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RESEARCH ARTICLE

Changes in arterial blood pressure after single oral administration of milk-casein-derived peptides in spontaneously hypertensive rats

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In this study we evaluated the short-term oral antihypertensive effect of several peptide sequences isolated from casein fractions, previously characterized as *in vitro* angiotensin-converting enzyme-inhibitors, in spontaneously hypertensive rats (SHR). Systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of the rats were measured by the tail cuff method before administration and also 2, 4, 6, 8 and 24 h post-administration. The sequences LVYPFTGPIPN, HLPLP, IAK, YAKPVA and WQVLPNAVPAK showed a clear decrease in SBP and DBP in SHR. HPHPHLSF caused a significant decrease of the DBP in the SHR, but this sequence did not modify the SBP of these animals in a significant manner. KKYNVPQL did not modify SBP in the SHR, and caused a slight, but significant and maintained, decrease in DBP in these animals. SBP and DBP returned to baseline values 24 h post-administration of all peptides. In conclusion, these peptides are bioactive ingredients with potential benefit in the prevention and treatment of hypertension or other associated disorders.

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1 Introduction

Elevated blood pressure is one of the several critical risk factors for the development of cardiovascular disease. A frequently used intervention for the treatment of high blood pressure has been the use of angiotensin-converting enzyme (ACE) inhibitors. ACE inhibitors decrease the production of the vasoconstrictor hormone angiotensin II, while simultaneously slowing the breakdown of bradykinin, a peptide that decreases vascular tone, promotes endothelial production of nitric oxide and other vasculoprotective factors. Therefore, the inhibition of ACE can produce an antihypertensive effect.

Dietary interventions are very important to control hypertension. Food-derived peptides and among them milk derived peptides, are capable of modulating specific biological functions, such as blood pressure. In fact, many food protein-derived sequences exerting ACE-inhibitory activity *in vitro* have been described [1–3]. Some of these sequences have also been tested *in vivo*, proving their antihypertensive effect. Thus, it has been demonstrated that certain food peptides can decrease arterial blood pressure in hypertensive animals after single oral doses [4–10] and after long-term administration [11–14]. In addition, some of the food-derived peptides with ACE-inhibitory activity effectively reduced systolic blood pressure (SBP) and diastolic blood pressure (DBP) in hypertensive patients (for recent reviews see [15–17]). In general terms, the results of these studies have highlighted an important lack of correlation between the *in vitro* ACE inhibitory activity and the *in vivo* action,

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Abbreviations: ACE, angiotensin-converting enzyme; **CN,** casein; **DBP,** diastolic blood pressure; **SBP,** systolic blood pressure; **SHR,** spontaneously hypertensive rats

because the former does not take into consideration the physiological transformations that determine the bioavailability of the peptides and because there might be other mechanisms different from ACE inhibition implicated in the antihypertensive effect [18]. It is therefore important to test the *in vivo* effect of the ACE-inhibitory peptide sequences to establish their possible usefulness and their physiological relevance.

The purpose of this study was to investigate the short-term oral antihypertensive effect of several peptide sequences isolated from casein (CN) fractions, previously characterized as *in vitro* ACE-inhibitors, in spontaneously hypertensive rats (SHR). Two of the studied sequences (LVYPFTGPIPN and HLPLP) derive from β-CN. In particular, LVYPFTGPIPN derives from caprine kefir [19] and the sequence HLPLP had previously been identified as the minimum active fragment of peptide LHLPLP which was found in a fermented milk [9, 20]. Other sequences (IAK, YAKPVA, WQVLPNAVPAK and HPHPHLSF) were identified in an ovine κ-CN hydrolysate [21], and the last studied sequence (KKYNVPQL) is an αs_1-CN-derived peptide identified in Manchego cheese [22].

2 Material and methods

2.1 Drugs and peptides

Captopril (Sigma, USA), a known ACE-inhibitor with recognized effect as antihypertensive agent, was used in this study. We also used the following synthetic peptides: β-CN f(58–68) = LVYPFTGPIPN, β-CN f(134–138) = HLPLP, κ-CN f(22–24) = IAK, κ-CN f(61–66) = YAKPVA, κ-CN f(76–86) = WQVLPNAVPAK, κ-CN f(98–105) = HPHPHLSF and αs_1-CN f(100–109) = KKYNVPQL. These peptide sequences were prepared by conventional Fmoc solid phase synthesis with a 431A peptide synthesizer (Applied Biosystem,Überlingen, Germany), by the Unitat de Pèptids of Barcelona University, according to the method described by Atherton and Sheppard [23]. Their purity (>90%) was verified in our laboratory by reverse phase high performance liquid chromatography and tandem mass spectrometry, as described previously [24]. Captopril and the mentioned peptides were dissolved in distilled water to be administered to the rats.

2.2 Experimental procedure in rats

We have used 17–20-wk-old male SHR weighing 314 ± 3 g. These animals were obtained from Charles River Laboratories España S.A. They remained at a temperature of 23°C with 12 h light/dark cycles, and consumed tap water and a standard diet for rats (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. All the above-mentioned products were orally administered by gastric intubation, between 9 and 10 a.m., to the rats. The administered doses were: LVYPFTGPIPN 10 mg/kg, HLPLP 7 mg/kg, IAK 4 mg/kg, YAKPVA 6 mg/kg, WQVLPNAVPAK 7 mg/kg, HPHPHLSF 10 mg/kg and KKYNVPQL 10 mg/kg. The doses were estimated based on the *in vitro* ACE inhibitory activity of each synthetic peptide. Distilled water served as negative control, and captopril 50 mg/kg served as positive control. We always administered 1 mL/rat of water, and when a compound was orally given, 1 mL/rat of an appropriate solution of this compound was also administered. We measured the SBP and the DBP of the rats by the tail cuff method before administration and also 2, 4, 6, 8 and 24 h post-administration. Before the measurement, the rats were kept at 30°C for 15 min to make the pulsations of the tail artery detectable. The original method for measuring arterial blood pressure using the tail cuff provides only SBP values [25], but the equipment used in this study, LE 5001 (Letica, Hospitalet, Barcelona, Spain), has a high sensitivity pulse transducer coupled with an accurate microprocessor program, and allows us to distinguish between SBP and DBP. To establish the value of SBP and DBP, five measurements were taken, and the average of all of them was obtained. To minimize stress-induced variations in blood pressure all measurements were taken by the same person in the same peaceful environment. Moreover, to guarantee the reliability of the measurements we established a training period of 2 wk before the actual trial time, and during this period the rats were accustomed to the procedure.

All the above-mentioned experiments were performed as authorized for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

2.3 Statistical analysis

The results are expressed as mean values ± SEM for six to eight experiments, and were analyzed by a two-way ANOVA, using the GraphPad Prism 4 software. In addition, in order to compare the different treatments and to assess the effect of time within each treatment, some data were also analyzed by a one-way ANOVA. Differences between the groups were assessed by the Bonferroni test and we always consider the differences between the means to be significant when $p<0.05$.

3 Results

Before administration of the different products, the SHR showed SBP and DBP values of 213.4 ± 2.8 and 155.7 ± 4.3 mm Hg, respectively. The values of SBP and DBP obtained in the rats before the administration of tap water and those obtained after this administration were very much alike. Captopril caused the greatest antihypertensive effect. This drug led to a clear decrease in the SBP and in the DBP of the SHR, and the maximum effect in these variables was respectively observed 6 and 4 h post-administration.

Figure 1 shows the blood pressure lowering effect of β-CN-derived peptides (LVYPFTGPIPN and HLPLP). Both sequences show a clear decrease in SBP and DBP in SHR, and the maximum effect in the SBP was observed in both cases 4 h post-administration. The shorter β-CN peptide HLPLP caused the maximum decrease in the DBP 2 h post-administration, while LVYPFTGPIPN achieved the maximum DBP effect 4–6 h post-administration. After the moment of the maximum effect, the values of SBP and DBP progressively increased, and in both cases these variables reached values very similar to those of the water rats 24 h post-administration. In the case of the LVYPFTGPIPN sequence, the recovery of the SBP was faster than that of the DBP, but in the case of the HLPLP the recovery of the DBP was faster than that of the SBP.

The changes in arterial blood pressure of κ-CN-derived peptides (IAK, YAKPVA, WQVLPNAVPAK and HPHPHLSF) are shown in Fig. 2. The sequences IAK, YAKPVA and WQVLPNAVPAK also caused a significant decrease in the SBP and in the DBP in SHR. The antihypertensive effect was similar to that observed with

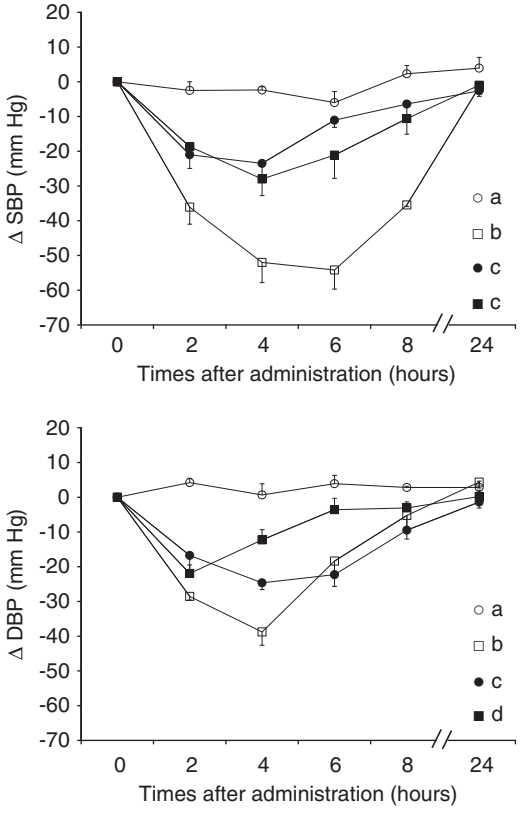

Figure 1. Decreases in SBP and DBP caused in SHR by the administration of water (○) (SBP a; DBP a), captopril (50 mg/kg) (□) (SBP b; DBP b), LVYPFTGPIPN (10 mg/kg) (●) (SBP c; DBP c) and HLPLP (7 mg/kg) (■) (SBP c; DBP d). The data represent the mean values ± SEM for 6–8 rats. Different letters represent statistical differences ($p < 0.05$). *p*-Value is estimated by two-way ANOVA.

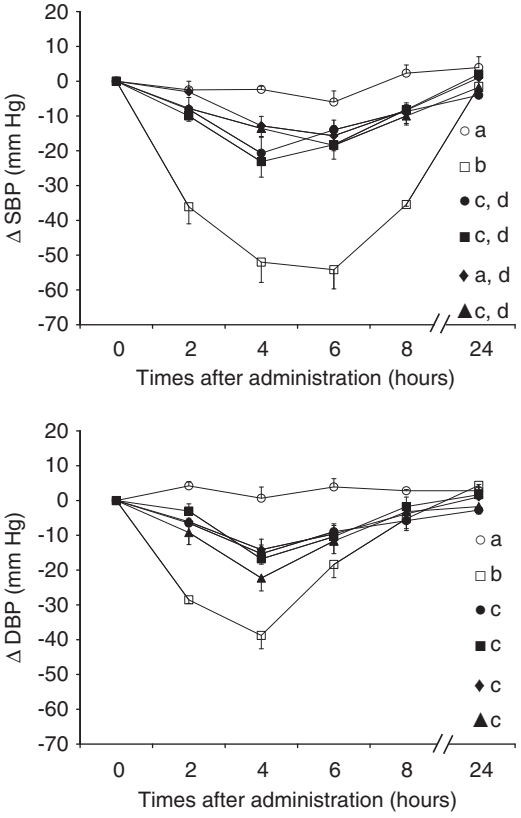

Figure 2. Decreases in SBP and DBP caused in SHR by the administration of water (○) (SBP a; DBP a), captopril (50 mg/kg) (□) (SBP b; DBP b), IAK (4 mg/kg) (●) (SBP c, d; DBP c), YAKPVA (6 mg/kg) (■) (SBP c, d; DBP c), HPHPHLSF (10 mg/kg) (♦) (SBP a, d; DBP c) and WQVLPNAVPAK (7 mg/kg) (▲) (SBP c, d; DBP c). The data represent the mean values ± SEM for six to eight rats. Different letters represent statistical differences ($p < 0.05$). *p*-Value is estimated by two-way ANOVA.

β-CN-derived peptides, and the maximum decreases in SBP and DBP caused by IAK, YAKPVA and WQVLPNAVPAK were obtained 4–6 h post-administration. HPHPHLSF caused a significant decrease of the DBP in the SHR, but this sequence did not modify the SBP of these animals in a significant manner. SBP and DBP returned to baseline values 24 h post-administration of all the κ-CN-derived peptides.

Figure 3 shows the blood pressure lowering effect of the α_{s1}-CN-derived peptide KKYNVPQL. This sequence did not modify SBP in the SHR, and caused a slight, but significant and maintained, decrease in DBP in these animals.

Table 1 resumes the characteristics of the studied peptides and the results obtained with these sequences.

4 Discussion

Most of studies performed to determine the anti-hypertensive effects of ACE-inhibitory peptides have used

Table 1. Characteristics of the studied peptides and results obtained with these sequences

Protein fragment	Sequence	Molecular mass	IC_{50} (mM)	Oral dose (mg/kg)	−ΔSBP (mm Hg)[a)]	−ΔDBP (mm Hg)[a)]
β-CN f(58–68)	LVYPFTGPIPN	1216.4	27.9[19]	10	28±4.8	24.6±1.9
β-CN f(134–138)	HLPLP	575.7	21.6[20]	7	23.5±4.1	21.9±2.4
κ-CN f(22–24)	IAK	330.2	15.7[21]	4	20.7±4.8	15.3±3.1
κ-CN f(61–66)	YAKPVA	647.3	14.3[21]	6	23.1±4.4	16.8±2.9
κ-CN f(76–86)	WQVLPNAVPAK	1221.7	10.1[21]	7	18.44±1.6	22.3±3.7
κ-CN f(98–105)	HPHPHLSF	970.5	28.9[21]	10	15.7±2.9	14.1±3.1
αs1-CN f(102–109)	KKYNVPQL	988.6	77.1[22]	10	11.5±3.4	9.7±1.1

IC_{50}, concentration needed to inhibit 50% of ACE activity. References are indicated in square brackets.
a) Maximum effect.

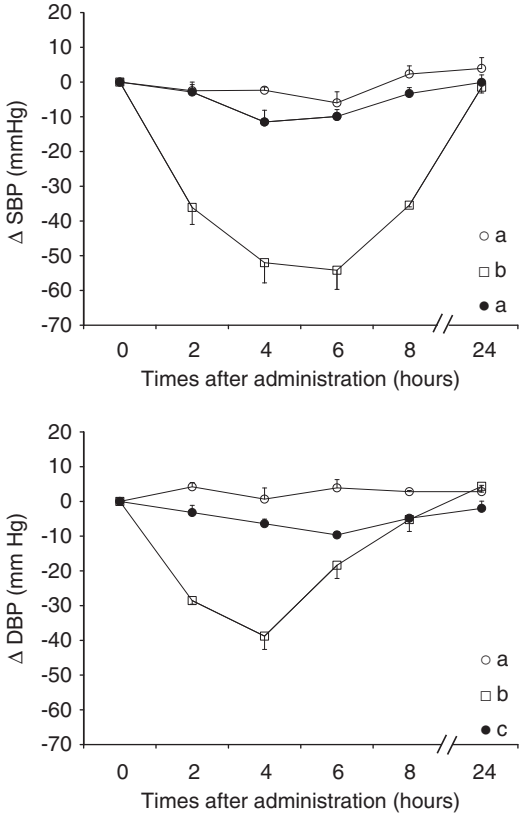

Figure 3. Decreases in SBP and DBP caused in SHR by the administration of water (○) (SBP a; DBP a), captopril (50 mg/kg) (□) (SBP b; DBP b), KKYNVPQL (10 mg/kg) (●) (SBP a; DBP c). The data represent the mean values±SEM for six to eight rats. Different letters represent statistical differences ($p<0.05$). *p*-Value is estimated by two-way ANOVA.

SHR rats [2, 26, 27]. *In vivo* effects are usually tested in this strain, because SHR constitute an accepted model for human essential hypertension [26]. The development of hypertension in these animals is very similar to that in man. In both cases hypertension appears at an early age, there is a family history of this pathology and it is worsened by a sodium-rich diet [28]. Milk is one of the most important sources of bioactive peptides, and in this study we have investigated the effect of several milk-derived peptides, that showed *in vitro* ACE inhibitory activity, in SHR.

It was not surprising to appreciate that the greatest decreases in SBP and DBP were observed when captopril was administered, because this drug is a potent ACE-inhibitor with an IC_{50} value much lower (0.02 μM) than the studied peptides [29]. In fact, we used captopril as positive control, and therefore we wanted a clear effect with this drug that could be considered, in principle, the maximal effect caused by the inhibition of ACE *in vivo*. Nevertheless, we and others have described peptide sequences that caused decreases in SBP and DBP higher or similar to those of captopril or zofenopril [4, 7, 9]. These peptides demonstrated potent ACE-inhibitory properties, and some of them inhibited ACE *in vivo* [30–32], but other mechanisms different from ACE inhibition, such as vasodilator or antioxidant activity, have been also reported to explain the antihypertensive effect of these sequences [10, 33–37].

On the other hand, it has been postulated that the digestion process after oral ingestion of peptides is intimately related to their potentially antihypertensive properties. In fact, peptides without ACE-inhibitory activity and antihypertensive properties, can release after oral ingestion other minor peptides which present blood pressure lowering effects. We have to bear in mind that the antihypertensive activity of the peptidic sequences when they are orally administered, is dependent on their ability to reach the target site without being degraded and inactivated by intestinal or plasma peptidases. Perhaps the sequences IAK and HLPLP, which are short peptides, were responsible for the observed antihypertensive effect. In particular, peptide HLPLP derives from LHLPLP, a sequence which was found in milk fermented with *Enterococcus faecalis* [9]. This peptide with an additional leucine at the *N*-terminus survives simulated gastrointestinal digestion [38] but it was hydrolysed by cellular peptidases to HLPLP prior to transport across a Caco-2 cell layer [20]. Therefore, the observed effects of HLPLP could probably be due to the *in vivo* inhibition of ACE caused by this peptide in SHR. The other sequences showed low values of IC_{50} (LVYPFTGPIPN, YAKPVA, WQVLPNAVPAK, HPHPHLSF and KKYNVPQL), but they are probably too long to be orally absorbed. Specifically,

peptide KKYNVPQL is hydrolyzed by gastrointestinal proteases into several fragments causing a significant decrease in the ACE inhibitory activity [39]. The cleavage of these sequences could nevertheless occur in the gastrointestinal tract, and other oligopeptides are probably responsible for the antihypertensive effect observed when these long peptides are orally administered.

In conclusion, we have demonstrated the antihypertensive properties of different milk-derived peptides studied in SHR. The results obtained indicate that the ACE-inhibitory peptides assayed, or shorter forms generated by gastrointestinal digestion, can be efficiently absorbed through the intestine of the animals in an active form. Studies in animals, however, are required to evaluate the impact of gastrointestinal digestion on the stability and bioactivity of the peptides that we have studied, and to clarify if other mechanisms different from the inhibition of ACE could be implicated in their antihypertensive activity. In conclusion, these peptides are bioactive constituents with potential benefit in the prevention and treatment of hypertension or other associated disorders.

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The authors have declared no conflict of interest.

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