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Local expression of AP/AngIV/IRAP and effect of AngIV on glucose-induced epithelial transport in human jejunal mucosa

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Abstract

Background: Recently it was shown that the classic renin-angiotensin system (RAS) is locally expressed in small intestinal enterocytes and exerts autocrine control of glucose transport. The aim of this study was to investigate if key components for the Angiotensin III (AngIII) and IV (AngIV) formation enzymes and the AngIV receptor, insulin-regulated aminopeptidase (IRAP), are present in the healthy jejunal mucosa. A second aim was to investigate AngIV effects on glucose-induced mucosal transport in vitro.

Material and methods: Enteroscopy with mucosal biopsy sampling was performed in healthy volunteers. ELISA, Western blotting and immunohistochemistry were used to assess the protein levels and localization. The functional effect of AngIV was examined in Ussing chambers.

Results: The substrate Angiotensin II, the enzymes aminopeptidases-A, B, M as well as IRAP were detected in the jejunal mucosa. Immunohistochemistry localized the enzymes to the apical brush-border membrane whereas IRAP was localized in the subapical cytosolic compartment in the enterocyte. AngIV increased the glucose-induced electrogenic transport in vitro.

Conclusion: The present study indicates the presence of substrates and enzymes necessary for AngIV formation as well as the receptor IRAP in the jejunal mucosa. The functional data suggest that AngIV regulates glucose uptake in the healthy human small intestine.

Keywords

Aminopeptidase, angiotensin III, angiotensin IV, IRAP, human jejunal epithelium, Ussing chambers

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Introduction

Metabolism refers to all life-maintaining chemical processes of cells, and glucose is one of the fundamental energetic substrates for most cell types. Intestinal glucose uptake is mediated by the sodium-glucose co-transporter 1 (SGLT1) situated at the luminal surface of the enterocytes, whereas the facilitative glucose transporter (GLUT2) is considered to provide basolateral exit for glucose.[1,2] The rate of glucose transport across the intestinal epithelium is dependent on various signals.[3] Pancreatic hormones such as insulin[4,5] and glucagon,[6] and gastrointestinal hormones like cholecystokinin,[7] gastric inhibitory polypeptide[8] and glucagon-like peptide-2[9] are known to regulate the rate of enterocyte glucose transport. The renin angiotensin system (RAS) is not only an endocrine system, but is also locally expressed in the enterocytes,[10] and has been demonstrated in rats to exert autocrine inhibition of intestinal glucose transport via the Angiotensin II (AngII) type 1 receptor

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(AT1R).[11–13] Recent data from our laboratory confirm that this mechanism is operational also in the human jejunum.[14] In addition, we were able to demonstrate that activation of the AngII type 2 receptor (AT2R) enhanced glucose-induced epithelial transport. Thus, with regard to SGLT1-mediated transport in the healthy human jejunal mucosa, there appears to be a classic antagonistic organization between the two AngII sub-receptors.[15]

However, in addition to the classic angiotensin-converting enzyme (ACE)-AngII axis acting on AT1R and AT2R, there are evidently a number of other angiotensins with biological actions, for example, the ACE2-Ang(1–7) axis acting primarily via the Mas receptor.[13] Another RAS pathway starts with formation of Angiotensin III (AngIII) from degradation of AngII by glutamyl aminopeptidase (AP-A; EC 3.4.11.7), cleaving the Asp1-Arg2 bond.[16,17] Angiotensin IV (AngIV) is then generated by the enzyme arginine aminopeptidase (AP-B; EC 3.4.11.6) or alanyl aminopeptidase (AP-M; also termed aminopeptidase-N; EC 3.4.11.2), eliminating arginine from the N-terminus of AngIII.[18] AngIV binds to the AngIV receptor (AT4R), which has been identified as insulin-regulated aminopeptidase (IRAP; EC 3.4.11.3).[19] IRAP is expressed in various organs such as brain, adrenal gland, kidney, lung and heart.[20,21] There are data suggesting that peptides such as vasopressin, oxytocin, cholecystokinin-8 and somatostatin are cleaved by IRAP in association with memory-enhancing properties.[22] Furthermore, IRAP is co-expressed with the glucose transporter GLUT4 in adipocytes and myocytes, thus enhancing insulin-regulated glucose uptake.[23] In a recent study we showed that the AP/AngIV/IRAP axis was expressed and functional in the human esophageal mucosa.[24] However, no data are available describing the effects of the AP/AngIV/IRAP axis in the human small intestine.

The aim of the present study was therefore to elucidate expression and localization of AP-A, AP-B, AP-M and the AngIV-receptor IRAP in the jejunal mucosa of healthy individuals. The second aim was to investigate if AngIV influences glucose-induced SGLT1-mediated transport across human jejunal epithelial specimens mounted in Ussing chambers.

Material and methods

Ethics statement

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee at the University of Gothenburg, Sweden, and by the Regional Ethical Review Board in Gothenburg. All individuals were informed verbally and in writing and signed a consent form. The investigation was performed at the Department of Gastrosurgical Research and Education at the Sahlgrenska University hospital in Gothenburg, Sweden.

Study participants

Sixteen participants (n = 16, mean age 26 years, range 18–31 years, eight females) 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 normal health status.

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 10 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.

Western blot analysis of jejunal mucosa

These specimens had been snap-frozen in liquid nitrogen and kept frozen. During the analysis, the specimens were thawed, sonicated and homogenized in a PE buffer (10 mM potassium phosphatase buffer, pH 6.8 and 1mM ethylenediaminetetraacetic acid (EDTA)) containing 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; Boehringer Mannheim, Mannheim, Germany) and protease inhibitor cocktail tablet Complete (Roche Diagnostics AB, Stockholm, Sweden). The homogenates of each sample were centrifuged (10,000× g, 10 minutes at 4°C) and the supernatants were analyzed regarding the protein content by using the Bradford method. The samples were diluted in sodium dodecyl sulfate (SDS) buffer, and heated at 70°C for 10 minutes before being loaded onto NuPage 10% Bis-Tris gel, and the proteins in each sample were separated with gel electrophoresis using 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Invitrogen AB, Lidingo, 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 incubated with the respective primary antibody AP-A, B, M, IRAP and the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). The membranes were incubated overnight at 4°C. To enhance the detection signal, a secondary antibody

Table 1. Antibodies used in Western blot analyses and immunohistochemistry.

Primary antibody	Catalog number	Supplier
AP-A	sc-18065	Santa Cruz Biotechnology, Heidelberg, Germany
AP-B	ab119761	Abcam, Cambridge, UK
AP-M	sc-166270	Santa Cruz Biotechnology, Heidelberg, Germany
IRAP	sc-365051	Santa Cruz Biotechnology, Heidelberg, Germany
GAPDH	IMG-5143A	IMGENEX, San Diego, CA, USA

AP-A, AP-B and AP-M: aminopeptidase A, B and M, respectively; IRAP: insulin-regulated aminopeptidase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

conjugated with alkaline phosphatase was linked to the primary antibody. The secondary antibody was detected by a chemoluminescent light (reagent CDP-Star, Tropix, Bedford, MA, USA), which was captured by a Chemidox XRS cooled charge-couple device camera and analyzed using Quantity One software (BioRad Laboratories, Hercules, CA, USA). GAPDH was used as a control for equal loading, and for each tested sample the optical density of primarily antibody/GAPDH represents the results. Each time the membrane was incubated with a new primary antibody, the previous antibody has been removed with stripping buffer (Re-Blot Plus Mild Solution (10x), Millipore, Temecula, CA, USA). The stripping order was as follows: AP-A (160 kDa), AP-B (75 kDa), AP-M (140 kDa), GAPDH (36 kDa) and IRAP (140 kDa).

Enzyme immunoassay

AngII levels were investigated by enzyme-linked immunosorbent assay (ELISA) analysis. The proteins were solubilized in the same way as for the Western blot samples described above. The ELISA plate and reagents were prepared and the analysis performed according to the manufacturer's instructions (angiotensin II enzyme immunoassay kit A05880-96 wells, Spi Bio Bertin Pharma, Montigny le Bretonneux, France). The principle of this enzyme immunoassay is that immobilized anti-AngII antibodies in the bottom of each of the 96 wells bind to potential AngII peptides in the solubilized biopsy samples. The antibody-AngII complex forms a yellow compound together with a tracer (acetyl- cholinesterase) and a chromogen (Ellman's reagent) and each sample's concentration is colorimetrically determined (absorbance at 405 nm, TECAN, Salzburg, Austria) subsequently compared with a prepared standard curve on the same ELISA plate. Concentration of AngII is then expressed in relation to total protein amount for each tested sample (pg/mg tissue protein). The limit of detectability was 0.09 pg/mg tissue protein and coefficient of variation (CV) was 3.9%.

Immunohistochemistry

The following method was used to present the intraepithelial location of alternative RAS components in the jejunal mucosa. Mucosa specimens were fixed in neutral buffered 4% formaldehyde, dehydrated, embedded in paraffin and then cut and mounted on the glass slides. The sections for immuhistochemistry were deparaffinized and boiled in 50 mM citrate buffer (pH 6.0) for 15 minutes for antigens retrieval. The catalyzed signal amplification (CSA) II biotin-free tyramide signal amplification staining system kit (Dako Sweden AB, Stockholm, Sweden) was used for the immunohistochemistry protocol. After blockade of endogenous biotin (15 minutes) and peroxidase activity (15 minutes), the slides were pre-incubated with the primary antibodies (AP-A, B, M and IRAP) in a dilution of 1:500 overnight (antibodies are described in Table 1). The negative control sections were incubated with wash buffer instead of the primary antibody. To enhance the detection signal, the slides were incubated with the biotinylated secondary antibody (goat anti-mouse/donkey anti-goat). This was followed by sequential incubation with an amplification reagent (fluorescyl-tyramide hydrogen peroxide) in darkness and anti-fluorescein-horseradish peroxidase (HRP), 15 minutes each. The color was developed using 3, 3' diaminobenzidine.

Ussing chamber experiments

To investigate the in vitro effects of AngIV, Ussing chambers experiments were performed. Jejunal biopsies from healthy individuals 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, and later they were 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). Initially, the serosal Ussing chamber reservoirs were filled with 5 ml Krebs, while the Krebs solution on the luminal side was kept glucose free to allow study of the effects of adding glucose (see below). The solution was oxygenated and stirred with a gas flow (95% O_2 and 5% CO_2) and kept at 37°C. The 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 (Iep) was obtained using Ohm's law (Iep = PD/Rep). The UPM has been described in detail elsewhere.[25] 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

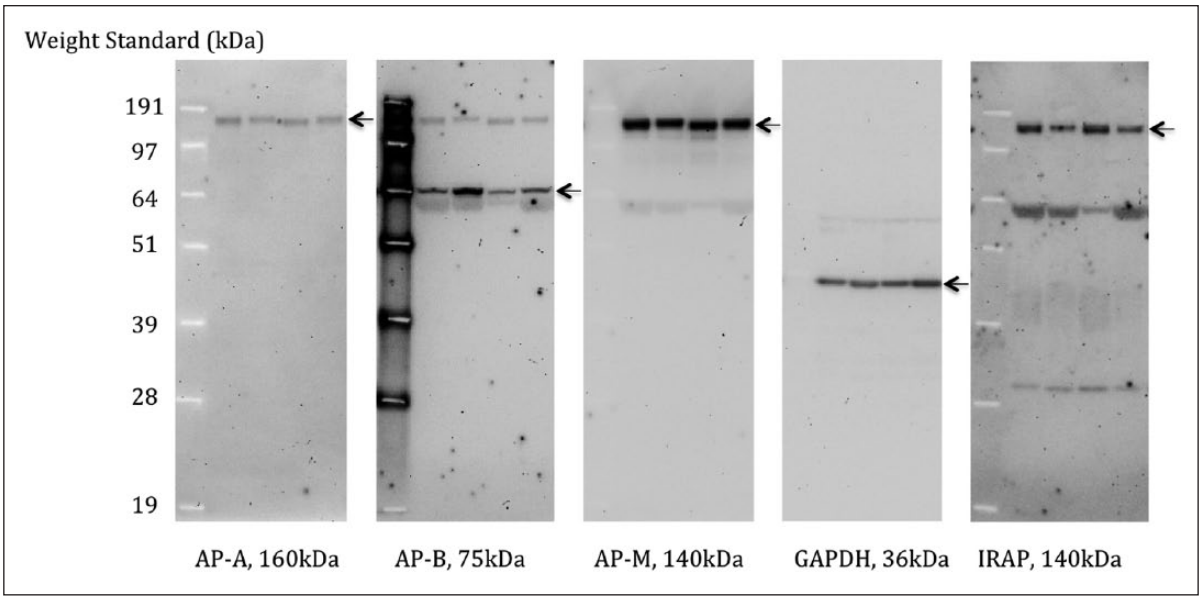

Figure 1. Representative Western blot protein bands (see arrow) demonstrate immunoreactivity to AP-A, AP-B, AP-M and IRAP, as well as the loading control GAPDH, in samples of healthy human jejunal mucosa. The first lane shows the weight standard followed by samples taken from a female and a male in turns. AP-A, AP-B and AP-M: aminopeptidase A, B and M, respectively; IRAP: insulin-regulated aminopeptidase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

assessed from the discharge curve and the magnitude of the applied current, was 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 protocol. After an equilibration period of 10 minutes, basal values were recorded over 15 minutes. 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 AngIV peptide (10^{-6}M) (Sigma-Aldrich, Stockholm, Sweden), a run-in period of 10 minutes was allowed. To induce an electrogenic response, D-glucose (Sigma Chem. Inc, St Louis, MO, USA) was then added to the luminal compartment at a concentration of 10 mM. To be included in the analysis, at least two biopsies from each individual had to be successfully mounted, allowing one to serve as a control and the other(s) to be used for pharmacological intervention. In addition to the AngIV peptide 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.

Statistical analysis

Kruskal-Wallis and Mann-Whitney *U* test for independent variables and Friedman's and Wilcoxon's signed rank test for related variables were used to analyze the differences. All analyses were performed using Prism 6 for Max OS X (GraphPad Inc, LaJolla, CA, USA). A p value of <0.05 was considered significant. The data are presented as mean ±SEM. The number of individuals was denoted as n and the number of preparations/observation as N.

Results

Expression of alternative RAS components in the human jejunal mucosa

The peptide AngII was detected in all samples and range between 0.15 and 15.4 pg/mg protein tissue (see Table 2, as supplementary information). The expression of the proteins AP-A, AP-B, AP-M and the IRAP were identified by Western blotting in all samples of jejunal mucosa (Figure 1). The protein expression of AP-A, AP-B, AP-M and the IRAP did not differ quantitatively between females and males. Immunohistochemistry showed staining for the enzyme AP-A, AP-B, AP-M and IRAP in most of the enterocytes lining the villi. Staining for AP-A and AP-M were distinctly observed in the enterocyte brush-border membrane (Figure 2(a) and (e)). Immunoreactivity to AP-B was predominantly localized to the apical cytosolic compartment and to the brush-border membrane, but some staining was also localized basolaterally in the enterocytes (Figure 2(c)). Staining for IRAP was particularly localized to the apical cytosolic compartment in the enterocytes (Figure 2(g)).

Glucose-induced electrogenic transport and AngIV effects in vitro

Data from 14 participants allowed analyses (i.e. at least two successful Ussing chamber preparations from the same individual). The Ussing chambers were initially

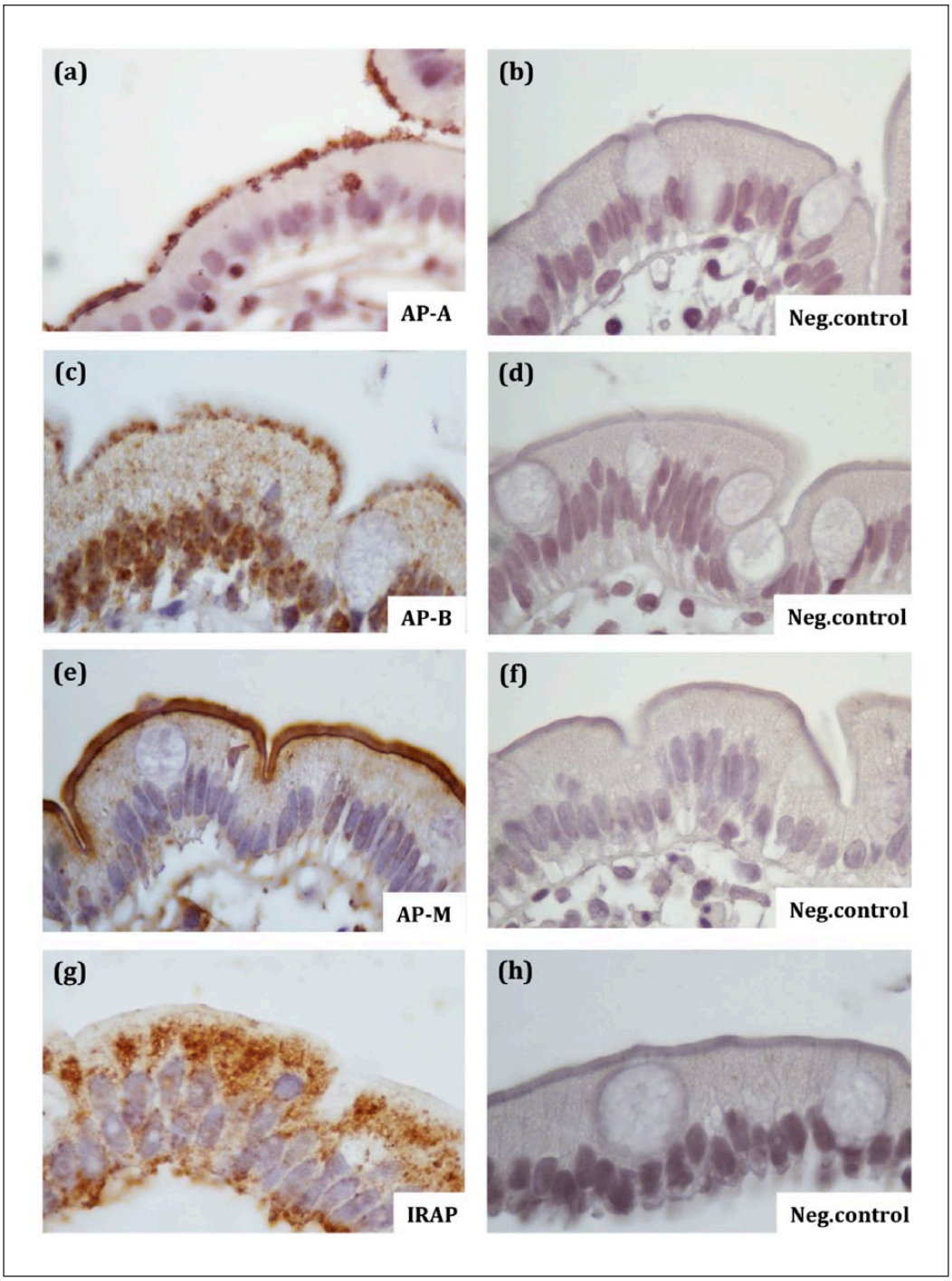

Figure 2. Representative examples of immunohistochemical staining (brown) of the AP-A, AP-B, AP-M and IRAP with corresponding negative controls (magnification 100×). Immunoreactivity to AP-A (a), AP-B (c) and AP-M (e) could be detected preferably at the apical brush-border membrane in the enterocytes. IRAP (g) was localized in the subapical cytosolic compartment. No immunoreactivity was detected in the negative controls (b, d, f and h). Background stained with hematoxylin eosin. AP-A, AP-B and AP-M: aminopeptidase A, B and M, respectively; IRAP: insulin-regulated aminopeptidase.

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 PD ranging from 0.51 to 4.69 mV (luminal side negative), Rep ranging from 2.6 to 13.0 Ω*cm^2 and Iep ranging from 88 to 511 mA/cm^2 (n = 14, N = 30). When D-glucose (10 mM) was added to the luminal compartment in untreated control preparations, the Iep increased by 28% from the individual baseline, whereas the Rep decreased marginally (Figure 3). In the presence

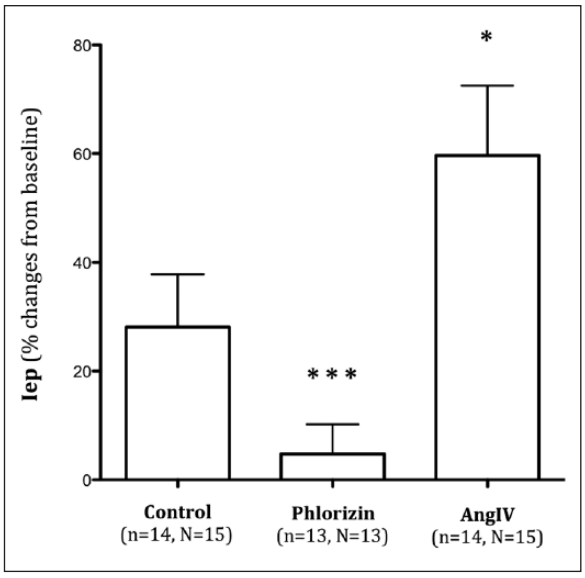

Figure 3. Jejunal mucosal specimens mounted in Ussing chambers. The graphs show the glucose-induced response regarding epithelial current (Iep) following exposure to 10 mM D-glucose (at the luminal side) in controls and in the presence of phlorizin (0.3 mM) and AngIV (10^{-6} M). Values are given as mean ± SEM. The number of individuals is indicated by *n* and preparations by *N*. Significant differences are denoted by *<0.05, and ***<0.001 vs. control (*p* value for related variables using Wilcoxon's signed rank test). AngIV: Angiotensin IV.

of the selective SGLT1-inhibitor phlorizin, the addition of glucose was associated with a very small Iep response reaching only 5% above baseline (p value < 0.001). In the AngIV-treated preparations, the Iep responses after the addition of glucose were significantly larger with a magnitude of 60% (p value = 0.043) compared to controls (Figure 3). Rep was not significantly influenced by AngIV. The Iep values before and after adding glucose are shown in Table 3 as supplementary information.

Discussion

In this study, we examined the expression of alternative RAS AP/AngIV/IRAP axis as well as the effects of AngIV on intestinal glucose uptake (i.e. the glucose-induced SGLT1-mediated electrogenic transport). The results confirmed the presence of the enzymes AP-A, AP-B, AP-M, as well as of substantial amounts of the substrate peptide AngII, indicating that both AngIII and AngIV can be produced locally in the human small intestinal mucosa. Moreover, the addition of the AngIV-peptide in vitro resulted in markedly increased glucose-induced electrogenic transport. Thus, the present results support that a local alternative RAS AP/AngIV/IRAP may exert a regulatory impact on glucose uptake in the human small intestine.

RAS is a potent regulatory system, exerting effects on the intracellular, tissue, organ as well as systemic level.[26] The plasticity and complexity of the RAS is spectacular with its various pathways, with compensatory and feedback mechanisms, and with various components having counteracting effects.[16] For decades, AngII was considered as the end product and the only bioactive peptide of the RAS. However, later studies have revealed biological activity of several other angiotensin fragments. For example, a growing body of evidence suggests that the ACE2-Ang-(1–7)-Mas receptor axis, being activated primarily in pathological conditions, has opposite actions to those of the ACE-AngII-AT1R system in many tissues, including the heart, liver and kidney.[27] One interesting example of this is the finding by Wong et al. where experimental (streptozotocin-induced) type-1 diabetes in rats resulted in a lack of AT1R-mediated inhibition of SGLT-1, resulting in an un-controlled glucose uptake.[12] In this situation the expression of the ACE2/Ang(1–7)/MasR axis was up-regulated resulting in reduced epithelial glucose uptake.[13] The authors interpreted the up-regulation of ACE2/Ang(1–7)/MasR axis as a compensatory response to the lowered classic RAS-/AT1R-mediated inhibition of mucosal glucose absorption.[13] Apparently, the RAS system provides at least two pathways for the local control of enterocyte glucose transport, and in the present paper we propose the existence of a third one: the AngIV/IRAP axis. The AngIV-peptide has recently drawn attention since it exerts a wide range of effects such as processes underlying learning and memory[22] and inflammatory responses.[28] AngIV is formed during the degradation of the AngII by first having cleaved up to the AngIII by the enzyme AP-A.[16] The AP-B or AP-M, which has previously been shown in the intestinal mucosa,[29,30] then removes Arg residue of AngIII to produce AngIV. AngIV exerts its action via binding with high affinity to the IRAP also known as AngIV receptor, but is also a weak agonist for the AngII-receptors.[19–21,31] IRAP is a type II integral membrane protein and member of the zinc-dependent aminopeptidase family[32] and is expressed in different cell types, for example, coloncytes,[33] macrophages[34] and muscle cells.[35] IRAP is co-localized with the insulin-regulated glucose transporter GLUT4 in several tissue types and therefore AngIV might interact with the uptake of glucose.[23] In response to insulin both IRAP and GLUT4 translocate to the plasma membrane, thereby enhancing the glucose uptake into the cell.[36] Some studies in the brain suggest that AngIV mediates its effects via the inhibition of the enzymatic activity of IRAP and its ability to cleave the N-terminal amino acid from several peptides, thereby causing extracellular accumulation of neuropeptide substrates such as oxytocin, vasopressin, cholesystokinin-8 and somatostatin.[22] At the moment three hypotheses have been put forward to explain the role of IRAP in the physiological effects of AngIV: (i) inhibition of the enzymatic activity of IRAP, (ii) AngIV binding to IRAP may increase the translocation of IRAP and GLUT4 and (iii) activation of IRAP acting as a receptor and causing

intracellular signaling.[21] IRAP has also been shown to interact in the adaptive immune responses to pathogens in both mast cells and macrophages, independent of AngIV activation.[34,37] On the other hand, AngIV has the possibility to bind and activate the hepatocyte growth factor receptor named c-Met.[38] The rate of delivery or formation, as well as elimination, are other important determinants for activity that in turn are dependent on the actual expression pattern of the RAS-associated enzymes. AngIV has been reported to be short-lived and completely degraded within five minutes.[31] In the present investigation on young healthy individuals, the substrate peptide AngII (by ELISA) and its degradation enzymes AP-A, AP-B and AP-M (by Western blotting) were all observed indicating that both AngIII and AngIV can be produced locally in the human jejunal mucosa. AngIII has the affinity to bind to both the AT1R and AT2R and has similar effects to AngII.[39] AngIV is a unique ligand in the RAS system because of its ability to bind to IRAP. In addition to IRAP being detected by Western blotting, the immunohistochemistry staining also showed the presence of IRAP at an apical cytosolic localization, suggesting a role for IRAP in regulation of membrane transport processes. The potential functionality in the intestinal mucosa of this receptor was supported by a pronounced presence of the principal AngIV-generating enzymes AP-A, AP-B and AP-M preferably at the brush-border membrane in the enterocytes. Taken together, the present investigation indicates that an alternative RAS AP/AngIV/IRAP axis is present and may play a functional role in the human small intestine.

It is well established that intestinal uptake of glucose (and sodium) is mainly mediated by SGLT1 localized at the luminal (apical) surface of the enterocyte and further delivered to the portal blood via basolaterally located GLUT2.[3] To characterize SGLT1-mediated transport in the human jejunal mucosa, we used mini-Ussing chambers, which have previously been validated.[25] If correctly mounted, i.e. no leakage, the mucosa spontaneously gives rise to a distinct luminal-negative PD signal (used to ensure tissue viability) as well as Rep. Changing the luminal glucose concentration from zero to 10 mM induces a mucosa-generated electrical current (Iep) that in this system is mainly due to SGLT-1 mediated transport as confirmed by sensitivity to phlorizin. Interestingly, addition of the natural IRAP-receptor ligand AngIV significantly increased the glucose-induced electrogenic response in the present study. Currently, there is no conclusive evidence as to how AngIV interacts with, for example, IRAP, leading to the above-described effects. As mentioned above, AngIV is an active member of the RAS with its own effects.[21,22] Some of the effects of AngIV may be mediated by an interaction with AT1R and/or AT2R, or by a mixture of all three receptors (AT1R, AT2R, IRAP), possibly depending on tissue type and expression pattern.[22] Thus, the present results cannot exclude the possibility that the effects of AngIV are mediated via the AT2R in line with our previous work showing that AT2R activation increased the glucose-induced electrogenic response in a similar setting.[14]

Pharmacological analyses using stable and highly selective AngIV analogs that have been developed should considerably improve the outcomes of future studies.[31] Such an AngIV analog, preferably one that does not bind to AT1R,[31] and use of a random hexapeptide to evaluate if the enterocytes react via an unknown mechanism, may certainly contribute to elucidating the mechanism of AngIV and which functions actually are IRAP dependent.

RAS has a dynamic local expression pattern in relation to challenges of tissue integrity and apparently so also in the small intestinal mucosa.[13,40] It can be suspected that mucosal expression and the function of RAS during pathophysiological conditions, for example, in diabetes or inflammatory bowel disease, differ considerably from the healthy (physiological) individuals studied in the present study. Thus, in order to extend the influence of the classic and alternative RAS pathways under certain pathological mucosal conditions, or diabetes, in humans further investigation is warranted.

In summary, the present study indicates the presence of substrates and enzymes necessary for AngIV formation as well as the receptor IRAP in the jejunal mucosa. Furthermore, functional data suggest that AngIV regulates glucose uptake in the healthy human small intestine.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Kellett GL, Brot-Laroche E, Mace OJ, et al. Sugar absorption in the intestine: The role of GLUT2. *Annu Rev Nutr* 2008; 28: 35–54.
2. Wright EM, Loo DD and Hirayama BA. Biology of human sodium glucose transporters. *Physiol Rev* 2011; 91: 733–794.
3. Thazhath SS, Wu T, Young RL, et al. Glucose absorption in small intestinal diseases. *Expert Rev Gastroenterol Hepatol* 2014; 8: 301–312.
4. Pennington AM, Corpe CP and Kellett GL. Rapid regulation of rat jejunal glucose transport by insulin in a luminally and vascularly perfused preparation. *J Physiol* 1994; 478: 187–193.
5. Tobin V, Le Gall M, Fioramonti X, et al. Insulin internalizes GLUT2 in the enterocytes of healthy but not insulin-resistant mice. *Diabetes* 2008; 57: 555–562.
6. Debnam ES and Sharp PA. Acute and chronic effects of pancreatic glucagon on sugar transport across the brush-

border and basolateral membranes of rat jejunal enterocytes. *Exp Physiol* 1993; 78: 197–207.
7. Hirsh AJ and Cheeseman CI. Cholecystokinin decreases intestinal hexose absorption by a parallel reduction in SGLT1 abundance in the brush-border membrane. *J Biol Chem* 1998; 273: 14545–14549.
8. Cheeseman CI and Tsang R. The effect of GIP and glucagon-like peptides on intestinal basolateral membrane hexose transport. *Am J Physiol* 1996; 271(3 Pt 1): G477–G482.
9. Au A, Gupta A, Schembri P, et al. Rapid insertion of GLUT2 into the rat jejunal brush-border membrane promoted by glucagon-like peptide 2. *Biochem J* 2002; 367: 247–254.
10. Spak E, Björklund P, Helander HF, et al. Changes in the mucosa of the Roux-limb after gastric bypass surgery. *Histopathology* 2010; 57: 680–688.
11. Wong TP, Debnam ES and Leung PS. Involvement of an enterocyte renin-angiotensin system in the local control of SGLT1-dependent glucose uptake across the rat small intestinal brush border membrane. *J Physiol* 2007; 584: 613–623.
12. Wong TP, Debnam ES and Leung PS. Diabetes mellitus and expression of the enterocyte renin-angiotensin system: Implications for control of glucose transport across the brush border membrane. *Am J Physiol Cell Physiol* 2009; 297: C601–C610.
13. Wong TP, Ho KY, Ng EK, et al. Upregulation of ACE2-ANG-(1–7)-Mas axis in jejunal enterocytes of type 1 diabetic rats: Implications for glucose transport. *Am J Physiol Endocrinol Metab* 2012; 303: E669–E681.
14. Casselbrant A, Malinauskas M, Marschall HU, et al. Angiotensin II exerts dual actions on sodium-glucose transporter 1-mediated transport in the human jejunal mucosa. *Scand J Gastroenterology* 2015; 50: 1068–1075.
15. de Gasparo M, Catt KJ, Inagami T, et al. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 2000; 52: 415–472.
16. Fyhrquist F and Saijonmaa O. Renin-angiotensin system revisited. *J Intern Med* 2008; 264: 224–236.
17. Wright JW, Krebs LT, Stobb JW, et al. The angiotensin IV system: Functional implications. *Front Neuroendocrinol* 1995; 16: 23–52.
18. Vauquelin G, Michotte Y, Smolders I, et al. Cellular targets for angiotensin II fragments: Pharmacological and molecular evidence. *J Renin Angiotensin Aldosterone Syst* 2002; 3: 195–204.
19. Albiston AL, McDowall SG, Matsacos D, et al. Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. *J Biol Chem* 2001; 276: 48623–48626.
20. Chai SY, Fernando R, Peck G, et al. The angiotensin IV/AT4 receptor. *Cell Mol Life Sci* 2004; 61: 2728–2737.
21. Vanderheyden PM. From angiotensin IV binding site to AT4 receptor. *Mol Cell Endocrinol* 2009; 302: 159–166.
22. Stragier B, De Bundel D, Sarre S, et al. Involvement of insulin-regulated aminopeptidase in the effects of the renin-angiotensin fragment angiotensin IV: A review. *Heart Fail Rev* 2008; 13: 321–337.
23. Keller SR, Scott HM, Mastick CC, et al. Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from Glut4 vesicles. *J Biol Chem* 1995; 270: 23612–23618.
24. Björkman E, Edebo A, Fändriks L, et al. Angiotensin IV and the human esophageal mucosa: An exploratory study in healthy subjects and gastroesophageal reflux disease patients. *J Renin Angiotensin Aldosterone Syst*, pp. 1–8. Epub ahead of print 22 January 2014. DOI: 10.1177/1470320313512389
25. Björkman E, Casselbrant A, Lundberg S, et al. In vitro assessment of epithelial electrical resistance in human esophageal and jejunal mucosae and in Caco-2 cell layers. *Scand J Gastroenterol* 2012; 47: 1321–1333.
26. Kumar R, Thomas CM, Yong QC, et al. The intracrine renin-angiotensin system. *Clin Sci (Lond)* 2012; 123: 273–284.
27. Bader M. ACE2, angiotensin-(1–7), and Mas: The other side of the coin. *Pflugers Arch* 2013; 465: 79–85.
28. Ruiz-Ortega M, Esteban V and Egido J. The regulation of the inflammatory response through nuclear factor-kappab pathway by angiotensin IV extends the role of the renin angiotensin system in cardiovascular diseases. *Trends Cardiovasc Med* 2007; 17: 19–25.
29. Alfalah M, Wetzel G, Fischer I, et al. A novel type of detergent-resistant membranes may contribute to an early protein sorting event in epithelial cells. *J Biol Chem* 2005; 280: 42636–42643.
30. Naim HY, Joberty G, Alfalah M, et al. Temporal association of the N- and O-linked glycosylation events and their implication in the polarized sorting of intestinal brush border sucrase-isomaltase, aminopeptidase N, and dipeptidyl peptidase IV. *J Biol Chem* 1999; 274: 17961–17967.
31. Nikolaou A, Van Den Eynde I, Tourwé D, et al. [3H] IVDE77, a novel radioligand with high affinity and selectivity for the insulin-regulated aminopeptidase. *Eur J Pharmacol* 2013; 702: 93–102.
32. Rogi T, Tsujimoto M, Nakazato H, et al. Human placental leucine aminopeptidase/oxytocinase. A new member of type II membrane-spanning zinc metallopeptidase family. *J Biol Chem* 1996; 271: 56–61.
33. Matsumoto H, Nagasaka T, Hattori A, et al. Expression of placental leucine aminopeptidase/oxytocinase in neuronal cells and its action on neuronal peptides. *Eur J Biochem* 2001; 268: 3259–3266.
34. Nikolaou A, Stijlemans B, Laoui D, et al. Presence and regulation of insulin-regulated aminopeptidase in mouse macrophages. *J Renin Angiotensin Aldosterone Syst* 2014; 15: 466–479.
35. Esteban V, Ruperez M, Sánchez-López E, et al. Angiotensin IV activates the nuclear transcription factor-kappaB and related proinflammatory genes in vascular smooth muscle cells. *Circ Res* 2005; 96: 965–973.
36. Bogan JS. Regulation of glucose transporter translocation in health and diabetes. *Annu Rev Biochem* 2012; 81: 507–532.
37. Saveanu L, Babdor J, Lawand M, et al. Insulin-regulated aminopeptidase and its compartment in dendritic cells. *Mol Immunol* 2013; 55: 153–155.
38. Kawas LH, McCoy AT, Yamamoto BJ, et al. Development of angiotensin IV analogs as hepatocyte growth factor/Met modifiers. *J Pharmacol Exp Ther* 2012; 340: 539–548.
39. Ardaillou R. Active fragments of angiotensin II: Enzymatic pathways of synthesis and biological effects. *Curr Opin Nephrol Hypertens* 1997; 6: 28–34.
40. Ewert S, Sjoberg T, Johansson B, et al. Dynamic expression of the angiotensin II type 2 receptor and duodenal mucosal alkaline secretion in the Sprague-Dawley rat. *Exp Physiol* 2006; 91: 191–199.