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Analgesic and anti-edematogenic effects of oral trypsin were abolished after subdiaphragmatic vagotomy and spinal monoaminergic inhibition in rats

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ABSTRACT

Aims: Rheumatoid arthritis brings great burdens to the patients. In addition to the highly expensive treatment, they are commonly associated with severe side effects. In such context, the research for safe and affordable treatments is needed.
Main methods: Arthritis was induced by CFA (0.5 mg/mL) in female wistar rats. Trypsin was given p.o. (2.95 mg/kg; 2 mL) 24 h after the intra-articular CFA injection. Articular incapacitation was measured daily by counting the paw elevation time (PET; s) during 1-min periods of stimulated walk, throughout the 7-days after intra-articular CFA injection. Articular diameter (AD) was accessed just after each PET measurement, taken the difference between naïve and diseased knee-joint diameter (cm).
Key findings: The present study showed that orally administered trypsin was able to reduce nociception and edema, effects that could be observed throughout the evaluation period. These effect, however, were not observed in animals underwent subdiaphragmatic vagotomy, suggesting a vagal mediation for trypsin effects. Likewise, these effects were blocked in rats which received intrathecal injection of the neurotoxins 5,7-dihydroxytryptamine or 6-hydroxydopamine, suggesting the involvement of spinal amines from axon terminals.
Significance: The present study proposes that oral trypsin may cause vagal activation, followed by the activation of descending inhibitory pathways and such mechanism may lead to a novel approach for the treatment of arthritis.

1. Introduction

Rheumatoid arthritis (RA) is an auto-immune disease characterized by articular inflammation, hyperalgesia, leukocyte infiltration, and angiogenesis. Incapacitation due to pain and articular rigidity are the major complaints of RA patients. The pharmacological and biological options for its treatment can only modify its symptomatology and brings additional burden to the patients, either due to their toxicity or due to their high cost [1,2]. In this scenario, electrical stimulation of the vagus nerve is suggested as an innovative approach, since it seems to reduce the arthritic symptoms, as well as preventing its development, which could be safer [3–6]. Vagal stimulation was shown to reduce several inflammatory parameters [7,8] as well as nociception [9–11], being this last possibly mediated by the monoaminergic neurotransmission in the spinal cord [12,13].

However, the subdiaphragmatic vagal branches innervating the gastrointestinal tract are intimately connected with structures such as the *lamina propria* and myenteric plexus, being able to receive the influence of substances in the lumen [14,15]. Such anatomical characteristic may provide a better way to chemically activate vagal afferents, by orally-given substances, instead the invasive direct electrical stimulation.

Among the several pharmacological receptors in the visceral afferents, PAR-2 is thought to be expressed in mesenteric nerve terminals [14] and may be an interesting target for several reasons. PAR-2 activation can stimulate vagal afferents either directly or indirectly, due to the secondary release of inflammatory mediators possessing sensitizing effects upon sensory fibers [16–21]. Trypsin is a well-known endogenous PAR-2 and PAR-4 activator, although PAR-2 can also be activated by mast cell tryptase and PAR-4 by plasmine as well, among several other proteases [22,23,24].

Early in the 1950s, Parenzyme®, an oily suspension of trypsin designed for intravenous injection, was marketed for the treatment of inflammation such as phlebitis, ocular inflammation, and traumatic wounds [25,26]. The success of this product led to the release of several presentations, including an oral formulation of trypsin, Orenzyme®. However, parenzyme and related compounds were eventually discontinued in the US market, which was followed by other countries. The main reason was that, by the 1970s, more restrictive laws for renewing drug licensing came to require proof of effectiveness in large

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clinical trials besides rigorous safety data [26]. Unfortunately, drug companies were no longer interested in such an investment in favor of other new compounds that were arriving.

As a result, the clinical efficacy of such oral enzymes is unknown, and even less their mechanism of action. A study done for 3–7 weeks with 63 patients suggested that a combination of bromelain, trypsin, and rutin could be as effective as diclofenac in the symptomatic relief of osteoarthritis of the knee [27]. Taking these data into account, the present study aimed to evaluate the effectiveness of oral trypsin in an immune-related arthritis model in rats, and the possible involvement of the vagus nerve and central mechanisms in its action.

2. Materials and methods

2.1. Animals

The experiments were performed on female *Rattus norvegicus* of Wistar strain rats, from 70 to 90 days old, weighting 180–200 g during the time course of the study. They were housed in a temperature-controlled room (20 ± 1 °C) under a 12–12 h light/dark cycle with free access to water and food. Animals came from de Central Vivarium of the institution (UFSC) housed in a group of five animals *per* home cage lined with sawdust. This study followed the ethical guidelines of the International Association for the Study of Pain (IASP, 1983), was previously approved by the local ethics committee for animal use (CEUA-UFSC: PP00723), and it complies with the ARRIVE guidelines.

2.2. Arthritic incapacitation measurement

Arthritic nociception was evaluated by the rat knee-joint incapacitation test [28]. The rats were placed on a revolving cylinder (30 cm diameter; 3 rpm) for 1 min periods, while a computer-assisted device measured the total time during which the right hind paw was not in contact with the cylinder surface, yielding the paw elevation time (PET, s). Before the knee-joint injection of CFA stimulus, the PET was 10 s on average. CFA increases PET only in the affected limb.

An exclusion criteria was adopted where the animals not presenting a minimum PET value of 20 s after the rechallenge were not used, since with such lower PET an analgesic effect could not be detected. Thus, all experimental groups started with 10 animals so we could maintain an average of 7–8 animals in each. All remaining animals were distributed in the experimental groups according to the mean PET values in order to homogenize the starting incapacitation level. In addition, each home box contained one animal of each experimental group.

2.3. Articular diameter increase

In order to quantify the inflammatory edema induced by CFA, the rats were restrained in the supine position and a micrometer was used to measure the articular diameter (in centimeters) of the inflamed knee-joint, through the medio-lateral axis, at three points along the proximo-distal direction. The highest value was chosen.

2.4. Subdiaphragmatic vagotomy

Based on a previously described procedure [29], the animals were anesthetized with xylazine:ketamine mixture (5:90 mg/kg, i.p.) and underwent bilateral vagotomy, having their ventral and dorsal branches of the vagus nerve dissected. Sham-operated animals were submitted to the same procedure, but without vagus nerve dissection. After sutured, the animals received antibiotics shots (Shotapen®, Virbac; 100 μL/animal) and were allowed four days before the experimental session.

2.5. Intrathecal injections

Intrathecal drug injections were performed at the lumbar level of the spinal cord according to the method described elsewhere [30]. In short, the animals were anesthetized with isoflurane (2% in oxygen) and a 29-gauge needle was carefully inserted between the L5–L6 vertebrae space until a flick of the rat's tail was observed. This reflex indicates that the spinal channel has been reached. Injections did not exceed 20 μL.

2.6. Drugs and treatments

5,7-dihydroxytryptamine (5,7-DHT), 6-hydroxydopamine (6-OHDA), dexamethasone, and porcine trypsin were purchased from Sigma-Aldrich do Brasil Ltda (São Paulo, SP, Brazil). Trypsin (2.95 mg/kg; 1 mL) and dexamethasone (4 mg/kg; 1 mL) were diluted in saline and were given p.o. 24 h after intra-articular inflammatory stimulation. The animals fasted for 12 h before oral treatments. The neurotoxins 6-OHDA (10 μg/20 μL) and 5,7-DHT (20 μg/20 μL) were diluted in an ascorbic acid-containing PBS solution at 2% w/v, and injected intrathecally four days before arthritis induction.

2.7. Experimental design

The arthritis-like condition was induced using Complete Freund Adjuvant (*Mycobacterium butyricum*, DIFCO; 0.5 mg/mL). Animals were first immunized with a subcutaneous injection of 50 μL in the base of the tail, and seven days later, they received an equal dose of CFA in the right knee-joint (rechallenge), producing a monoarthritis in this joint. Immediately before the rechallenge, arthritic incapacitation and articular diameter were evaluated, and then daily after that for seven consecutive days. This procedure was a modification from our previous work [31], since we had observed that enough immunization could be occurring in a shorter time.

Oral trypsin was given to animals that successfully responded to the arthritis induction, 24 h after the rechallenge. Surgical or spinal cord injection procedures were never done in the same animals, and were carried out 3–4 days before the second exposure to CFA, in different animals. The experimenter was blinded about the treatments but not about the vagotomized group.

2.8. Allometric extrapolation

The allometric method enables the extrapolation of doses among individuals with different body mass [32], and was used to calculate an equivalent dose of trypsin for rats as that used in humans.

$$\frac{[\mathrm{MBR}\ (\mathrm{m}) \div \mathrm{total\ dose}] \times \mathrm{MBR}\ (\mathrm{t})}{\mathrm{Weight}\ (\mathrm{t})} = \mathrm{Dose/kg}$$

Given the metabolic basal rate (MBR) of the animal model (human; m = 10,373) and of the animal target (rat; t = 26.7), the total dose used in the animal model can be extrapolated to the weight of the animal target. The parenzyme® total daily dose according to the original patient information leaflet was 320,000 IU/day. Assuming that 1500 IU of purified trypsin is equivalent to 1 mg, the total dose was approximately 200 mg. Doing all substitutions in the formula, we reached to the final dose for rats of nearly 2.95 mg/kg.

2.9. Statistical analysis

All statistical analyses were carried out using the software Statistica 7.0®. Data were expressed as the mean ± SEM as shown in the figures. The PET and AD time-course curves were compared by two-way ANOVA followed by Duncan's *post hoc* test when a P level of <0.05 was detected. In this test, the P level indicated refers to the whole time-course curve (treatment).