Inhibitory effect of α_{S1} - and α_{S2} -casein hydrolysates on angiotensin I-converting enzyme in human endothelial cells in vitro, rat aortic tissue ex vivo, and renovascular hypertensive rats in vivo

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ABSTRACT

A great number of milk-derived peptides have been shown to exhibit angiotensin converting enzyme (ACE) inhibitory properties and thus potential utility in the regulation of blood pressure. The present work aimed to investigate the effects of 2 milk trypsin hydrolysates from α_{S1} - and α_{S2} -casein (CH1 and CH2, respectively) on ACE activity evaluated in human umbilical vein endothelial cells (HUVEC) in vitro, rat aortic tissues ex vivo, and renovascular hypertensive rat in vivo. Incubation of HUVEC and rat aortic tissues with CH1 or CH2 induced a concentration-dependent inhibition of hydrolysis of the ACE substrate hippuryl-histidyl-leucine (HHL), the hydrolysates being much less potent than perindopril (an ACE inhibitor). However, in contrast to perindopril, CH1 and CH2 failed to modify angiotensin I-induced aortic ring vasoconstriction. The HPLC profiles of rat plasma after intragastric administration were variable among individuals but none of the observed peaks corresponded to peptides comprising CH1 or CH2 or to fragments of these peptides. During 4 wk of cardiovascular monitoring, in hydrolysate-fed renovascular hypertensive rats, systolic blood pressure weakly decreased compared with the control group. However, the CH1-fed hypertensive rats exhibited a decrease of heart rate during the nocturnal period of activity. To conclude, our results show that CH1 and

CH2 inhibited ACE activity in HUVEC and rat aortic tissue but failed to antagonize the aortic-constricting effects of the natural agonist angiotensin I. Moreover, we demonstrated that CH1, to a greater extent than CH2, can slightly affect cardiovascular parameters although the ingested bioactive peptides could not be detected in the blood.

Key words: angiotensin-converting enzyme, milk trypsin hydrolysate, blood pressure

INTRODUCTION

Cardiovascular disease is the leading cause of death in most Western countries. Hypertension is an established independent risk factor for coronary heart disease, atherosclerosis, heart and kidney failure, and stroke. Hypertension is a key component in cardiovascular diseases, and epidemiological studies have shown that a small reduction in blood pressure (about 10 mmHg) can be associated with significant benefits in the population. Angiotensin converting enzyme (**ACE**) plays an important role in the regulation of arterial blood pressure by catalyzing the conversion of angiotensin I to the vasoconstrictor angiotensin II (Skeggs et al., 1956). Inhibition of ACE, which decreases angiotensin II plasma levels, is currently one of the most efficient tools used to fight hypertension, and several ACE inhibitors such as captopril, enalapril, and perindopril are currently used as first-line treatment (Bicket, 2002). During the last 2 decades, many studies have focused on the dietary prevention of hypertension development, with a particular interest in the biological activity of peptides and proteins derived from milk casein. In this context,

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several peptide preparations obtained from enzymatic digestion of casein have been investigated for their ACE inhibitory activity (for a review, see Huth et al., 2006). Moreover, some of them were shown to have antihypertensive activity against established hypertension in spontaneously hypertensive rats (Masuda et al., 1996; Nurminen et al., 2000) and in humans (Messaoudi et al., 2005; Mizuno et al., 2005; Cadée et al., 2007). Bioactive peptides, as specific protein fragments, are produced during digestion of milk in the gastrointestinal tract or during food processing (Meisel and Fitzgerald, 2003; Korhonen and Pihlanto, 2007). Their bioactivity is based on inherent amino acid composition and sequence, and the ACE inhibitory activity was mainly attributed to α_{S1} - and α_{S2} -casein fractions (Tauzin et al., 2002). However, the ACE inhibitory characteristics of these preparations have been mostly demonstrated in vitro using purified peptides and purified ACE, which does not always imply an antihypertensive effect in vivo. To reach their target sites after digestion or serve as a prodrug with in vivo activation, the ACE inhibitory peptides must first be absorbed in their intact form from the intestine and resist degradation by plasma peptidases. In contrast, their efficiency is based on their ability to reach, in their active form, the ACE anchored in the plasma membrane of the target cells.

We investigated 2 experimental milk trypsin hydrolysates from α_{S1} (Lecouvey et al., 1997; Nabhan et al., 2004) and α_{S2} bovine casein (Tauzin et al., 2002), named casein hydrolysates 1 and 2 (**CH1** and **CH2**, respectively), previously shown to inhibit pure human ACE in vitro with a synthetic substrate. The purpose of the present work was to follow the biological activity of CH1 and CH2 in the main steps leading from a chemical inhibitor to an efficient in vivo bioactive diet component. Study components included the evaluation of 1) ACE activity at the cell level using a primary culture model of endothelial cells from human umbilical vein (**HUVEC**), 2) ACE activity at the organ level using angiotensin-induced vasoconstriction of rat aortic ring, 3) plasma content in the components of CH1 and CH2 in vivo in rats by proteomic investigation, and 4) the efficiency of CH1 and CH2 in vivo in a rodent model of noninherited hypertension.

MATERIALS AND METHODS

Materials

Fetal bovine serum (**FBS**) and human serum (**HS**) were purchased from Biowest (Nuaille, France) and from the National Blood Transfusion Center (Villejuif, France), respectively. Endothelial cell growth supplement (**ECGS**) was purchased from BD Biosciences (Le

Pont de Claix, France), and M199 medium was purchased from Invitrogen (Cergy-Pontoise, France). All other chemical products were purchased from Sigma (Saint-Quentin Fallavier, France). Perindopril was a gift from W. Copalu (Servier, France).

Test Products

The CH1 and CH2 test products (200 g) were obtained after hydrolysis by trypsin of 2 casein substrates enriched in α_{S1} -CN and α_{S2} -CN, respectively, and prepared by Ingredia (Arras, France) according to Tauzin et al. (2002). The CH1 and CH2 hydrolysates were desalted by electrodialysis and then lyophilized.

Peptide Identification

The CH1 and CH2 products were analyzed by reverse phase (**RP**)-HPLC onto a Symmetry C18 column (2.1 i.d. $\times$ 150 mm, 5- μ m particle size, 100 Å porosity; Waters, Milford, MA) kept at 40°C. The column was connected to an HPLC (Hewlett Packard, 1100 Model, Agilent Technology, Massy, France). Detection was performed at 214 and 280 nm with a G1315A photodiode array detector (Agilent Technology). Quantities of 100 μ g of spray-dried hydrolysate were solubilized in 0.2% trifluoroacetic acid (**TFA**), filtered through 0.45- μ m polyvinylidene fluoride filters (Pall Corporation, Saint Germain-en-Laye, France), and injected. After a 3-min isocratic elution with 4% (vol/vol) acetonitrile containing 0.1% (vol/vol) TFA, a linear gradient was applied for 67 min at a flow rate of 0.25 mL/min until the solution reached 40% acetonitrile containing 0.1% (vol/vol) TFA. The molecular masses of CH1 and CH2 peptides were determined by on-line coupling of RP-HPLC with an electrospray source ionization-mass spectrometer (Perkin Elmer-Sciex API III⁺, Sciex, Thornhill, Ontario, Canada).

The separated peptides were sprayed through a 75- μ m i.d. fused-silica capillary inserted into a stainless steel capillary maintained at a 4.8 kV potential. Liquid nebulization was assisted by a coaxial airflow along the sprayer usually adjusted to the range of 0.3 to 0.5 MPa. The potential orifice (100 μ m i.d.) located at the interface between the sprayer and the mass analyzer was 75 V. A 1.2 L/min countercurrent flow of N₂ between the electrospray ionization source and the nozzle was used to prevent solvent cluster and to aid desolvation. Molecular masses were determined from the measured m/z (step size 0.33, dwell-time 0.5 ms). Assignment of the sequences was assisted by the use of the Bio multiview 1.3.1 package (Sciex). The identification of the peptides was based on the theoretical masses of the tryptic peptides from bovine α_{S1} -CN and α_{S2} -CN. Some sequences

were confirmed by tandem mass spectrometry (MS/MS) analysis with a API III⁺ (Sciex) as described previously (Gagnaire et al., 1996). Collected peptides were dried with a speed-vacuum and solubilized in 50% (vol/vol) acetonitrile containing 0.2% (vol/vol) formic acid. They were delivered to the sprayer by a syringe infusion pump (model 22, Harvard Apparatus, South Natick, MA) at a flow rate of 5 μ L/min.

Cell Culture

Human umbilical vein endothelial cells were isolated from fresh cord veins (Jaffe et al., 1973). Briefly, the cord veins were cannulated and flushed with 50 mL of PBS (1.37 M NaCl, 5.2 mM Na₂HPO₄, 40.2 mM KCl, 1.47 mM KH₂PO₄, 111 mM glucose, pH 6.5). Type I collagenase (273 U/mg) in PBS medium (0.1% wt/vol) was then infused for 5 min at 37°C, and detached cells were collected and centrifuged at $200 \times g$ for 5 min. The cell pellet was resuspended in M199 basal medium containing 20% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cells were grown on 0.1% gelatin precoated flask at 37°C in a humidified 5% CO₂ incubator. When they reached 80 to 90% confluence, the cells were trypsinized and cultured in basal M199 medium supplemented with 15% FBS, 5% HS, 2 mM glutamine, 37.5 μ g/mL ECGS, 50 U/mL heparin, and penicillin-streptomycin. The cells were used only until the third passage to avoid downregulation of ACE expression (Balyasnikova et al., 1998). The HUVEC were seeded in 6-well plates at a density of 10,000 cells/cm². Because endothelial cells express ACE in a confluence-dependent manner, the assessment of ACE activity was done at the maximal ACE level; that is, 4 or 5 d after confluence.

Determination of ACE Inhibition

The inhibitory effect of hydrolysates on ACE activity was determined in living HUVEC and in rat aortic tissue with a synthetic substrate of ACE, hippuryl-histidyl-leucine (HHL; Cushman and Cheung, 1971). The HUVEC were incubated in the cell incubator at 37°C in saline Puck medium containing 6.1 mM glucose and 3.4 mM HHL, in the absence or presence of perindopril (from 1 nM to 10 mM) or a casein hydrolysate (0.01 to 10,000 mg/L). Aortic tissues were incubated in the same medium, in the absence or presence of perindopril (0.1 mM) or a hydrolysate (10 to 1,000 mg/L). After 2 h, the incubation medium of HUVEC or aortic tissue was transferred into a fresh tube and the reaction was stopped by the addition of 5 μ M captopril, 100 mM EDTA, and 0.067% TFA. Assays were performed

in triplicate and repeated in 3 or 4 independent cell cultures or rat aortas, respectively.

The hippuric acid released from HHL was quantified by RP-HPLC onto a Symmetry C18 column (2.1 mm i.d. $\times$ 150 mm, 3.5 μ m, 100 Å; Waters) kept at 37°C. The column was connected to an Alliance 2695 model HPLC (Waters). The samples (10 to 100 μ L according to protein content) were filtered through 0.45- μ m polyvinylidene fluoride filters. A linear gradient from 8.0 to 12.5% acetonitrile in 0.1% TFA was applied for 9 min (flow rate 0.25 mL/min). Detection was achieved at 228 nm with a Waters 2996 photodiode array detector, and ACE activity was expressed as the percentage of the maximal activity obtained in control medium. In HUVEC, the inhibitory potency of CH1 and CH2 was measured by the 50% maximal inhibitory concentration (IC₅₀), obtained after logarithmic linearization of the curve [% activity/(1 - % activity)] = f [CH1 or CH2 (mg/L)].

Influence of Hydrolysates on Rat Aortic Vasoconstriction Induced by Angiotensin I

Aortic rings were prepared from 280- to 320-g male Wistar rats (Charles River Laboratories, L'Arbresle, France). The rats were anesthetized with pentobarbital (60 mg/kg i.p.). Thoracic aortas were dissected free and placed in ice-cold Krebs-Henseleit solution containing NaCl (116.3 mM), KCl (5.4 mM), CaCl₂ (1.8 mM), NaH₂PO₄ (1.04 mM), MgSO₄ (0.83 mM), NaHCO₃ (19 mM), and glucose (5.5 mM), pH 7.4. The blood vessels were cleaned of connective tissue and cut into 2- to 3-mm rings. The rings were suspended isometrically between 2 stainless steel hooks in organ chambers containing 5 mL of Krebs solution at 37°C and continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide. The aortic rings were attached to a force-displacement transducer connected to an amplifier (Bionic Instrument, Phymep, France) and a data acquisition system (PowerLab, AD Instruments Ltd., Bella Vista, New South Wales, Australia) to record tension changes using Chart version 5 data analysis software (AD Instruments Ltd.). After a stabilization period, the rings were stretched in a stepwise manner and were allowed to equilibrate at 2 g over a period of 1 h, during which the Krebs-Henseleit solution was changed every 15 min. The arterial rings were then challenged with KCl (60 mM), to evaluate their functional integrity. After the developed tension reached the peak value, the rings were washed with Krebs solution and then allowed to stabilize for 40 min. Subsequently, the rings were contracted with phenylephrine (3 μ M), and the presence of a functional endothelium was confirmed by

the relaxation induced by acetylcholine (ACh, 1 μ M). Endothelium was considered intact when ACh caused at least a 75% relaxation of the ring. Preliminary dose-response curves led to the choice of 2 concentrations of angiotensin I (1 and 0.1 μ M), which elicited an increase in aortic tension of 850 ± 1.3 mg and 480 ± 3 mg, respectively. In the first series of experiments, after washout and renewed stabilization, the rings were incubated for 15 min with CH1 or CH2 (10 to 1,000 mg/L) or perindopril (1 nM to 0.1 mM), before angiotensin I (0.1 or 1 μ M) administration. In the second series of experiments, testing the time course of the effects, the rings were incubated for 30, 60, 90, and 120 min with CH1, CH2 (1 g/L), or incubation medium, before administration of angiotensin I (0.1 μ M). Angiotensin I-induced contraction of isolated aortic rings was expressed as the percentage of the contraction induced by KCl (60 mM) on each aortic ring. The inhibitory effect of perindopril on angiotensin I-induced vasoconstriction was expressed as the percentage of the contraction induced by angiotensin I in the absence of perindopril; $E_{\max}$ indicated the maximal inhibition induced by perindopril (0.1 mM). The concentration of perindopril that produced half of its maximal inhibition, the IC_{50} , expressed as the negative log mol/L ($-\log IC_{50}$), was derived from log-logit transformations of individual concentration-response curves.

Animals

All animals received attentive care, and study protocols complied with the institutional guidelines for animal research (NIH pub. no. 85-23, National Research Council, revised 1996). The Animal Care and Use Committee of the Faculty of Pharmacy, University of Paris-Sud 11, approved the protocols. The animal holding facilities are registered under agreement number A 92-019-01, and the main authors are authorized to manage experiments on animals (agreement level I, ref. 92-261, 2006-09-12).

Absorption Assay

In the first assay, 24 fasting male Wistar rats (200–250 g; Charles River Laboratories) with permanent aortic catheters were force-fed with a single intragastric dose of CH1 or CH2 (doses 100 and 500 mg/kg, $n = 6$ per hydrolysate and per dose) in 10% glucose saline. Blood samples (1 mL) were collected on heparin from the aorta, first at 24 h before force-feeding (time 0) and then 5 to 90 min thereafter. The samples were immediately centrifuged ($3,000 \times g$, 4 min) to collect the plasma and then stored at -80°C before further analysis of the plasma peptide content by RP-HPLC. In the second

assay, 6 rats were force-fed CH1 or CH2, once, at 500 mg/kg. Blood samples were taken at 5, 10, and 20 min after feeding. Precipitation of proteins was obtained by mixing cold acetonitrile (800 μ L) and plasma (400 μ L). After shaking (90 s) and centrifugation (4 min, $3,000 \times g$), the supernatant (peptide fraction) was freeze-dried and stored at -20°C . The proteolytic activity in the samples was evaluated according to Todorova (2000) before the drying step. In parallel, to evaluate the rate of degradation of peptides in the plasma, 20 μ L of CH1 or CH2 (2 mg/mL in water) was added to 100 μ L of plasma and incubated at 37°C for 0, 5, 15, and 30 min. The reaction was stopped and the proteins were precipitated by the addition of 200 μ L of cold acetonitrile and centrifugation. The supernatant was freeze-dried and diluted with 120 μ L of HPLC buffer A, and 20 μ L was injected into the HPLC system. Measurement of the degradation rate of peptides in plasma was performed as follows: 20 μ L of CH1 or CH2 (20 mg/mL) was incubated at 37°C with 100 μ L of plasma. Samples were taken once every 5 min for 30 min and the peptide content was analyzed by HPLC as described above. The degradation rate of the peptide mixtures was evaluated by measuring the sum of the peak surfaces.

Purification and Identification of the Peptides

The freeze-dried fractions were resuspended in 50 μ L of solvent A (water/TFA 0.1%) and analyzed by RP-HPLC. The column, the temperature of which was held at 40°C , was a Jupiter Proteo C12, 90A, 4 μ m, 2×150 mm (Phenomenex, Torrance, CA) with a guard column. The samples were eluted with a linear gradient of solvent B (water/acetonitrile 60%/TFA 0.1%) in solvent A going from 5 to 60% in 50 min after 5 min of isocratic elution with solvent B 5%, at a flow rate of 0.3 mL/min. The absorbance was monitored at 215 nm. The injection volume was 20 μ L. The peaks were collected and analyzed by Edman sequencing on an automatic sequencer (494A, Applied Biosystems, Courtaboeuf, France) and by matrix-assisted laser desorption ionization–time-of-flight MS (Voyager DE-STR, Applied Biosystem), using α -cyano-4-hydroxycinnamic (α -CHCA) as the matrix.

Pathophysiological Investigations

Renal artery stenosis was surgically induced in rat to obtain the Goldblatt 2 kidney-1 clip (2K1C) model (Goldblatt, 1948) as described previously (Rousseau et al., 2001). This model is known to be fully modulated by ACE inhibitors (Rousseau et al., 2001). Briefly, 5-wk-old male Wistar rats ($n = 48$; Charles River Laboratories) were maintained at a constant tempera-

ture ($24 \pm 1^\circ\text{C}$) with a 12-h dark/light cycle. After 1 wk of acclimation, the rats (weighing 230 ± 10 g) were anesthetized (pentobarbital 40 mg/kg, i.p.) for application of a 0.2-mm-i.d. silver clip on the left renal artery ($n = 40$) or for sham operation ($n = 8$). All rats were then returned to individual cages and fed a standard rat chow (UAR, Villemoisson-sur-Orge, France) for 4 consecutive postsurgical weeks. After these 4 wk, blood pressure measurements were made to evaluate the establishment of hypertension to select the rats for the nutritional experiment. Systolic blood pressure (SBP) was measured in the morning on vigil and restrained rats (prewarmed for 10–15 min at 28°C) by tail-cuff (Powerlab, Ad Instrument) as described previously (Rousseau et al., 2001, 2003; Robbez Masson et al., 2008). Briefly, the rats were held in a plastic tube, and their tails were extended through a rubber cuff that was subsequently inflated with air. The pressure level at which the first pulse appeared after blood flow had been interrupted with the inflated cuff was designated the SBP. At least 10 determinations were made per animal, and the mean of the lowest 5 values was taken as the SBP level (Duarte et al., 2001). Only animals with a SBP >150 mmHg 4 wk after clipping were used for the dietary treatment (Edmunds et al., 1989). About 30% of the operated rats were withdrawn from the study, either because they failed to develop hypertension (due to development of a collateral artery) or because they developed malignant hypertension with a significant risk of death during the experimental period (Edmunds et al., 1989). The

remaining hypertensive (HT) rats were then randomly assigned to 3 dietary groups [control (HT-CON), HT-CH1, and HT-CH2 groups, $n = 8$ respectively], which were fed the different experimental diets (Table 1) for 5 wk; the sham-operated rats were maintained on the CON diet. Fifteen additional operated HT rats from all dietary groups and 3 sham-operated rats underwent a second surgery to implant a telemetry transmitter in the abdominal cavity (TL11M2-C50-PXT, DSI, St. Paul, MN) as described previously (Rousseau et al., 2003; Robbez Masson et al., 2008). Next, the rats were assigned to 1 of the 3 dietary groups ($n = 5/\text{group}$) and the sham-operated rats were maintained on the CON diet. Blood pressure (BP) and heart rate (HR) were monitored daily for 4 wk, starting 1 wk after surgery. The digital BP signals were sampled for 10 s once every 10 min. Night recordings were distinguished from day recordings. Heart rate, mean BP (MBP), SBP, and diastolic blood pressure (DBP) were recorded continuously, and pulse pressure (PP) was calculated from SBP and DBP [where $\text{PP} = (\text{SBP} - \text{DBP})$; Rousseau et al., 2003; Robbez Masson et al., 2008]. The interval between the Q wave on the electrocardiogram complex and the onset of the aortic BP pulse on the BP waveform was defined as the QA interval; this served as a noninvasive index to evaluate cardiac contractility by telemetry in awake animals (Cambridge and Whiting, 1986). Both BP recordings and the extracted cardiac contractility index (QA interval) were collected from vigil rats by telemetry during the night and day periods.

Table 1. Formulation of the experimental diets¹

Ingredient	Diet, ² g/kg of diet (% energy)		
	CON	CH1	CH2
Sucrose	100 (9.5)	100 (9.5)	100 (9.5)
Cornstarch	530 (50.5)	530 (50.5)	530 (50.5)
Cellulose	50	50	50
Mineral mixture ³	40	40	40
Vitamin mixture ³	10	10	10
Soy protein isolate ⁴	180 (17.1)	171 (16.3)	171 (16.3)
L-Cystine	5 (0.5)	5 (0.5)	5 (0.5)
Choline bitartrate	5 (0.5)	5 (0.5)	5 (0.5)
α_{S1} bovine casein ⁵ (CH1)	0	9 (0.8)	0
α_{S2} bovine casein ⁵ (CH2)	0	0	9 (0.8)
Cocoa butter ⁶	40 (8.6)	40 (8.6)	40 (8.6)
Sunflower seed oil ⁷	40 (8.6)	40 (8.6)	40 (8.6)

¹The basal mix of diets was homemade as pellets by the Unité de Préparation des Aliments Expérimentaux (UPAE, INRA, France) based on a formulation adapted from AIN-93 (Reeves et al., 1993).

²CON = rats fed diet without casein hydrolysate; CH1 = diet with casein hydrolysate from α_{S1} -CN; CH2 = diet with casein hydrolysate from α_{S2} -CN.

³Mineral AIN-93M and vitamin AIN-93VX mixtures, respectively; MP Biomedicals, Illkirch, France.

⁴MP Biomedicals.

⁵ α_{S1} and α_{S2} bovine casein hydrolysates (CH1 and CH2); Ingredia, Arras, France.

⁶Barry-Callebaut, Meulan, France.

⁷Lesieur, Antony, France.

Figure 1. Reverse phase-HPLC profiles of casein hydrolysates CH1 (a) and CH2 (b). The peptides known to be angiotensin converting enzyme inhibitors or their precursors (see Table 1) were identified by MS analysis and are indicated in the figure.

At the end of the dietary treatment, the rats were killed, and blood was collected and placed in an inhibitor cocktail of proteases and EDTA and centrifuged. The plasma was stored at -80°C for later biochemical analysis. Heart and kidneys were collected, rinsed in cold saline, and weighed. The EDTA-plasma angiotensin II level was determined using an angiotensin II enzyme immunoassay kit (cat. no. A05880, SPI-BIO, Montigny le Bretonneux, France) after methanolic extraction on phenyl cartridge (ref. no. D30004, SPI-BIO; Volland et al., 1999).

Data Analysis

For the *in vitro* and *ex vivo* investigations, the results were expressed as mean $\pm$ SEM. Statistical significance was tested with ANOVA followed by Bonferroni post-test. The data were expressed as mean $\pm$ SEM, $n = 5$ (physiological data) or $n = 8$ (biochemical data). Differences were considered significant when $P < 0.05$. A repeated-measures ANOVA was used to analyze the longitudinal time course follow-up of the cardiovascular parameters (SBP, DBP, PP, and HR) to determine any further increase in BP after the beginning of the dietary treatment. In the hypertensive groups, the data (SBP, DBP, MBP, PP, HR, and QA) recorded in the last 2 d of each week during the dietary treatment (wk 1, 2, 3, and 4) were evaluated using a GLM ANOVA followed by a Bonferroni (all pairwise) multiple comparison test when statistical significance was observed ($P < 0.05$). Statistical analyses were carried out using the NCSS 2000 program (NCSS statistical software, Kaysville, UT).

RESULTS

Identification of ACE Inhibitory Peptides in CH1 and CH2

The HPLC elution profiles of CH1 and CH2 are presented in Figure 1a and 1b, respectively. The sequences of the most important peptide peaks identified in the HPLC elution profiles of CH1 and CH2 were obtained. Identification of the peptides revealed that most of them are already known as ACE inhibitory peptides or are possible precursors of ACE inhibitory peptides (Table 2). This first result highlights the high content of the hydrolysates used in this study in peptides endowed with ACE inhibitory activity and their value for *ex vivo* and *in vivo* experiments.

ACE Activity in HUVEC and Rat Aortic Tissue: Effects of CH1 and CH2

HUVEC. As shown in Figure 2, HUVEC incubated in control medium time-dependently hydrolyzed the peptide substrate HHL in a linear fashion for up to 2 h. This hydrolysis was completely abolished by the ACE inhibitor perindopril (10 mM). When the cells were incubated for 2 h with CH1 or CH2 (0.01–10,000 mg/L), HHL hydrolysis decreased in a dose-dependent manner, as demonstrated in Figure 3 by the inhibition standard curves. The mean of IC_{50} values obtained for CH1 and CH2 in 3 independent experiments were 94.8 ± 6 and 64.8 ± 8 mg/L ($P = 0.027$), respectively. Perindopril tested in the same conditions decreased HHL hydrolysis in a concentration-dependent manner, with an IC_{50} value of 189 nM (data not shown).

Figure 2. Time course of hippuric acid liberation in human umbilical vein endothelial cells incubated in control medium (white bars) or in medium containing perindopril (10 mM; black columns). Results are expressed as the percentage of hippuric acid liberated by cells at time 0. Data are mean $\pm$ SEM of 3 independent cell cultures in triplicate.

Rat Aortic Tissue. As shown in Figure 4, CH1 and CH2 also induced dose-dependent inhibition of HHL hydrolysis in rat aortic tissues, with a maximal inhibitory effect of $79.7 \pm 1.2\%$ and $83.9 \pm 0.7\%$ with 1,000 mg/L of CH1 and CH2, respectively. Under the same conditions, perindopril (0.1 mM) induced strong inhibition of hippuric acid release ($99.7 \pm 0.1\%$; $n = 9$; data not shown). No difference was detected between CH1 and CH2 effects on ACE activity.

Effects of Hydrolysates on Angiotensin I-Induced Rat Aortic Ring Vasoconstriction. The influence of CH1 and CH2 was first tested on aortic ring vasoconstriction induced by angiotensin I (1 μM). At any concentration, CH1 and CH2 failed to modify the angiotensin I-induced aortic vasoconstriction after 15 min of incubation (data not shown). Under the same conditions, perindopril induced a concentration-dependent inhibition of constriction, with a log(IC₅₀) value

Table 2. Sequence of the main peptides present in casein hydrolysates CH1 and CH2 and information from the literature regarding their angiotensin converting enzyme inhibitory activity (50% maximal inhibitory concentration, IC₅₀)

Peak number ¹	Identification	AA sequence	Reference	IC ₅₀ (μM)
CH1				
1	α_{S1} -CN (f194–199)	TTMPLW	Maruyama et al., 1987	16
			Pihlanto-Leppälä et al., 1998	51
			Tauzin et al., 2002	12
2	α_{S1} -CN (f23–34)	FFVAPFPEVFGK	Maruyama and Suzuki, 1982	77
			Sekiya et al., 1992	77
			Tauzin et al., 2002	18
CH2				
1	α_{S2} -CN (f182–188) = α_{S2} -CN (f182–184) precursor			ND ²
2	α_{S2} -CN (f182–184)	TVY	Tauzin et al., 2002	15
3	α_{S2} -CN (f206–207)	YL	Mullaly et al., 1996	122
4	α_{S2} -CN (f81–91)	ALNEINQFYQK	Tauzin et al., 2002	264
5	α_{S2} -CN (f25–41) 1P and 2P = α_{S2} -CN (f25–32) precursor			ND
	α_{S2} -CN (f25–32)	NMAINPSK	Tauzin et al., 2002	60
6	α_{S2} -CN (f92–98)	FPQYLQY	Tauzin et al., 2002	14
7	α_{S2} -CN (f174–181)	FALPQYLK	Tauzin et al., 2002	4.3
8	α_{S2} -CN (f99–113)	LYQGPIVLNPWDQVK	Tauzin et al., 2002	ND

¹Peak numbers refer to labeled peaks in Figure 1a and 1b.

²ND = not detected.

Figure 3. a) Concentration-dependent inhibition by casein hydrolysates CH1 and CH2 (0.01–10,000 mg/L) of angiotensin converting enzyme (ACE) activity in human umbilical vein endothelial cells; b) determination of the 50% maximal inhibitory concentration (IC_{50}) values of CH1 and CH2, after logarithmic linearization. Each curve represents an individual experiment performed in triplicate. The mean $\pm$ SEM of IC_{50} values of 3 separate culture experiments are reported in the results section.

Figure 4. Effects of casein hydrolysates CH1 and CH2 (0.01–1 g/L) on angiotensin converting enzyme (ACE) activity in rat aortic tissue. Results are expressed as percentage of the maximal enzymatic activity, defined as that obtained in absence of hydrolysate in the medium of incubation. Data are mean $\pm$ SEM of 4 independent experiments.

of $7.03 \pm 0.53\%$ ($n = 5$) and an $E_{\max}$ value of $74.34 \pm 6.29\%$ ($n = 5$; data not shown). Because the inefficacy of hydrolysates may result from ACE saturation in the presence of angiotensin I ($1 \mu\text{M}$), their influence was tested in further experiments on aortic rings submitted to $0.1 \mu\text{M}$ angiotensin I. Under these new conditions, CH1 and CH2 were unable to affect angiotensin I-induced vasoconstriction (Figure 5a), which was still inhibited by 0.1 mM perindopril. We further investigated the influence of aortic ring incubation time with CH1 and CH2 on their effects on angiotensin I-induced vasoconstriction. Irrespective of the time of incubation (30, 60, 90, or 120 min), the contractile response to

angiotensin I was not influenced by either CH1 or CH2 (1 g/L ; Figure 5b).

CH1 and CH2 in Plasma After Digestion

The HPLC profiles of rat plasma after intragastric administration varied among individuals, but none of the main peaks observed in the plasma corresponded to peptides included in CH1 or CH2 or to fragments of these peptides (data not shown). Comparison of the chromatograms shows a large uniformity of the major peaks. In contrast, we observed great intersample variability of the minor peaks. Unfortunately, the peaks that showed significant surface changes disappeared rapidly, suggesting rapid turnover of these molecules in the plasma.

Some peaks with surface modifications during kinetics were analyzed by MS and Edman sequencing, but no peptide could be identified. The estimate of the level of proteolytic activity affecting CH1 and CH2 in the plasma indicated a degradation rate of 50% for CH1 and 60% for CH2 after 30 min. After 5 min, several peptides of the hydrolysates had totally disappeared from the plasma. In contrast, new peaks appeared, possibly resulting from degradation. The rapid rate of hydrolysis in plasma and the limitation of detection sensitivity could explain why no peptide from the CH1 and CH2 hydrolysates was found in the blood samples (Figure 6).

Effects of CH1 and CH2 on Blood Pressure In Vivo

As expected, all of the Goldblatt 2K1C rats exhibited established hypertension compared with the sham-

Figure 5. Contracting response to angiotensin I ($0.1 \mu\text{M}$) on rat aortic rings under control conditions after a 15-min incubation period with a range of concentrations of casein hydrolysates CH1 or CH2 (CH; 0.001–1 g/L) or with perindopril (P, 0.1 mM) (panel a) or after a range of incubation durations (30–120 min) with CH1, CH2 (1 g/L), or with control medium (CON) (panel b). Results are expressed as percentage of each ring maximal contraction induced by 60 mM KCl. Data represent the mean $\pm$ SEM of 5 experiments.

Figure 6. Measurement of the extent of the casein hydrolysate CH1 and CH2 peptide degradation in plasma by following the sum of all HPLC peak areas (■, CH1; ▲, CH2).

operated rats at the beginning of the dietary treatment (data not shown). The hypertension persisted after the 4-wk dietary treatment (Table 3). This hypertension was accompanied by a significant cardiac hypertrophy ($1,302 \pm 6.1$, $1,277 \pm 18.3$, and $1,279.5 \pm 10.1$ mg of heart weight for the HT-CON, HT-CH1, and HT-CH2 groups, respectively) compared with the sham-operated rats ($1,023.7 \pm 26.8$ mg). The heart weight/BW ratio was also significantly affected by pathology (3.3 ± 0.08 , 3.1 ± 0.17 , and 3.3 ± 0.28 for the HT-CON, HT-CH1, and HT-CH2 groups, respectively) compared with the sham-operated rats (2.1 ± 0.07). However, no statistically significant difference was seen between the dietary groups of the HT rats. During the 4 wk of cardiovascular monitoring, none of the dietary groups displayed any significant further increase in SBP, DBP (Figure 7a and 7b), MBP, or PP (Figure 7c and 7d).

Throughout the course of the dietary treatment, SBP, DBP, MBP, and PP (Figure 7) tended to decrease transiently or significantly decreased in the CH1-fed rats mainly during the night period after 2 wk ($P = 0.07$, $P = 0.09$, $P = 0.06$, and $P = 0.03$, for SBP, DBP, MBP, and PP, respectively) and after 3 wk ($P = 0.06$, $P = 0.05$, $P = 0.08$, $P = 0.003$, respectively). However, after 4 wk of diet, the parameters returned to values similar to those of the HT-CON rats. The cardiac parameters are shown on Figure 8. The HT-CH1 rats exhibited a significant decrease in nocturnal HR (with $P = 0.0029$) and an increase in nocturnal QA interval ($P = 0.0148$) from wk 3 of dietary treatment onward. In contrast, CH2 induced only a transient trend of PP decrease dur-

ing the second ($P = 0.05$) and a significant PP decrease during the third week of dietary treatment ($P = 0.02$), and this effect disappeared by the end of the experiment because of a decrease of PP observed in HT-CON rats. Throughout the experiment, SBP tended to decrease by 11 mmHg in the HT-CH1 group and by 6 mmHg in HT-CH2 group over the 4 wk of dietary treatment, compared with the HT-CON group. However, statistical significance of the effect was never reached. After 4 wk of dietary treatment, analysis of plasma angiotensin II revealed no statistically significant difference between the dietary groups of hypertensive rats (127 ± 42 , 93 ± 22.8 , and 78 ± 16.4 pg/mL of plasma from the HT-CON, HT-CH1, and HT-CH2 groups, respectively) because of high individual variability, but these values were high compared with those from sham-operated rats (65 ± 8.7 pg/mL).

DISCUSSION

The aim of this study was to investigate the influence of 2 milk protein trypsin hydrolysates (CH1 and CH2) on ACE activity in biological ex vivo models such as a HUVEC line and rat aortic tissue and on angiotensin I-induced rat aortic ring vasoconstriction. The ability of several peptides originating from milk casein to inhibit ACE has been widely investigated with the goal of using milk derivatives as potential dietary regulators of blood pressure. Although the activity of such peptides is easy to demonstrate in assay in vitro, their efficiency in vivo is more difficult

Table 3. Hemodynamic cardiovascular parameters during night and day periods from sham-operated rats versus male hypertensive (HT) Goldblatt 2K1C rats receiving casein hydrolysate CH1 or CH2 or no casein hydrolysate (CON group) after 4 wk of dietary treatment¹

Parameter ²	Sham-operated rat (control diet)	HT-CON	HT-CH1	HT-CH2
SBP (mmHg)				
Night	128 ± 0.3	217 ± 5.2‡	201 ± 5.8‡	196 ± 4.2‡
Day	120 ± 0.3‡	209 ± 5.4‡	200 ± 5.4‡	193 ± 4.4‡
DBP (mmHg)				
Night	95 ± 0.3	188 ± 4.0‡	179 ± 3.3‡	176 ± 2.6‡
Day	88 ± 0.3‡	183 ± 3.7‡	178 ± 3.0‡	173 ± 2.9‡
MBP (mmHg)				
Night	110 ± 0.3	160 ± 4.0‡	151 ± 3.6‡	146 ± 2.9‡
Day	105 ± 0.3‡	157 ± 4.1‡	150 ± 3.3‡	145 ± 3.7‡
PP (mmHg)				
Night	33 ± 0.2	57 ± 1.0‡	50 ± 2.3*‡	50 ± 1.0‡
Day	32 ± 0.1	54 ± 1.0‡	49 ± 2.3‡	49 ± 0.4‡
HR (beats/min)				
Night	399 ± 1.4	429 ± 5.6‡	369 ± 11.3*	403 ± 5.8
Day	344 ± 1.7‡	365 ± 10.7‡‡	368 ± 11.7‡	345 ± 11.2‡
QA (ms)				
Night	38 ± 0.4	30 ± 0.3‡	32 ± 0.6*‡	30 ± 0.7
Day	40 ± 0.2‡	33 ± 0.4‡‡	32 ± 0.6‡	32 ± 0.6‡‡

¹Data are mean ± SEM of the hemodynamic parameters over the last 3 d of dietary treatment, n = 5.

²SBP = systolic blood pressure; DBP = diastolic blood pressure; MBP = mean blood pressure; PP = pulse pressure; HR = heart rate; QA = extracted cardiac contractility index.

* = dietary effect compared with CON group (within the HT groups only), $P < 0.05$; ‡ = day-night difference for each considered parameter (within each group), $P < 0.05$; ‡‡ = surgery-induced hypertension compared with sham-operated rats, $P < 0.01$.

to assess, because no clear link between in vitro and in vivo activities has been established.

In Vitro Assays

The HPLC analysis of CH1 and CH2 peptides released by trypsin digestion revealed the presence of several peptides previously identified as ACE inhibitors. Moreover, the precursors of the peptides α_{S2} -CN (f25–32), α_{S2} -CN (f182–184), α_{S2} -CN (f25–41)-1P, α_{S2} -CN (f25–41)-2P, and α_{S2} -CN (f182–188) were also identified. The inhibitory activity of the peptides, as described in Table 2, was established in vitro with purified ACE. In the present study, we evaluated ACE inhibition efficiency of a rough preparation of these different peptides. In human endothelial cells, CH1 and CH2 inhibited ACE activity with IC_{50} values of 94.8 ± 6 and 64.3 ± 8 mg/L, respectively. According to steric exclusion chromatography, the average molecular masses of CH1 and CH2 were close to 1,150 and 1,390 Da, respectively (data not shown), which correspond to IC_{50} values of 82 and 46 μ M, respectively. Consequently, CH1, CH2, and individual peptide IC_{50} values (Table 2) were of the same order of magnitude. In contrast, CH1 and CH2, respectively, were 400 and 250 times less powerful than perindopril at inhibiting ACE, as previously reported for milk-derived lactoferricin-related peptides (Centeno et al., 2006).

Ex Vivo Assay

Based on these data collected at the cellular level, we investigated the effects of CH1 and CH2 at the organ level under more physiological conditions, using whole rat aorta. Hydrolysates CH1 and CH2 dose-dependently inhibited ACE activity in rat aortic tissue with IC_{50} values between 0.1 and 1 g/L (corresponding to 87–870 μ M for CH1 and 72–720 μ M for CH2). In fact, the hydrolysates are much less potent in this tissue compared with captopril (IC_{50} values between 7 and 23 nM; Cushman and Cheung, 1971; Pilhantho-Leppala et al., 1998) and 10 times less potent than in the HUVEC model. This difference in potency may be associated with a limited hydrolysate access to ACE in aortic tissues compared with HUVEC (Vermeirssen et al., 2004).

In contrast with these inhibitory effects, CH1 and CH2 did not affect angiotensin I-induced aortic ring vasoconstriction. This discrepancy could reflect different ACE inhibition efficacies of the hydrolysates, depending on the substrate, which was synthetic (HHL) in the ACE activity evaluation and biological (angiotensin I) in functional investigations. In the competition for binding to ACE, the affinity of the physiological substrate angiotensin I for ACE may be much higher than that of milk-derived peptides. This may not be the case for hippuric acid. These results agree with data from Centeno et al. (2006), who reported that some lactoferricin-related peptides with in vitro ACE inhibition

Figure 7. Evolution of blood pressure parameters during day (left side) and during the night period (right side) from male hypertensive Goldblatt 2K1C rats receiving casein hydrolysate (CH)1 ($\circ$), CH2 (Δ), or no casein hydrolysate ($\bullet$) for 4 wk. Upper panels show systolic (SBP) and diastolic (DBP) blood pressure; lower panels show mean blood pressure (MBP) and pulse pressure (PP). Data are expressed as means $\pm$ SEM of blood pressure parameters recorded during each period per day (n = 6).

activity cannot reduce the vasoconstrictive response of isolated aortic rings to angiotensin I. Moreover, several milk-derived peptides, including IPP, VPP, and KV-LPVP, had no effect on blood pressure in rats infused with angiotensin I (Fuglsang et al., 2003), whereas they potently inhibited *in vitro* HHL hydrolysis and were reported as hypotensive reference compounds (Nakamura et al., 1995; Maeno et al., 1996). Other authors also outlined the lack of correspondence between ACE inhibitory activity *in vitro* and antihypertensive effect *in vivo* (Li et al., 2004; Vermeirssen et al., 2004). Some hypotheses have been proposed that these milk-derived peptides do not act directly as acute ACE inhibitors but through other pathways or after long-term treat-

ment (Fuglsang et al., 2003). Indeed, feeding spontaneously hypertensive rats with goat milk hydrolysates for 12 wk resulted in an improvement of the impaired endothelium-dependent relaxation response to ACh (Geerlings et al., 2006). Another explanation is linked to the dual action of ACE, which is responsible for both the conversion of angiotensin I to angiotensin II and the inactivation of bradykinin, a potent vasodilator that stimulates NO release and prostacyclin (prostaglandin I₂) production in endothelium. Some active peptides may interfere preferentially with either angiotensin or bradykinin sites. This view is supported by Centeno et al. (2006), who studied rabbit carotid artery and showed that lactoferricin-derived peptides exhibit a capacity to

Figure 8. Evolution of the cardiac parameters during the day (left side) and during the night period (right side) from male hypertensive Goldblatt 2K1C rats receiving casein hydrolysate (CH)1 (○), CH2 (△), or no casein hydrolysate (●) for 4 wk. Upper panels show heart rate (HR, beats/min) and lower panels show QA interval (ms). Data are expressed as means $\pm$ SEM of cardiac parameters recorded during each period per day ($n = 6$). * $P < 0.05$ = dietary effect, significantly different from control (CON) group.

inhibit angiotensin I conversion but have no effect on bradykinin. These data emphasize the complexity of trying to predict the complex action of these numerous compounds. In our study, the effect of CH1 and CH2 on bradykinin metabolism was not evaluated and may warrant further investigation.

In Vivo Assay: Absorption Assay

We investigated the presence of peptides from CH1 and CH2 in the plasma of force-fed rats. Bioactive peptides have already been characterized from *in vivo* digests (Vermeirssen et al., 2004). Examples of such

peptides found intact in the plasma after gastrointestinal digestion are extremely rare in the literature, although the bioactive peptides IPP and VPP were detected in the heat-treated solubilized fractions of abdominal aorta in spontaneously hypertensive rats after the administration of sour milk (Masuda et al., 1996). In the present work, no peptide from CH1 or CH2 could be detected in blood samples. The most likely explanation of this observation is the rapid degradation of the peptides by proteolytic enzymes. We have observed *in vitro* that more than 50% of the peptides from the hydrolysates were degraded in 30 min. Moreover, resistance to proteolysis is highly variable among

peptides, and some are completely degraded in less than 5 min. Yamamoto (1997) reported that dipeptides strongly inhibiting ACE *in vitro* are often weakly active or inactive *in vivo*. That author attributed the loss of activity to peptide degradation *in vivo*. Other examples of *in vivo* hydrolysis of peptides can be found in the literature (Bechgaard et al., 1997; Endo et al., 1997; Fuglsang et al., 2004), and these data are in agreement with our results. Nevertheless, if short peptides can be totally degraded, degradation of larger peptides can give rise to new active peptides. Angiotensin II can be degraded into 4 peptides that inhibit ACE (Matsufuji et al., 1995). We have indeed observed the appearance of new peaks during the degradation kinetics of the hydrolysates. In addition, we cannot exclude the possibility that the circulating peptides were below the limit of detection. The rats were fed up to 500 mg/kg (i.e., 125 mg per rat daily). Our experiments revealed an IC_{50} close to 100 mg/L for CH1 and CH2, which is the limit of detection by the HPLC technique. In a clinical study, after patients consumed milk or yogurt, 2 long peptides, κ -caseino-glycopeptide (CMP) and the N-terminal peptide of α_{S1} -CN, were detected in plasma by immunochemistry, a much more sensitive and specific technique (Chabance et al., 1998). After subjects ingested 500 mL of milk (about 160 g of proteins, a protein charge 4-fold higher than in the present study), the authors found only 2 mg/L of CMP. In another study (Fosset et al., 2002), rats were fed 750 mg of CMP and the maximum concentration detected in the blood was 7 μ g/mL after 60 min. Regardless of length, some bioactive peptides are able to pass through the blood but their concentration remain very low (Gardner, 1988).

In Vivo Assay: Pathophysiological Investigation

The Goldblatt 2K1C rat is a model of hypertension related to vasoconstriction status because of high plasma renin concentration caused by a lack of appropriate excretion of water and sodium by the kidneys (Guédon et al., 1979). In addition to modeling hypertension, this model is known to develop cardiac left ventricular hypertrophy (Korner, 1995) and arteriosclerosis of the large arterial trunks (Bennett et al., 1993; London et al., 1998). Two months after clipping, the stimulation of the renin-angiotensin system, as well as high plasma renin concentration, persists (Michel et al., 1986). This explains why in the Goldblatt 2K1C model, BP can be controlled by ACE inhibitors (Campbell et al., 1995; Rousseau et al., 2001). However, our results show that in this model, BP could not be controlled by CH1 or CH2. Although CH1 and CH2 inhibited ACE *in vitro* as observed with other peptides, this inhibition was

200-fold weaker compared with that induced by captopril (Fuglsang et al., 2003). The HT-CH1 and HT-CH2 rats exhibited lower levels of angiotensin II compared with the HT-CON rats, but the concentration remained higher than that in sham-operated rats. This suggests that these hydrolysates may have a nonselective effect or may be partial agonists of ACE. Under our experimental conditions, CH1 and CH2 were unable to induce a significant and durable (long-lasting) hypotensive effect. In a randomized study on hypertensive patients, SBP and DBP were significantly decreased by the active tripeptides IPP and VPP without reaching normalization of BP values (Seppo et al., 2003). Conversely, casein hydrolysates containing VPP and IPP were recently reported to improve vascular endothelial function without any effect on SBP in hypertensive patients (Hirota et al., 2007). In the present study, SBP and other BP parameters tended to decrease with CH1, but not with CH2, which was unable to normalize blood pressure. The effect on SBP was weak compared with the dose-dependent effect obtained using the reference ACE inhibitor perindopril in a previous study (Rousseau et al., 2001). In agreement with these results, other authors reported the observation of weak transient effects on BP in rats (Fuglsang et al., 2003). However, our study was conducted in a model of severe hypertension (close to 200 mmHg in SBP), which would normally require strong pharmaceutical intervention rather than a dietary treatment. The severity of the pathology could explain the weak efficiency of bioactive peptides that could be weakly specific but retain very high IC_{50} . Although weak, the hypotensive effect was demonstrated in this study as in others (Maeno et al., 1996), based on a dose-dependent inhibition of ACE. This appears to be a constant in the differentiation between pharmacology and nutrition. The former is associated with therapy for established pathology, whereas the latter is considered in the context of prevention. This relationship has been reviewed in the field of hypertension and dietary fatty acids (Grynberg, 2005). This view is supported by several clinical studies that reported the efficacy of casein hydrolysate in moderate hypertension (Mizushima et al., 2004; Mizuno et al., 2005; Sano et al., 2005; Cadée et al., 2007), with no effect or side-effects in normotensive patients.

Interestingly, the effect of CH1 on BP parameters was accompanied by a robust cardiac effect more conclusive on HR and QA. Heart rate variations represent a validated marker of stress, and α_{S1} -casein hydrolysate (CH1), with its HR-lowering effect, appeared to be beneficial in healthy low- and high-stress responders. These antistress properties of CH1 may suggest a better target for CH1 than established hypertension (Guesdon et al., 2006; Messaoudi et al., 2005).

CONCLUSIONS

In conclusion, the casein hydrolysates used in this study significantly inhibited ACE as evaluated on a purified enzyme, human endothelial cells, and rat arterial tissue, although they were characterized by dramatically higher IC₅₀ values compared with current drugs. Unfortunately, per os administration in rats was unable to affect established hypertension except for a transient decrease in some BP parameters that was too weak to be considered a health benefit. This weak effect can be attributed both to the rapid degradation of the active components absorbed from the homogenates and to their weak inhibitory capacity compared with pharmaceutical drugs. To compensate for both degradation and weak activity, a large amount of peptide would have to be consumed to meet in vivo inhibition constants as required by a chronic pathology. In contrast, long-term treatment and early prevention may be the next logical scientific target for such a nutritional approach, provided that true clinical prevention studies can be carried out. Additionally, the present work has contributed to providing a basis for a preclinical strategy to assess (and compare) the influence of dietary compounds on blood pressure and thus to optimize the protocol for intervention studies in humans.

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