ABSTRACT

Previous studies have demonstrated that milk fermented with Enterococcus faecalis decreases the systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of spontaneously hypertensive rats. In this study, we evaluated the antihypertensive activity of the following peptide sequences: LHLPLP, LHLPLPL, LVYPFPGPIPNSLPQNIIPP, VLGVPVRGPFP, and VRGPFPIIV. These peptides isolated from E. faecalis-fermented milk showed in vitro angiotensin I-converting enzyme-inhibitory activity. Because the most potent angiotensin I-converting enzyme-inhibitory sequences were LHLPLP and LVYPFPGPIPNSLPQNIIPP, we administered different doses of these peptides to spontaneously hypertensive rats. High doses of the remaining sequences were also administered to these animals. Water served as a negative control and captopril as a positive control. All products were administered orally. The SBP and DBP were measured before administration and also at 2, 4, 6, 8, and 24 h after administration. Before administration of the different products, spontaneously hypertensive rats showed SBP and DBP values of 218 ± 2.5 and 157 ± 5.9 mmHg, respectively (n = 30). The sequences LHLPLP, LVYPFPGPIPNSLPQNIIPP, VLGVPVRGPFP, and VRGPFPIIV caused clear and significant decreases in SBP, DBP, or both in the animals. In particular, the antihypertensive effect could be clearly established when 2 or 3 mg/kg of LHLPLP was administered. These 2 doses of LHLPLP showed similar antihypertensive properties. Four hours after administration of captopril or the highest doses of the different peptides, the decreases in the SBP and DBP (mmHg) were as follows: captopril (SBP = 52 ± 5.8, DBP = 38.8 ± 3.8), 3 mg/kg of LHLPLP (SBP = 25.3 ± 8.2, DBP = 29.5 ± 7.6), 6 mg/kg of LVYPFPGPIPNSLPQNIIPP (SBP = 14.9 ± 3.7, DBP = 8.7 ± 4.4), 10 mg/kg of LHLPLP (SBP = 7.7 ± 4.1, DBP = 9.4 ± 3.1), 10 mg/kg of VLGVPVRGPFP (SBP = 16.2 ± 5.8, DBP = 21.64 ± 3.2), and 10 mg/kg of VRGPFPIIV (SBP = 16.05 ± 2.74, DBP = 9.19 ± 3.49). The results obtained suggest that the sequences LHLPLP, LVYPFPGPIPNSLPQNIIPP, VLGVPVRGPFP, and VRGPFPIIV could be responsible, at least in part, for the antihypertensive properties described for E. faecalis-fermented milk.

Key words: angiotensin I-converting enzyme inhibitory peptide, antihypertensive peptide, arterial blood pressure, spontaneously hypertensive rat

INTRODUCTION

Angiotensin I-converting enzyme (ACE) is a key enzyme in the regulation of peripheral blood pressure. It cleaves the carboxyl-terminal dipptide from angiotensin I to produce the potent vasopressor octapeptide angiotensin II, and it inactivates the vasodilator bradykinin by the sequential removal of 2 carboxyl-terminal dipeptides (Laragh et al., 1972; Soffer, 1976). The in vitro inhibition of angiotensin II formation has been used for screening therapeutic antihypertensive agents, and many of the drugs that are presently used to control hypertension are, in fact, ACE inhibitors.

In 1979 food proteins were also discovered to be precursors of numerous ACE-inhibitory peptides (Oshima et al., 1979). In particular, many studies have investigated the antihypertensive properties of different ACE-inhibitory fragments released by enzymatic hydrolysis during milk fermentation. Lactobacillus helveticus generally has higher proteolytic activity than other lactic acid bacteria. In this context, Nakamura et al. (1995b) demonstrated that the peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) represented most of the ACE-inhibitory fragments released by enzymatic hydrolysis during milk fermentation. Lactobacillus helveticus generally has higher proteolytic activity than other lactic acid bacteria. In this context, Nakamura et al. (1995b) demonstrated that the peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) represented most of the ACE-inhibitory activity of Calpis sour milk (Calpis Co., Ltd., Tokyo, Japan), which is prepared by fermenting skim milk with a starter containing L. helveticus and Saccharomyces cerevisiae. Calpis sour milk had antihypertensive effects in spontaneously hypertensive rats (SHR) after a single oral administration (Nakamura et al., 1995a), and these 2 tripeptides also demonstrated an antihypertensive effect in SHR by inhibiting ACE in target...
Moreover, long-term oral intake of IPP and VPP, or a sour milk product fermented by *L. helveticus* LBK-16H containing these tripeptides, attenuated the development of hypertension in young, prehypertensive SHR (Sipola et al., 2001, 2002). In addition, some of the known food-derived peptides with ACE-inhibitory activity effectively reduced arterial blood pressure in hypertensive patients (Hata et al., 1996; Kawasaki et al., 2002; Seppo et al., 2003).

Our research group has demonstrated that 4 strains of *Enterococcus faecalis* are especially significant as producers of ACE-inhibitory peptides other than IPP and VPP (Muguerza et al., 2006). In fact, we were able to identify several peptide sequences in these fermented products, and some of these peptides exerted potent ACE-inhibitory activity (Quirós et al., accepted). These ACE-inhibitory sequences, and also the *E. faecalis* strains that can produce these bioactive sequences, were included in a patent presented by Muguerza et al. (2004). We have also demonstrated that the fermented milk produced by using these selected *E. faecalis* strains produces an acute antihypertensive effect in SHR after a single oral administration (Muguerza et al., 2006). Moreover, the long-term oral intake of this *E. faecalis*-fermented milk attenuated the development of hypertension in SHR (Miguel et al., 2005). This milk did not modify the arterial blood pressure of Wistar-Kyoto rats, which are used as the normotensive control of the SHR (Muguerza et al., 2006). The aim of this study was to evaluate the possible antihypertensive effect of the peptides with potent in vitro ACE-inhibitory activity that have been identified in *E. faecalis*-fermented milk.

### Materials and Methods

#### Drugs and Peptides

Captopril (Sigma, St. Louis, MO), a known ACE inhibitor, was used in this study. The following synthetic peptides were also used: LHLPLP, LHLPLPL, LVYPFPGPIONSLPVQNNP, VLGPVVRGPFP, and VRGPPPIIV. These peptide sequences were prepared by conventional 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis with a 431A peptide synthesizer (Applied Biosystems Inc., Überlingen, Germany) by the Unitat de Peptides of Barcelona University, according to the method described by Atherton and Sheppard (1989). Their purity (>90%) was verified in our laboratory by reversed-phase HPLC tandem mass spectrometry according to the method described by Gómez-Ruiz et al. (2003). The drug and the peptides were dissolved in distilled water before being administered to the rats.

**Experimental Procedure in Rats**

In this study, we used 17- to 20-wk-old male SHR weighing 300 to 350 g. These animals were obtained from Charles River Laboratories España S.A. (Barcelona, Spain). The rats remained at a temperature of 23°C with 12-h light–dark cycles, and consumed tap water and a standard rat diet (A04; Panlab, Barcelona, Spain) ad libitum during the experiments.

Four different doses of LHLPLP (0.5, 1, 2, and 3 mg/kg) and 2 different doses of LVYPFPGPIONSLPVQNNP (3 and 6 mg/kg) were administered to the SHR. High doses (10 mg/kg) of the remaining sequences were also administered to these animals. The peptides (1 mL of the corresponding water solution) were orally administered by gastric intubation between 0900 and 1000 h. To carry out similar experiments, captopril (50 mg/kg) served as the positive control and 1 mL of distilled water served as the negative control. The systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of the rats were measured by the tail cuff method before administration of the different products and also 2, 4, 6, 8, and 24 h after administration. The original method for measuring arterial blood pressure using the tail cuff (Buñag, 1973) provides only SBP values, but the equipment used in this study (LE 5001; Letica, Hospital, Barcelona, Spain) had a high-sensitivity pulse transducer coupled with an accurate microprocessor program, which allowed us to distinguish between SBP and DBP. The indirect measurement of blood pressure with this equipment is basically sphygmonanometric, and the process is the same as that used in blood pressure measurements in humans. Before the measurement, the rats were held at 30°C for 10 min to make the pulsations of the tail artery detectable, and the values of SBP and DBP were obtained by the average reading from 3 measurements.

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

#### Statistical Analysis

The results are expressed as mean values ± standard errors of the mean for a minimum of 6 rats. They were analyzed by 2-way ANOVA using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). In addition, to compare the different treatments and to assess the effect of time within each treatment, some data were also analyzed by one-way ANOVA, and the differences between groups were assessed by the Bonferroni test. The differences between means were considered to be significant when P < 0.05.
RESULTS

Before administration of the different products, the SHR showed SBP and DBP values of 218 ± 2.5 and 157 ± 5.9 mmHg, respectively (n = 30). All the SBP and DBP values obtained after administration of the distilled water were very similar to those obtained before administration (1-way ANOVA). Captopril decreased SBP and DBP in the SHR (P < 0.05 vs. water). The maximum decreases in SBP and DBP caused by captopril were observed from 4 to 6 h and from 2 to 4 h, respectively, after administration of this drug (1-way ANOVA). The peptide LHLPPL also decreased SBP and DBP in the SHR (P < 0.05 vs. water), with the effect on DBP of this peptide being dose dependent. In particular, the antihypertensive effect of LHLPPL was clearly established when 2 or 3 mg/kg of this peptide was administered. The effects of these 2 doses of LHLPPL were very similar (P > 0.05) and were clearly observed between 2 and 8 h postadministration (1-way ANOVA). The decrease in SBP caused by 2 or 3 mg/kg of LHLPPL was smaller than the decrease in this variable caused by captopril (P < 0.05). However, at the highest doses used, this peptide produced a decrease in DBP similar to the decrease in this variable caused by the drug (P > 0.05; see Figure 1).

The sequence LVYPFPGPINSLPQNIPP also caused a clear and significant decrease in the SBP and in the DBP of the SHR (P < 0.05 vs. water). Nevertheless, the effect of this sequence on these variables was less accentuated than the effect of captopril (P < 0.05; see Figure 2). The sequences VLGVPVRGPFP and VRGFPPIIV caused a significant decrease in the DBP of the SHR (P < 0.05 vs. water), but these peptides did not modify the SBP of the animals (P > 0.05 vs. water). The sequence LHLPPL caused a very slight, nonsignificant change in the SBP and DBP of the SHR (P > 0.05 vs. water; see Figure 3).

At 24 h after administration of the different products, the values of SBP and DBP were very similar to those obtained before administration (1-way ANOVA).

DISCUSSION

We investigated the antihypertensive activity of different ACE-inhibitory peptides isolated from E. faecalis-fermented milk. The ACE-inhibitory activity of these peptides was analyzed in our laboratory by a modification of the method used by Cushman and Cheung (1971). This is an in vitro test, which permitted us to obtain the concentrations of the different products that inhibited 50% of the ACE activity (IC50). Nevertheless, it is important to test the effect of the ACE-inhibitory compounds in vivo to establish their possible usefulness against hypertension. We must bear in mind that when administered orally, their antihypertensive activity depends on their ability to reach the target site without being degraded or inactivated by intestinal or plasma peptidases.

These experiments demonstrated that the majority of the peptides investigated (LHLPPL, LVYPFPGPINSLPQNIPP, VLGVPVRGPFP, and VRGFPPIIV) could effectively reduce SBP, DBP, or both in SHR. The doses of these peptides were established based on the IC50 values previously obtained by our research group (Quiros et al., accepted). Thus, we administered low doses of the sequences LHLPPL and LVYPFPGPINSLPQNIPP, because these sequences had low IC50 values (5.4 and 5.3 μM, respectively). High doses (10 mg/kg) of the other 3 sequences were used, because all of them had IC50 values higher than 130 μM.

Most of the peptide fragments with ACE-inhibitory activity that have been isolated from hydrolysates of food materials, among them the peptide sequences VPP and IPP, and also effective antihypertensive sequences isolated from milk fermented with E. faecalis CECT 5728 (LHLPPL, LVYPFPGPINSLPQNIPP, and VLGVPVRGPFP), have a Pro group in the carboxy termini (Cheung et al., 1980). In fact, structure–activity correlations indicate that the C-terminal tripeptide sequence plays a very important role in the ACE-inhibitory activity, with the presence of aromatic AA or imino acids such as Pro enhancing the inhibition (Cheung et al., 1980). The superiority of Pro as the C-terminal residue is probably attributable to a rigid ring structure of this AA that may lock the carboxyl group into a conformation favorable for interaction with the positively charged residue at the active site of the enzyme (Cushman et al., 1977). The antihypertensive effect observed when the sequences LHLPPL or VLGVPVRGPFP were administered to the SHR was probably due to the in vivo inhibition of ACE by these peptides in the animals. The sequence LVYPFPGPINSLPQNIPP, with a low IC50 value, also has a Pro group as the C-terminal residue, but this peptide is too long to be absorbed orally. The cleavage of the sequence LVYPFPGPINSLPQNIPP to generate IPP, a known ACE-inhibitory peptide, could nevertheless occur in the gastrointestinal tract, and this tripeptide or other derived peptide fragments could be responsible for the antihypertensive effect observed when this long peptide is administered orally. The sequences VRGFPPIIV and LHLPPL, which do not have a Pro group in the carboxyl terminus, and which had the highest IC50 values when the ACE-inhibitory activities were evaluated in vitro (630 and 432.7 μM, respectively), had minor effects on the arterial blood pressure of the SHR. In spite of this, the sequence VRGFPPIIV caused a significant decrease in the DBP of these animals. In this context,
we should point out that, according to Cheung et al. (1980), the existence of hydrophobic AA, such as Phe and Ile, close to the C-terminal residue might also positively influence the binding to ACE. Sekiya et al. (1992) also carried out determinations of the ACE-inhibitory activity of different peptides derived from food proteins and suggested that the peptides with an IC$_{50}$ value between 100 and 500 $\mu$M could have physiological activity. Nevertheless, other researchers later indicated that only the different peptides obtained from food proteins
Figure 2. Decrease in systolic blood pressure (SBP) and diastolic blood pressure (DBP) caused in spontaneously hypertensive rats by the administration of water (○), captopril (■; 50 mg/kg; Sigma, St. Louis, MO), or 2 different doses of the peptide sequence LVYPFPQPNSLP-QNIPP: 3 (◆) and 6 mg/kg (▲). The data represent the mean values ± SEM for a minimum of 6 rats. a $P < 0.05$ vs. water; b $P < 0.05$ vs. captopril; c $P < 0.05$ vs. 3 mg/kg of LVYPFPQPNSLPQNNP. P estimated by a 2-way ANOVA.

with an IC$_{50}$ value lower than 100 $\mu$M showed in vivo effects (Fujita and Yoshikawa, 1999). One can therefore assume that the antihypertensive potency depends on the in vitro efficacy to inhibit ACE. In particular, we can highlight the fact that the antihypertensive properties of the peptides used in this study were predictable in principle, because the sequence LHLPLP, which had an IC$_{50}$ value lower than 100 $\mu$M, was the most effective sequence in vitro and in vivo.

In this study, it was not surprising that in most cases the greatest decreases in SBP and DBP were observed when captopril was administered because this drug is
Figure 3. Decrease in systolic blood pressure (SBP) and diastolic blood pressure (DBP) caused in spontaneously hypertensive rats by the administration of different products: water (○), captopril (◆; 50 mg/kg; Sigma, St. Louis, MO), the peptide sequence LHLPLPL (●; 10 mg/kg), the peptide sequence VLGPVRGFPF (▲; 10 mg/kg), or the peptide sequence VRGPFPIIV (■; 10 mg/kg). The data represent the mean values ± SEM for a minimum of 6 rats. a $P < 0.05$ vs. water; b $P < 0.05$ vs. captopril. $P$ estimated by a 2-way ANOVA.

A potent ACE-inhibitor, with an IC$_{50}$ value much lower (0.02 µM) than those of the peptides studied (Fujita and Yoshikawa, 1999). Nevertheless, we must note that the decreases in the DBP observed when 2 or 3 mg/kg of LHLPLP was administered were very similar to the decreases observed in this variable when 50 mg/kg of captopril was administered. We used captopril as the positive control, because we wanted to obtain a clear
Moreover, antioxidant-rich diets significantly reduced the effect of these peptides on isolated tissues and by their slower elimination (Fujita and Yoshikawa, 1999). Another possibility is that ACE inhibition might not be the only mechanism of action to explain the antihypertensive effect of LHLPLP or the sequence used in this study. Certain antihypertensive effects (Fujita et al., 1995, 1996; Kato et al., 1995; Mata et al., 1999). The effect of these peptides on isolated arteries should, in fact, be evaluated in the future. Moreover, antioxidant-rich diets significantly reduced the arterial blood pressure in SHR (Akpaaffiong and Taylor, 1998; Soares de Moura et al., 2002; Rodríguez-Iturbe et al., 2003), and we cannot discount the radical-scavenging activity of these peptides because we have already demonstrated this activity for some peptides isolated from food proteins (Dávalos et al., 2004). We should also point out that the effect of LHLPLP was specific to the hypertensive state, because this peptide (Aleixandre et al., 2004; Muguerza et al., 2004), as the E. faecalis-fermented milk (Muguerza et al., 2004, 2006) did not modify the arterial blood pressure of Wistar-Kyoto rats.

In conclusion, most of the sequences investigated (LHLPLP, LVYPFPGPISLPQNIPI, VLGPVRGGPFP, and VRGPFGPIIV), and the sequence LHLPLP in particular, effectively decreased arterial blood pressure in SHR and could, at least in part, be responsible for the antihypertensive properties previously described for E. faecalis-fermented milk. The present study reinforces the idea of using E. faecalis-fermented milk as a functional food in the nonpharmacological treatment of hypertension. Nevertheless, we must bear in mind that some differences between rodents and humans exist in bowel structure and function, and also in microflora. It is therefore evident that before routine clinical use of this milk or its antihypertensive peptides, it would be necessary to carry out clinical studies to demonstrate the efficiency of these peptides and to guarantee their safe use in healthy subjects and hypertensive patients. More studies in animals would also be advisable to evaluate the impact of gastrointestinal digestion on the stability and bioactivity of the peptides we investigated, and to clarify whether other mechanisms different from the inhibition of ACE could be implicated in their antihypertensive activity.

ACKNOWLEDGMENTS

This study was supported by Leche Pascual S.A. (Madrid, Spain) and by an Instituto Nacional de Investigaciones Agropecuarias-Ministerio de Ciencia y Tecnología (INIA-MCYT) project CAL01-046-02. We also thank Manuel Bas Caro, technician in pharmacology, for excellent care of the rats and collaboration in administering the different products.

REFERENCES


Hata, Y., M. Yamamoto, M. Ohni, K. Nakajima, Y. Nakamura, and T. Takano. 1996. A placebo-controlled study of the effect of sour...