAs were more abundant in small than large intestine with the exception of that of the AA antiporters ASCT2 (SLC1A5) and y^+LAT2 (SLC7A6) [catalytic subunit of 4F2hc (SLC3A2)] that displayed a higher gene expression in the ascending colon. (b) The luminal transporter mRNAs showed equal expression levels along the duodenum and terminal ileum except the one of the cationic AA and cystine exchanger subunit $b^{0,+}$AT (SLC7A9) that was higher in the terminal ileum (Fig. 4a). (c) Basolateral transporter subunit mRNAs of LAT1 (SLC7A5), y^+LAT1 (SLC7A7) and y^+LAT2 were equally expressed along the duodenum and terminal ileum, whereas the mRNAs of the transporters/transporter subunits LAT2 (SLC7A8), 4F2hc (SLC3A2), LAT4 (SLC43A2) and TAT1 (SLC16A10), showed a lower gene expression level in the distal ileum than in the duodenum (Fig. 4b). (d) The mRNA encoding ACE was higher in the terminal ileum, whereas that of ACE2 was equal along the small intestine (Fig. 4a).

Intestinal gene expression of ACE2, B^0AT1, SIT1, PAT1 and PEPT1 in patients treated with ACEIs

Mucosal biopsies from 42 patients referred for gastroduodenoscopy were analysed. Nine of these patients were treated with ACE-Inhibitors, 13 with AT_1-receptor blockers,

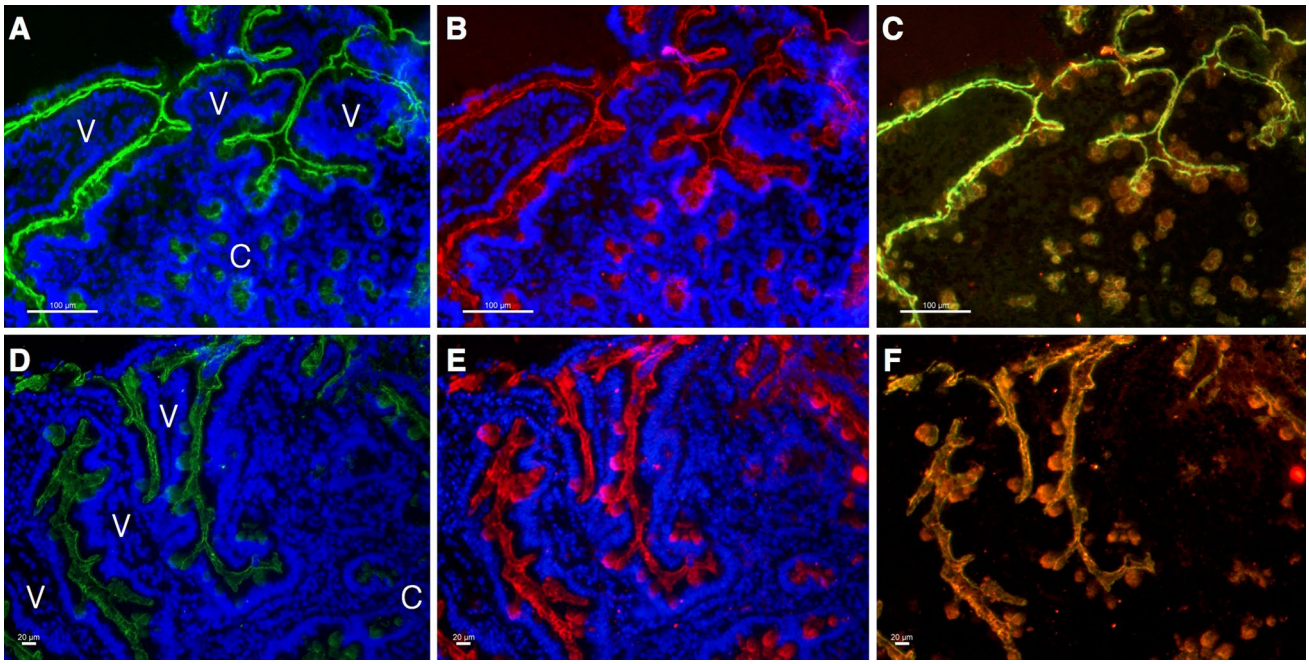


Fig. 3 Immuno-localization of ACE2 with B^0AT1 and SIT1 in human small intestine. Representative tissue specimens from duodenum show B^0AT1 (**a** *green*) and ACE2 (**b** *red*) co-localising (**c** *yellow*) at the brush-border membrane of enterocytes lining small intestinal villi (marked by the *letter* '*V*'). The imino transporter SIT1 (**d** *green*) and ACE2 (**e** *red*) co-localise (**f** *yellow*) at the apical membrane of epithelial cells lining duodenal villi ('*V*'). Cellular DNA (**a**, **b**, **d**, **e**; DAPI) is shown in blue to display the nuclei. Crypts are marked by the letter '*C*' (**a**, **d**). Pictures were taken at ×20 magnification. The *white bars* represent 100 µm (**a**–**c**) or 20 µm (**d**–**f**), respectively

22 had no RAS-active treatment and none was treated with both medications.

Levels of mRNA expression of all gene products assessed were very similar in duodenum parts II and III, whereas clear differences between duodenum and terminal ileum and between small and large intestine were observed for several genes (Fig. 4). The impact of ACEI and ARB treatment on mucosal gene expression was assessed for duodenal samples (means of all available biopsies). In patients treated with ACEIs the mean duodenal mRNA expression level of ACE2 was increased 1.9-fold when compared to non-treated controls and that of the SLC transporters B^0AT1, PEPT1 and PAT1 1.7-, 1.6- and 1.6-fold, respectively. No significant differences in ACE2, B^0AT1, PEPT1, PAT1 or SIT1 expression levels were observed in patients treated with ARBs (Fig. 5a). None of the other genes assessed, including SIT1, showed significant differences between groups (data of the other genes not shown).

To test whether the ACEI-induced effects on intestinal ACE2, B^0AT1, PAT1 and PEPT1 expression are correlated with each other, we determined the Pearson correlation of respective mean duodenal mRNA levels in all patients ($n = 44$) quantifying the strength of the linear association. Gene expression of intestinal ACE2 strongly correlated with that of B^0AT1 (correlation coefficient 0.83, confidence interval 0.70–0.90, $p < 0.0001$), and PEPT1 (correlation coefficient 0.80, confidence interval 0.63–0.89, $p < 0.0001$). The correlation of ACE2 with PAT1 (correlation coefficient 0.34, confidence interval 0.02–0.60, $p < 0.05$) was quite small and only weakly significant (Fig. 5b–d).

Promoter analysis

As the mRNA expression of ACE2, B^0AT1, and PEPT1 correlated with each other, we performed a comparative promoter analysis to identify common regulatory sequences. Comparison of the proximal promoter regions of relevant transcripts of these three genes revealed a common promoter module with three conserved transcription factor binding sites (TFBS) (SORY_EBOX_MIZ1_modules). Screening of the human promoter database for the occurrence of this module revealed its presence only in six other genes, namely HUS1, HIP1, CASR, GALC, ZNF555, and VPS13C. A second analysis including all potential promoter regions of ACE2, B^0AT1, and PEPT1 detected a single additional module with five conserved TFBS (HOXC_FKHD_HOMF_HOMF_RORA_module) that was found in a single other gene (CDV3). The expression and potential co-regulation of these genes sharing promoter modules with ACE2, B^0AT1 and PEPT1 was tested in the small intestine biopsies. Only four of these seven genes displayed an mRNA expression level reliably quantifiable by qPCR

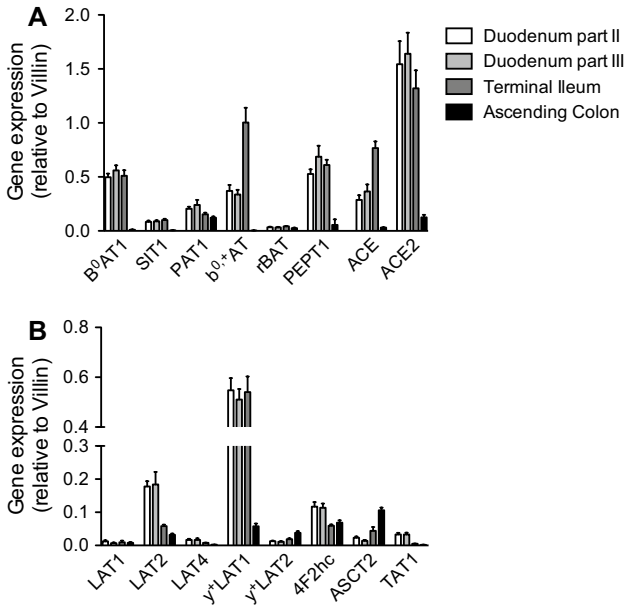


Fig. 4 Relative mRNA abundance of AA- and peptide transporters, ACE and ACE2 along the human intestine. **a** Apical (facing the lumen of the gut) AA- and peptide transporters, ACE and ACE2. **b** Basolateral (facing the extracellular space) AA transporters. Each *bar* indicates the mean relative mRNA expression ± SEM [normalised to villin ($2^{Ct(Villin) - Ct(target)}$)]. mRNA expression of each gene is shown at four different intestinal localizations: duodenum, part II (*white bars*), part III (*light grey bars*), terminal ileum (*dark grey bars*) and ascending colon (*black bars*)

and none of them was increased in ACEI-treated patients (data not shown), indicating that the effect described above for ACE2, B^0AT1 and PEPT1 transcripts is selective for these luminal membrane proteins.

Transcription factors HNF1a and HNF4a

Transcription factors HNF1a and HNF4a were shown to activate transcription of the B^0AT1 gene SLC6A19 in villus enterocytes (Tumer et al. 2013). Gene expression of these transcription factors was hence tested in patients treated with ACEI and in controls. Small intestinal gene expression of HNF1a was about 20-fold lower when compared to SLC6A19, whereas the HNF4a gene was expressed about fourfold lower than SLC6A19. None of these transcription factors was increased in patients treated with ACE-inhibitors (data not shown), indicating that the effect for B^0AT1, ACE2 and PEPT1 transcripts underlies another regulatory mechanism.

Discussion

The ACE2 carboxypeptidase is an important player of the systemic RAS and is also expressed at the surface of the small intestine (Camargo et al. 2009; Kowalczuk et al. 2008). In enterocytes, ACE2 is required for the expression of the major neutral amino acid transporter B^0AT1 the defect of which causes Hartnup disorder. A similar interaction with ACE2 has been proposed for the L-proline transporter SIT1 the defects of which contributes to iminoglycinuria (Broer et al. 2008). The deficiency of ACE2 impairs amino acid absorption in the mouse, in particular of tryptophan, and thereby increases susceptibility to intestinal inflammation (Hashimoto et al. 2012). In rodent heart, kidney and aorta, treatment with ACEIs and/or ARBs was shown to increase ACE2 expression (Chappel and Ferrario 2006; Ferrario and Varagic 2010; Igase et al. 2005).

This study addressed the question of the role of ACE2 in human intestine. First we characterised its functional interaction with the proline transporter SIT1 in *X. laevis* oocytes. Then we demonstrated the axial expression of ACE2 along human intestine and also that of ACE, AA- and peptide transporters. Finally we showed that patients treated with ACE-inhibitors express higher transcript levels of intestinal ACE2, amino acid transporters B^0AT1 and PAT1 and peptide transporter PEPT1, a novel finding that suggests the possibility that ACEI impact on the absorption of other drugs and amino acids.

Intestinal ACE2 and its role as accessory protein for luminal amino acid transporters

ACE2 has multiple roles in the human body (Kuba et al. 2010). It is an enzyme that is expressed in various cells of many organs and is key to the regulation of local and systemic angiotensin II levels. Next to its role within local and systemic renin-angiotensin systems, ACE2 has also the function of an intestinal brush-border peptidase involved in protein digestion, similar to ACE. Indeed, high ACE2 expression levels are found at the luminal surface of small intestinal enterocytes (Camargo et al. 2009) and also of kidney proximal tubule cells (Kowalczuk et al. 2008). In small intestine, its role at the brush-border membrane is that of cleaving single carboxy-terminal AAs from nutrient proteins/peptides and also of interacting within digestive complexes comprising AA transporters, such as B^0AT1 (Fairweather et al. 2012). Whereas intestinal B^0AT1 surface expression depends on the presence of ACE2 (Camargo et al. 2009), its interacting partner in the kidney, collectrin (Danilczyk et al. 2006), does not have a peptidase domain (Kuba et al. 2010). It is possible that B^0AT1 evolutionary first interacted with ACE2 within a complex of proteins involved in intestinal absorption named ‘metabolon’ by Broer and co-workers (Fairweather et al. 2012). If this was the case, the newer kidney structural partner collectrin could have become necessary to separate the AA transport associated function from that of degrading kidney proximal

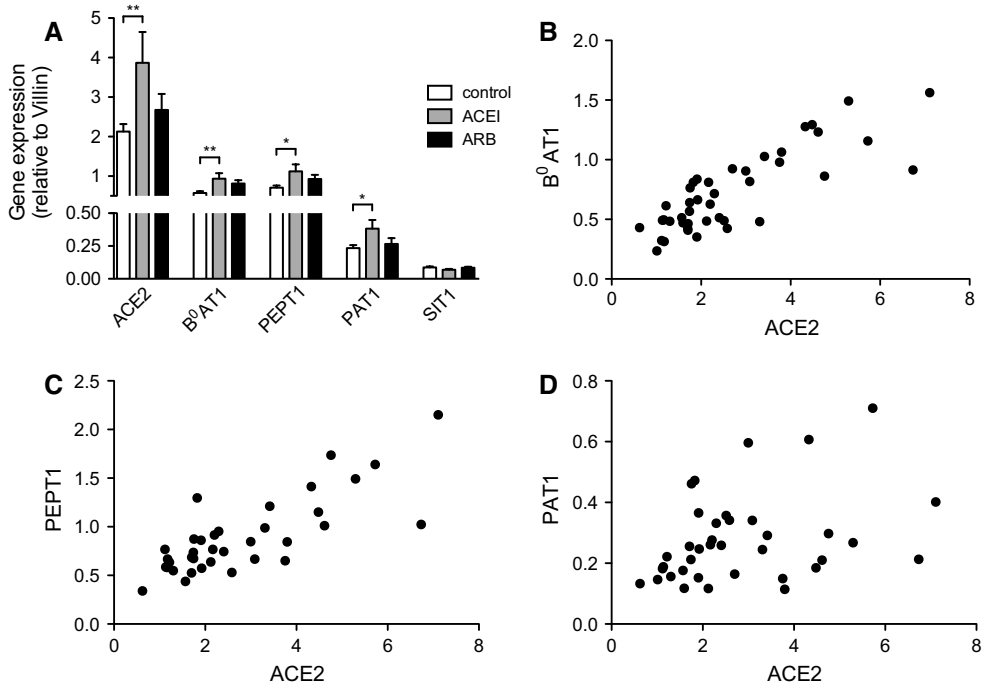


Fig. 5 **a** Effect of ACEIs and ARBs on human duodenal ACE2 and transporter gene expression. Mean duodenal mRNA expression [normalised to villin ($2^{Ct(Villin) - Ct(target)}$)] of ACE2, AA transporters B^0AT1 and PAT1 and peptide transporter PEPT1 in control patients (control; *white bars*) vs. patients treated with ACE-inhibitors (ACEI; *grey bars*) vs. patients treated with angiotensin II AT_1 receptor blockers (ARB; *black bars*). $*p < 0.05$; $**p < 0.01$ (Bonferroni corrected p values from a mixed mode including the experiment number as random factor to take into account the grouping structure of the data caused by the data acquisition in 4 independent experiments). **b–d** Correlation analysis of ACE2 with B^0AT1, PEPT1 and PAT1 mRNA expression. Mean duodenal gene expression of ACE2 with B^0AT1, PEPT1 and PAT1, respectively, was correlated. ACE2 and B^0AT1: correlation coefficient 0.83, CI 0.70–0.90, $p = 1.38 \times 10^{-11}$ (Fig. 4b). ACE2 and PEPT1: correlation coefficient 0.80, CI 0.63–0.89, $p = 8.56 \times 10^{-9}$ (Fig. 4c). ACE2 and PAT1: correlation coefficient 0.34, CI 0.02–0.60, $p = 0.037$ (Fig. 4d)

tubule angiotensin II. Similarly to B^0AT1, the system IMINO transporter SIT1 has been suggested to depend on intestinal ACE2 and proximal tubule collectrin expression (Danilczyk et al. 2006; Singer et al. 2012). Indeed, high-affinity intestinal L-proline absorption was shown to be reduced in *ACE2* knock-out mice (Singer et al. 2012), and *collectrin* knock-out mice showed reduced proximal tubule SIT1 expression and consequently prolinuria (Danilczyk et al. 2006). We hereby show, using the *Xenopus* oocyte expression system, a functional interaction of human SIT1 with both accessory human proteins ACE2 and collectrin, as previously demonstrated for human B^0AT1 (Kowalczuk et al. 2008). A functional interaction of SIT1 with ACE2 had previously not been observed when using the mouse orthologs in *Xenopus* oocytes, presumably because of the high transport activity induced by mouse SIT1 in the absence of an exogenous accessory protein (Kowalczuk et al. 2008). An analogous species difference had been previously observed for the functional expression of B^0AT1 in *Xenopus* oocytes (Seow et al. 2004).

Digestive complexes may alter transport kinetics of AA transporters, as shown for the aminopeptidase N and B^0AT1. Such an effect can be explained by a change in local AA concentration, but was not observed in the case of the monocarboxypeptidase ACE2 and B^0AT1 (Fairweather et al. 2012). Similarly, we show here that the apparent affinity of SIT1 for L-proline was not changed by co-expression of either ACE2 or collectrin. Maximal transport rate (V_{max}) was higher upon co-expression of these accessory proteins, and ACE2 increased oocyte plasma membrane sorting of SIT1 transporter, as shown by immunofluorescence staining and Western blot analysis. Interestingly, the apparent affinity of human SIT1 for L-proline was 2–4 times higher than previously reported for the mouse (Kowalczuk et al. 2005), rat (Takanaga et al. 2005) or opossum (Ristic et al. 2006) orthologues, a species-specific difference for which we have no explanation.

Axial distribution of intestinal ACE, ACE2, amino acid and peptide transporters

Whereas the gene expression along the human intestine has been described for some transporters (Kim et al. 2002; Meier et al. 2007; Terada et al. 2005), to date information

about the axial distribution of AA transporters along the digestive tract has been derived from animal studies (Dave et al. 2004; Ramadan et al. 2006; Takanaga et al. 2005). Moreover, the data that is available from the human gut is difficult to interpret, since tissue specimens from different axial localizations originated from different patients and thus local expression differences might reflect discrepancies between patient groups (Terada et al. 2005). This is the first study summarising the longitudinal gene expression of several luminal and basolateral peptide and AA transporters and of ACE and ACE2 along the human digestive tract within one single group of patients. We show that the mRNA expression level of most luminal transporters is equal along the small intestine [duodenum (parts II and III) and terminal ileum] with the exception of the cysteine and dibasic AA exchanger subunit $b^{0,+}$AT. Together with the glycoprotein rBAT, this catalytic subunit forms a heteromeric AA exchanger composed of two subunits covalently linked together (Chillaron et al. 2010; Font-Llitjos et al. 2007). In murine intestine, both transporter subunits were shown to be expressed at a higher level in ileum than in jejunum and duodenum (Dave et al. 2004). Similarly, we found higher levels of the transporter subunit $b^{0,+}$AT mRNA in the distal small intestine in humans. In contrast, mRNA levels of the heavy chain rBAT were equal all along the small intestine. Basolateral 4F2hc (CD98) and luminal rBAT are the only members of the SLC3 family of heteromeric AA transporter heavy chains that (as glycoprotein subunits) associate with different members of the SLC7 family (of catalytic subunits; also called light chains), including luminal $b^{0,+}$AT and basolateral LAT1, LAT2, y^+LAT1 and y^+LAT2 (Fernandez et al. 2002; Verrey et al. 2000). In small intestine enterocytes and proximal tubule kidney cells, the heterodimer $b^{0,+}$AT-rBAT mediates the apical entry of L-cystine (Feliubadalo et al. 1999) and cationic AAs including L-arginine (Arg). Here we show that the basolateral antiporter y^+LAT1 that exchanges cationic AAs against neutral AAs and Na^+ (Palacin et al. 2005) is highly expressed all along the human small intestine up to the ileum and thus most likely represents the exit pathway for transcellular cationic AA transport. In contrast, the expression of the other y^+L-type transporter y^+LAT2 is very low, suggesting that this catalytic subunit is not of functional importance for the transepithelial cationic AA absorption. Furthermore, the mRNA expression of the basolateral AA transporters considered to be part of the neutral AA transport machinery (LAT2, LAT4 and TAT1) (Mariotta et al. 2012) appear to be expressed at a lower level in the terminal ileum than in the duodenum and presumably the jejunum, in contrast to y^+LAT1. This differential expression may reflect the fact that the largest load of neutral AAs is already absorbed before the ileum such that the efflux capacity of ileal enterocytes for neutral AAs is not anymore that high. In contrast, the cationic AA absorption via luminal $b^{0,+}$AT-rBAT and basolateral y + LAT1 is highly developed in the terminal ileum, an observation for which we have no good explanation.

Luminal transporters—with the exception of the low affinity small neutral amino/imino acid transporter 1 (PAT1) (Anderson et al. 2004)—showed very low or negligible transcript expression values in the large intestine. From the tested basolateral amino acid transporters, only the neutral and cationic AA exchanger y^+LAT2-4F2hc (Broer et al. 2000) and small neutral AA and L-glutamine (Gln) antiporter ASCT2 (Broer et al. 2011) were considerably expressed in the large intestine. Epithelial colonic cells show rapid renewal and transport large amounts of water and electrolytes. Colonocytes therefore need high amounts of energy and use AAs (especially Gln and L-glutamate (Glu)) from the circulation to supply the citric acid cycle (Blachier et al. 2009; Scheppach et al. 1996). Thus, it seems likely that basolateral exchangers ASCT2 and y^+LAT2-4F2hc provide colonocytes with Gln and other amino acids for their energy needs.

Whereas the dipeptidase ACE showed highest gene expression in the terminal ileum, mRNA levels of the monocarboxypeptidase ACE2 and of its interaction partners B^0AT1 and SIT1 were equal along the human small intestine. ACE2 protein co-localised with the transport proteins B^0AT1 and SIT1 to the brush-border membrane of small intestinal enterocytes on villi. Staining of B^0AT1 appeared to be stronger on top of the villi when compared to the signal in the crypts, supporting the notion that this surface protein is important for the function of mature enterocytes. This finding agrees with previous observations made in the rodent small intestine, showing a clear crypt-to-tip expression gradient along the villi for various AA transporters, including B^0AT1 (Camargo et al. 2009; Dave et al. 2004; Tumer et al. 2013).

ACE-inhibitors increase expression of ACE2 and luminal amino acid and peptide transporters

The regulation of SLC transporter expression in intestine has not been studied extensively. Physiological factors including developmental stage, diet, starvation, circadian rhythm and pathophysiological situations such as inflammatory bowel disease have been suggested to modulate intestinal SLC transporter mRNA expression. Regulatory mechanisms that may mediate these changes include transcriptional activation, RNA stabilisation and epigenetic regulation (Douard et al. 2008; Dyer et al. 1997; Naruhashi et al. 2002; Pan and Hussain 2009).

In the present study we show that the administration of ACE-inhibitors increases small intestinal ACE2 gene expression. As yet, ACE2 up-regulation by ACEI has

been reported only for other organs and in rodents (Ferrario et al. 2005; Igase et al. 2005). Small intestinal ACE2 mRNA was not different in patients treated with ARBs when compared to controls. In contrast, gene expression of small intestinal ACE2 was increased in ACEI-treated patients. Interestingly, gene expression levels of luminal AA transporters B^0AT1, PAT1 and of the peptide transporter PEPT1 were also increased in patients treated with ACE-inhibitors. The up-regulation of ACE2 mRNA correlated with that of B^0AT1, PEPT1 and PAT1 gene expression, indicating that these four gene products are regulated in parallel. Comparative promoter analysis detected two common promoter modules, which, however, appear not to suffice for mediating the observed co-regulation. Furthermore, transcription factors HNF1a and HNF4a, known to activate the transcription of the B^0AT1 gene SLC6A19 (Tumer et al. 2013), did neither seem to cause this up-regulation. This ACEI-induced regulation did not impact on steady state blood plasma and urine AA concentrations (data not shown).

This study shows the brush-border membrane co-localization of the carboxypeptidase ACE2 and the amino acid transporters B^0AT1 and SIT1, as well as the axial distribution of ACE, ACE2 and most known amino acid and peptide transporters expressed in the small intestine. The gene expression levels of B^0AT1, PAT1 and PEPT1 are additionally shown for the first time to be modulated by treatment with ACE-inhibitors. Since the AA transporter PAT1 and the peptide transporter PEPT1 are known to transport different drugs including Vigabatrin, 5-aminolevulinic acid (PAT1) (Anderson et al. 2004), Betalactam antibiotics and Valacyclovir (PEPT1) (Brandsch 2009), we may speculate that treatment with ACE-inhibitors might impact on the absorption kinetics of these medications. Additionally, since *ACE2*-deficient mice lacking brush-border membrane B^0AT1 expression were shown to be more susceptible to intestinal inflammation because of impaired local L-tryptophan (Trp) homeostasis (Hashimoto et al. 2012), it also may be that treatment with ACEIs and consecutive increase of intestinal ACE2 and B^0AT1 reduces susceptibility to intestinal inflammation, especially in conditions of low plasma Trp levels such as observed in states of malnutrition as in anorexia nervosa (Attia et al. 2005).

Acknowledgments The authors thank Mital Dave for cloning the human SIT1 cDNA. RNV was supported by a grant for the Doctorate of Medicine and of Philosophy (MD-PhD) students from the Swiss National Foundation. The laboratory of FV is supported by Grant 130471 of the Swiss National Foundation and the Swiss National Centre of Competence in Research Kidney Control of Homeostasis.

Conflict of interest The authors declare that they have no competing financial interests.

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