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Effect of intraperitoneally administered hydrolyzed whey protein on blood pressure and renal sodium handling in awake spontaneously hypertensive rats

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Abstract

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The present study evaluated the acute effect of the intraperitoneal (*ip*) administration of a whey protein hydrolysate (WPH) on systolic arterial blood pressure (SBP) and renal sodium handling by conscious spontaneously hypertensive rats (SHR). The *ip* administration of WPH in a volume of 1 ml dose-dependently lowered the SBP in SHR 2 h after administration at doses of 0.5 g/kg (0.15 M NaCl: 188.5 ± 9.3 mmHg vs WPH: 176.6 ± 4.9 mmHg, N = 8, P = 0.001) and 1.0 g/kg (0.15 M NaCl: 188.5 ± 9.3 mmHg vs WPH: 163.8 ± 5.9 mmHg, N = 8, P = 0.0018). Creatinine clearance decreased significantly (P = 0.0084) in the WPH-treated group (326 ± 67 μL min⁻¹ 100 g body weight⁻¹) compared to 0.15 M NaCl-treated (890 ± 26 μL min⁻¹ 100 g body weight⁻¹) and captopril-treated (903 ± 72 μL min⁻¹ 100 g body weight⁻¹) rats. The *ip* administration of 1.0 g WPH/kg also decreased fractional sodium excretion to 0.021 ± 0.019% compared to 0.126 ± 0.041 and 0.66 ± 0.015% in 0.15 M NaCl and captopril-treated rats, respectively (P = 0.033). Similarly, the fractional potassium excretion in WPH-treated rats (0.25 ± 0.05%) was significantly lower (P = 0.0063) than in control (0.91 ± 0.15%) and captopril-treated rats (1.24 ± 0.30%), respectively. The present study shows a decreased SBP in SHR after the administration of WPH associated with a rise in tubule sodium reabsorption despite an angiotensin I-converting enzyme (ACE)-inhibiting *in vitro* activity (IC₅₀ = 0.68 mg/mL). The present findings suggest a pathway involving ACE inhibition but measurements of plasma ACE activity and angiotensin II levels are needed to support this suggestion.

Key words

- Arterial blood pressure
- Hydrolysis
- Renal function
- Urinary sodium excretion
- Whey protein isolate

Introduction

Peptides derived from food proteins can regulate physiological functions in the digestive, neural and cardiovascular systems (1-3). A functional food can be defined as a dietary ingredient that affects its host in a targeted manner so as to exert positive effects that may eventually justify certain health claims. The term functional food encompasses a very broad range of products from foods generated around a particular functional ingredient (e.g., stanol-enriched margarine) to staple, everyday foods enriched with a nutrient not usually present to any great extent (e.g., folic acid enriched bread or breakfast cereals). Peptides derived from soybean and pork proteins can suppress the increase in serum cholesterol after a meal (4,5), while those derived from casein hydrolysates treated with pepsin promote calcium absorption and are used as functional foods, i.e., they contain ingredients that have health-promoting properties that extend beyond their immediate nutritional value. Peptides with hypotensive activity have been identified in hydrolysates of gelatin, casein, maize endosperm protein, and fish muscle (6-9), and are believed to be useful as functional dietary ingredients for hypertensive patients. This activity is attributable mainly to the inhibition of angiotensin-I-converting enzyme (ACE), which plays a prominent role in the regulation of arterial blood pressure by converting the inactive decapeptide angiotensin I to a strong vasoconstrictor octapeptide, angiotensin II, while at the same time inactivating the vasodilator and natriuretic nonapeptide, bradykinin (10,11).

Milk proteins are precursors of peptides with various biological activities, including opioid activity and immunomodulatory and antihypertensive actions (12). The antihypertensive effect of these peptides has also been related to the inhibition of ACE (13), and has been studied in spontaneously hypertensive rats (SHR) and humans (14,15).

These biologically active peptides are released from milk proteins by enzymatic hydrolysis during gastrointestinal digestion, milk fermentation (16) or hydrolysis *in vitro*. The most common way to produce bioactive peptides is to release them by limited hydrolysis using pancreatic enzymes, mainly trypsin. However, other enzymes and combinations of proteases have been used to generate bioactive peptides. Several studies have used alcalase to obtain antihypertensive hydrolysates from many protein sources (17-19). This enzyme is an industrial alkaline protease that is very stable in organic solvents and serves as a catalyst for the resolution of N-protected amino acids in aqueous solution and organic solvents, and is used to prepare optically pure peptides.

For many years, dietary interventions as a non-pharmacological approach for treating hypertension have focused on the intake of electrolytes. However, according to Martin (20), manipulation of the dietary protein content could also be useful in the non-pharmacological treatment of hypertension. However, to the best of our knowledge, no study has investigated the hypotensive and renal effects of protein hydrolysates. In the present study, the *in vitro* ACE-inhibiting activity of a whey protein hydrolysate (WPH) and its effect on blood pressure, renal function and renal handling of sodium were investigated in conscious SHR.

Material and Methods

The experiments were conducted on male SHR (270-300 g) that were allowed free access to tap water and standard rat laboratory chow (Purina rat chow: Na⁺ content: 135 ± 3 μEq/g; K⁺ content: 293 ± 5 μEq/g). To evaluate the effect of the *ip* administration of WPH on systolic blood pressure (SBP) and renal function, non-anesthetized rats were randomly assigned to one of three groups: 1) rats receiving vehicle solution *ip* (0.15 M NaCl, N = 6), 2) captopril-treated

rats (captopril, 10 mg/kg, *ip*, N = 6), and 3) WPH-treated rats (WPH, at 1.0 g/kg, *ip*, N = 8) in a volume of 1 mL. The general guidelines established by the Brazilian College for Animal Experimentation (COBEA) were followed throughout the study. The doses of WPH used during the renal function tests were chosen after a preliminary dose-response study to assess the effects of WPH on SBP in a separate group of rats.

Experimental design

To assess the effect of the dose of WPH on SBP compared to control (0.15 M NaCl) and captopril administration, the rats received non-cumulative *ip* injections in a volume of 1 mL containing different doses of WPH (0.25, 0.5, 0.75, and 1.0 g/kg). The SBP was measured 2 h later in conscious, restrained rats (N = 8 for each dose or experimental group) by a tail-cuff method using an electrospigmomanometer (Narco Bio-Systems, Austin, TX, USA). This indirect approach allows repeated measurements and yields values with a close correlation (correlation coefficient = 0.975) to those obtained by direct intra-arterial recordings (21).

For the renal function studies, the rats were weighed and housed individually in metabolic cages. These experiments were done at the same time for each group. Fourteen hours before the renal tests, 60 mmol LiCl/100 g body weight was given by gavage. After an overnight fast, each non-anesthetized rat received tap water by gavage (5% of body weight), followed by a second load of the same volume 1 h later. Twenty minutes after the second load, 0.15 M NaCl (control), 1.0 g WPH/kg body weight or 10 mg captopril/kg body weight (the latter two dissolved in 1 mL of 0.15 M NaCl) was administered *ip* and spontaneously voided urine was collected over a 2-h period. The voided urine passed through a funnel in the bottom of the cage into a graduated centrifuge tube. At the end of the experiment,

blood samples were drawn by cardiac puncture, and urine and plasma samples were taken for analysis.

Preparation of whey protein hydrolysate

Whey protein isolate (Davisco, Le Sueur, MN, USA) was first denatured to 65°C for 15 min under conditions described below. The whey protein isolate suspension (10% on a protein weight basis) was hydrolyzed with alcalase (Novo Nordisk Biochem Inc., Franklinton, NC, USA) in a pH-Stat apparatus (Mettler Toledo, Columbus, OH, USA). Hydrolysis was performed using an enzyme-to-substrate protein ratio of 0.01 (v/w) at 60°C. The suspension was maintained at pH 7.0 by the continuous addition of 1 N NH₄OH until 10% of the substrate was hydrolyzed. At the end of the incubation, the solution was heated to 90°C for 10 min to stop enzyme activity and the hydrolysate was freeze-dried. The extent of hydrolysis was measured on the basis of NaOH uptake.

Inhibitory effect of the whey protein hydrolysate on ACE activity

The *in vitro* inhibition of ACE activity by WPH (N = 24) was assessed by capillary electrophoresis using a modification of the method of Cushman and Cheung (22) previously described by Costa et al. (23). The reaction was carried out by incubating ACE (4 mU, 100 µL; Sigma, St. Louis, MO, USA) and hippuryl-His-Leu (3.8 mM, 100 µL; Sigma) in 100 mM sodium borate containing 300 mM NaCl, pH 8.3, for 30 min at 37°C. Different concentrations of the hydrolysate dissolved in 50 µL borate buffer were mixed with the ACE solution before the addition of hippuryl-His-Leu to start the reaction. The reaction was stopped by adding acetonitrile (250 µL). The samples were mixed and injected directly into the silica capillary (52 cm x 75 µm ID) in the hydrodynamic mode at a pressure of 50 mbar. The

voltage applied was 10 kV for 30 min. The hippuric acid released was detected at 228 nm using a diode array detector. The IC_{50} value was defined as the concentration of hydrolysate (mg/mL) required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE).

Biochemical analyses

Plasma and urine sodium, potassium and lithium concentration were measured by flame photometry (Micronal, B262, São Paulo, SP, Brazil), while the creatinine concentrations and WPH osmolarity were determined spectrophotometrically (Instruments Laboratory, Genesys V, Lexington, MA, USA) and with a wide-range osmometer (Advanced Instruments Inc., Needham Heights/Medkan Heights, MA, USA), respectively.

Renal function calculations and statistics

The tail blood pressure measurements and renal function results are reported as the mean \pm SEM per 100 g body weight. Renal clearance was calculated using a standard formula ($C = UV/P$) based on the plasma

creatinine and lithium levels. Creatinine clearance (CCr) was used to estimate the glomerular filtration rate and lithium clearance (CLi^+) was used to assess proximal tubule sodium reabsorption. Fractional sodium ($FENa^+$) and potassium (FEK^+) excretions were calculated as CNa^+/CCr and CK^+/CCr , respectively, where CNa^+ and CK^+ are the sodium and potassium clearances. The fractional proximal ($FEPNa^+$) and post-proximal ($FEPPNa^+$) sodium excretions were calculated as $CLi^+/CCr \times 100$ and $CNa^+/CLi^+ \times 100$, respectively (24,25).

Data were analyzed statistically by the unpaired Student *t*-test. The level of significance was set at $P < 0.05$.

Results

In vitro inhibition of ACE activity by whey protein hydrolysate

Figure 1 shows a chromatogram of the ACE reaction mixture containing WPH at several concentrations. Complete separation of hippuric acid and hippuryl-His-Leu was achieved with this method. The amount of hippuric acid detected decreased as the concentration of WPH increased, indicating the inhibition of ACE by WPH (Figure 1B-D). The IC_{50} for WPH (0.68 mg/mL, $N = 8$) indicated that the hydrolysate had moderate ACE-inhibitory activity that was comparable to that reported by Byun and Kim (18) ($IC_{50} = 0.629$ mg/mL) for a gelatin hydrolysate obtained by digestion with alcalase, and to that for milk hydrolyzed by a purified yeast protease ($IC_{50} = 0.42$ mg/mL).

Hypotensive and renal effect of intraperitoneally administered whey protein hydrolysate on SHR

The experiments were performed by *ip* injection of 0.15 M NaCl (control) or 215 mOsm/kg H_2O protein hydrolysate solutions (pH ~7.5) of distilled water containing 0.2964

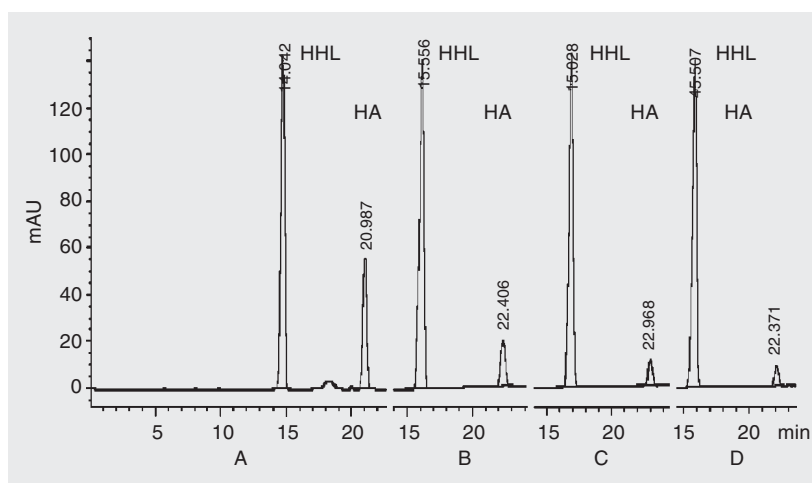


Figure 1. Capillary electrophoresis separation of the angiotensin I-converting enzyme reaction mixture incubated without whey protein hydrolysate (WPH; A) and with WPH at concentrations of 0.25 mg/mL (B), 0.5 mg/mL (C), and 1.0 mg/mL (D; $N = 8$ for each concentration). HHL = hippuryl-His-Leu; HA = hippuric acid.

and 0.1482 g/mL of 98.8% WPH (at 1.0 and 0.5 g/kg, respectively).

Figure 2A shows that the WPH significantly and dose-dependently lowered the SBP in SHR 2 h after the administration of 0.5 g WPH/kg (control: 188.5 ± 9.3 mmHg vs WPH: 176.6 ± 4.9 mmHg, $P = 0.001$) and 1.0 g WPH/kg (control: 188.5 ± 9.3 mmHg vs WPH: 163.8 ± 5.9 mmHg, $P = 0.0018$). This decrease in SBP was similar to that observed after treatment with captopril (vehicle: 196.1 ± 3.3 mmHg vs captopril: 153.1 ± 2.9 mmHg, $P = 0.01$). The maximum percent decrease in SBP after administration of the vehicle solution, captopril and WPH was -2.2 ± 0.17 , -21.3 ± 2.3 and $-28.1 \pm 3.6\%$, respectively.

Figure 2B-F shows the effect of the vehicle solution, captopril and WPH on CCr and renal Na^+ and K^+ handling in SHR. The glomerular filtration rate estimated by CCr decreased significantly in SHR treated with 1.0 g of WPH/kg body weight ($326 \pm 67 \mu\text{L min}^{-1} 100 \text{ g body weight}^{-1}$) compared to those receiving 0.15 M NaCl solution ($890 \pm 26 \mu\text{L min}^{-1} 100 \text{ g body weight}^{-1}$) and captopril ($903 \pm 72 \mu\text{L min}^{-1} 100 \text{ g body weight}^{-1}$, $P = 0.0084$). The *ip* administration of 1.0 g WPH/kg body weight also decreased FENa^+ to $0.021 \pm 0.019\%$ compared to 0.126 ± 0.041 and $0.66 \pm 0.015\%$ in vehicle- and captopril-treated rats, respectively ($P = 0.033$). Similarly, the FEK^+ in WPH-treated rats ($0.25 \pm 0.05\%$) was significantly lower ($P = 0.0063$) than in control ($0.91 \pm 0.15\%$) and captopril-treated rats ($1.24 \pm 0.30\%$), respectively.

The decrease in urinary sodium and potassium excretion caused by WPH (1.0 g/kg body weight, *ip*) was accompanied by a significant decrease in proximal (control: 75.3 ± 15 vs WPH: $12.7 \pm 5\%$) and post-proximal (control: 0.43 ± 0.08 vs WPH: $0.16 \pm 0.076\%$) sodium excretion when compared to 0.15 M NaCl- and captopril-treated rats (FEPNa^+ : $141.3 \pm 20\%$; FEPPNa^+ : $0.22 \pm 0.065\%$).

Discussion

Considerable resources have been devoted to studying the potential hypotensive effects of milk protein-derived peptides in SHR and hypertensive human volunteers (26). We have, for the first time, studied the effect of protein hydrolysates on renal function. The present study demonstrated that the *ip* administration of WPH caused a marked decrease in SBP and glomerular filtration rate (Figures 1 and 2). This route of administration was required by the peptide nature of the agent being administered which could not be oral. SBP was reduced 2 h after the administration of WPH, and this de-

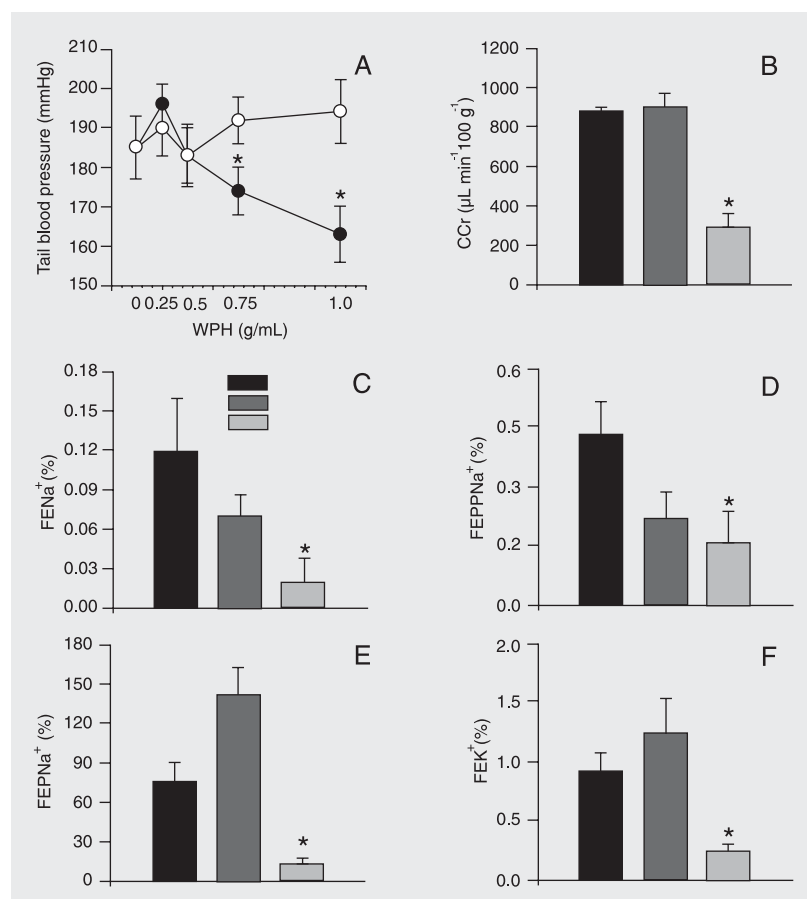


Figure 2. Effect of intraperitoneal administration of whey protein hydrolysate (WPH; 1.0 g/kg body weight) on arterial blood pressure (vehicle: open circles; WPH: closed circles, A), creatinine clearance (CCr, B), fractional excretion of sodium (FENa^+ , C), post-proximal (FEPPNa^+ , D), and proximal (FEPNa^+ , E) fractional excretion of sodium, and fractional excretion of potassium (FEK^+ , F). * $P < 0.05$ vs control (vehicle) group (Student *t*-test).

crease was sustained for up to 4 h after injection (data not shown). The WPH also transiently but significantly decreased the urinary fractional sodium and potassium excretion, with a simultaneous fall in the fractional proximal and post-proximal urinary sodium excretion (Figure 2).

WPH may influence tubular sodium reabsorption by a direct action on tubular sodium transport or by hemodynamically mediated mechanisms involving a reduction in medullary blood flow. Although neither renal blood flow nor renal vascular resistance was measured in the present study, the decreased glomerular filtration rate, estimated by CCr, suggested hemodynamic changes in the glomerular arteriolar vasculature. The present data support a role for tubular mechanisms in the conservation of sodium and water and show an antinatriuretic response resulting from a compensatory natriuretic tubular action associated with a modified glomerular filtration and decreased arterial blood pressure. Nevertheless, the persistent decrease in urinary sodium excretion produced after WPH may override the dramatic reduction in CCr in these rats. Under our experimental conditions, the fractional potassium excretion was also decreased after WPH administration. Many factors have been proposed as being important for the renal excretion of potassium including blood pH, potential across the luminal membrane, sodium delivery to the distal tubule, and urinary flow rate (27). In the present study, the decreased kaliuresis in WPH-treated rats may be explained by a remarkable decrease of sodium delivery to distal tubules as a consequence of striking potassium reabsorption before distal nephron segments.

The precise mechanism underlying the reduction in SBP caused by WPH in SHR is unknown. The long-term control of arterial pressure is dominated by renal control of the fluid and electrolyte balance (28,29). The interpretation of the results of urinary sodium excretion in adult hypertensive rats is

complicated by the interdependency of renal salt excretion and increased arterial pressure. Several reports have shown that the basal rates of ion excretion are similar in normotensive rats and in SHR with established hypertension (28,30,31). However, when the renal perfusion pressure is reduced to the range observed in normotensive rats, the urinary sodium excretion of SHR decreases (28). The kidneys of SHR require a higher arterial pressure than those of normotensive rats to excrete the same amount of salt under basal conditions. The present data are consistent with this view. The decrease in SBP in SHR after the administration of WPH (Figure 2) was associated with a rise in proximal and post-proximal sodium reabsorption despite a supposed fall in *in vitro* ACE activity (Figure 1).

The doses of hydrolysate used here produced responses similar to those observed by Fujita and Yoshikawa (32) for a peptide derived from digested thermolysin of dried Bonito in hypertensive rats. Sipola et al. (33) showed that peptides derived from whey proteins relaxed mesenteric arteries. A similar relaxation in SHR could explain the hypotension seen here after the *ip* administration of WPH. WPH obtained by digestion with alcalase may be a potentially useful source of peptides for functional foods because of its ability to inhibit *in vitro* ACE, and lower SBP in SHR (Figures 1 and 2).

It was recently shown, for example, that α -lactorphin (34,35) reduced blood pressure in SHR and normotensive WKy rats in a dose-dependent manner following subcutaneous administration. However, the arterial blood pressure-reducing effect was absent in the presence of naloxone, indicating that the hypotensive effect was mediated through the vasodilatory action of binding to opiate receptors (14). Furthermore, the hypotensive effects of milk protein hydrolysates may also be due in part to the high levels of biologically available calcium present in these products (36). The hypotensive effects

of high calcium, low fat dairy product diets have been well documented (37).

The present results provide further insights into the cardiovascular and renal effects of peptides from whey protein on global renal function in non-anesthetized, unre-

strained rats. Further studies are needed to measure the plasma ACE activity and angiotensin II levels in rats during the peak hypotensive response. Until these studies are done, the present findings are only suggestive of a pathway involving ACE inhibition.

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