PROJETO

INFLUÊNCIA DA ANGIOTENSINA(1-7) NA ABSORÇÃO JEJUNAL DE ÁGUA EM RATO.

Relatório Final

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1) **Artigo diretamente relacionado:**

2) **Outro Artigo Completo:**

Os trabalhos foram apresentados em Congressos Nacional e Internacional com resumos publicados.

1) Absorção jejunal de tripsina em ratos demonstrado por immunofluorescência.
XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental FeSBE agosto 2002, Salvador, Bahia, Brasil.

2) Effect of angiotensin-(1-7) on jejunal absorption of water in rats.
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Effect of angiotensin-(1–7) on jejunal absorption of water in rats


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Abstract

The effect of angiotensin-(1–7) on jejunal water absorption in rats was investigated. The jejunal sac of anesthetized rats was filled with two ml of tyrode solution containing 3.7 MBq of tritiated water. A femoral vein was cannulated for administration of peptides and drugs. Infusion of Ang-(1–7) at the dose of 0.7 ng/kg/min produced a significant increase in jejunal water absorption compared to control (32% increase). The Ang-(1–7) antagonist A-779 abolished the effect of Ang-(1–7) on water absorption. A reduction of the Ang-(1–7) effect was also produced by treatment with the AT1 receptor antagonist, losartan, or the AT2 receptor antagonist, PD123319. The increase in jejunal water absorption produced by Ang-(1–7) was blocked by the nitric oxide synthase inhibitor, L-NAME, and by indomethacin. These data suggest that the effect of Ang-(1–7) on the jejunal loop is mediated by activation of a multiple angiotensin receptors and/or by an atypical angiotensin receptor. Furthermore, the effect of Ang-(1–7) on jejunal water absorption is mediated by nitric oxide and by a cyclooxygenase-dependent mechanism. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Angiotensin-(1–7); A-779; L-NAME (Nω-nitro-L-arginine methyl ester); Losartan; Tritiated water

1. Introduction

The amino terminal heptapeptide, angiotensin (Ang)-1–7, is a biologically active component of the renin-angiotensin system with several biological actions mainly opposite to those of Ang II [6,7,16]. Processing of Ang I by endopeptidases (neutral-endopeptidase 24.11, prolyl-endopeptidase) or of Ang II by prolyl endopeptidase or carboxypeptidase generates Ang-(1–7) [16]. The effects of Ang-(1–7) are mediated by binding to specific Ang-(1–7) receptors-sensitive to the Ang-(1–7) analog D-Ala3–Ang-(1–7) (A-779) [16,17].

Several studies have shown that Ang-(1–7) may contribute to the physiological role of the renin-angiotensin system in hydroelectrolyte homeostasis, especially by demonstrating that the kidney is an important target organ for its actions [11,19–21]. The renal action of Ang-(1–7) appears to be largely dependent on the experimental conditions [16]. However, the observation that chronic infusion of an Ang-(1–7) antagonist in rats produces diuresis and natriuresis suggests that the renal action of Ang-(1–7) mainly involves an increase in water and sodium absorption by the kidney [19].

Hydroelectrolytic balance control includes, in addition to changes in water and electrolytes excretion, modulation of drinking and gastrointestinal absorption. It is well known that Angiotensin II is a potent dipogenic peptide [16]. Additionally, it has been described that Angiotensin II increases jejunal water absorption acting on the subtype-2 (AT2) Ang II receptor and inhibits absorption via the subtype-1 (AT1) Ang II receptor [13]. It is well established that Ang-(1–7) is devoid of a central dipogenic effect [6,7,16]. However, possible biological actions of Ang-(1–7) influencing water absorption in the gastrointestinal system have not been studied. Thus, the present study was undertaken to evaluate the effects of Ang-(1–7) on jejunal water absorption.

2. Materials and methods

2.1. Animals

The experiments were conducted on male Wistar rats (from CEBIO-Centro de Bioterismo do Instituto de Ciências Biológicas da UFMG) weighing 180–220 g (N ≥ 5 per
group) with free access to water. The animals were fasted overnight before use.

2.2. Surgical procedure

After the induction of anesthesia with 40 mg/kg of thiopental administered intraperitoneally (i.p.), a polyethylene catheter (od, 0.61 mm) was inserted via the femoral vein into the inferior vena cava. This venous cannula was connected to a constant-rate infusion pump through which isotonic saline with or without drugs was infused at the rate of 20 μl/min. Another catheter was introduced via the femoral artery in order to collect blood samples (0.1 ml) at five-min intervals.

2.3. Measurement of jejunal water absorption

The abdominal cavity was opened through a midline incision, and the proximal end of the jejunum (below the duodenojejunal ligament) was isolated, while maintaining its nerve and blood supplies. Two cannulas were introduced and tied to the extremities of the loop for perfusion. The 15 cm isolated intestinal segment was washed thoroughly with Tyrode solution at the rate of 0.5 ml/min for 15 min and gently emptied, and the two extremities were tied to produce a closed jejunal segment or sac. A 15-min resting period was allowed to elapse in order to equilibrate the jejunal lumen, and during this time inhibitors were administered i.v. (20 μl/min) when necessary. Tyrode solution was prepared as follows: 137 mM NaCl, 2.7 mM KCl, 1.36 mM CaCl₂, 0.49 mM MgCl₂, 11.9 mM NaHCO₃, and 5 mM D-glucose, pH 8 (buffered by HCO₃⁻).

After the 15-min resting period, the jejunal sac was filled with 2 ml of Tyrode solution containing 3.7 MBq of tritiated water (Amersham-Life Science, 185 MBq/ml-5.0 mCi/ml) and an aliquot (5 μl) was removed from the tritiated water solution as an initial control. The intestinal sac was returned to the abdominal cavity in order to preserve normal temperature and avoid dehydration. Control animals were infused with saline (20 μl/min) and experimental animals were infused with saline containing the appropriate drugs over a period of 30 min. The effect of several inhibitors on the response to Ang-(1-7) was then tested. Blood samples (0.1 ml each) were collected from the femoral artery at 5-min intervals for 30 min with no volume replacement since the total volume collected was 0.6 ml per rat, which represents only 6% of the average blood volume (10 ml) of a rat weighing 200 g. Two 25-μl aliquots of each sample were assayed for tritiated water by liquid scintillation. The samples were transferred to 12 x 75-mm centrifuge tubes containing: 10 μl NCS solubilizer, 10 μl of an alcoholic KOH solution (100 ml alcoholic solution of 0.50 M KOH containing 1.0 ml of 10% ammonium oxalate, m/v), 4.0 ml of Bray (7.2 g PPO, 0.18 g POPOP dissolved in 1200 ml toluene and 600 ml Triton X-100) [4]. The samples were spun at 3000 rpm for 5 min, and the supernatant was then analyzed. Samples of tritiated water were counted with a Liquid Scintillation Counter (Pakard Instruments model 2550 TR/LL) and counts per minute were converted to disintegrations per minute using a store quench curve specially constructed for this experiment. At the end of the experiment the intestinal sac was removed from the animal, emptied, and heated to 80°C for 48 h to obtain tissue dry weight. The results were expressed as percentage of total radioactivity injected into the intestinal loop/jejunal dry weight (g).

2.4. Drugs

Ang II, Ang-(1-7) and A-779 were obtained from Bachem, Torrance, CA (Batch # 25691, Batch # 2888, and Batch # 2888, respectively). The concentration of peptides in the solutions used in the experiments was checked by radioimmunoassay (RIA) using random samples [2]. PD 123,177 (AT₂ receptor antagonist) and the losartan AT₁ receptor antagonist were from Parke-Davis and DuPont-Merck, respectively. Nω-nitro-L-arginine methyl ester (L-NAME), indomethacin, and NCs solubilizer were purchased from Sigma Chemical Co, Saint-Louis, MO. Thionembutal was obtained from Abbott. All other chemicals used were of the highest purity available.

2.5. Statistical analysis

Data were analyzed statistically by the Kruskal Wallis test for the area under a curve of each rat [15], with the level of significance set at P < 0.05. The results are reported as mean ± SEM.

3. Results

3.1. Pattern of the effect of Ang-(1-7) on jejunal water absorption

The time course of the effect of i.v. infusion of Ang-(1-7) on jejunal water absorption is shown in Fig. 1. Panel A. Ang-(1-7) significantly stimulated jejunal water transport above control (N = 7) when infused at a dose of 0.7 ng/kg.min (N = 5) (P < 0.05). Fig. 1, Panel B shows the averaged values of water absorption for the control group and for the groups submitted to i.v. infusion of Ang-(1-7) or Ang II. At the rate of 0.7 ng/kg.min, Ang-(1-7) significantly increased water absorption compared to the other groups, except Ang-(1-7) at 7 ng/kg.min. On the other hand, angiotensin II at similar rates did not influence jejunal water absorption in our preparation.

3.2. Influence of angiotensin antagonists on the effect of Ang-(1-7)

The effect of antagonists on the action of Ang-(1-7) is depicted in Fig. 2. The selective Ang-(1-7) receptor antag-
Fig. 1. Panel A: Effect of Ang-(1–7) on jejunal water absorption. Ang-(1–7) was administered i.v. at doses of 7.00 ng/kg/min (●, N = 5), 0.70 ng/kg/min (○, N = 5), or 0.07 ng/kg/min (△, N = 5); control (•, N = 7). The jejunal sac was filled with two ml of Tyrode solution containing 3.7 MBq of tritiated water. Values are mean ± SEM jejunal water absorption measured in blood samples by liquid scintillation. The assay was performed as described in Materials and Methods. * (P < 0.05) compared to the control group. Panel B: Effect of Ang-(1–7) and Angiotensin II on jejunal water absorption. Histograms represent mean ± SEM jejunal water absorption measured as area under a curve for each rat. The asterisk indicates that the response is significantly different from that of the other groups (P < 0.05), except Ang-(1–7) at 7 ng/kg/min. Ang II was administered i.v. at doses of 7.00 ng/kg/min (N = 5), or 0.70 ng/kg/min (N = 7).

Fig. 2. Influence of angiotensin antagonists on the effect of Ang-(1–7) on jejunal water absorption. Histograms represent mean ± SEM jejunal water absorption measured as area under a curve for each rat. Open bars are values for the control group, which represent vehicle or vehicle plus Ang antagonist. Solid bars are values for the experimental groups which represent Ang-(1–7) plus Ang antagonist. Control (N = 7); Ang-(1–7) 0.70 ng/kg/min (N = 5); A-779 (100 ng/kg i.v.) plus Ang-(1–7) 0.7 ng/kg/min (N = 6); A-779 (100 ng/kg i.v.) plus isosorbide saline (N = 8); losartan (1 mg/kg i.v.) plus Ang-(1–7) 0.7 ng/kg/min (N = 5); losartan (1 mg/kg i.v.) plus isosorbide saline (N = 5); PD 123.177 (750 ng/kg i.v.) plus Ang-(1–7) 0.07 ng/kg/min (N = 7); PD 123.177 (750 ng/kg i.v.) plus isosorbide saline (N = 4). Responses to the peptide were determined 15 min after the administration of the receptor antagonist. The asterisk indicates that the response was significantly different from that of the Ang-(1–7) group (*P < 0.01). † indicates that the response was significantly different from that of the control group (**P < 0.01). † † P < 0.01 compared to the inhibitor alone.

3.3 Influence of indomethacin and L-NAME on the effect of Ang-(1–7)

The role of arachidonic acid metabolite formation in mediating or modulating the responses to Ang-(1–7) was studied by evaluating the effect of the cyclooxygenase inhibitor, indomethacin, on the responses to Ang-(1–7). Indomethacin (5.0 mg/kg i.v.) alone reduced jejunal water absorption when compared with the control group (P < 0.01). The jejunal water absorption observed in the presence of Ang-(1–7) in indomethacin-treated animals was significantly smaller than that observed under control conditions (Fig. 3).

In order to determine if the formation of nitric oxide (NO) could be involved in the modulation or mediation of the responses to Ang-(1–7), the effect of L-NAME on the responses to the peptide was also investigated.

As shown in Fig. 3, L-NAME significantly reduced the effect of Ang-(1–7) on jejunal water absorption when administered 15 min before the peptide (0.70 ng/kg/min). The
The stimulatory effect of Ang-(1-7) on jejunal water absorption was blocked by A-779. This result suggests that the action of Ang-(1-7) on jejunal water transport is receptor-mediated. Indeed, several studies have provided evidence that the effects of Ang-(1-7) are mediated by a specific receptor [16,17]. Additionally, the observation that A-779 alone inhibited jejunal water absorption suggests that endogenous Ang-(1-7) participates in the modulation of jejunal water absorption.

An intriguing observation was the fact that in rats treated with losartan, Ang-(1-7) decreased rather than increased water transport. It is difficult to reconcile this observation with the current data available in the literature [13,18]. It has been shown that in blood vessels Ang-(1-7) can antagonize Ang II by low affinity binding to AT1 receptors. In addition, it has been shown that losartan blocked the antidiuretic effect of Ang-(1-7) in water loaded rats [1]. A similar blockade was observed with A-779. Further, in isolated rat atria losartan blocked the noradrenaline-releasing activity of Ang-(1-7) [7]. However, in the present study we observed that in the presence of losartan-treatment Ang-(1-7) actually induced a decrease in water absorption. An inhibitory role for the A-779-sensitive receptor in this effect can be essentially ruled out based on the blockade of the stimulatory effect of Ang-(1-7) by A-779, and by the decrease in water transport observed with A-779 by itself. A similar argument is valid for AT2 receptor (see below). Further studies are obviously necessary to clarify this observation.

We have observed that the angiotensin subtype 2 (AT2) antagonist PD 123 177 reduced jejunal water transport. This result is in agreement with previous studies suggesting a role for AT2 receptors in water absorption [13]. However, an effect of AT2 blockade on basal water absorption in the presence of angiotensin II was observed in sodium-restricted rats but not in animals receiving a normal sodium intake [13]. Differences in the extent of renin-angiotensin-system activation under our experimental conditions as compared to those used in previous studies [13] may explain this apparent discrepancy. Despite these differences, these data suggest a role for an AT2-mediated mechanism in jejunal water transport.

The effect of Ang-(1-7) on jejunal water transport was reduced by pre-treatment with the angiotensin subtype 2 antagonist PD 123 177. This observation is in keeping with several reports showing blockade of some Ang-(1-7) actions by AT2 receptors antagonists [16,18]. It remains to be clarified if these observations are due to interference of AT2 antagonists with typical or atypical receptors [16,18]. Indeed, Ang-(1-7) presents very low affinity for classic AT2 receptors [3]. Taken together, our observations illustrate the complexity of the relationship between angiotensin-(1-7) and angiotensinergic receptors [16,18].

In our experimental model, the number of capillaries and the rate of water diffusion may be considered constant. Moreover, the permeability of the jejunal loop can be

data also show that the nitric oxide synthase inhibitor significantly reduced jejunal water absorption by itself.

4. Discussion

The present study demonstrates that the heptapeptide Ang-(1-7) elicited an increase in jejunal water absorption. This effect was observed with an intermediate dose (0.70 ng/kg/min), while at ten-fold lower or higher rate no significant increase in jejunal water absorption was observed. These observations indicate that Ang-(1-7) causes an increase in jejunal water absorption with a tendency to a bell-shaped dose-response curve, as also reported for other preparations [7,9]. Although the mechanism of this phenomenon has not been investigated, induction of a turn-off signal such as production of PGE2 or internalization of receptors, at higher concentrations of this angiotensin, are probably involved. Contrasting with previous finding [13], we could not demonstrate an increase in water absorption in response to Ang II in our preparation. This discrepancy is probably related to methodological differences. In addition, since AT1 receptors inhibit and AT2 receptors stimulate water absorption, differences in the relative concentrations of AT1 and AT2 receptors in our animals as compared to those used in the previously published study should also be considered [13].
changed by a direct peptide influence on the jejunum or an action via the innervation of the jejunum. The association of this fact with vasodilatation in the mesenteric vascular bed can produce an increase in jejunal water absorption. One possible mechanism by which Ang-(1-7) may cause vasodilation is by the release of a vasodilating prostanoid. In this regard, Ang-(1-7) has been reported to stimulate the release of prostaglandin (PG)E_2 and 6-Keto-PGF_1α, the metabolite of prostacyclin, via activation of a receptor subtype distinct from AT_1 and AT_2 [8]. In the present study the action of Ang-(1-7) on jejunal water absorption was reduced by the blockade of cyclooxygenase, as compared to the control group. However, indomethacin by itself reduced water absorption, preventing a more clear interpretation of our data. On the other hand, the jejunal water absorption induced by Ang-(1-7) was abolished by nitric oxide synthase inhibition, suggesting a role for NO release in mediating this Ang-(1-7) effect. This observation is in accordance with previous studies showing that at the cellular level, Ang-(1-7) stimulates the release of NO [7,12,14]. Involvement of NO-related signals has also been suggested for the stimulatory effect of AT_2 receptors on jejunal water transport [5]. An additional interpretation of this result could be that the vasooconstriction produced by L-NAME treatment may interfere with the action of Ang-(1-7). Future studies using in vitro preparations would clarify this possibility.

One may argue that Ang-(1-7) or the other drugs could alter kidney function, vascular permeability or some other factor involved in water regulation in the extracellular fluid compartment, resulting in a less accurate measurement of absorption from the jejunal sac fluid. Ang-(1-7) given to euvoletic rats has no renal effects or a slight natriuretic/diuretic effect [16,18]. Thus, the renal effect would actually "decrease" the effect of Ang-(1-7) on jejunal water absorption. Indomethacin and L-NAME may produce anti-diuretic effects that would not mask an effect of Ang-(1-7) on water absorption. On the other hand, losartan could produce diuresis. However, this was not enough to introduce an artifactual decrease in water absorption (see Fig. 2). In addition, the Ang-(1-7) effects on kidney function are virtually blocked by this antagonist [1]. Finally PD 123 177 at the dose used has no described effect on kidney function. Concerning vascular permeability, none of the drugs used has been report to increase it. We are not aware of any other action of Ang-(1-7) that could mask its effect on jejunal water transport.

In conclusion, the results obtained in the present study indicate that Ang-(1-7) increases jejunal water absorption through a complex mechanism involving multiple angiotensin receptors or, more likely, an atypical A-779-sensitive receptor. This effect seems to be dependent on an NO-related mechanism. Our results also support a role for endogenous Ang-(1-7) and Ang II AT_2 receptors in the modulation of jejunal water transport.

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of selective angiotensin (1–7) antagonist in normotensive and hyper-
angiotensin-(1–7) blocks renal actions of angiotensin-(1–7) in the
application of angiotensin-(1–7) on nephron function. Kidney Blood
Dear Dr. Borges,

It was very good to have your letter and the revised version of your manuscript. I am happy with the changes you have made and am delighted to accept the revised paper formally for publication.

I have made a number of minor amendments to your text in order to improve or clarify the English. I hope that this is what you wished me to do.

Thank you for sending your paper to the Histochemical Journal. We are very glad to have it and look forward to receiving further papers from you and your colleagues in the near future.

Yours sincerely,

[Signature]

Professor Peter J. Stoward
Route of jejunal mucosa absorption of trypsin demonstrated by Immunofluorescence

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Abstract

Previous results demonstrated porcine trypsin absorption in isolated jejunal loops from male Wistar rats by open-loop perfusion. In order to study its possible routes of absorption this work was carried out. Trypsin (0.5 mg/ml) was dissolved in tyrode solution and then was perfused at a rate of 0.5 ml/min, at 37°C, for 40 minutes. Using immunoperoxidase (PAP) and immunofluorescence techniques, strong reactivity of the enzyme to anti-TLCK-trypsin antibody was demonstrated throughout the enterocyte cytosol. The present data indicate that trypsin was absorbed by the enterocytes probably through a transcellular route.

Key words: Immunoperoxidase, immunofluorescence, trypsin, jejunal absorption.
Introduction

In a previous investigation, we studied the absorption of active trypsin through the jejunal wall and determined the presence of trypsin in the mesenteric vein (Borges et al., 1995). Trypsin was shown to be absorbed by the isolated jejunal loop of the rat by enzyme-linked immunosorbent assay (ELISA). Histological and ultrastructural examination of the jejunal mucosa before and after perfusion revealed that the brush-border, basement membrane, and junctional complexes were fully preserved, thus eliminating the possibility that trypsin might have destroyed the structures, thereby reaching the blood circulation (Borges et al., 1995).

Proteinases have been used as anti-inflammatory drugs (Bazerque et al., 1972; Humphries, 1971; Innerfeld, 1957; Kryle et al., 1957; Martin, 1957; Silbert, 1957). A possible anti-inflammatory effect of intraduodenally administered trypsin was investigated using the paw edema and pleurisy models of carrageenan-induced inflammation in rats. No anti-inflammatory effect was detected by plethysmography or on the basis of pleural leukocyte migration in treated animals compared to a sham group. Evans blue dye protein leakage into the peritoneal cavity, as a measure of vascular permeability, demonstrated a pro-inflammatory effect of trypsin. These results indicate that trypsin may be acting not as an anti-inflammatory agent but by accelerating the inflammatory process, thereby reducing the duration of the process (Borges et al., 2000).

Therefore, it appears to be relevant to demonstrate the routes of trypsin absorption throughout the intestinal epithelium, expanding our previous findings. With this purpose we perfused a trypsin solution into the small intestine of the rat and used immunoperoxidase and immunofluorescence techniques to study its absorption across the intestinal enterocyte layer.
Materials and Methods

Antibodies against trypsin

Rabbit polyclonal antibodies against TLCK-trypsin (porcine trypsin inhibited by reaction with tosyl-L-lysyl-chloromethyl-ketone) were produced as described by Arnon & Schechter (1966).

Jejunal perfusion

Two groups of male Wistar rats weighing (180-220 g) were used: group 1 (control) rats were perfused with tyrode solution (N=5); group 2 (experimental) rats were perfused with trypsin solution (N=5). The abdominal cavity of animals under sodium pentobarbital anesthesia (40 mg/kg) was opened through a midline incision. A 15-cm segment of jejunum below the duodenojejunal ligament was isolated while maintaining its nerve and blood supplies. Two cannulas were introduced and tied to the extremities of the loop for perfusion. The abdominal wall was then closed in order to preserve normal temperature and to avoid tissue dehydration. The cannulas were exteriorized through the extremities of the abdominal suture (Borges et al., 1995).

A Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM CaCl₂, 0.49 mM MgCl₂, 11.9 mM NaHCO₃, and 5 mM D-glucose) at 37°C, pH 8 (buffered by HCO₃⁻) was perfused, at a rate of 0.5 ml/min for 20 min in order to equilibrate the jejunal lumen. Tyrode solution containing 0.5 mg/ml of porcine trypsin (Sigma, USA) was perfused for an additional 45 min. Other rats were similarly perfused using Tyrode solution only (controls).
Immunoperoxidase

The unlabeled-antibody peroxidase-antiperoxidase (PAP) staining method (Sternberger et al., 1970) was used for identification of trypsin immunoreactivity in tissue sections. Tissue fragments from the jejunum were washed with tyrode solution and collected for histological and immunoperoxidase studies after fixation in Bouin's fluid containing 2% acetic acid for 18 to 24 hours. Sections 5 μm thick were obtained from each tissue block. Phosphate-buffered saline (0.01 M, pH 7.2) was employed throughout as the washing buffer after each step and as the diluent. The incubation of the sections with the primary antibody was carried out at 4°C overnight. The other incubations were carried out at room temperature for 30 minutes. Briefly, the PAP method steps were as follows: (1) incubation in 0.3% hydrogen peroxide to block endogenous peroxidase activity; (2) incubation with 1:30 normal porcine serum to reduce nonspecific binding by the second antibody; (3) incubation with the primary antiserum TLCK-trypsin (titer, 1:000); (4) incubation with porcine anti-rabbit immunoglobulin (Dako, Copenhagen) (titer, 1:180); (5) incubation with rabbit PAP (Miles-Yeda Ltd, Rehovot, Israel) (titer, 1:250); (6) incubation with 25 mg/dl of 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide solution for five to ten minutes under microscopic control, and (7) hematoxylin (Merck, Brazil) counterstaining, dehydration, clearing, and mounting. For negative controls, normal rabbit serum was employed instead of the primary antiserum. Intraductal pancreatic secretion of normal rat pancreas was used as positive control for trypsin-like immunoreactivity.
Immunofluorescence

Samples of the same tissues (jejunum) were washed with tyrode solution, embedded in OCT and snap frozen in isopentane in a beaker immersed in liquid nitrogen and kept frozen at -70°C. Sections 8 μm thick were obtained from each block of frozen unfixed tissue in the cryostat at -30°C. Then, the samples were fixed with ice-cold acetone for 15 min, blocked with 3 % BSA in PBS solution (1 hour), labeled with 1:25 dilution of the rabbit polyclonal antiserum TLCK-trypsin for one hour, and stained with (goat) fluorescein-conjugated anti-rabbit secondary antibody for 45 minutes (Calbiochem, USA) (titer, 1:100) (Leite et al., 1999). For negative controls, normal mouse serum (titer, 1:400) in PBS solution with 3 % BSA (Sigma) was employed instead of the primary antibody. Specimens were mounted in fluoroGuard Antifade reagent (Bio-Rad). Labeled tissue was examined by fluorescence microscopy.

Results

Immunoperoxidase staining did not show immunoreactivity inside the enterocytes, nevertheless a material exhibiting trypsin-like immunoreactivity was identified along the luminal border of the intestinal surface cells. Intracellular immunoreactivity was not seen in immunoperoxidase staining sections (Fig. 1).

Immunofluorescence of the jejunum tissues from trypsin-treated-animals revealed trypsin-like immunoreactive sites (Fig. 2). The labeling was strong in the columnar epithelium that did express trypsin-like material. On the other hand, the control rats perfused with tyrode solution, or negative controls (jejunal mucosa of
trypsin-perfused-animals without primary antibody or jejunal mucosa of tyrode-perfused-animals without primary antibody) were negative (Fig. 2).

Discussion

Proteolytic enzymes are able to increase the permeability of the mucosal epithelium and, hence, facilitate their own absorption by a mechanism of self-enhanced paracellular diffusion (Bock et al., 1998; Kolac et al., 1996). However, this process was examined by measuring the transepithelial electrical resistance and the fluorescent transport markers across the intact monolayer. Previous studies have demonstrated that after intestinal perfusion, trypsin was transferred across the mucosa to the blood circulation in which small levels of the enzyme were detected (Borges et al., 1995). Besides, the morphological examination of the tissue at the end of the trypsin perfusion experiment showed that no damage was induced in the tissue and that the integrity of the intestinal wall was maintained. Nevertheless, the route of trypsin absorption was not demonstrated

In the present study, we investigated the sites of trypsin immunoreactivity all along the mucosa after its perfusion through the intestinal lumen. Trypsin was recognized by the anti-TLCK-trypsin antibody by both the immunofluorescence or immunoperoxidase techniques. The nature of the trypsin absorbed, either as an intact molecule or as fragmented peptides, could not be defined. No structure in the intracellular compartments of the enterocytes was specifically labeled by the immunoperoxidase technique. Intracellular trypsin-like immunoreactivity in intestinal mucosa was not showed by immunoperoxidase staining, probably due to the destruction of intraepithelial reactivity by the fixation and paraffin embedding processes. Besides the quantity of trypsin absorption by enterocytes was relatively
low, i.e. about 6\% of the total perfused trypsin (Borges, et al., 1995). Immunofluorescence was used in order to increase the recovery of receptor-binding sites that can survive in frozen tissue sections. The Immunofluorescence technique demonstrated the presence of trypsin-like immunoreactivity in the villi enterocytes. This immunoreactivity was not detected in the absence of specific antibody. In summary, trypsin-like immunoreactivity was found inside the enterocytes after trypsin perfusion, expanding our previous results and indicating a transcellular transport of this macromolecule.
**Figure Legends**

**Fig. 1** A- Paraffin section of the rat jejunal mucosa after trypsin infusion, immunostained with an anti-trypsin antibody. Strong trypsin-like immunoreactivity is seen along the epithelial border. B- Lack of immunoreactivity in the epithelial border of control rat perfused with tyrode solution. A and B Immunoperoxidase x 400.

**Fig. 2** Immunofluorescence micrographs using cryosections of the rat jejunum stained with anti-trypsin antibody. A- Rat perfused with trypsin solution; columnar epithelium expresses trypsin-like material. B- Control rat perfused with tyrode solution. C- Negative control, jejunal mucosa of trypsin-perfused-animals without primary antibody. D- negative control, jejunal mucosa of tyrode-perfused-animals without primary antibody (D). (A,B,C,D) x 400.
REFERENCES


Effect of angiotensin-(1-7) on jejunal absorption of water in rats

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The effect of angiotensin-(1-7) on jejunal water absorption was investigated in rats. The jejunal sac of anesthetized rats was filled with two ml of tyrode solution containing 3.7 MBq of tritiated water. A femoral vein was cannulated for administration of peptides and drugs. Water absorption peaked at approximately 15 minutes after tritiated water administration. Infusion of Ang-(1-7) at the dose of 0.7 μg/kg min produced a significant increase in jejunal water absorption (8.16 ± 0.35)% H2O/jejunal loop dry weight (g) as compared to the control (6.27 ± 0.63)% H2O/jejunal loop dry weight (g). The Ang-(1-7) antagonist A-79 abolished the effect of Ang-(1-7) on water absorption (4.81 ± 0.28)% H2O/jejunal loop dry weight (g). A similar effect was produced by the AT1 receptor antagonist losartan. The increase in jejunal water absorption produced by Ang-(1-7) was also blocked by the nitric oxide synthase inhibitor, L-NAME (4.60 ± 0.34)% H2O/jejunal loop dry weight (g) while indomethacin did not change the Ang-(1-7) effect. These data suggest that the effect of Ang-(1-7) on the jejunal loop is mediated by activation of a losartan-sensitive selective receptor. Furthermore, the effect of Ang-(1-7) on jejunal water absorption is mediated by a nitric oxide-related mechanism and is not affected by cyclooxygenase pathway products.

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ABSORÇÃO JEJUNAL DE TRIPSINA EM RATOS DEMONSTRADO POR IMMUNOFLUORESCÊNCIA. Borges, E. L.; Leite, M. de F.; Barbosa, A. J. A.; Alves, J. B.; Fisiologia e Biofísica, UFMG; Morfologia, UFMG.


Métodos e Resultados: Foram utilizados ratos Wistar (N=5), anestesiados com pentobarbital sódico e submetidos a uma laparotomia mediana para exposição e isolamento de um segmento de 15 cm de alça jejunal. Solução de Tyrode (pH 8.0), contendo tripsina na concentração de 0,5 mg/mL foi infundida (0,5 ml/min) através de alça jejunal isolada por 40 minutos. Forte imunoreatividade tripsina-like foi observada na borda epitelial de specimens coradas pela Peroxidase. Intensa marcação pela fluorescência também foi observada no epitélio colunar expressando a presença de material tripsina-like, nos ratos perfundidos com tripsina. Na ausência do anticorpo primário e nos ratos perfundidos com solução de Tyrode essa marcação não foi evidenciada.

Conclusões: Esses resultados mostram evidências a favor da absorção trancelular da tripsina pelo enterócito.

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