Peptides 59 (2014) 63–69

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Angiotensin IV induced contractions in human jejunal wall musculature *in vitro*

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ARTICLE INFO

Article history:
Received 5 May 2014
Received in revised form 7 July 2014
Accepted 8 July 2014
Available online 16 July 2014

Keywords:
Angiotensin III
Angiotensin IV
IRAP
Human jejunal wall musculature

ABSTRACT

Angiotensin II (AngII) has been reported to mediate contractile actions in rats and human jejunal wall musculature. However, except for one report showing the angiotensin IV (AngIV) contractile effects on the internal anal sphincter of rats, no data is available describing the action of AngIV on smooth muscle in human small intestine. The aim of this study was to investigate the expression and localization of the enzymes responsible to AngIV formation, as well as the receptor, and to elucidate the contractile function of AngIV in the muscular layer of human jejunum *in vitro*. Jejunal smooth muscle was taken from 23 patients undergoing Roux-en-Y gastric bypass surgery and was used to record isometric tension *in vitro* in response to AngIV alone and in the presence of losartan or PD123319. ELISA, western blot and immunohistochemistry were used to investigate the expression and localization of key components for AngIV formation: the enzymes aminopeptidases-A, B, M, and the AngIV receptor insulin-regulated aminopeptidase (IRAP). AngIV elicited concentration-dependent contraction in both longitudinal and circular smooth-muscle preparation. Presence of losartan abolished AngIV-induced contraction, but not PD123319. The main peptide AngII, as well as the enzymes aminopeptidases-A, B and M was detected in all muscle samples. Immunohistochemistry localized the enzymes and IRAP in the myenteric plexus between longitudinal and circular muscle layers. The present study indicates that all enzymes necessary for AngIV formation exist in human jejunal smooth muscle and that the contractile action elicited by AngIV is primarily mediated through the AngII type 1 receptor.

1. Introduction

The classical renin–angiotensin system (RAS) certainly known as endocrine system is one of the body's most powerful regulators of body fluid balance and having a role in the cardiovascular system [18,22]. In addition, recent research has also recognized RAS to be locally expressed with paracrine/autocrine functions [19]. The key mediator of the RAS, the octapeptide angiotensin II (AngII), has been reported to mediate contractile actions *via* the AngII type 1 receptors (AT1R) and type 2 receptors (AT2R) of rat and human small intestinal wall musculature and esophagus *in vitro* [4,12,29].

The angiotensin IV (AngIV) is a peptide of alternative RAS, which is formed during the degradation of AngII (see Fig. 1). Primarily, AngIII is produced from the metabolism of AngII by aminopeptidase A (AMP-A), which cleaves the Asp1-Arg2 bound. Accordingly, AngIV is generated by the enzyme aminopeptidase B (AMP-B) or aminopeptidase M (AMP-M), due to elimination of the amino acid arginine from the N-terminus of AngIII sequence [5,16,34].

The specific binding site of AngIV is characterized as an "AT4 receptor". A recent study has displayed a specific binding site of AngIV and its localization in different tissues and cells [30]. The AT4 receptor has currently been identified as the insulin-regulated aminopeptidase (IRAP) [1].

The AngIV has revealed having a wide range of physiological functions [30]. The AngIV acting through IRAP has shown to reduce the degradation of neuropeptides at the spinal cord in rats [7], to improve associative learning and memory performance in a mouse model [21], to have a protective action on AngII-induced cardiac cell apoptosis and proliferation and collagen synthesis of cardiac fibroblasts [33].

There is also evidence that AngIV has effects on smooth muscles. AngIV has shown to have vasodilatation effects in porcine pulmonary arteries [6], vasorelaxation response in pulmonary artery

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http://dx.doi.org/10.1016/j.peptides.2014.07.008
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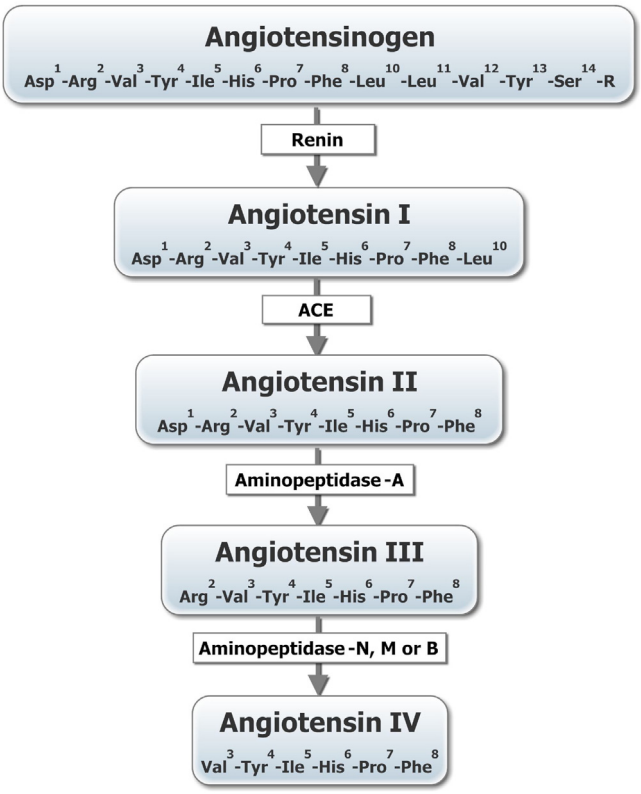

Fig. 1. Overview of the chemical structure and the enzymes involved in the synthesis of angiotensin II and its degradations fragments angiotensins III and IV.

[23], as well as vasoconstrictive effect in isolated rat basilar artery without unspecific interference of AT1 and AT2 receptors [13]. However, except for one report from de Godoy et al. [10], showing the AngIV-contractile effects on the internal anal sphincter on rats, no data is available describing the action of AngIV on smooth muscles in human small intestinal.

The first aim of the present study was to elucidate the expression and localization of an alternative RAS, the enzymatic capability responsible to AngIV formation as well as IRAP in the human small intestinal muscular wall. The second aim was to investigate the existence for contractile function of AngIV in the muscular layer of human jejunum *in vitro*.

2. Materials and methods

2.1. Ethics

The study was approved by the Ethical Committee of Göteborg University as well as by the Regional Ethical Review Board in Gothenburg (Dnr: 261-13) and was performed in accordance with the Declaration of Helsinki. All patients were informed verbally and in writing and signed a consent form before inclusion into the study.

2.2. Preparation of surgically resected material

Intestinal tissue of full wall thickness was taken from patients ($n = 23$, mean age 46, range 21–65, 16 females) undergoing gastric by-pass surgery for morbid obesity at Sahlgrenska University Hospital, Gothenburg, Sweden. Of these 23 patients, two patients were taking medicine for hypertension; one patient was taking Candesartan (angiotensin II type 1 receptor blocker, 50 mg 1×) and one was taking Enalapril (angiotensin converting enzyme inhibitor, 10 mg 1×). A full-wall specimen was resected from the jejunum between the gastro-entero and the entero-entero anastomosis as the loop was divided to create a Roux-en-Y construction as already reported by Spak et al. [28]. Tissue specimens from the muscular layer were taken for western blot analyses, immunohistochemistry, enzyme immunoassay and contractility experiments *in vitro* as described below.

2.3. Western blot analysis

The specimens were snap-frozen in liquid nitrogen and kept for later western blot analysis. During analysis, the specimens were thawed, sonicated and homogenized in a PE buffer (10 mM potassium phosphate buffer, pH 6.8 and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Boehringer Mannheim, Mannheim, Germany). The homogenates of each sample were centrifuged (10,000 × *g*, 10 min at 4 °C) and the supernatants analyzed regarding the protein content by using the Bradford method. The sample were diluted in SDS (sodium dodecyl sulfate buffer), and heated at 70 °C for 10 min before being loaded onto a NuPage 10% Bis–Tris gel, and the proteins in each sample separated with gel electrophoresis using 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 an iBlot (Invitrogen AB). Membranes were incubated with the respective primary antibody (aminopeptidases A, B, M, IRAP, and the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) (Table 1). Membranes were incubated overnight at 4 °C. To enhance detection signal, a secondary antibody (goat anti-rabbit or mouse and donkey anti-goat) conjugated with alkaline phosphatase was linked to the primary. The secondary antibody was detected by a chemo luminescent light (reagent CDP-Star, Tropix, Bedford, MA, USA), which was captured (Chemidox XRS cooled charge-couple device camera) and analyzed using the software program Quantity One (camera and software from Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as control for equal loading and for each tested sample the optical density of primarily antibody/GAPDH represent the results. Each time the membrane was incubated with a new primary antibody, previous antibody had been removed with a stripping buffer (Re-Blot Plus Mild Solution (10×), Millipore, Temecula, CA, USA).

2.4. Enzyme immunoassay

AngII levels were investigated by ELISA analysis. The proteins were solubilizes in the same way as for the western blot samples described above. The ELISA plate and reagents were prepared and the analysis performed depending 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 based on that immobilized anti-AngII antibodies in the bottom of each 96 well bind to potential AngII peptides in the solubilized biopsy samples. The antibody–AngII complex forms a yellow compound together with a tracer (acetylcholinesterase) 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.

2.5. Immunohistochemistry

Muscular specimens were fixed in neutral buffered 4% formaldehyde, dehydrated, embedded in paraffin and then were mounted on glass slides. Sections for immunohistochemistry were

Table 1
Antibodies used in western blot analyses and immunohistochemistry.

	AMP-A	AMP-B	AMP-M	IRAP	GAPDH
Primary antibody	(N-20)	[2G3]	CD13 (A-5)	(E-12)	(5143A)
Company	sc-18065	ab119761	sc-166270	sc-365051	IMGENEX
Secondary antibody	Donkey anti-goat	Goat anti-mouse	Goat anti-mouse	Goat anti-mouse	Goat anti-rabbit
Company	sc-2020	sc-2008	sc-2008	sc-2008	sc-2007

Abbreviations: AMP-A, AMP-B and AMP-M: aminopeptidases A, B and M, respectively; IRAP R: insulin-responsive aminopeptidase receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; sc: Santa Cruz; ab: Abcam.

deparaffinized and then boiled in 50 mM citrate buffer (pH 6.0) for 15 min for antigens retrieval. The CSA II biotin free tyramide signal application staining system kit (Dako Sweden AB, Stockholm, Sweden) was used for the immunohistochemistry protocol. After blockade of endogenous biotin (15 min) and peroxidase activity (15 min), slides were pre-incubated with serum block followed by incubation with primary antibodies (aminopeptidases A, B, M, and IRAP) in dilution of 1:500 overnight (antibodies are described in Table 1). Negative control sections were incubated with wash buffer instead of the primary antibody. To enhance detection signal, the slides were incubated with a biotinylated secondary antibody (goat anti-mouse/donkey anti-goat). This was followed by sequential incubations with amplication reagent (fluorescyl-tyramide hydrogen peroxide) in darkness and anti-fluorescein-HRP, 15 min each. The color was developed using 3.3′-diaminobenzidine.

2.6. Contractile experiments in vitro

The tissue was immediately submerged in oxygenated (95% O_2–5% CO_2) Krebs solution (4.69 mM KCl, 2.52 mM $CaCl_2$, 1.16 mM $MgSO_4$, 1.01 mM NaH_2PO_4, 25 mM $NaHCO_3$, 118.07 mM NaCl, 11.10 mM glucose) chilled on ice. The musculature, including the circular and longitudinal layers, were separated from the mucosa/submucosa by means of sharp dissection and then cut into approximately 2×10 mm strips orientated along either the longitudinal or circular muscle layers (both layers were kept intact due to preserve the myenteric plexus). The intestinal strips were when placed in warm (37 °C) oxygenated Krebs solution in separate 25 mL wells (Letica automated organ bath, AD instruments Pty, Hastings, UK) anchored by a metallic hook at the lower end and attached by silk suture at the upper end to a force transducer for isometric recording of muscular activity (PowerLab®, AD Instruments Pty). The muscular strips were pre-stretched to a tension of 1 g and allowed to equilibrate for 45 min to establish spontaneous contractions at a stable frequency. Losartan (10^{-6} M) (Sigma–Aldrich, Stockholm, Sweden), PD123319 (10^{-6} M) (Sigma–Aldrich) or Krebs solution (vehicle) was added and a baseline period of 15 min was allowed. AngIV (Sigma–Aldrich) was when added stepwise in concentration of 1×10^{-7} M, 5×10^{-7} M, 1×10^{-6} M and 5×10^{-6} M. The strips were washed, and bethanechol (Sigma–Aldrich), a non-selective muscarinic agonist resistant to cholinesterase degradation was added at a concentration of 10^{-4} M to obtain a reference contraction [9]. A contractile response was defined as the difference between baseline tension and the tension obtained during each AngIV concentration. The magnitude was expressed as percentage of a maximal reference contractions obtained by bethanechole. Because the equipment allowed up to eight parallel preparations, untreated strips were run in parallel with strips subjected to investigational compounds. It follows that each strip was only subjected to one drug and one subsequent series of AngIV concentrations and that all strips at one experimental run were taken from the same individual. In a subset of experiments, tetrodotoxin 10^{-6} M (Sigma–Aldrich) were administered 15 min before the first dose of AngIV. AngII (Sigma–Aldrich) in concentration 10^{-10} M to 10^{-5} M was used as contractile response reference.

2.7. 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 for analyzing the differences. The statistical software program SPSS 19.0 was used (SPSS, Chicago, IL, USA) and a p-value of ≤ 0.05 was considered significant. Data are presented as mean ± SEM. The number of individuals was denoted n and preparations/observation N.

3. Results

3.1. Expression of RAS components in the human jejunal muscular layer

The peptide AngII was detected in all samples and range between 32.48 and 153.44 pg/ml (data not shown). Western blot analysis showed presence of the alternative RAS components AMP-A, AMP-B, AMP-M and IRAP in all muscular preparations (Fig. 2).

Immunohistochemistry revealed expression of the enzyme AMP-B (Fig. 3A and B) and AMP-M (Fig. 3C) principally in the myenteric plexus localized between longitudinal and circular muscle layers. Staining for IRAP was found in the muscle cells of both the longitudinal and circular muscle layer and in the myenteric plexus (Fig. 3D and E). No immunoreactivity was detected for AMP-A (data not shown) or in the negative control sections (Fig. 3F).

3.2. Intestinal muscle contractility in vitro

Jejunal longitudinal and circular smooth-muscle preparation both displayed concentration-dependent contractile responses to AngIV as well as to AngII (some AngII-contractile results have been presented in a previous publication [29]) (Fig. 4). The maximal contractile responses of AngIV occurred at 5×10^{-6} M and were significantly smaller in circular smooth-muscle preparations compared to longitudinal from the same individual ($p = 0.019$, Fig. 5). The maximal increase in longitudinal smooth-muscle tension in relation to the individual bethanechol-reference contraction was 23.3 ± 5.0%, and for circular smooth muscle preparations 13.7 ± 5.0% (Fig. 5). A typical recording of the contractile responses

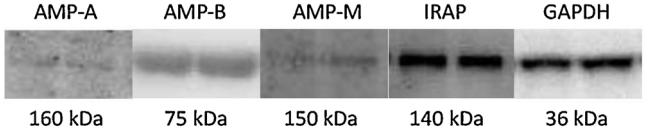

Fig. 2. Protein bands as displayed on the western blot membrane. The figure shows expressions of the aminopeptidase A (AMP-A), aminopeptidase B (AMP-B), aminopeptidase M (AMP-M), IRAP and the loading control GAPDH in the muscular layer of human jejunum.

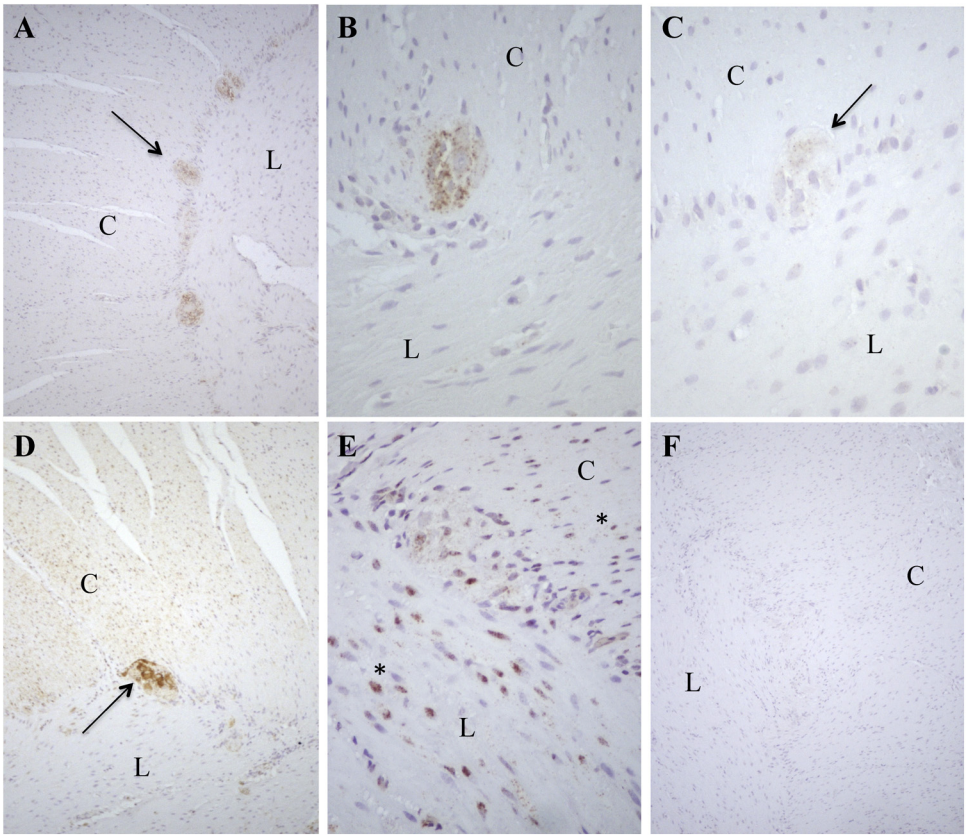

Fig. 3. Localization of angiotensins III and IV enzymes and IRAP in the human jejunal smooth-muscle. The Panels (**A and B**) show immunohistochemical staining of AMP-B in the myenteric plexus (arrow) localized between longitudinal (denoted L) and circular (denoted C) muscle layers (magnification 10 and 4×). The panel (C) shows faint staining of AMP-M in the myenteric plexus (arrow) (magnification 40×). The panels (**D and E**) show staining of IRAP in the muscle cells (indicated *) of both the longitudinal and circular muscle layer. The arrow indicates the myenteric plexus, which also shows distinct staining (magnification 10 and 40×). The panel (F) negative control for IRAP (magnification 10×).

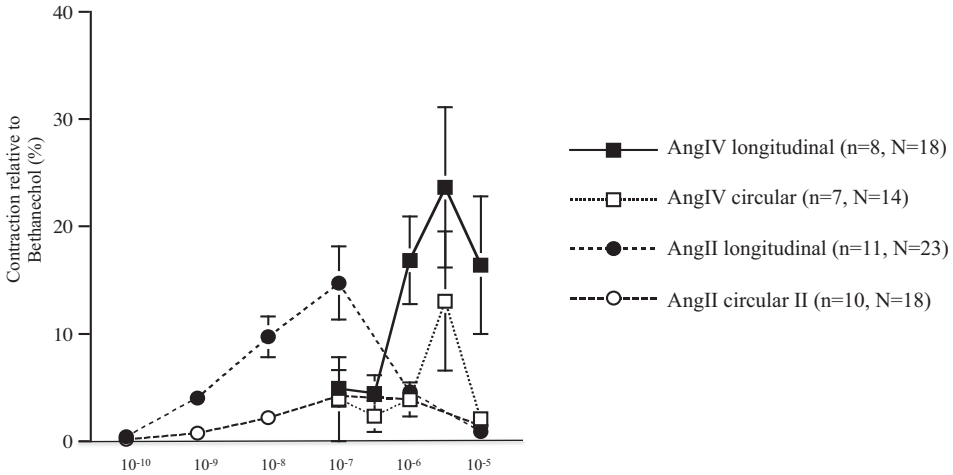

Fig. 4. Contractile response to angiotensin IV (squares) and angiotensin II (circles) in longitudinal (black) and circular (white) muscle strips. Data are presented as percentages of an individual bethanechol-induced contraction and are plotted as group mean ± SEM.

to AngIV in absence and presence of AngII receptor antagonists is shown in Fig. 6A and B. Presence of the AT1R antagonist losartan at 1×10^{-6} M abolished the AngIV-induced contraction in both longitudinal ($p = 0.01$, Fig. 7A) and circular muscle preparations ($p = 0.05$, Fig. 7B). In contrast, the AT2R antagonist PD123319 at 1×10^{-6} M have no effect on the concentration-responses curve by AngIV (Fig. 7A and B). Neither does tetrodotoxin influence the AngIV-elicited contraction significantly (Fig. 7).

4. Discussion

The expression of the enzymes AMP-A, AMP-B and AMP-M was observed in the human jejunal muscular tissue. The existence of the peptide AngII and the above enzymes, suggests local production of the peptide AngIII as well as AngIV. The AngIV receptor IRAP was also expressed in the muscle cells and in the myenteric plexus in the muscle layer. This study represents the first evidence that

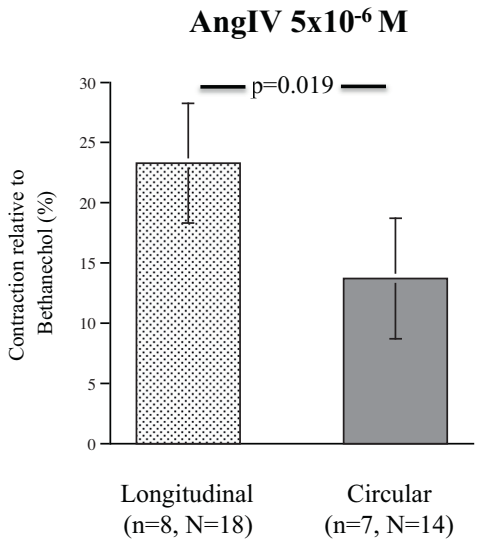

Fig. 5. Contractile response to angiotensin IV (AngIV) at the concentrations of 5×10^{-6} M in human jejunal longitudinal (18 strips from 8 patients) and circular (14 strips from 7 patients) smooth-muscle preparations. Data are presented as percentages of an individual bethanechol-induced (10^{-4} M) contraction, and are plotted as group means ± SEM. The *p*-value denotes significant different in related variables (Wilcoxon's signed rank test).

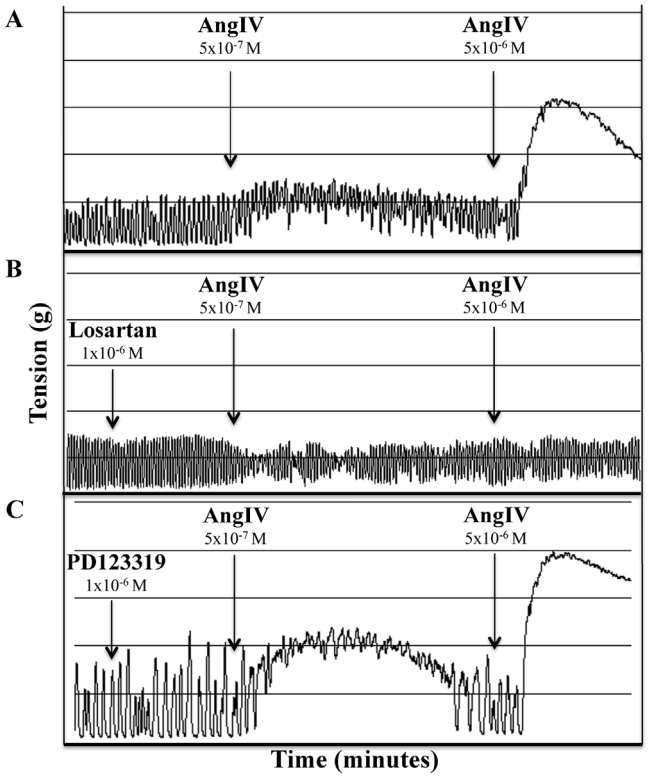

Fig. 6. Typical recording of the contractile response to angiotensin IV (AngIV) in two different concentrations (A), and after addition of the angiotensin II type 1 receptor antagonist losartan (B) or angiotensin II type 2 receptor antagonist PD123319 (C). Muscle strip from human longitudinal jejunum.

the peptide AngIV has contractile function on the human small intestinal wall musculature.

Recent studies have shown that the peptide AngII has the potential effect of smooth muscle contraction in animals [2,9,11,17,20,27,32], as well as on human gastro-intestinal muscle wall [4,12,14,29]. A previous study from our laboratory showed protein expression of AT1 and AT2 receptors, and gene expression of ACE and angiotensinogen in the human jejunal muscle wall and a concentration-dependent muscle contraction in response to AngII [29]. This study, however, is the first to measure the active peptide AngII responsible for the contractile effects in human jejunal muscular samples. Levels of AngII have previously been described in rat small intestinal [10]. Until recently, AngII was considered the only biologically active product of the RAS system. Because of this, only one paper has elucidated the physiological role of muscular contraction to other RAS fragments [10]. The peptide AngIV is formed during the degradation of the AngII (see Fig. 1), by first having cleaved up to the AngIII [25]. The aminopeptidase B or M then removes Arg residue of AngIII to produce AngIV [16].

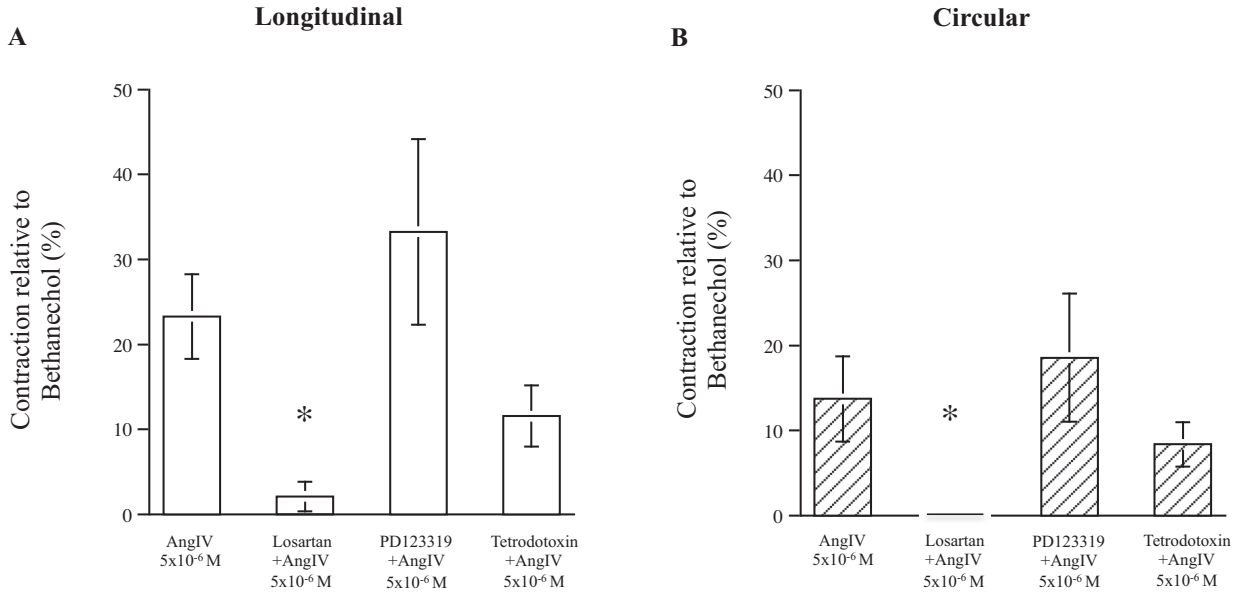

Fig. 7. Contractile response to angiotensin IV (AngIV) in human jejunal longitudinal (A) and circular (B) smooth muscles in controls (given only AngIV) or muscle-strips pretreated by the antagonists losartan (10^{-6} M) and PD123319 (10^{-6} M), as well as the neurotoxin tetrodotoxin (10^{-6} M). Effects of AngIV are expressed as % of the contraction induced by 10^{-4} M bethanechol. Data are presented as mean ± SEM. * denotes significant difference as compared to control (Mann–Whitney *U*-test).

In fact, the AngII-enzyme immunoassay, used in this study, has cross reactivity up to 36% for AngIII and AngIV. It follows that it cannot be completely ruled out that some of the detected AngII instead is AngIII or AngIV. Moreover, the existence of the three aminopeptidases A, B, and M that were observed by western blotting could also be the strong evidence indicating that both AngIII and AngIV can be produced locally in the human jejunal muscular tissue. The immunohistochemistry showed AMP-B and AMP-M principally in the myenteric plexus, indicating that AngIV can be formed locally, between jejunal longitudinal and circular muscle layers. Another possibility is that AMP-B and M are expressed in the vasculature surrounding myenteric plexus, suggesting for example involvement in the enzymatic cascade of RAS regulating local microcirculation [26]. AMP-B is widely distributed in a number of tissues, including endocrine and non-endocrine cell types, as well as in neural components [8]. AMP-A was found by western blot, but not by immunohistochemistry which could be due to various technical reasons (*e.g.* the antigen retrieval was not optimal) [3]. Taken into consideration these immunoreactivity-based observations suggest that angiotensins beyond AngII can be active in the human small intestinal muscular tissue. In addition, the AngIV receptor, IRAP, was also detected in myenteric plexus, and in the muscles cells. This finding hereby suggests, that locally produced AngIV simply can reach its receptor – IRAP.

The present *in vitro* contractility experiments demonstrated a concentration-dependant muscle contraction in response to AngIV. The maximal response occurred at 5×10^{-6} M with a threshold response at 1×10^{-7} M. It is interesting to note that AngII produced concentration-dependent contractions in the lower range of concentrations (from 0.1 nM to 100 nM) and subsequently decreases in the higher range, whereas AngIV produced concentration-dependent contraction at higher concentrations (from 100 nM to 500 nM). AngII may be a more potent activator of smooth muscle contractions, but this study shows that the degradation product AngIV also has the ability to produce an effect on muscle contraction. Differences in sensitivity [14,27] and in the functional significance [4,29] to AngII between the circular and longitudinal musculature have previously been observed. Our *in vitro* study also showed differences in the contractile significance between the circular and longitudinal musculature to AngIV. The maximal increase in longitudinal smooth-muscle tension in relation to the individual bethanechol-reference contraction was almost the double for circular smooth muscle. Spak et al. showed higher contractile response to AngII in longitudinal jejunal smooth muscle preparation compared to circular smooth muscle [29]. The AT1R antagonist losartan abolished the AngIV-induced contraction in both longitudinal and circular muscle, but was unaffected by the AT2R antagonist PD123319. These results suggest that AngIV cause contraction mainly *via* activation of the AT1R. According to De Godoy et al. AngI and AngII are the most effective in eliciting contraction, followed by AngIII in the rat anal sphincter [10]. Moreover, they showed that AngIII-induced actions are attributable to AT1 receptor activation, because losartan, but not PD123319, causes selective antagonism of AngIII effects. On the other hand, they found that AngIV produce only a minor contractile effect in the rat anal sphincter but no information over which receptor was given [10]. AngIV binds with high affinity to the "AT4R", or the insulin-regulated aminopeptidase, IRAP, which is pharmacologically distinct from the AT1R and AT2R [8]. AngIV has been suggested to exert its physiological effects by inhibiting the enzymatic activity of IRAP resulting in, for example, accumulation of neuropeptide substrates of this enzyme (*e.g.* vasopressin and cholecystokinin) [30]. However, AngIV has also been describing to exert its effects over the classical angiotensin receptor subtype, the AT1 receptor [30]. In this respect, our *in vitro* results strongly indicate that the action of AngIV is mediated *via* the AT1R.

AngII has previously been described to elicit a direct action on human jejunal smooth muscle contractions *via* the AT1R, independent of an intramural neural link [29]. In the present study the neurotoxin tetrodotoxin had no significant effects on AngIV induced contractions, strongly indicating that the site of action of AngIV is on the muscle cells and without intrinsic neuronal mediation. Accordingly, the function of IRAP, expressed in the muscle layer, remains to be elucidated.

Interesting, AngII has also been shown to excite enteric neurons in myenteric plexus *via* the AT1R expressed in the plexuses neurons [31]. If IRAP, also expressed in the myenteric plexus, could be involved in mediating excitation of neuron, in the same line as AT1R, or more probably *via* accumulations of other neuropeptides in response to AngIV-inhibition, remains to be elucidated.

Experimentation on *human* intestinal specimens is complicated because of the limited availability of *normal* tissue sample. In the present study, we used specimens from patients undergoing Roux-en-Y gastric bypass surgery. During this operation, it is possible to get full-wall specimens of jejunum. The RAS is activated in several diseases related to obesity particularly hypertension and diabetes [15], and obese patients are frequently using well-established pharmaceuticals like AT1R-blockers or ACE-inhibitor [24]. A recent study from our laboratory reported that there is an AT1R-dependent component of the peristaltic contraction force following swallowing *in vivo* [4]. In the present study, two patients were taking RAS-medication that in fact could influence the results. ACE activity has been shown to be critical in the biosynthesis of AngII and related peptides [10]. Future investigations are needed to evaluate potential physiological and pathophysiological consequences of local formation of AngIV in the human small intestinal muscular layer.

To summarize, the present investigation demonstrates the existence of a local alternative RAS in the wall musculature of human jejunum. Moreover, this study demonstrates AngIV-induced contraction of both longitudinal and circular muscle preparations. Pharmacological manner *in vitro* indicate, that the contractile action by AngIV is primarily mediated *via* AngII type 1 receptor.

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