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ORIGINAL ARTICLE

Angiotensin II exerts dual actions on sodium-glucose transporter 1-mediated transport in the human jejunal mucosa

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Abstract

Objectives. Intestinal glucose absorption is mainly mediated via the sodium-glucose transporter 1 (SGLT1) at the apex of the enterocytes, whereas the glucose transporter 2 (GLUT2) provides a basolateral exit. It has been shown in rats that Angiotensin II (AngII), the principal mediator of renin-angiotensin system (RAS), inhibits jejunal SGLT1-mediated glucose absorption. The aim of the present study was to investigate if a similar mechanism exists also in the human jejunal mucosa. ***Material and methods.*** Enteroscopy with mucosal biopsy sampling was performed in 28 healthy volunteers. Functional assessments were performed in Ussing chambers using a pharmacological approach. Western blotting and immunohistochemistry were used to assess the presence of the AngII type 1 (AT1R) and type 2 receptor (AT2R), as well as the glucose transporters SGLT1 and GLUT2. ***Results.*** Exposure of the mucosa to 10 mM glucose elicited a ≈50% increase in the epithelium-generated current (Iep). This glucose-induced electrogenic response was sensitive to the competitive SGLT1 inhibitor phlorizin, but not to AngII when given alone. AngII combined with the AT2R blocker PD123319 markedly inhibited the response. AngII in combination with the AT1R antagonist losartan tended to increase the electrogenic response, whereas direct activation of AT2R using the agonist C21 significantly enhanced the mucosal response to glucose. The AT1R and AT2R as well as SGLT1 and GLUT2 were detected inside the human enterocytes. ***Conclusions.*** The pharmacological analysis indicated that activation of AT1R inhibits, whereas activation of AT2R enhances SGLT1-mediated glucose transport in the human jejunal mucosa.

Key Words: *angiotensin II type 2 receptor, glucose transporters, human, intestinal, jejunum, renin-angiotensin system, Ussing chamber*

Introduction

Small intestinal glucose absorption plays a pivotal role in glycemic control. Textbooks teach that the main pathway for intestinal glucose uptake into the enterocytes is via sodium-linked co-transport employing the phlorizin-sensitive sodium-glucose transporter 1 (SGLT1), a symporter located apically in the enterocytes. The sodium-glucose transport is driven by a sodium gradient caused by a basolaterally located active sodium-potassium exchanger, whereas glucose exits the enterocyte through the glucose transporter 2 (GLUT2) being situated basolaterally. These principal glucose transporters of the enterocyte have been extensively studied over the last decades and an intricate regulation of their activity and expression has been established [1,2]. However, most of the studies have been conducted on rodent models or in cell cultures, and several paradigmatic features have not yet been confirmed in man [3]. The present investigation was an attempt to test in human jejunal mucosa if the hormone Angiotensin II (AngII) has

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potential to influence epithelial glucose transport as described in rats by Wong et al [4,5]. AngII is the principal mediator of the renin-angiotensin system (RAS) being well-known for its involvement in fluid/electrolyte homeostasis and blood pressure control. Most RAS components, for example the prohormone angiotensinogen, the angiotensin-forming enzymes and angiotensin-receptors, can be expressed also locally in many tissues and can exert paracrine actions influencing, for example epithelial transport and inflammation, or contribute to tissue remodeling [6]. Wong et al. showed in rats that the small intestinal epithelial brush border membrane has the capacity for local formation of AngII. The authors also demonstrated that activation of the AngII type 1 receptor (AT1R) inhibited SGLT1-mediated glucose absorption [4]. Furthermore, this mechanism was shown to be downregulated in experimental (streptozotocin-induced) type-1 diabetes in association with a relocation of GLUT2 to the brush border of the enterocytes resulting in an un-controlled glucose uptake [5]. We and others have previously shown that also the human jejunum harbors Ang II receptors of both subtypes [7,8], but potential impact on glucose transport has not yet been investigated in man. The aim of the present study, therefore, was to assess the effect of AngII via either receptor subtype on glucose-induced SGLT1-mediated epithelial transport *in vitro*. Jejunal mucosae from healthy volunteers were mounted in Ussing chambers and were evaluated using a pharmacological approach. A secondary aim was to confirm the presence of AT1R and AT2R as well as the principal glucose transporters in the jejunal epithelium under study.

Material and methods

Ethics statement

The study was performed in accordance with the Declaration of Helsinki and was approved by the Regional Ethical Review Board in Gothenburg, Sweden (no: 810-11). All study participants were informed verbally and in writing and signed a consent form. The investigation was performed at the Department of Gastrosurgical Research & Education, at the Sahlgrenska Universityhospital in Gothenburg, Sweden.

Study participants

Eligible participants were recruited from previous studies or by advertising on notice boards locally at the hospital and at the medical school. A structured interview (focusing on general health, potential gastrointestinal disorders, and previous abdominal surgery) was used to confirm a normal health status. Inclusion criteria were: voluntary participation, self-reported general healthy state, age between 18 and 65 years, body mass index (BMI) between 18 and 25 kg/m^2. Exclusion criteria were: overweight or obesity (BMI >25 kg/m^2); history of drug abuse; use of prescription medication within the previous 14 days (with the exception of contraceptives); pregnancy or breast feeding, or potentially childbearing women not using adequate birth control (e.g. IUD, barrier method, oral contraceptive, abstinence); in the investigator's judgment, clinically significant abnormalities at the screening examination or in the laboratory test results. Twenty-eight healthy volunteers (13 females) with a mean age 24 years (range 19–32 years), with a BMI of 23.1 (range 19–26.5 kg/m^2) participated in the study.

Enteroscopy

The healthy volunteers arrived at the hospital's endoscopy unit in the morning after an overnight fast. After conscious sedation with midazolam and alfentanil, an enteroscope (*i.e.* a thin-calibered pediatric colonoscope) was introduced into the gastroduodenum and proximal jejunum. Eight to twelve biopsies were harvested in the jejunum approximately 50 cm distal to the ligament of Treitz. Four to six jejunal biopsies were either snap frozen or chemically fixated for later expression analyses. The remaining biopsies were prepared for functional assessments in mini-Ussing chambers.

Ussing chamber experiments

Jejunal biopsies were immediately immersed in ice-cold oxygenated (95% O_2 and 5% CO_2) Krebs solution with the following composition (in mM): 118.07 NaCl; 4.69 KCl; 2.52 $CaCl_2$; 1.16 $MgSO_4$; 1.01 NaH_2PO_4; 25 $NaHCO_3$; and 11.10 glucose. They were then mounted in mini-Ussing chambers that had a biopsy insert with a diameter of 2 mm and a square area of 0.034 cm^2 (Warner instruments, Hamden, CT, USA). After mounting, each half chamber was filled with 5 mL Krebs solution, bathing both the mucosal and serosal sides of the specimen. The solution was maintained at 37°C and continuously oxygenated with 95% O_2 and 5% CO_2 and stirred by gas flow in the chambers. Initially, the Krebs solution on the luminal side was held glucose free to allow study of effects of adding glucose (see below). Potential difference (PD) was measured with a pair of matched calomel electrodes (REF401, Radiometer analytical, Denmark). The Ussing Pulse Method (UPM) was used to determine the tissue's epithelial electrical

resistance (Rep). The epithelial net ion current (I_{ep}) was obtained using Ohm's law (I_{ep} = PD/Rep). The UPM has been described in detail elsewhere [9]. Briefly, the method is based on the concept that the epithelium acts as a capacitor and resistor coupled in parallel. Short current pulses charge the epithelial capacitor and when the current ends, the capacitor is gradually discharged. The epithelial voltage response as assessed from the discharge curve and the magnitude of the applied current were used for calculation of Rep. The data were collected using an amplifier and specially constructed software developed in LabView (National Instruments, Austin, TX, USA).

Experimental procedures. After an equilibration period of 10 min, basal values were recorded over 15 min. To be eligible for experimentation, each preparation had to exhibit a lumen-negative PD of at least 0.5 mV at baseline. After serosal addition of the AT1R antagonist losartan (10^{-6} M) (Merck, NJ, USA), or of the AT2R antagonist PD123319 (10^{-6} M) (Sigma-Aldrich, Stockholm, Sweden), or the AT2R agonist C21 (10^{-6} M) [10], or vehicle (Krebs solution = Control), a run-in period of 5 min was allowed. AngII was then added to the serosal compartment at a concentration of 10^{-6} M. To induce an electrogenic response, D-glucose (Sigma Chem. Inc., St Louis, MO, USA) was added to the luminal compartment after another 5 min at a concentration of 10 mM. Due to an expected short tissue viability (≈60–90 min), all AngII receptor agonists and antagonists were given at a single concentration (10^{-6} M) based on results from previous studies on human esophageal preparations *in-vitro* [9,11]. To be included in the analysis, at least two biopsies from each individual had to be successfully mounted, allowing one to serve as time-control and the other(-s) to be used for pharmacological intervention. In addition to the AngII receptor binding compounds, the SGLT1-inhibitor phlorizin was used at a luminal concentration of 0.3 mM (Sigma-Aldrich) to ascertain that the glucose-induced response was mediated by SGLT1.

Western blot analyses

Specimens were snap-frozen in liquid nitrogen and kept frozen for later Western blot analysis. The frozen specimens were sonicated in a PE buffer (10 mM potassium phosphate buffer, pH 6.8 and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS: Boehringer Mannheim, Mannheim, Germany) and protease inhibitor cocktail tablet Complete (Roche Diagnostics AB, Stockholm, Sweden). The homogenate was then centrifuged (10,000 *g* for 10 min at 4°C) and the supernatant was analyzed for protein content by the Bradford method and stored at −70°C [12]. The samples were diluted in SDS buffer and heated at 70°C for 10 min and then loaded on a NuPage 10% Bis-Tris gel. Electrophoresis was run using a MOPS buffer (Invitrogen AB, Lidingö, Sweden). The gel was loaded with a prestained molecular weight standard (SeeBlue, NOVEX, San Diego, CA, USA). After the electrophoresis, the proteins were transferred to a polyvinyldifluoride transfer membrane (Hybond, 0.45 μm, RPN303F, Amersham, Buckinghamshire, UK) using the iBlot dry blotting system (Invitrogen AB). The membranes were than incubated with specific antibodies targeting AT1R and AT2R, as well as the glucose transporters SGLT1 and GLUT2 (for specification see Table I). An alkaline phosphatase conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibody (Santa Cruz) and CDP-Star (Tropix, Bedford, MA, USA) were used as a substrate to identify immunoreactive proteins by means of chemiluminescense. Images were captured by a Chemidox XRS cooled CCD camera and analyzed with Quantity One software (Bio-Rad laboratories, Hercules, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control for equal loading for each tested sample. Data are presented as the ratio between optical density of primary antibody and of GAPDH in each sample. The membrane was stripped for new primary antibodies using stripping buffer (Re-Blot Plus Mild Solution (10X), Millipore, Temecula, CA, USA).

Table I. Antibodies used in Western blots and immunohistochemistry.

Primary antibody	Catalogue number	Supplier
AT1R	N-10	Santa Cruz Biotechnology, Heidelberg, Germany
AT2R	H-143	Santa Cruz Biotechnology, Heidelberg, Germany
GAPDH	IMG-5143A	Imgenex, BioSite, San Diego, CA, USA
GLUT2	ab85715	Abcam, Cambridge, UK
SGLT1	ab14686	Abcam, Cambridge, UK

Abbreviations: AT1R and AT2R = angiotensin II type 1 and type 2 receptor, respectively; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; GLUT2 = glucose transporter 2; SGLT1 = sodium-glucose cotransporter 1.

Immunohistochemistry

Immunohistochemistry was used to demonstrate the intraepithelial location of AngII receptors in the

jejunal mucosa. Antigen retrieval was performed on deparaffinized slides by boiling them in 10 mM citrate buffer (pH 6.0). After blockade of peroxidase activity, the slides were pre-incubated with serum block followed by incubation with respective primary antibody (Table I; minimal staining of at least three slides from separate individuals per antibody) over night according to the suppliers' recommendations. Negative control sections were incubated with normal mouse/rabbit IgG instead of primary antibody. To enhance the detection signal, the slides were incubated with a biotinylated secondary antibody (goat anti-rabbit/goat anti-mouse) and the protein-antibody complex was detected using horseradish peroxidase-streptavidin and developed with the color 3,3′-diaminobenzidine (Dako K1497/K1501 staining systems). All samples were background stained with hematoxylin (Santa Cruz Biotechnology, Heidelberg, Germany).

Statistics

Normal distribution of the data sets was confirmed using the Shapiro-Wilk test. Differences between groups were investigated using ANOVA and if indicated, contrasted with the un-paired t test. All analyses were performed using Prism 6 for Mac OS X (GraphPad Inc, LaJolla, CA, USA). A *p*-Value below 0.05 was considered significant. In the presentation below, the number of individuals is denoted with '*n*' and the number of Ussing preparations with '*N*'.

Results

Glucose-induced electrogenic transport in vitro

Data from 18 subjects allowed analyses (i.e. at least two successful Ussing chamber preparations from the same individual). The Ussing chambers were initially prepared with a glucose-free medium at the mucosal side (baseline condition). At baseline with a glucose-free luminal medium, the mucosal preparations spontaneously produced an epithelial potential difference (PD) ranging from 0.69 to 2.7 mV (luminal side negative), epithelial electrical resistance (Rep) ranging from 4.1 to 11.3 $\Omega^{\star}cm^2$ and epithelial current (Iep) ranging from 96 to 482 $\mu A/cm^2$ ($n = 18$, $N = 18$). When D-glucose (10 mM) was added to the luminal compartment in untreated control preparations Iep increased by ≈50% from the individual baseline, whereas Rep decreased marginally (Figure 1). In the presence of the selective SGLT1-inhibitor phlorizin, the addition of glucose was associated with a very small Iep response reaching only 6% above baseline ($p < 0.001$).

Effects of angiotensin II

In the AngII-treated preparations, the addition of glucose raised Iep with a magnitude (44%) not different from time controls ($p = 0.46$). When AngII was combined with the AT2R selective antagonist PD123319, the addition of glucose was associated with smaller Iep responses (mean 17% above baseline) compared to those when AngII was given alone ($p = 0.031$). The combination of AngII and the AT1R antagonist losartan was associated with a somewhat larger Iep response (mean +57% above baseline) compared with AngII alone, but the difference did not attain statistical significance. The Iep responses to glucose in preparations treated with the combination of AngII and the AT2R agonist C21 reached ≈75% above baseline (Figure 1). These responses were significantly larger compared to those with AngII alone ($p = 0.038$) or with the combination of AngII and PD123319 ($p = 0.001$) (Figure 1). Rep was not significantly influenced by any of the tested compounds (Figure 1, upper panel).

Mucosal expression of components of AngII receptors

Western blotting exhibited AT1R and AT2R at expected molecular weights in all examined samples of jejunal mucosa (Figure 2, upper panels). Immunohistochemistry showed staining for AT1R and AT2R in association to blood vessels and in some mesenchymal cells located in the lamina propria. Both AT1R and AT2R were markedly stained in the apical cytosolic compartment in most of the enterocytes of the villi, whereas the crypt cells were less stained (Figure 2A and B). Immunoreactivity to AT1R was found also in the enterocyte brush border of some examined villi, then with a less marked cytosolic staining (Figure 2A, middle panel). AT1R staining was also detected at some epithelial cells with a shape corresponding to enteroendocrine cells (Figure 1A).

Glucose transporters

Immunoblotting indicated that both SGLT1 and GLUT2 were well expressed in all samples of the human jejunum (Figure 3, upper panels). Immunoreactivity to SGLT1 was predominantly localized to the apical cytosolic compartment and to the brush border membrane of the villous enterocytes (Figure 3 lower panel). It was noted that several of the examined villi lacked SGLT1 brush border staining (Figure 3A, middle panel). As expected, GLUT2 was localized basolaterally in the enterocytes (Figure 2B). Most examined villi exhibited staining in the supranuclear

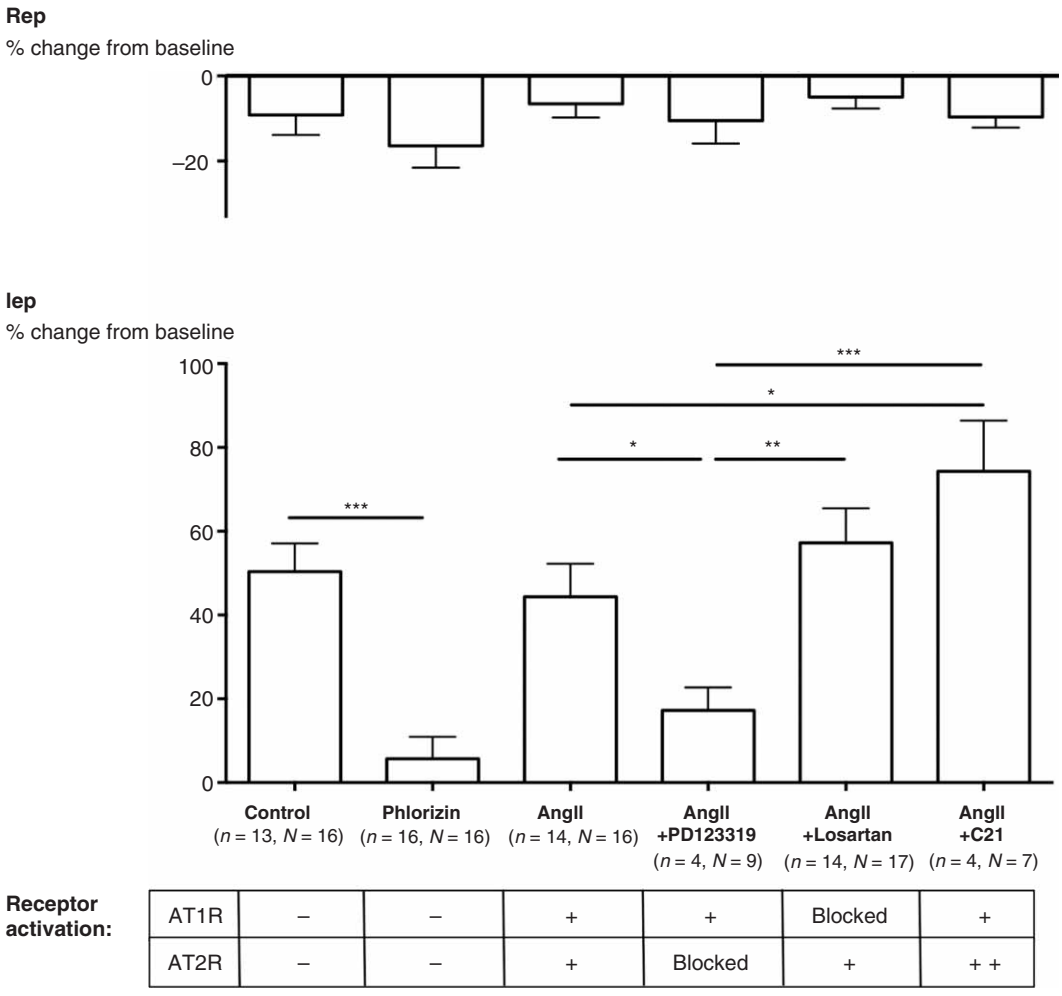

Figure 1. Jejunal mucosal specimens mounted in Ussing chambers. The upper graphs show % change from the individual baseline regarding epithelial electrical resistance (Rep) and epithelial current (Iep) following exposure to 10 mM D-glucose (at the luminal side) in untreated controls and in the presence of phlorizin (0.3 mM). The glucose-induced response was also tested in the presence of 10^{-6} M AngII, as well as AngII in combinations with PD123319 (10^{-6} M), losartan (10^{-6} M) or C21 (10^{-6} M). Values are given as mean ± SEM, the number of individuals is indicated *n* and preparations *N*. Significant differences are denoted by * at $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$. The lowest graph illustrates the expected AngII receptor activation following the various pharmacological treatments.

cytosolic compartment of the enterocytes. The crypts cells were generally very little stained (not shown).

Discussion

To characterize SGLT1-mediated transport in the human jejunal mucosa, we used mini-Ussing chambers and assessed the phlorizin-sensitive epithelium-generated electrical current (Iep) in response to glucose exposure. If correctly mounted (i.e. no leakage detected and with a viability in terms of a distinct PD signal), changing the luminal glucose concentration from zero to 10 mM raised the Iep by ≈50% indicating the activation of an electrogenic transport process. Presence of the compound phlorizin markedly reduced or abolished this glucose-induced response confirming that the transport mechanism in action was mainly SGLT1 dependent [13]. In contrast to the data from rat experiments by Wong et al. [4], the addition of AngII alone in the present study on human mucosa did not inhibit glucose transport (i.e. the glucose induced electrogenic response). The combined presence of AngII and the AT2R-selective pharmacological blocker PD123319 (thus a condition compatible with a selective AT1R stimulation as indicated in Figure 1, lower panel) resulted in a markedly reduced glucose induced response. The reasonable explanation is that AngII, having similar affinity to its two subreceptors, in the presence of a selective AT2R antagonist predominantly activates the still un-opposed AT1-receptor that in turn mediates inhibition of the electrogenic response to glucose. However, AngII combined with the selective AT1R antagonist losartan (thus compatible with a

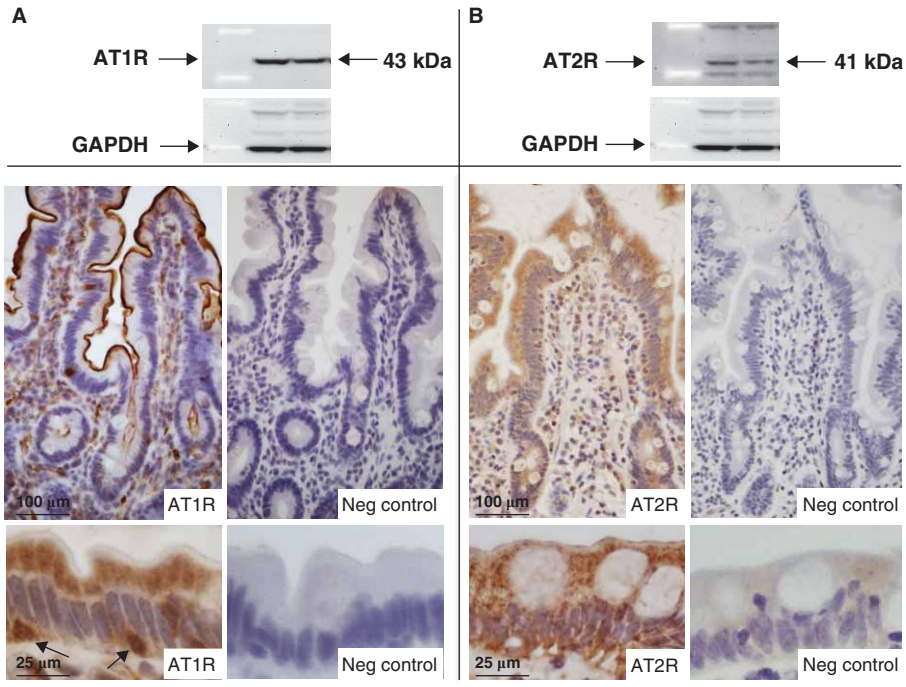

Figure 2. Protein expression analyses of AT1R (panels A) and AT2R (panels B) in human jejunal mucosal biopsies. Upper panels show representative western blots from two subjects. The middle panels show representative examples of immunohistochemical staining (brown) of AT1R and AT2R with corresponding negative controls (*n b* both projections are background stained with hematoxylin). Arrows indicate AT1R immunoreactivity at cells with a shape typical of enteroendocrine cells. Note that AT1R immunoreactivity could be detected in association to the apical brush border membrane (A, middle panel), but occasionally only in the subapical cytosolic compartment (A, lower panel). Arrows indicate enteroendocrine-like cells with dense AT1R-immunoreactivity.

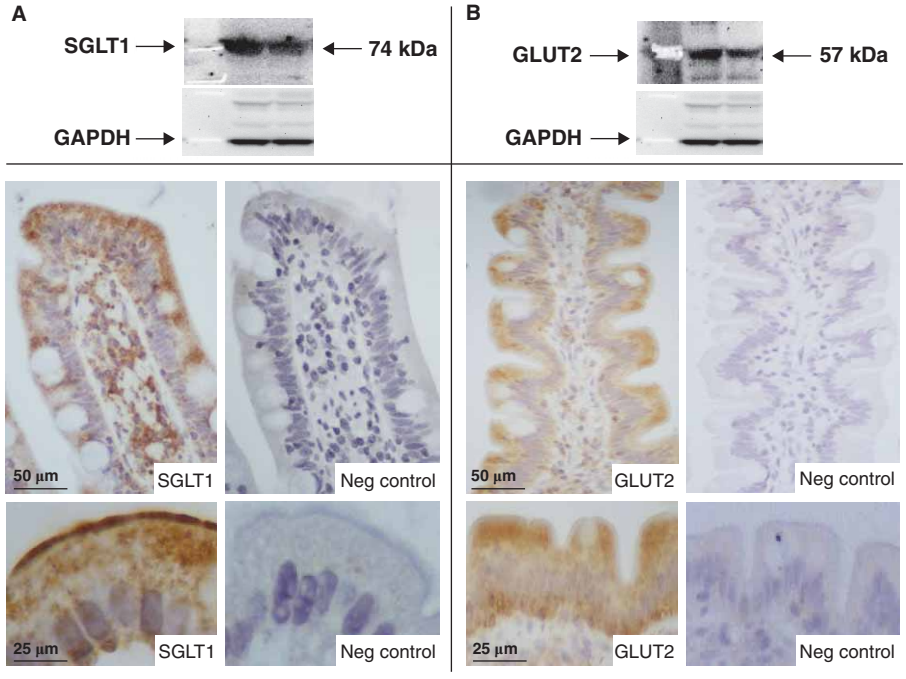

Figure 3. Expression analyses of SGLT1 (panels A) and GLUT2 (panels B) in human jejunal mucosal biopsies. The upper panels show representative western blots from two subjects. The middle panels show representative examples of immunohistochemical staining (brown) of SGLT1 and GLUT2 with corresponding negative controls (*n b* both projections are background stained with hematoxylin). Note that the apical brush-border membrane occasionally lacked distinct SGLT1-staining (A, middle panel) and that SGLT1 and GLUT2 were frequently detected in the supranuclear cytosolic compartment.

AT2R stimulation) did not result in a significant effect, although there was a border-significant trend towards increased Iep. Thus, this experiment did not support an AT2R-mediated effect. Still, when AT2Rs were activated by the presence of the receptor-selective non-peptide agonist C21, the electrogenic response was significantly enhanced. The latter finding thus speak in favor of that AT2R-activation reinforces the SGLT1-mediated electrogenic response. Taken together, the pharmacological analysis indicated that AT1R activation inhibits jejunal glucose/sodium absorption, whereas the AT2Rs exert the opposite action. Such a Yin-Yang principle of the two AngII subreceptors is well-known and has been demonstrated in several tissues and also in the gut [6,11,14–18]. Simultaneous activation of the opposing AT1R and AT2R also explains why addition of AngII *per se* did not result any change in Iep.

The failure of AngII in combination with losartan to significantly activate the epithelial transport is intriguing and deserves some consideration. Theoretically, the AT1R blockade should result in that AngII predominantly activates the AT2R (see Figure 1, lower panel). It may be that losartan at 10^{-6}M was at a subthreshold concentration for "shunting" the ligand AngII towards the AT2R. An alternative explanation is that the number of AT2R in the tissue was low. It is obvious that future studies must include concentration–response relationships and ideally also quantitative assessment of the AngII receptor subtypes on an individual level. The latter may be of importance as the expression of AngII receptors is variable and particularly so regarding AT2R being preferably expressed in conditions characterized of tissue restitution and anti-inflammatory actions [19]. In the present study, we confirmed protein expression of both AngII receptor types and SGLT1 in the mucosal biopsies supporting the results from the pharmacological analyses. In addition to the expected association to vascular structures, immunoreactivities to both AT1R and AT2R were also found in the supranuclear cytosolic compartment of the enterocytes, thus in close association to the glucose transporters. Immunoreactivity to AT1R was occasionally observed also in the brush border as reported to be the case in the rat [4,5]. However, the brushborder localization was not a consistent finding in the presently examined human jejunal samples indicating a possible species difference. Interestingly, some epithelial cells with an enteroendocrine morphological appearance exhibited AT1R-immunoreactivity. Such AT1R-bearing endocrine cells have previously been observed in the human gastric antrum [20] suggesting that AngII can influence the synthesis and liberation of gastrointestinal hormones. In the present study, the glucose transporter SGLT1 was only occasionally localized to the apical brush border membrane of the enterocytes. Furthermore, a marked supranuclear vesicular staining was observed in many enterocytes. A cytosolic localization of SGLT1 below the brush border membrane is not according to the paradigm (see e.g. 8) but has indeed been previously reported in human enterocytes [2,21]. It can be speculated that the long-lasting fasting period preceding the enteroscopic procedure with an associated lack of luminal glucose may be associated with an internalization of the glucose transporter. Such a phenomenon has been reported during energy deficit in cholangiocytes [22] and may hypothetically be present also in the intestinal mucosa in the interdigestive state of intermittent feeders like the human being. In the present study, we found that immunoreactivity to GLUT2 was consistently localized to the basolateral membrane, but staining was found also in the cytosol between the nuclei and the brush border membrane. A relocalization of GLUT2 from basolateral to apical localization has been claimed to occur in the postprandial state to maximize glucose uptake [1,5,23]. The cytosolic localization of GLUT2 in the present study can, as above suggested for SGLT1, represents the picture in the interdigestive state. However, intracellular trafficking of GLUT2 has been seriously questioned and has become a controversial topic in this field of science [2,24]. Parts of the controversy can be species dependent and the human small intestinal mucosa has to be further investigated in this regard.

The potential clinical relevance of AT1R and AT2R in the small intestinal mucosa is so far unknown. Several clinical studies show that interference with the AngII system improves insulin resistance and diabetes, partly with unknown mechanisms of action [25]. Considering the important role of intestinal glucose absorption in glycemic control the actions of enterocyte AngII receptors certainly deserve attention in future research.

In summary, the present investigation in the healthy human jejunal mucosa provides data indicating an AT1R mediated inhibition of SGLT1-mediated glucose uptake, whereas activation of AT2R instead enhances this transport.

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