Cardiovascular Effects of Fermented Milk Containing Angiotensin-
Converting Enzyme Inhibitors Evaluated in Permanently
Catheterized, Spontaneously Hypertensive Rats

Anders Fuglsang,1* Dan Nilsson,2 and Niels C. B. Nyborg3
Royal Danish School of Pharmacy, Institute of Pharmacology, 2100 Copenhagen Ø,1 Novo Nordisk A/S,
2670 Måløv,2 and Chr. Hansen A/S, 2970 Hørsholm,3 Denmark

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In this study, two strains of Lactobacillus helveticus were used to produce fermented milk rich in angiotensin-
converting enzyme (ACE) inhibitors. In vitro tests revealed that the two milks contained competitive inhibitors
of ACE in amounts comparable to what has been obtained in previously reported studies. The two milks were
administered by gavage to spontaneously hypertensive rats that had had a permanent aortic catheter inserted
through the left arteria carotis, and mean arterial blood pressure and heart rate were monitored from 4 to 8 h
after administration. Unfermented milk and milk fermented with a lactococcal strain that does not produce
inhibitors were used as controls. Highly significant blood pressure effects were observed; i.e., milk fermented
with the two strains of L. helveticus gave a more pronounced drop in blood pressure than the controls.
Significant differences in heart rate effects were detected with one of the strains.

Angiotensin-converting enzyme (EC 3.4.15.1) (ACE) is
known to play a role in the regulation of blood pressure in
animals, including humans. The enzyme converts angiotensin I
into angiotensin II, the former being an inert peptide and the
latter being a pressor agent. The enzyme is also responsible for
the breakdown of bradykinin, which is a dilatory peptide. The
enzyme is thus an obvious drug target in treatment of certain
cardiovascular diseases, including hypertension. Lactic acid
bacteria are known to produce enzymes of the enzyme in
various amounts during fermentation (5, 10, 13). The inhibitors
are formed by the bacterial proteinases when the lactic acid
bacteria hydrolyze milk proteins, mainly casein, into peptides,
which can be used as nitrogen sources necessary for growth. All
of the ACE inhibitors known to date that are formed in milk
during fermentation are peptides that act as competitive inhibi-
tors, such as Ile-Pro-Pro and Val-Pro-Pro (11). In some cases
it has been demonstrated that fermented milk rich in inhibitory
substances can lower systolic blood pressure in spontaneously
hypertensive rats (SHR) after oral administration (11, 13, 14).

Since the inhibitors formed are peptides, which are formed
by degradation of caseins, proteolysis plays an important role.
In theory, both specificity and overall proteolytic activity may
have a significant role in the formation. The fact that the
proteolytic systems of lactic acid bacteria are quite unspecific,
i.e., that they cleave, for instance, caseins at a high number of
places (for a review, see reference 8), combined with the fact
that ACE can be inhibited by many different peptidic struc-
tures (for instance, Val-Pro-Pro [11], Ile-Tyr [15], and Lys-Val-
Leu-Pro-Val-Pro [9], all of which inhibit ACE in the micro-
MATERIALS AND METHODS
Bacteria and fermentations. Lactobacillus helveticus CHCC637 and L. helveti-
cus CHCC641 were propagated in heat-treated 9.5% reconstituted skim milk,
and fermentations were carried out at 37°C overnight, using a 1% inoculum.
Lactococcus lactis CHCC1448 was propagated similarly but at 30°C. The three
strains were obtained from the Chr. Hansen Culture Collection (Chr. Hansen
Permanent catheterization of rats. Twelve-week-old male SHR were obtained from Mollegaard & Bombholtgard A/S, Ejby, Denmark, and had free access to tap water and standard Altromin pellet food. Lights were on from 6 a.m. to 6 p.m. The rats were allowed to acclimatize at least 7 days before being used in the experiment. Under narcopt anaesthesia with midazolam (Alpharma, Oslo, Norway) and fentanyl (Hypnorm; Janssen, Belgium), the rats had tygon tubing (Copenhagen University, Institute of Pharmacology, Copenhagen, Denmark) inserted in the aorta through the left carotid artery. The distal end of the tubing protruded from the neck. The rats were given 3 days of postoperative pain treatment by subcutaneous meloxicam injections (Metacam Vet; Boehringer Ingelheim GmbH, Ingelheim, Germany). The catheters were flushed with saline at up to 48-h intervals in order to keep them free of clots and were sealed with a fluid consisting of glucose (50%), heparin (500 U/ml; Leo Pharmaceuticals, Ballerup, Denmark), and streptokinase (10,000 U/ml; Aventis Behring, Danderyd, Sweden).

On the day of the experiment, the rats were placed in a restrainer and the external end of the catheter was flushed with saline and connected to a pressure transducer (P23XL; SpectraMed, Bilthoven, The Netherlands) for recording of MAP. The pulsatile signal was recorded using a DAQ801/DaqEd data acquisition system (Quatech Inc., Ohio) running at a 50-Hz sampling rate. After recording of their baseline MAP and heart rate, the rats were administered fermented milk (10 ml/kg of body weight) by gavage and put back in the cage. Four hours after the feeding they were put into the restrainer again and had their MAP continuously measured for 4 h. As a negative control, unfermented milk was used along with milk fermented by CHCC1448 (the latter being a strain that produces fermented milk with very little ACE inhibition). This control more closely resembles fermented milk and did not cause significant ACE inhibition in vivo in a previous study.

Data analysis. BeePee v2 (a 32-bit Windows application programmed by Anders Fuglsang) was used to extract data for the heart rate and MAP from the data acquisition files. GraphPad Prism 2.01 (GraphPad Inc.) was used to perform two-way analysis of variance on the blood pressure and heart rate data. This program was also used to fit data for the inhibition in vitro to the Michaelis-Menten equation and thus to obtain concentration-response curves and 50% inhibitory concentrations (IC50). A P of <0.05 was considered statistically significant.

RESULTS

Figure 1 shows the dose-response curves of in vitro ACE inhibition of the four types of milk used in the study. The two strains of *L. helveticus* clearly produce fermented milk with ACE-inhibitory properties. The data fit with a maximal effect of approximately 100% inhibition, which indicates that the mode of inhibition is competitive. The ACE-inhibitory IC50 (indicators of potency towards the enzyme) are listed in Table 1 along with data for optical density at 600 nm, peptide content, and pH. Note that the IC50 given in microliters signifies the volume that inhibits the enzyme by 50% under assay conditions where the total volume is 300 ml and is therefore a measure of the concentration-dependent pharmacological property called potency. The IC50 given in milligrams per milliliter tells how many milligrams of the peptide pool per milliliter are needed to inhibit ACE by 50% and is therefore an indicator of how specific the obtained peptide pool is against ACE, regardless of whether or not the peptides are present in large or small amounts (4, 7). Figure 2 shows the plot of ΔMAP versus time, where ΔMAP is the initial MAP minus the MAP at a specific time point. Figure 3 shows the change in heart rate calculated the same way. Graphically, the curves for the test groups display lower ΔMAP than do the curves for their controls. Both the blood pressures and heart rates are statistically significantly different from those for the controls, as detailed in

**TABLE 1. Comparison of milk and bacterial strains**

<table>
<thead>
<tr>
<th>Strain or milk type</th>
<th>OD600</th>
<th>pH</th>
<th>Peptide content (mg/ml)</th>
<th>IC50 (µl)</th>
<th>IC50 (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. helveticus</em> CHCC637</td>
<td>6.0</td>
<td>3.3</td>
<td>4.90</td>
<td>3.53</td>
<td>9.8</td>
</tr>
<tr>
<td><em>L. helveticus</em> CHCC641</td>
<td>5.0</td>
<td>3.4</td>
<td>4.73</td>
<td>3.41</td>
<td>16.5</td>
</tr>
<tr>
<td><em>L. lactis</em> CHCC1448</td>
<td>3.1</td>
<td>4.3</td>
<td>2.28</td>
<td>1.65</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Unfermented milk</td>
<td>1.0</td>
<td>6.7</td>
<td>0.27</td>
<td>0.21</td>
<td>80</td>
</tr>
</tbody>
</table>

a OD600, optical density at 600 nm; ND, not determined.  
b The left column is based on data obtained with casein peptone as the standard; the right column is based on data obtained with casein tryptone as the standard. Calkulation based on the casein peptone standard.
Table 2. The reason why differences are shown rather than actual values is that there was a large variability between the individual blood pressures and heart rates among the rats.

DISCUSSION

The two strains of *L. helveticus* produce competitive inhibitors (maximum inhibition according to the fit is approximately 100%), which is in accordance with earlier reports on the mode of action for milk-derived inhibitors of ACE. The peptide content, pH, and optical density resemble those previously obtained with other bacteria (13).

The peptide content, though difficult to interpret directly because of the lack of a standard that reflects the real distribution of peptidic molecular weights of the peptides liberated by the action of bacterial proteinases, was lowest in the two controls as expected. Calculation of IC50 in milligrams per milliliter yields a number that is characteristic of the peptide pool formed and is thus an indicator of the amount of peptide pool necessary to inhibit ACE by 50% under the present assay conditions. In theory, differences in the peptide pools might therefore be detected by this value. Using nonparametric statistics could be a way of detecting differences in proteolytic specificity among strains. This, however, would call for a higher number of observations than present in this study. The data for optical density, pH, and peptide content are similar to those in previous reports on a strain of *L. helveticus*, designated CP790, that had the ability to produce inhibitors (11). Milk fermented with this strain proved highly hypotensive in a tail cuff study, with a drop of up to about 30 mm Hg in systolic blood pressure in SHR (11, 13). As blood pressure-lowering agents do not change MAP and systolic pressure equally, the data presented here cannot be directly compared to those from reports on systolic effects. It is interesting, however, that we detect signif-

**TABLE 2. Comparison of two *L. helveticus* strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Avg difference (ΔMAP, mm Hg)</th>
<th>Avg difference (ΔHR, bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. helveticus</em> CHCC637</td>
<td>−12****</td>
<td>−14****</td>
</tr>
<tr>
<td><em>L. helveticus</em> CHCC641</td>
<td>−11****</td>
<td>−13****</td>
</tr>
</tbody>
</table>

* The average blood pressure and heart rate effects of the two types of ACE-inhibiting rich milk are given. **, *P* < 0.01; ****, *P* < 0.0001; NS, not statistically significant; and ΔHR, bpm, change in heart rate, beats per minute.

* The left column data are relative to those for the unfermented milk control; the right column data are relative to those for the *L. lactis* CHCC1448 control.
satory stimulation of the sympathetic nervous system, thereby raising heart rate directly. In contrast to this, calcium channel blockers in the treatment of hypertension may affect heart rate; significant effects on the heart rate. It is well known that drugs used for hypertension do not yield identical probability values versus the two controls. The reason for this could well be differences in the pool of active substances affect other targets. The present results therefore allow us only to conclude that the antihypertensive effects observed with ACE-inhibitory fermented milk may be mediated by a decreased heart rate but do not absolutely prevent us from excluding dilatory effects in the periphery. Regional flow studies may be a way of assessing such effects.

From Table 2 it is also clear that the fermented milk produced by L. helveticus CHCC637 and L. helveticus CHCC641 does not yield identical probability values versus the two controls. The reason for this could well be differences in the pool or potency of the obtained sample, as Table 1 suggests. It is also worth noting that the heart rate effect was insignificant versus that of unfermented milk but significant versus that of the L. lactis CHCC1448 product. The reason must be differences in the chemical composition of these two controls, such as differences in pH or peptide content.

The data in this study do not answer some very fundamental questions, such as when the effects are most pronounced or how long the duration of action is. It is not within our present permission to measure the animals outside the interval of 4 to 8 h after oral administration. During the 4 h in the restrainer when MAP and the heart rate are being measured, the animals do not have access to water or food. This situation is far from real life, and so the data presented may not accurately reflect the pharmacological potential of the samples. This problem could be overcome in the future by using radiotelemetry.

All blood pressure curves (both tests and controls) seem to decrease with time, which may be explained by the fact that the animals lose body fluid during the 4 h. Another possibility is that the rats gradually acclimatize to being in the experiment over these 4 h. In that case, the initial blood pressures measured here do not reflect the factual baseline blood pressures, and the significant figures could instead indicate an altered ability to acclimatize during acute stress (as imposed by the experimental procedure).

An obvious future objective is to evaluate the two types of fermented milk in hypertensive animals using both tail cuffs and radiotelemetry. Studies on the plasma half-lives of ACE-inhibitory, milk-derived peptides are under way in order to elucidate and characterize the true mechanism of action of antihypertensive peptides formed during milk fermentation.

ACKNOWLEDGMENTS

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REFERENCES