Year: 2015

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

Vuille-Dit-Bille, Raphael N ; Camargo, Simone M ; Emmenegger, Luca ; Sasse, Tom ; Kummer, Eva ; Jando, Julia ; Hamie, Qeumars M ; Meier, Chantal F ; Hunziker, Schirin ; Forras-Kaufmann, Zsafia ; Kuyumcu, Sena ; Fox, Mark ; Schwizer, Werner ; Fried, Michael ; Lindenmeyer, Maja ; Götze, Oliver ; Verrey, François

Abstract: Sodium-dependent neutral amino acid transporter B(0)AT1 (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(0)AT1 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 AT1 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(0)AT1 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(0)AT1 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.

DOI: <https://doi.org/10.1007/s00726-014-1889-6>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-110231>

Journal Article

Updated Version

Originally published at:

Vuille-Dit-Bille, Raphael N; Camargo, Simone M; Emmenegger, Luca; Sasse, Tom; Kummer, Eva; Jando, Julia; Hamie, Qeumars M; Meier, Chantal F; Hunziker, Schirin; Forras-Kaufmann, Zsafia; Kuyumcu, Sena; Fox, Mark; Schwizer, Werner; Fried, Michael; Lindenmeyer, Maja; Götze, Oliver; Verrey, François (2015). Human intestine luminal ACE2 and amino acid transporter expression increased by ACE-inhibitors. *Amino acids*, 47(4):693-705.

DOI: <https://doi.org/10.1007/s00726-014-1889-6>

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

Raphael N. Vuille-dit-Bille¹, Simone M. Camargo¹, Luca Emmenegger¹, Tom Sasse¹, Eva Kummer¹, Julia Jando¹, Qeumars M. Hamie¹, Chantal F. Meier¹, Schirin Hunziker¹, Zsofia Forras-Kaufmann², Sena Kuyumcu², Mark Fox², Werner Schwizer², Michael Fried², Maja Lindenmeyer¹, Oliver Götze², François Verrey¹

¹Institute of Physiology and Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland, ²Division of Gastroenterology and Hepatology, University Hospital of Zurich, Zurich, Switzerland

Corresponding author

François Verrey

Institute of Physiology, University of Zurich

Winterthurerstrasse 190

8057 Zurich, Switzerland

Tel: +41 44 635 5044

Fax: +41 44 635 6814

verrey@access.uzh.ch

Abstract

Sodium-dependent neutral amino acid transporter B⁰AT1 (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 B⁰AT1 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 AT₁ 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 analyzed 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 B⁰AT1 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 B⁰AT1 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 B⁰AT1; SIT1; ACE2, angiotensin converting enzyme inhibitors; intestine

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 B⁰AT1 (broad neutral AA transporter 1, SLC6A19) is also expressed in kidney proximal tubule and its defect was shown to cause Hartnup disorder (Kleta et al. 2004; Seow et al. 2004). Intestinal B⁰AT1 expression and function depends on the presence of the accessory protein ACE2 (angiotensin converting enzyme 2) (Camargo et al. 2009; Kowalczyk 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; Kowalczyk 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 B⁰AT1 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 B⁰AT1 (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,

1 kidney and the aorta (Chappel and Ferrario 2006; Ferrario and Varagic 2010; Igase et al. 2005). However, it is
2 not known, whether ACEIs or ARBs also affect the expression of small intestine ACE2, which is involved – as a
3 carboxypeptidase – in protein digestion and – by interacting with B⁰AT1 and potentially SIT1 – in AA
4 absorption. Additionally, the knowledge about the axial distribution of AA- and peptide transporters along the
5 intestine is sparse and mainly originates from animal studies (Dave et al. 2004; Terada et al. 2005).
6
7

8
9 In this study we (i) addressed the question whether human ACE2 can interact functionally with the proline
10 transporter SIT1 using the *Xenopus laevis* oocyte expression system and (ii) whether it co-localizes with SIT1 as
11 with B⁰AT1 at the luminal surface of human intestinal mucosa. Furthermore (iii), we analyzed the axial
12 distribution of ACE, ACE2, amino acid- and peptide transporters in human intestine and (iv) assessed whether
13 ACEIs and ARBs potentially impact on ACE2 and AA transporter expression in the intestine of human patients.
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Materials and methods

Cloning and cRNA synthesis of transporters for expression in *Xenopus 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 EcoRI before inserting into the expression vector pBluescript-KSM. For the insertion of the Flag tag, SIT1 was excised with EcoRV and SpeI, ligated to Flag tag containing vector (FastBac-Flag, kindly provide by Thierry Hennet) and cut with SfoI and SpeI. The fusion protein of SIT1 with N-terminal Flag tag was returned to the expression vector using EcoRV and SpeI. SIT1 and SIT1-Flag constructs were linearized respectively with XbaI 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 *Xenopus 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 ng and 20 ng, SIT1 and collectrin: 5 ng 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 MgCl₂, and 1 mM CaCl₂) and pre-incubated at 25°C for 2 minutes. The uptake solution containing radiolabeled amino acids (2 µCi of radiolabeled tracer per group of oocytes) was added for 10 minutes 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 minutes. 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). Data were expressed as pmol/h*oocyte, and values obtained for non-injected oocytes were subtracted. Kinetic 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 AT₁-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 HPLC (High Performance Liquid Chromatography) 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 x 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/m². 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). Body mass index (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 μ m (tissue specimens) and 9 μ m 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 minutes in a wet chamber. For fixation, slides were immersed in Methanol (70%) for 90 seconds. After fixation, slides were washed (1 x 15 min, 2 x 5 min) with phosphate buffered saline (PBS, 0.1 M) (137.0 mM NaCl, 2.7 mM KCl, 12.0 mM HPO₄²⁻/H₂PO₄⁻). 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 hour (tissue specimens) and 3 hours (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 B⁰AT1 (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 x 5 min) and oocyte sections in PBS (0.2 M) (2 x 5 min) and PBS (0.1 M) (1 x 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 hour 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 minutes at RT (oocytes specimens). Tissue sections were washed with PBS (0.1 M) (3 x 5 min) and oocyte sections with PBS (0.2 M) (2 x 5 min) and PBS (0.1 M) (1 x 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) 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 homogenized 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 x through a 25 G needle. The solution was centrifuged at 100 x g for 10 minutes, 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). 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 hour at room temperature. To compare the loading, the membrane was stained with mouse anti-tubulin (Sigma, 1:5000) and alkaline phosphatase (AP)-conjugated anti-mouse antibody. Antibody binding was detected with the Luminata Classico Western HRP substrate (Merk-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 seconds at 6000 rpm using a Precellys® 24 tissue homogenizer (Bertin Technologies, France). The solution was centrifuged at 10'000 g for 5 minutes 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 x), MgCl₂ (5 mM), random hexamers (2.5 µM), deoxyNTP mix (500 µM each), RNase inhibitor (0.4 U x µl⁻¹), multiscribe reverse transcriptase enzyme (1.25 U x µl⁻¹), RNA template (33 ng x µl⁻¹) and RNase free water. All reactions were executed with negative controls (RT-) using the same protocol without adding the multiscribe reverse transcriptase 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 to 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 minutes), 60°C (15 seconds) and 72°C (1 minute). For analyzing 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 ΔC_t method (relative expression = $2^{-\Delta C_t}$, ΔC_t = average C_t value of target – average C_t value of reference).

Comparative promoter analysis

The proximal promoter regions of human ACE2, B⁰AT1 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 characterized 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) 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 *Xenopus laevis* oocytes

To test whether human ACE2 and human collectrin functionally interact with SIT1 – as shown for B⁰AT1 (Kowalczyk 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 2-fold, when ACE2 or collectrin were co-expressed with SIT1, whereas no significant Pro uptake was observed when ACE2 or collectrin RNA was injected alone (Figure 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 μ M) was similar in oocytes co-expressing ACE2 ($K_{0.5}$: 50.4 μ M) or collectrin ($K_{0.5}$: 60.0 μ M), respectively, as shown by the $K_{0.5}$ derived from Lineweaver-Burk analysis. In contrast, the maximal transport capacity (V_{max}) was more than two-fold higher in oocytes co-injected with ACE2 or collectrin, respectively (Figure 1B).

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 *Xenopus laevis* oocytes and visualized by immunohistochemistry (Figure 2A) and Western blotting of membrane lysates (Figure 2B). When co-expressed with human ACE2, human SIT1 transporter (green) produced a clearly stronger signal at the plasma membrane (Figure 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 (Figure 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 B⁰AT1, the co-expression of ACE2 also increased cell surface expression and transport function of SIT1.

Localization of amino acid transporters B⁰AT1 and SIT1 and of ACE2 in human small intestine

The distribution of the AA transporters B⁰AT1 (Figure 3A; green) and SIT1 (Figure 3D; green) and of ACE2 (Figure 3B and E; red) along the villi (V) and in the crypts (C) was visualized by immunohistochemistry. The two transporters co-localized with ACE2 along the brush-border membrane of duodenum (Figures 3C and F) and terminal ileum (not shown) enterocytes on villi. The signal of AA transporter B⁰AT1 (Figure 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 labeling with all antibodies and represent presumably goblet cells that are non-specifically stained. Whereas in the colon B⁰AT1 was not detected by immunofluorescence, ACE2 and SIT1 were labeled within colonic crypts where they co-localized 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 describe the data and do not refer to statistical *significant differences*. These measurements revealed distinct axial gene expression patterns (Figure 4): (i) 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. (ii) 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 b⁰+AT (SLC7A9) that was higher in the terminal ileum (Figure 4A). (iii) 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 (Figure 4B). (iv) The mRNA encoding ACE was higher in the terminal ileum, whereas that of ACE2 was equal along the small intestine (Figure 4A).

Intestinal gene expression of ACE2, B⁰AT1, SIT1, PAT1 and PEPT1 in patients treated with ACEIs

Mucosal biopsies from 42 patients referred for gastroduodenoscopy were analyzed. Nine of these patients were treated with ACE-Inhibitors, 13 with AT₁-receptor blockers, 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 (Figure 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 B⁰AT1, PEPT1 and PAT1 1.7-, 1.6- and 1.6-fold, respectively. No significant

1 differences in ACE2, B⁰AT1, PEPT1, PAT1 or SIT1 expression levels were observed in patients treated with
2 ARBs (Figure 5A). None of the other genes assessed, including SIT1, showed significant differences between
3 groups (data of the other genes not shown).

4 To test whether the ACEI-induced effects on intestinal ACE2, B⁰AT1, PAT1 and PEPT1 expression are
5 correlated with each other, we determined the Pearson correlation of respective mean duodenal mRNA levels in
6 all patients (n = 44) quantifying the strength of the linear association. Gene expression of intestinal ACE2
7 strongly correlated with that of B⁰AT1 (correlation coefficient 0.83, confidence interval 0.70 – 0.90, p < 0.0001),
8 and PEPT1 (correlation coefficient 0.80, confidence interval 0.63 – 0.89, p < 0.0001). The correlation of ACE2
9 with PAT1 (correlation coefficient 0.34, confidence interval 0.02 – 0.60, p < 0.05) was quite small and only
10 weakly significant (Figure 5B-D).
11
12
13
14
15
16
17

18 **Promoter analysis**

19 As the mRNA expression of ACE2, B⁰AT1, and PEPT1 correlated with each other, we performed a comparative
20 promoter analysis to identify common regulatory sequences. Comparison of the proximal promoter regions of
21 relevant transcripts of these three genes revealed a common promoter module with three conserved transcription
22 factor binding sites (TFBS) (SORY_EBOX_MIZ1_modules). Screening of the human promoter database for the
23 occurrence of this module revealed its presence only in six other genes, namely HUS1, HIP1, CASR, GALC,
24 ZNF555, and VPS13C. A second analysis including all potential promoter regions of ACE2, B⁰AT1, and PEPT1
25 detected a single additional module with five conserved TFBS
26 (HOXC_FKHD_HOMF_HOMF_RORA_module) that was found in a single other gene (CDV3). The
27 expression and potential co-regulation of these genes sharing promoter modules with ACE2, B⁰AT1 and PEPT1
28 was tested in the small intestine biopsies. Only four of these seven genes displayed an mRNA expression level
29 reliably quantifiable by qPCR and none of them was increased in ACEI-treated patients (data not shown),
30 indicating that the effect described above for ACE2, B⁰AT1 and PEPT1 transcripts is selective for these luminal
31 membrane proteins.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

48 **Transcription factors HNF1a and HNF4a**

49 Transcription factors HNF1a and HNF4a were shown to activate transcription of the B⁰AT1 gene SLC6A19 in
50 villus enterocytes (Tumer et al. 2013). Gene expression of these transcription factors was hence tested in
51 patients treated with ACEI and in controls. Small intestinal gene expression of HNF1a was about 20-fold lower
52 when compared to SLC6A19, whereas the HNF4a gene was expressed about 4-fold lower than SLC6A19. None
53 of these transcription factors was increased in patients treated with ACE-inhibitors (data not shown), indicating
54 that the effect for B⁰AT1, ACE2 and PEPT1 transcripts underlies another regulatory mechanism.
55
56
57
58
59
60
61
62
63
64
65

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; Kowalczyk et al. 2008). In enterocytes, ACE2 is required for the expression of the major neutral amino acid transporter B⁰AT1 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 characterized its functional interaction with the proline transporter SIT1 in *Xenopus 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 B⁰AT1 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, similarly 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 (Kowalczyk 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 B⁰AT1 (Fairweather et al. 2012). Whereas intestinal B⁰AT1 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 B⁰AT1 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 tubule angiotensin II. Similarly to B⁰AT1, 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 B⁰AT1 (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 B⁰AT1 in *Xenopus* oocytes (Seow et al. 2004).

Digestive complexes may alter transport kinetics of AA transporters, as shown for the aminopeptidase N and B⁰AT1. 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 B⁰AT1 (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 summarizing 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^{0} AT1 and SIT1 were equal along the human small intestine. ACE2 protein co-localized with the transport proteins B^{0} AT1 and SIT1 to the brush-border membrane of small intestinal enterocytes on villi. Staining of B^{0} AT1 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^{0} AT1 (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 stabilization 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 B⁰AT1, 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 B⁰AT1, 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 B⁰AT1 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 B⁰AT1 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 B⁰AT1, 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 B⁰AT1 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 B⁰AT1 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).

1
2
3
4
5
6
7
8
9
10
Acknowledgements

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.

11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
Conflict of interest

The authors declare that they have no competing financial interests.

Tables

Table 1 Patients data.

	Control ¹	ACEI ²	ARB ³	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 ⁴ [kg/m ²]	25.2 ± 0.75	26.4 ± 1.9	26.5 ± 1.5	n. s.	n. s.
MAP ⁵ [mmHg]	87.9 ± 2.5	87.1 ± 3.3	90.3 ± 3.9	n. s.	n. s.
ACE activity [U/l]	35.2 ± 3.4	14.8 ± 1.7	33.0 ± 4.4	**	n. s.
Renin [mU/l]	12.2 ± 1.8	33.1 ± 9.1	613 ± 392	n. s.	n. s.
Aldosterone [ng/l]	102 ± 14.2	69.6 ± 9.5	148 ± 32.9	n. s.	n. s.
Angiotensin I [ng/ml]	0.49 ± 0.083	0.43 ± 0.077	0.59 ± 0.17	n. s.	n. s.
Angiotensin II [pg/ml]	11.3 ± 3.4	9.8 ± 3.7	65.0 ± 40.1	n. s.	n. s.

¹patients without RAS-active medication, ²patients treated with ACE-inhibitors, ³patients treated with angiotensin II AT₁ receptor blockers, ⁴BMI = body mass index, ⁵MAP = mean arterial pressure. ** p < 0.01; n. s. = not significant (p > 0.05) (ANOVA with posthoc Dunnett's test)

Figure Legends

Fig. 1 Functional interaction of amino acid transporter SIT1 with accessory proteins ACE2 and collectrin expressed in *Xenopus laevis* oocytes. **A** L-proline (100 µM) uptake. *Xenopus 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 4 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 4 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 – 1000 μ M, were determined 2 - 3 days after injection. Each data point represents the mean transport rate $\pm$ SEM. (n = total of 24 oocytes from 4 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) μ M, $K_{0.5}$ SIT1 + ACE2: 50.4 (CI: 29.5 – 104.6) μ M, $K_{0.5}$ SIT1 + collectrin: 60.0 (CI: 26.5 – 296.7) μ M. Maximal transport capacity (V_{max}) was about 2-fold 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).

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** Cell surface expression of labelled proteins (hSIT1-flag) was analysed by Western blot. Membrane of non-injected oocytes (NI), oocytes expressing human SIT1-flag construct (S) and SIT1-flag + human ACE2 (hS+hA) from 3 different batches of oocytes were analysed. The loading of oocyte membranes was controlled using a β -Tubulin antibody. Each band represents lysates made from 9-33 oocytes. The three different lines (dotted, continuous and dashed) indicate different batches of oocytes used.

Fig. 3 Immuno-localization of ACE2 with B⁰AT1 and SIT1 in human small intestine. Representative tissue specimens from duodenum show B⁰AT1 (**A**; green) and ACE2 (**B**; red) co-localizing (**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-localize (**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 x magnification. The white bars represent 100 μ m (**A**, **B**, **C**) or 20 μ m (**D**, **E**, **F**), respectively.

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 $\pm$ SEM (normalized 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).

Fig. 5 A Effect of ACEIs and ARBs on human duodenal ACE2 and transporter gene expression. Mean duodenal mRNA expression (normalized to villin ($2^{Ct(Villin)-Ct(target)}$)) of ACE2, AA transporters B⁰AT1 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 AT₁ 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 B⁰AT1, PEPT1 and PAT1 mRNA expression. Mean duodenal gene expression of ACE2 with B⁰AT1, PEPT1 and PAT1, respectively was correlated. ACE2 & B⁰AT1: correlation coefficient 0.83, confidence interval 0.70 – 0.90, $p = 1.38 \times 10^{-11}$ (Figure 4B). ACE2 & PEPT1: correlation coefficient 0.80, confidence interval 0.63 – 0.89, $p = 8.56 \times 10^{-9}$ (Figure 4C). ACE2 & PAT1: correlation coefficient 0.34, confidence interval 0.02 – 0.60, $p = 0.037$ (Figure 4D).

References

- Anderson CM et al. (2004) H⁺/amino acid transporter 1 (PAT1) is the imino acid carrier: An intestinal nutrient/drug transporter in human and rat. *Gastroenterology* 127:1410-1422
- Attia E, Wolk S, Cooper T, Glasofer D, Walsh BT (2005) Plasma tryptophan during weight restoration in patients with anorexia nervosa. *Biol Psychiatry* 57:674-678 doi:S0006-3223(04)01288-0
- Blachier F, Boutry C, Bos C, Tome D (2009) Metabolism and functions of L-glutamate in the epithelial cells of the small and large intestines. *Am J Clin Nutr* 90:814S-821S doi:ajcn.2009.27462S
- Brandsch M (2009) Transport of drugs by proton-coupled peptide transporters: pearls and pitfalls. *Expert Opin Drug Metab Toxicol* 5:887-905 doi:10.1517/17425250903042292
- Broer A et al. (2011) Impaired nutrient signaling and body weight control in a Na⁺ neutral amino acid cotransporter (Slc6a19)-deficient mouse. *J Biol Chem* 286:26638-26651 doi:10.1074/jbc.M111.241323
- Broer A, Wagner CA, Lang F, Broer S (2000) The heterodimeric amino acid transporter 4F2hc/y+LAT2 mediates arginine efflux in exchange with glutamine. *Biochem J* 349 Pt 3:787-795
- Broer S et al. (2008) Iminoglycinuria and hyperglycinuria are discrete human phenotypes resulting from complex mutations in proline and glycine transporters. *J Clin Invest* 118:3881-3892 doi:10.1172/JCI36625
- Broer S, Palacin M (2011) The role of amino acid transporters in inherited and acquired diseases. *Biochem J* 436:193-211 doi:BJ20101912
- Camargo SM et al. (2009) Tissue-specific amino acid transporter partners ACE2 and collectrin differentially interact with hartnup mutations. *Gastroenterology* 136:872-882 doi:S0016-5085(08)01893-3
- Cartharius K et al. (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 21:2933-2942 doi:bt1473
- Chappel MC, Ferrario CM (2006) ACE and ACE2: their role to balance the expression of angiotensin II and angiotensin-(1-7). *Kidney international* 70:8-10 doi:10.1038/sj.ki.5000321
- Chillaron J, Font-Llitjos M, Fort J, Zorzano A, Goldfarb DS, Nunes V, Palacin M (2010) Pathophysiology and treatment of cystinuria. *Nat Rev Nephrol* 6:424-434 doi:nrneph.2010.69
- Daniel H (2004) Molecular and integrative physiology of intestinal peptide transport. *Annu Rev Physiol* 66:361-384 doi:10.1146/annurev.physiol.66.032102.144149
- Daniel H, Kottra G (2004) The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. *Pflugers Arch* 447:610-618 doi:10.1007/s00424-003-1101-4
- Danilczyk U et al. (2006) Essential role for collectrin in renal amino acid transport. *Nature* 444:1088-1091 doi:nature05475
- Dave MH, Schulz N, Zecevic M, Wagner CA, Verrey F (2004) Expression of heteromeric amino acid transporters along the murine intestine. *J Physiol* 558:597-610
- Douard V, Cui XL, Soteropoulos P, Ferraris RP (2008) Dexamethasone sensitizes the neonatal intestine to fructose induction of intestinal fructose transporter (Slc2A5) function. *Endocrinology* 149:409-423 doi:10.1210/en.2007-0906
- Dyer J, Hosie KB, Shirazi-Beechey SP (1997) Nutrient regulation of human intestinal sugar transporter (SGLT1) expression. *Gut* 41:56-59

- 1 Fairweather SJ, Broer A, O'Mara ML, Broer S (2012) Intestinal Peptidases Form Functional Complexes with
2 Neutral Amino Acid Transporter B0AT1. *Biochem J* doi:BJ20120307
- 3 Feliubadalo L et al. (1999) Non-type I cystinuria caused by mutations in SLC7A9, encoding a subunit (bo,+AT)
4 of rBAT. *Nat Genet* 23:52-57 doi:10.1038/12652
- 5
6 Fernandez E, Carrascal M, Rousaud F, Abian J, Zorzano A, Palacin M, Chillaron J (2002) rBAT-b(0,+AT)
7 heterodimer is the main apical reabsorption system for cystine in the kidney. *Am J Physiol Renal*
8 *Physiol* 283:F540-548 doi:10.1152/ajprenal.00071.2002
- 9
10 Ferrario CM, Trask AJ, Jessup JA (2005) Advances in biochemical and functional roles of angiotensin-
11 converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. *Am J Physiol*
12 *Heart Circ Physiol* 289:H2281-2290 doi:00618.2005
- 13
14 Ferrario CM, Varagic J The ANG-(1-7)/ACE2/mas axis in the regulation of nephron function. *Am J Physiol*
15 *Renal Physiol* 298:F1297-1305 doi:ajprenal.00110.2010
- 16
17 Font-Llitjos M et al. (2007) Slc7a9 knockout mouse is a good cystinuria model for antilithiasic pharmacological
18 studies. *Am J Physiol Renal Physiol* 293:F732-740 doi:10.1152/ajprenal.00121.2007
- 19
20 Frech K, Danescu-Mayer J, Werner T (1997) A novel method to develop highly specific models for regulatory
21 units detects a new LTR in GenBank which contains a functional promoter. *J Mol Biol* 270:674-687
22 doi:S0022-2836(97)91140-2
- 23
24 Hashimoto T et al. (2012) ACE2 links amino acid malnutrition to microbial ecology and intestinal
25 inflammation. *Nature* 487:477-481 doi:10.1038/nature11228
- 26
27 Igase M, Strawn WB, Gallagher PE, Geary RL, Ferrario CM (2005) Angiotensin II AT1 receptors regulate
28 ACE2 and angiotensin-(1-7) expression in the aorta of spontaneously hypertensive rats. *American*
29 *journal of physiology Heart and circulatory physiology* 289:H1013-1019
30 doi:10.1152/ajpheart.00068.2005
- 31
32 Kim DK et al. (2002) The human T-type amino acid transporter-1: characterization, gene organization, and
33 chromosomal location. *Genomics* 79:95-103 doi:10.1006/geno.2001.6678
- 34
35 Kleta R et al. (2004) Mutations in SLC6A19, encoding B0AT1, cause Hartnup disorder. *Nat Genet* 36:999-1002
- 36
37 Kowalczyk S, Broer A, Munzinger M, Tietze N, Klingel K, Broer S (2005) Molecular cloning of the mouse
38 IMINO system: an Na⁺- and Cl⁻-dependent proline transporter. *Biochem J* 386:417-422
39 doi:BJ20050100
- 40
41 Kowalczyk S, Broer A, Tietze N, Vanslambrouck JM, Rasko JE, Broer S (2008) A protein complex in the
42 brush-border membrane explains a Hartnup disorder allele. *FASEB J* 22:2880-2887 doi:fj.08-107300
- 43
44 Kuba K, Imai Y, Ohto-Nakanishi T, Penninger JM (2010) Trilogy of ACE2: a peptidase in the renin-angiotensin
45 system, a SARS receptor, and a partner for amino acid transporters. *Pharmacol Ther* 128:119-128
46 doi:S0163-7258(10)00141-5
- 47
48 Mariotta L et al. (2012) T-type amino acid transporter TAT1 (Slc16a10) is essential for extracellular aromatic
49 amino acid homeostasis control. *J Physiol* 590:6413-6424 doi:10.1113/jphysiol.2012.239574
- 50
51 Meier C, Ristic Z, Klauser S, Verrey F (2002) Activation of system L heterodimeric amino acid exchangers by
52 intracellular substrates. *EMBO J* 21:580-589
- 53
54
55
56
57
58
59
60
61
62
63
64
65

- Meier Y et al. (2007) Regional distribution of solute carrier mRNA expression along the human intestinal tract. *Drug Metab Dispos* 35:590-594 doi:dmd.106.013342
- Naruhashi K, Sai Y, Tamai I, Suzuki N, Tsuji A (2002) PepT1 mRNA expression is induced by starvation and its level correlates with absorptive transport of cefadroxil longitudinally in the rat intestine. *Pharm Res* 19:1417-1423
- Nishimura M, Naito S (2005) Tissue-specific mRNA expression profiles of human ATP-binding cassette and solute carrier transporter superfamilies. *Drug Metab Pharmacokinet* 20:452-477 doi:JST.JSTAGE/dmpk/20.452
- Palacin M et al. (2005) The genetics of heteromeric amino acid transporters. *Physiology* 20:112-124, 2005. doi:10.1152/physiol.00051.2004
- Pan X, Hussain MM (2009) Clock is important for food and circadian regulation of macronutrient absorption in mice. *Journal of lipid research* 50:1800-1813 doi:10.1194/jlr.M900085-JLR200
- Ramadan T, Camargo SM, Summa V, Hunziker P, Chesnov S, Pos KM, Verrey F (2006) Basolateral aromatic amino acid transporter TAT1 (Slc16a10) functions as an efflux pathway. *J Cell Physiol* 206:771-779 doi:10.1002/jcp.20531
- Ristic Z et al. (2006) Neutral amino acid transport mediated by ortholog of imino acid transporter SIT1/SLC6A20 in opossum kidney cells. *Am J Physiol Renal Physiol* 290:F880-887 doi:10.1152/ajprenal.00319.2005
- Romeo E et al. (2006) Luminal kidney and intestine SLC6 amino acid transporters of B0AT-cluster and their tissue distribution in *Mus musculus*. *Am J Physiol Renal Physiol* 290:F376-383
- Scheppach W et al. (1996) Effect of L-glutamine and n-butyrate on the restitution of rat colonic mucosa after acid induced injury. *Gut* 38:878-885
- Seow HF, Broer S, Broer A, Bailey CG, Potter SJ, Cavanaugh JA, Rasko JE (2004) Hartnup disorder is caused by mutations in the gene encoding the neutral amino acid transporter SLC6A19. *Nat Genet* 36:1003-1007 doi:10.1038/ng1406
- Singer D et al. (2012) Defective intestinal amino acid absorption in *Ace2* null mice. *Am J Physiol Gastrointest Liver Physiol* doi:ajpgi.00140.2012
- Takanaga H, Mackenzie B, Suzuki Y, Hediger MA (2005) Identification of mammalian proline transporter SIT1 (SLC6A20) with characteristics of classical system imino. *J Biol Chem* 280:8974-8984 doi:10.1074/jbc.M413027200
- Terada T et al. (2005) Expression profiles of various transporters for oligopeptides, amino acids and organic ions along the human digestive tract. *Biochem Pharmacol* 70:1756-1763 doi:S0006-2952(05)00635-0
- Tumer E, Broer A, Balkrishna S, Julich T, Broer S (2013) Enterocyte-specific regulation of the apical nutrient transporter SLC6A19 (B0AT1) by transcriptional and epigenetic networks. *J Biol Chem* 288:33813-33823 doi:10.1074/jbc.M113.482760
- Verrey F, Meier C, Rossier G, Kuhn LC (2000) Glycoprotein-associated amino acid exchangers: broadening the range of transport specificity. *Pflugers Arch* 440:503-512
- Wehkamp J et al. (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 53:1658-1664 doi:53/11/1658

Werner C, Poss J, Bohm M (2010) Optimal antagonism of the Renin-Angiotensin-aldosterone system: do we
need dual or triple therapy? Drugs 70:1215-1230 doi:10.2165/11537910-000000000-00000

Figure 1

Figure 2
A

B

Figure 3
[Click here to download high resolution image](#)

Figure 4

A

B

Figure 5

A **B** **C** **D**