Peptides 71 (2015) 72–76

Contents lists available at ScienceDirect

Peptides

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

In vitro ACE-inhibitory peptide KGYGGVSLPEW facilitates noradrenaline release from sympathetic nerve terminals: Relationship with the lack of antihypertensive effect on spontaneous hypertensive rats

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ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form 24 April 2015
Accepted 17 June 2015
Available online 25 June 2015

Keywords:
Antihypertensive
Angiotensin-converting enzyme
Bioactive peptides
Hypertension
Mechanisms of action
Whey

ABSTRACT

This study aimed to validate the antihypertensive activity of the angiotensin-converting enzyme (ACE)-inhibitor whey protein hydrolysate (WPH) obtained through the action of proteolytic enzymes from *Cynara Cardunculus*. The antihypertensive activity of WPH fractions containing peptides with molecular weight below 3 kDa (Whey < 3 kDa) and 1 kDa (Whey < 1 kDa) along with the antihypertensive activity of three potent ACE-inhibitory peptide sequences (DKVGINYW, DAQSAPLRVY and KGYGGVSLPEW), previously identified in WPH, were also investigated. In parallel, the influence of KGYGGVSLPEW (the most potent ACE-inhibitory peptide sequence) on AT_1 receptors (a common pharmacological target of antihypertensive therapies beyond ACE), was evaluated. The effect of WPH and fractions (300 mg/kg) and peptide sequences (5 mg/kg) on systolic, diastolic and mean blood pressure was evaluated by telemetry on spontaneously hypertensive rats (SHR), after single oral administration. Despite their ACE-inhibitory effect *in vitro*, neither WPH, Whey <3 kDa, Whey <1 kDa or peptide sequences exhibited antihypertensive activity. In addition, KGYGGVSLPEW was not only devoid of AT_1 receptor antagonism but, on the contrary, had a similar effect to that of Ang II by facilitating the noradrenaline release from sympathetic nerve terminals. *In vitro* ACE blockade does not always correlate with antihypertensive activity and food-derived peptides cannot be classified as antihypertensive agents based exclusively on *in vitro* assays. The absence of an antihypertensive effect may also be a result of the interaction of these compounds with other components of the systems involved in the blood pressure control.

1. Introduction

Hypertension, *i.e.* high blood pressure, is a major risk factor for cardiovascular diseases (CVDs). CVDs are the main cause of death worldwide and its control has a large impact in the health state of human populations.

In the management of hypertension, dietary and lifestyle interventions assume a special relevance [1]. In this regard, a number of food-derived peptides capable to reduce systolic (SBP) and diastolic blood pressure (DBP) in both animal models and humans has been identified based on the ability to inhibit angiotensin-converting enzyme (ACE) [2–5]. The best characterized antihypertensive

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http://dx.doi.org/10.1016/j.peptides.2015.06.005
0196-9781/

peptides are IPP and VPP derived from milk casein that are already used as functional ingredients for the blood pressure control [6].

Whey and whey components are used by the food industry in a wide variety of applications but still, only 50% of the whey production in the European Union is utilized for human consumption [7]. The remaining amount is sold at lower prices for incorporation into animal foods or is disposed as waste after removal of organic load. Nevertheless, whey is a rich source of proteins (β-lactoglobulin and α-lactalbumin) which represent about 20% of bovine milk total protein content [8]. As previously reported, whey proteins can also be hydrolyzed into ACE-inhibitory peptides through the action of cardosins presented in the aqueous extract of the flowers of the thistle (*Cynara cardunculus*) [9]. However, as reviewed by our group, *in vitro* ACE-inhibitory activity does not always correlate with antihypertensive activity and the biological activity of these whey derived peptides needs further validation [10]. Moreover, the putative antihypertensive effects of these peptides may be mediated by mechanisms other than ACE inhibition, such as angiotensin II (Ang II) type 1 receptor (AT_1) antagonism [10].

The aim of this study was to investigate the short-term oral antihypertensive effect of a mixture of bovine, ovine and caprine whey protein hydrolysate (WPH) brought about by *C. cardunculus* on a well characterized model to study hypertension – spontaneously hypertensive rats (SHR). The antihypertensive activity of two WPH fractions containing peptides with molecular weight (MW) below 3 kDa or 1 kDa along with the antihypertensive activity of three potent ACE-inhibitory peptide sequences (DAQSAPLRVY, DKVGINYW and KGYGGVSLPEW), previously identified in WPH, were also evaluated [9]. In parallel, the influence of KGYGGVSLPEW (the most potent ACE-inhibitory peptide encrypted in WPH) on an AT_1 receptor-mediated effect of Ang II was also investigated.

2. Methods

2.1. Preparation of whey protein hydrolysate and fractions

WPH was obtained from a mixture of bovine, ovine and caprine whey, according to the method previously described, with some modifications [9]. Briefly, the mixture of bovine (80%), caprine (18%) and ovine (2%) whey, kindly provided by Saloio (Torres Vedras, Portugal), was ultrafiltered with a 10 kDa cut-off membrane. The resulting retentate was further hydrolyzed with an aqueous extract of *C. cardunculus* (Formulab, Maia, Portugal) at an enzyme/substrate ratio of 3% (v/v). After 3 h of incubation at pH 5.2 and 55 °C, this protein hydrolysate was ultrafiltered with a 10 kDa cut-off membrane and a portion of the filtrate (WPH) was submitted to nanofiltration with 3 kDa or 1 kDa cut-off membrane. After freeze-drying, WPH and the fractions with MW below 3 kDa (Whey < 3 kDa) and 1 kDa (Whey < 1 kDa) were reconstituted in autoclaved water and used for *in vivo* experiments. DKVGINYW, DAQSAPLRVY and KGYGGVSLPEW were custom-synthetized by GenScript (Piscataway NJ, USA).

2.2. *ACE inhibitory activity* in vitro *assay*

The ACE-inhibitory activity was measured using the fluorimetric assay of Sentandreu and Toldrá modified by Quirós et al. [11,12]. Non-linear fitting to the data was performed to calculate the IC_{50} (concentration needed to inhibit 50% of ACE activity) of WPH and fractions, as previously reported by Quirós et al. [13]. The protein content of WPH and fractions was determined by bicinchoninic acid assay (Pierce, Rockford IL, USA), using bovine serum albumin as a standard.

2.3. Study of the influence of KGYGGVSLPEW on AT_1 receptors

To evaluate the effect of KGYGGVSLPEW on AT_1 receptor we studied the influence of this peptide on a classical AT_1-mediated Ang II effect, namely the facilitation of noradrenaline release from sympathetic nerve terminals in the rat left ventricle [14].

Adult male SHR ($n = 3$) were euthanized by decapitation after isoflurane anesthesia. Left ventricle was isolated and then sectioned into several slices on a Krebs-Henseleit solution, previously aired with 95% O_2/5% CO_2, containing in mM: NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; Na_2EDTA, 0.03; ascorbic acid, 0.57 and glucose, 11. Slices were incubated with 0.1 μM [^{3}H]-noradrenaline (40.5 Ci/ml; PerkinElmer, Waltham, MA, USA) for 60 min at 37 °C and then transferred to perifusion chambers (Brandel perifusion system, Brandel, Gaithersburg, MD, USA). Tissues were washed with Krebs-Henseleit solution for 90 min at a flow of 0.8 ml/min. After washing, the effluent was collected continuously during 100 min, for radioactivity estimation in 5-min samples. During this procedure, tissues were submitted to two different periods of electrical stimulation (1 Hz, 50 mA, 2 ms, 300 pulses) at 25 and 75 min. The first period of stimulation was made in the absence of drugs ($S_{control}$) and the second period was made in the presence of drugs to be tested (S_{drug}). To evaluate the influence of KGYGGVSLPEW (1 μM) on the facilitatory effect of Ang II (200 nM) on noradrenaline release induced by electrical stimulation, KGYGGVSLPEW was added to the perifusion system 25 min before S_{drug} in the presence of Ang II. In addition, to evaluate the influence of KGYGGVSLPEW alone (300 nM and 1 μM) on noradrenaline release induced by electrical stimulation, this compound was added 25 min before S_{drug} in the absence of Ang II. Experiments were performed in the presence of 12 μM cocaine to block noradrenaline uptake, except for the incubation period when tissues were preloaded with [^{3}H]-noradrenaline. The radioactivity of samples, an index of noradrenaline release, was quantified by liquid scintillation counting.

The spontaneous outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of loss per min). The overflow induced by electrical stimulation was calculated by the subtraction of the spontaneous outflow immediately before the stimulation from the total outflow during the stimulation period. The overflow was calculated as a fraction of the tissue content (fractional release). Effects of drugs are expressed as the ratio between the fractional release in the presence (S_{drug}) or in the absence ($S_{control}$) of drugs under study.

2.4. Blood pressure measurements by telemetry

2.4.1. Animals and housing

Telemetry experiments were performed on male SHR obtained from Charles River (L'Arbesle, France) weighing 336 ± 13 g (animals in the first set of experiments) and 308 ± 11 g (animals in the second set of experiments). Telemetry transmitters (TA11PA-C40, Data Sciences International (DSI), St. Paul, MN, USA) were implanted in the abdominal cavity of SHR by Charles River with catheterization of the abdominal aorta. Animals were allowed to recover for at least one week before the initiation of experiments. SHR were housed individually in ventilated cages with free access to tap water and food (2014 Teklad Global Diets, Harlan Laboratories, Inc., Spain). A 12 h light/dark cycle was maintained in the room, at a constant temperature (22–24 °C) and relative humidity. Protocols were carried out in strict accordance with recommendations in the Directive 2010/63/EU for the use of experimental animals. The animal experimental protocol was approved by the Ethics Committee of the Faculty of Medicine of University of Porto (ORBEA FMUP).