

**EFFECTS OF
MILK PRODUCTS AND MILK PROTEIN-DERIVED PEPTIDES ON
BLOOD PRESSURE AND ARTERIAL FUNCTION
IN RATS**

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals I–IV, and some unpublished data:

- I Nurminen M-L, Sipola M, Kaarto H, Pihlanto-Leppälä A, Piilola K, Korpela R, Tossavainen O, Korhonen H, Vapaatalo H. α -Lactorphin lowers blood pressure measured by radiotelemetry in normotensive and hypertensive rats. *Life Sci* 2000;66:1535–1543.
- II Sipola M, Finckenberg P, Vapaatalo H, Pihlanto-Leppälä A, Korhonen H, Korpela R, Nurminen M-L. α -Lactorphin and β -lactorphin improve arterial function in spontaneously hypertensive rats. *Life Sci* (in press).
- III Sipola M, Finckenberg P, Korpela R, Vapaatalo H, Nurminen M-L. Effect of long-term intake of milk products on blood pressure in hypertensive rats. *J Dairy Res* 2002;69:103–111.
- IV Sipola M, Finckenberg P, Santisteban J, Korpela R, Vapaatalo H, Nurminen M-L. Long-term intake of milk peptides attenuates development of hypertension in SHR. *J Physiol Pharmacol* 2001;52:745–754.

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ABBREVIATIONS

AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ANG I	Angiotensin I
ANG II	Angiotensin II
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BP	Blood pressure
cAMP	Cyclic 3',5'-adenosine monophosphate
cGMP	Cyclic 3',5'-guanosine monophosphate
COX	Cyclooxygenase
DBP	Diastolic blood pressure
DMSO	Dimethylsulfoxide
ECE	Endothelin-converting enzyme
EDHF	Endothelium-derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
Gly	Glycine
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
IC ₅₀	Inhibitory concentration 50%, the concentration at which 50% of enzyme activity is inhibited
Ile	Isoleucine
i.p.	Intraperitoneally
IPP	Isoleucine-proline-proline
L-NAME	N ^G -nitro-L-arginine methyl ester
Leu	Leucine
NA	Noradrenaline
NaCl	Sodium chloride
NO	Nitric oxide
NOS	Nitric oxide synthase
Phe	Phenylalanine
PI	Phosphoinositol
Pro	Proline
SBP	Systolic blood pressure
s.c.	Subcutaneously
SEM	Standard error of mean
SHR	Spontaneously hypertensive rat
SNP	Sodium nitroprusside
TEA	Tetraethyl ammonium tetrahydrate
Tyr	Tyrosine
Val	Valine
VPP	Valine-proline-proline
WKY	Wistar-Kyoto rat

ABSTRACT

The effects of milk products and milk protein-derived peptides on blood pressure and the development of hypertension were investigated. In addition, the effect of milk peptides on arterial function was evaluated. The mechanisms underlying these effects of milk products and peptides were studied. Spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) and Wistar rats were used in these experiments.

α -Lactorphin (Tyr-Gly-Leu-Phe), a synthetic tetrapeptide originally derived from milk whey protein α -lactalbumin, dose-dependently lowered blood pressure after single subcutaneous administration in adult SHR and WKY. The antihypertensive effect of this peptide was abolished by opioid receptor antagonist naloxone, suggesting an involvement of opioid receptors in the action of α -lactorphin. β -Lactorphin (Tyr-Leu-Leu-Phe), a synthetic tetrapeptide originating from milk whey protein β -lactoglobulin, also elicited an antihypertensive effect in SHR after subcutaneous administration.

Since α -lactorphin lowered blood pressure, it was also of interest to investigate whether α -lactalbumin could influence blood pressure in SHR. However, long-term oral administration of α -lactalbumin or peptic hydrolysate of α -lactalbumin did not influence the development of hypertension in young SHR.

In mesenteric arterial preparations of adult SHR, α -lactorphin and β -lactorphin improved endothelium-dependent relaxation response to acetylcholine (ACh). This effect was partly mediated via nitric oxide (NO) since NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) abolished the improved relaxation response. Cyclooxygenase (COX) inhibitor diclofenac and non-selective potassium channel inhibitor tetraethylammonium tetrahydrate (TEA) did not influence the improved relaxation response in the presence of the peptides, suggesting that neither vasodilatory prostanoids nor hyperpolarization were involved. β -Lactorphin also improved the endothelium-independent relaxation

induced by sodium nitroprusside (SNP). α -Lactorphin and β -lactorphin had no effects on arterial function in WKY.

Long-term oral intake of synthetic tripeptides IPP (isoleucine-proline-proline) and VPP (valine-proline-proline), which have originally been derived from milk caseins, attenuated the development of hypertension in young SHR. A similar effect was seen after long-term intake of fermented milk products containing the tripeptides. Fermented milk products also raised plasma renin activity. In a functional bioassay of angiotensin-converting enzyme (ACE) inhibitory activity in rat mesenteric arterial preparations, IPP inhibited angiotensin I-induced contraction but did not affect angiotensin II-induced contraction. These results suggest that ACE inhibition is involved in the antihypertensive effect of fermented milk products containing IPP and VPP. However, other factors, such as calcium, may have contributed to the effect since long-term intake of fermented milk products attenuated the development of hypertension more than the intake of tripeptides alone.

In conclusion, fermented milk products and milk protein-derived peptides lowered blood pressure, improved arterial function and attenuated the development of hypertension in SHR. In the acute antihypertensive effect of α -lactorphin, opioid receptors were involved. The improved endothelium-dependent vasorelaxation in SHR induced by α -lactorphin and β -lactorphin *in vitro* was mediated at least partly by NO. The attenuated development of hypertension in young SHR after long-term intake of milk protein-derived tripeptides IPP and VPP or fermented milk products containing the tripeptides may be caused by the ACE inhibitory activity of IPP and VPP. However, other factors, such as calcium, may also be involved in the action of fermented milk products.

1 INTRODUCTION

Hypertension is a major risk factor for cardiovascular diseases, such as coronary heart disease, congestive heart failure and stroke. By lowering high blood pressure with antihypertensive treatment, the incidence and severity of these complications can be decreased. In addition to pharmacological treatment, changes in lifestyle factors have beneficial effects in the treatment of elevated blood pressure and its complications. These factors may also have a favourable role in the prevention of hypertension. Non-pharmacological treatment of hypertension includes diminished use of salt (sodium chloride, NaCl) and alcohol, and decreased overweight. Increased intake of potassium, magnesium and calcium may also be advantageous. Recent recommendations for prevention and treatment of hypertension, e.g. the Sixth Joint National Committee Report on detection, evaluation and diagnosis of high blood pressure (Joint National Committee 1997), recommendations by the World Health Organization and the International Society of Hypertension (Chalmers *et al.* 1999), as well as by the Finnish Hypertension Society (2002), emphasize the role of non-pharmacological therapy, which should also be considered the foundation for treating hypertensive patients receiving antihypertensive medication.

Some epidemiological evidence exists that consumption of milk and other dairy products is associated with reduced blood pressure (Ackley *et al.* 1983; Garcia-Palmieri *et al.* 1984; McCarron *et al.* 1984; Reed *et al.* 1985; Trevisan *et al.* 1988; Abbott *et al.* 1996; Iso *et al.* 1999). Furthermore, increased consumption of milk products has lowered blood pressure in some intervention studies (Bierenbaum *et al.* 1987, 1988; Zemel *et al.* 1988; van Beresteijn *et al.* 1990; Buonopane *et al.* 1992; Appel *et al.* 1997). It is, however, difficult to relate any specific component of dairy products to the reduction of blood pressure since milk products are rich in calcium, potassium and magnesium, and low in sodium. In addition to electrolytes, milk is a good source of protein. High intake of dietary protein has been associated with reduced blood pressure levels (Reed *et al.* 1985; Stamler *et al.* 1996a, 1996b). Moreover, dietary proteins can

be enzymatically degraded to peptide fragments. Some milk protein-derived peptides have been reported to lower blood pressure (for reviews, see Takano 1998; Shah 2000).

The purpose of the present study was to investigate in spontaneously hypertensive rats (SHR), an experimental model of essential hypertension, whether milk products and milk protein-derived peptides can influence blood pressure or the development of hypertension. The effect of milk protein-derived peptides on arterial function was evaluated. The studies aimed at clarifying the mechanisms by which these peptides elicit their effects.

2 REVIEW OF THE LITERATURE

2.1 MILK AND BLOOD PRESSURE

Epidemiological evidence implies that consumption of milk and other dairy products is inversely related to blood pressure and the risk for hypertension (Table 1). The first National Health and Nutrition Examination Survey (NHANES I), a cross-sectional study with over 10 000 persons in the USA, found that a diet low in dairy products was predictive of hypertension (McCarron *et al.* 1984). Other large cross-sectional American or Italian population studies also found that consumption of whole milk was significantly lower in hypertensive than normotensive persons (Ackley *et al.* 1983; Trevisan *et al.* 1988). A cross-sectional study reported that Puerto Rican middle-aged men who drank no milk had two times the prevalence of hypertension of those consuming at least one litre of milk daily (Garcia-Palmieri *et al.* 1984). Another cross-sectional study with over 6 000 middle-aged men of Japanese ancestry living in Hawaii (Honolulu Heart Program) found that milk consumption was inversely associated with both systolic (SBP) and diastolic blood pressure (DBP) (Reed *et al.* 1985). In a 22-year follow-up of over 3 000 men in this cohort, non-drinkers of milk had twice the rate of thromboembolic stroke as compared with those who consumed half a litre of milk daily (Abbott *et al.* 1996). Intake of calcium from non-dairy sources was not associated with the reduced stroke risk, suggesting that other constituents of milk may be important. An inverse association between the risk of ischaemic stroke and dietary calcium intake was observed in a large prospective Nurses' Health Study with over 85 000 middle-aged American women (Iso *et al.* 1999). The increase in the risk of ischaemic stroke was limited to women with low calcium intake (<600 mg/d). The inverse association was stronger for dairy calcium than for non-dairy calcium.

However, the relation between dairy calcium intake and reduced risk of hypertension or stroke has not been found in all epidemiological studies. A small cross-sectional study with 300 women reported no significant association

TABLE 1. Epidemiological studies on consumption of dairy products and blood pressure.

Country	Population	Design	Main results	Reference
USA	5 050 M+F 30–79 y	cross-sectional	Whole milk consumption was lower in hypertensive than in normotensive men. Low dairy calcium intake was associated with elevated BP in men.	Ackley <i>et al.</i> 1983
USA (NHANES I)	10 372 M+F 18–74 y	cross-sectional	Reduced consumption of dairy products was related to hypertension.	McCarron <i>et al.</i> 1984
Puerto Rico	7 932 M 45–64 y	cross-sectional	Milk consumption was inversely associated with hypertension. An increment of approximately 500 ml milk/d was estimated to be equivalent to a 2 mmHg decrease in SBP.	Garcia-Palmieri <i>et al.</i> 1984
Hawaii (Honolulu Heart Program)	6 496 M 55–68 y	cross-sectional	Milk consumption was inversely associated with SBP and DBP. Dairy calcium intake was inversely associated with BP, non-dairy calcium was not.	Reed <i>et al.</i> 1985
USA	308 F 20–80 y	cross-sectional	Intake of dairy calcium was not associated with BP.	Sowers <i>et al.</i> 1985
Italy	5 049 M+F 20–59 y	cross-sectional	Daily consumption of whole milk was inversely associated with SBP.	Trevisan <i>et al.</i> 1988
Canada	423+505 F 26 y (mean)	case-control	Lower intake of dairy calcium in pregnant women with pre-eclampsia or gestational hypertension than in pregnant controls.	Marcoux <i>et al.</i> 1991
Hawaii (Honolulu Heart Program)	3 150 M 55–68 y	prospective, 22-y follow-up	Non-drinkers of milk experienced stroke at twice the rate of milk consumers (>500 ml milk/d). Intake of non-dairy calcium was not related to thromboembolic stroke.	Abbott <i>et al.</i> 1996
USA (Health Professionals' Follow-up Study)	43 738 M 40–75 y	prospective, 8-y follow-up	Intake of dairy calcium and total calcium intake were not associated with stroke risk.	Ascherio <i>et al.</i> 1998
USA (Nurses' Health Study)	85 764 F 34–59 y	prospective 14-y follow-up	Calcium intake was inversely associated with risk of ischaemic stroke. Risk was increased if calcium intake <600 mg/d. Inverse association was stronger for dairy than non-dairy calcium intake.	Iso <i>et al.</i> 1999

SBP, systolic blood pressure; DBP, diastolic blood pressure; BP, blood pressure; M, male; F, female; y, years; NHANES, National Health and Nutrition Examination Survey

between blood pressure and calcium intake from dairy sources (Sowers *et al.* 1985). In addition, a large Health Professionals' Follow-up Study with more than 40 000 men failed to observe this association (Ascherio *et al.* 1998).

Some small clinical intervention studies have also investigated the effect of milk or dairy products on blood pressure (Table 2). In a randomized cross-over study with 50 normotensive participants, supplemental daily calcium intake (1 150 mg) for eight weeks from different dairy products lowered SBP by 5 mmHg and DBP by 1 mmHg (Bierenbaum *et al.* 1988). In a double-blind study with young healthy women (n=60) whose habitual calcium intake was low (<500 mg/d), consumption of one litre of normal milk daily for six weeks lowered SBP by 5 mmHg but did not significantly affect DBP (van Beresteijn *et al.* 1990). In the same study, consumption of mineral-poor milk with one-tenth of calcium, one-third of potassium and one-twentieth of magnesium of the normal milk lowered SBP by 2.3 mmHg. This suggests that components other than minerals in milk also have beneficial effects on blood pressure. In another open study with 82 normotensive men and women, daily skim milk supplementation (1.14 litres) for eight weeks lowered SBP by 4.7 mmHg and DBP by 4.5 mmHg (Buonopane *et al.* 1992). A recent double-blind cross-over study with 38 normotensive subjects found that replacing habitual consumption of any kind of liquid milk with two servings of skim milk (on average an extra 184 ml of skim milk daily) for four weeks reduced SBP by 3 mmHg (Hilary Green *et al.* 2000). In a group receiving skim milk enriched with calcium, SBP decreased by 4 mmHg, whereas consumption of skim milk enriched with both calcium and potassium decreased SBP by 8 mmHg (Hilary Green *et al.* 2000). However, all studies have not demonstrated a beneficial effect of milk on blood pressure. For example, in a randomized open trial with healthy middle-aged or elderly participants (n=204), an increase in skim milk or 1% milk intake by 3 cups (750 ml) per day had no effect on blood pressure (Barr *et al.* 2000).

More evidence on the advantageous effect of dairy products on blood pressure is provided by the recent randomized intervention study Dietary Approaches to Stop Hypertension (DASH), with almost 500 normotensive or mildly

TABLE 2. Intervention studies on consumption of dairy products and blood pressure.

Country	Subjects Age	Design Duration	Dietary intervention	Results	Reference
USA	162 M+F, HT+NT 21–65 y	open 12 weeks	~1 litre calcium-fortified skim milk (1 400 mg calcium/d) In hypertensive individuals (n=27)	SBP/DBP –4/–3 mmHg SBP/DBP –9/–5 mmHg	Bierenbaum <i>et al.</i> 1987
USA	15 M+F, HT diabetic adults	randomized parallel, open 4 weeks	600 mg/d calcium supplementation as yoghurt 600 mg/d calcium supplementation as calcium carbonate	SBP –14 mmHg SBP no effect	Zemel <i>et al.</i> 1988
USA	50 M+F, NT 21–65 y	randomized cross-over open 8 weeks	Yoghurt (~250 ml), cottage cheese (115 g), and 1% milk (~500 ml) daily (supplemental dairy calcium intake 1 150 mg/d) Usual diet with orange juice instead of milk	SBP –5 mmHg SBP no effect	Bierenbaum <i>et al.</i> 1988
Netherlands	60 F, NT 19–23 y	randomized double-blinded parallel 6 weeks	Normal milk (1 litre/d) “Mineral-poor” milk (1 litre/d) Calcium intake from other foods <500 mg/d	SBP –5 mmHg SBP –2 mmHg	van Beresteijn <i>et al.</i> 1990
USA	82 M+F, NT 21–73 y	parallel, open 8 weeks	~1 litre skim milk/d Usual diet	SBP/DBP –5/–5 mmHg BP no effect	Buonopane <i>et al.</i> 1992
USA	13 M, HT 46–75 y	parallel, open 4 weeks	Calcium intake from dairy 1 500 mg/d Calcium intake from dairy 400 mg/d	BP no effect BP no effect	Kynast-Gales & Massey 1992
USA	38 M+F, NT Over 40 y	randomized cross-over double-blinded 4 weeks	Skim milk (~500 ml/d) High-calcium skim milk (~500 ml/d) High-calcium skim milk with potassium (~500 ml/d)	SBP –3 mmHg SBP –4 mmHg SBP –8 mmHg	Hilary Green <i>et al.</i> 2000
USA	204 M+F, NT 55–85 y	randomized parallel, open 12 weeks	Increased skim or 1% milk consumption by 750 ml/d Usual diet with milk consumption <375 ml/d	SBP/DBP –2/–1 mmHg SBP/DBP –3/–1 mmHg	Barr <i>et al.</i> 2000

SBP, systolic blood pressure; DBP, diastolic blood pressure; NT, normotensive; HT, hypertensive; M, male; F, female; y, years

hypertensive subjects who did not take antihypertensive medication (Appel *et al.* 1997). In this study, consumption of a diet rich in fruits and vegetables for 8 weeks lowered SBP by 2.8 mmHg and DBP by 1.1 mmHg. When low-fat dairy products were added to this diet (the combination diet), the blood pressure-lowering effect was pronounced: SBP was lowered by 5.5 mmHg and DBP by 3.0 mmHg. Among hypertensive subjects, the reductions in SBP and DBP were even greater (11.4 mmHg and 5.5 mmHg, respectively). Sodium content of the diet was rather low (3 g/d), but no differences were present between the groups in sodium content, body weight or alcohol consumption. The DASH II study showed that the antihypertensive effect of the diet containing low-fat dairy products was even stronger when sodium content was reduced to 1.5 g/d (Sacks *et al.* 2001). In this study, SBP was lowered by 7.1 mmHg in normotensive subjects.

To summarize, data from the epidemiological and clinical studies show that dairy products may have a positive effect on blood pressure and its complications (for review, see Massey 2001). In general, the magnitude of the blood pressure-lowering effect was 2–5 mmHg in SBP. The effect was stronger in hypertensive than in normotensive subjects. Whether the beneficial effect of milk products is related to calcium and other electrolytes or to some other components of milk has not yet been fully elucidated, but some evidence exists that calcium is not the only component of milk that has a favourable effect on blood pressure.

2.2 COMPONENTS OF MILK AND BLOOD PRESSURE

2.2.1 Calcium and blood pressure

The blood pressure-lowering effect associated with intake of milk and other dairy products has often been attributed to calcium (Ackley *et al.* 1983; Bierenbaum *et al.* 1988; van Beresteijn *et al.* 1990).

In hypertensive animals, such as spontaneously hypertensive rats (SHR), dietary calcium supplementation (1.5% calcium chloride) attenuates the development of hypertension in young prehypertensive animals (Pörsti *et al.* 1990; Wuorela *et al.* 1992; Civantos *et al.* 1999). In addition, supplementary calcium lowers blood pressure in adult SHR, whereas calcium deprivation results in increased blood pressure in SHR (for review, see Schleiffer & Gairard 1995).

An inverse relationship between dietary calcium and blood pressure in humans has been observed in several epidemiological studies (for reviews, see Geleijnse & Grobbee 2000; Miller *et al.* 2000). A meta-analysis of observational studies on dietary calcium intake estimated that an increase of 100 mg in daily calcium intake would decrease SBP by 0.39 mmHg and DBP by 0.35 mmHg (Birkett 1998). In some epidemiological studies, a reduced risk of hypertension has been associated with dairy calcium but not with calcium from other sources (Abbott *et al.* 1996; Iso *et al.* 1999).

The beneficial effect of calcium on blood pressure has also been demonstrated in many intervention studies (for review, see Kotchen & McCarron 1998). A recent meta-analysis of 42 randomized controlled trials with a total of 3 500 subjects found that calcium supplementation (daily calcium intake >1 000 mg) for at least two weeks leads to small reductions in both SBP (1.4 mmHg) and DBP (0.8 mmHg) (Griffith *et al.* 1999). The reductions in SBP and DBP achieved with dietary calcium were at least as great as those obtained with non-dietary calcium supplementation (Griffith *et al.* 1999). Although the beneficial effect of increased calcium intake has been observed in both normotensive and hypertensive subjects, individuals with an increased risk of hypertension (e.g. African-Americans, pregnant women) or with a low habitual intake of calcium are suggested to be more likely to respond to an increased calcium intake with a decrease in blood pressure than other individuals (for review, see Miller *et al.* 2000).

2.2.2 Potassium and blood pressure

In addition to calcium, dairy products also contain other minerals, e.g. potassium, which may have a beneficial effect on blood pressure (for review, see Kotchen & McCarron 1998).

In SHR, the accelerated development of hypertension induced by a high-sodium diet (3–8% NaCl) can be attenuated by an increased intake of potassium (8% potassium chloride) (Sato *et al.* 1991; Ellis *et al.* 1992). However, when the amount of sodium in the diet is moderate (0.4–0.7% NaCl), the advantageous effect of potassium on the development of hypertension is not as evident. In SHR without a sodium-load, potassium supplementation (1–8% potassium chloride) has lowered blood pressure and reduced the development of hypertension in some experimental settings (Wu *et al.* 1998; Jin *et al.* 1999), but a beneficial effect has not been observed in all studies (Sato *et al.* 1991; Ellis *et al.* 1992).

In the third cross-sectional National Health and Nutrition Examination Survey (NHANES III) in the USA, with over 17 000 individuals aged 20 years or older, SBP and DBP were negatively associated with potassium intake (Hajjar *et al.* 2001). The prospective Nurses' Health Study found that low potassium intake may contribute to an increased risk of ischaemic stroke (Iso *et al.* 1999). Data from the American Health Professionals' Follow-up Study suggests that an increased intake of potassium may decrease the risk of stroke in middle-aged and elderly American men (Ascherio *et al.* 1998).

A meta-analysis of 33 randomized controlled clinical trials with a total of 2 600 participants also provides evidence of the protective effect of potassium on blood pressure (Whelton *et al.* 1997). SBP lowered by 3 mmHg and DBP by 2 mmHg with a median daily potassium dosage of 2.9 g. In a study with 300 normotensive women with low habitual intake of potassium (2.4 g/d), supplemental potassium (1.6 g/d) had a modest blood pressure-lowering effect (2.0 mmHg in SBP and 1.7 mmHg in DBP) (Sacks *et al.* 1998). The blood pressure-lowering effect of

potassium has been particularly apparent in hypertensive patients and in those concurrently exposed to a high intake of sodium (for review, see Karppanen 1991). Thus, in the light of current knowledge, an increased intake of potassium may be useful in the treatment of elevated blood pressure especially when sodium intake is high.

2.2.3 Magnesium and blood pressure

Magnesium is another possible antihypertensive factor in milk. However, the beneficial effect of magnesium on blood pressure is more controversial than that of calcium and potassium (for review, see Kotchen & McCarron 1998).

In some experimental studies, magnesium supplementation (0.5–1% Mg) has decreased blood pressure in mature SHR and inhibited the development of hypertension in young SHR and in adult stroke-prone SHR (Wolf *et al.* 1987; Adachi *et al.* 1994). In addition, the accelerated development of hypertension induced by high sodium intake (2.7% Na) in SHR can be attenuated by concurrent supplemental potassium and magnesium (1.5% and 0.1%, respectively) (Mervaala *et al.* 1992). However, a protective effect of magnesium has not been found in all experimental studies (Overlack *et al.* 1987; Evans *et al.* 1990; Mäkyinen *et al.* 1995).

An overview of observational studies on dietary magnesium intake and blood pressure suggests that increased magnesium is related to reduced blood pressure (Mizushima *et al.* 1998). The cross-sectional Atherosclerosis Risk in Communities (ARIC) study, with 15 000 middle-aged American participants, found an inverse association between dietary magnesium intake and both SBP and DBP (Ma *et al.* 1995). In addition, magnesium intake in the prospective Nurses' Health Study in the USA was related to a reduced risk of hypertension development (Iso *et al.* 1999). In the NHANES III, however, magnesium was not associated with any changes in blood pressure (Hajjar *et al.* 2001).

The results from clinical intervention trials evaluating the effect of magnesium on blood pressure have also been somewhat inconsistent (for review, see Saris *et al.* 2000). An overview of eight placebo-controlled studies detected no blood pressure-lowering effect of magnesium supplementation (240–500 mg/d) (Witteaman & Grobbee 1990). More recently, in a randomized cross-over intervention study, magnesium supplementation (486 mg/d) in middle-aged hypertensive subjects (n=60) lowered SBP by 3.7 mmHg and DBP by 1.7 mmHg (Kawano *et al.* 1998). In a study with 300 normotensive women, magnesium supplementation (336 mg/d) had no effect on blood pressure (Sacks *et al.* 1998). Taken together, magnesium may have an advantageous effect on blood pressure, but thus far, epidemiological studies or intervention trials have produced no convincing evidence.

2.2.4 Proteins and blood pressure

In addition to various minerals, milk is rich in protein. Milk proteins are divided into caseins and whey proteins. Caseins, which comprise approximately 80% of total protein content in bovine milk, are in turn divided into α -, β - and κ -caseins. Major whey proteins, α -lactalbumin and β -lactoglobulin, account for 2–5% and 7–12% of the total protein in bovine milk, respectively (Wong *et al.* 2000).

Some epidemiological studies suggest an inverse association between protein intake and blood pressure (for reviews, see Obarzanek *et al.* 1996; He *et al.* 1999). Large cross-sectional studies, such as the Honolulu Heart Program with 6 000 subjects (Reed *et al.* 1985) and the Intersalt Study with 10 000 subjects (Stamler *et al.* 1996a), found an inverse relationship between dietary protein intake and both SBP and DBP. The Intersalt Study estimated that SBP and DBP were on average 3.0 mmHg and 2.5 mmHg lower in persons with a high dietary protein intake than in those whose intake was lower (81 vs. 44 g/d) (Stamler *et al.* 1996a). In the prospective Nurses' Health Study, the intake of animal protein was inversely associated with the risk of haemorrhagic stroke (Iso *et al.* 2001). Nevertheless, all epidemiological studies have not shown the

beneficial relation between high intake of dietary protein and blood pressure. For instance, the NHANES III found that a diet low in protein was associated with reduced SBP (Hajjar *et al.* 2001).

A few intervention trials evaluating the effect of dietary proteins on blood pressure have been performed (for review, see Obarzanek *et al.* 1996). Very recently, an eight-week randomized controlled trial was performed in mildly hypertensive men and women (n=36) aged at least 20 years, who received antihypertensive medication (Burke *et al.* 2001). When compared to low-protein diet (12.5% of energy), a diet supplemented with soy protein (protein intake 25% of energy) lowered SBP by 5.9 mmHg and DBP by 2.6 mmHg (Burke *et al.* 2001). The Multiple Risk Factor Intervention Trial (MRFIT) conducted in the USA with over 11 000 middle-aged men at high risk of coronary heart disease found that intake of total protein was inversely associated with DBP (Stamler *et al.* 1996b). This study was a randomized primary prevention trial in which diet counselling and antihypertensive drug treatment were given to an intervention group to reduce mortality in coronary heart disease. Some aspects in the other intervention trials make interpretation of their results difficult (for review, see Obarzanek *et al.* 1996). For example, most of the studies were conducted in normotensive subjects, in whom a possible blood pressure-lowering effect might be difficult to detect. Furthermore, none of the trials was specifically designed to test the hypothesis that high protein intake lowers blood pressure. Moreover, sample sizes were relatively small (n=13–69 per study) to allow detection of slight changes in blood pressure. These clinical studies cannot, therefore, confirm the inverse relation between dietary protein and blood pressure.

The underlying mechanism by which dietary proteins influence blood pressure is unknown. One hypothesis is that proteins rich in specific amino acids may result in high concentrations of these amino acids in specific brain regions or in blood vessel walls, thus evoking a vasodepressor response (for review, see Obarzanek *et al.* 1996). Tryptophan and glycine, for instance, have elicited a depressor response in animals (for review, see Nurminen *et al.* 1998). Supplementation with taurine has normalized blood pressure in experimental hypertension models, but it

has not influenced blood pressure in normotensive rats (for review, see Niittynen *et al.* 1999). Intravenous administration of arginine has reduced blood pressure in hypertensive subjects, but oral administration has not induced any changes in blood pressure in men with coronary artery disease, even though endothelial function did improve (Niittynen *et al.* 1999). Another possibility is the fragmentation of milk proteins into short-chain peptides, which may influence blood pressure.

2.2.5 Milk protein-derived peptides and blood pressure

Milk proteins are degraded into numerous peptide fragments by enzymatic hydrolysis. These peptides have been described to possess a variety of biochemical and physiological properties since 1979, when the first milk peptides with an opioid-like activity were discovered (Brantl *et al.* 1979; Henschen *et al.* 1979; Zioudrou *et al.* 1979). Other properties of milk protein-derived peptides include angiotensin-converting enzyme (ACE) inhibitory activity as well as mineral binding, antithrombotic, antimicrobial and immunomodulatory properties (Table 3) (for reviews, see FitzGerald & Meisel 2000; Gill *et al.* 2000; van Hooijdonk *et al.* 2000; Rutherford & Gill 2000; Vegarud *et al.* 2000).

The cardiovascular effects of milk protein-derived peptides have not been extensively studied to date, but along with other components of milk, they appear to have beneficial effects on blood pressure (for reviews, see Groziak & Miller 2000; Pfeuffer & Schrezenmeir 2000).

TABLE 3. Selected bovine milk protein-derived peptides and their properties.

Property	Peptide (amino acids)	Source	Effects	Reference
OPIOID-LIKE ACTIVITY	β -Casomorphin-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile)	β -Casein (f60–66)	Inhibits contraction in guinea pig ileum assay and in mouse vas deferens assay	Brantl <i>et al.</i> 1979; Henschen <i>et al.</i> 1979
	α_{s1} -Exorphin (Arg-Tyr-Leu-Gly-Tyr-Leu)	α_{s1} -Casein (f90–95)	Inhibits stimulated mouse vas deferens, binds to opioid receptors in radioreceptor assay	Loukas <i>et al.</i> 1983
	α -Lactorphin (Tyr-Gly-Leu-Phe)	α -Lactalbumin (f50–53)	Inhibits contraction in guinea pig ileum assay, binds to opioid receptors in radioreceptor assay	Yoshikawa <i>et al.</i> 1986; Antila <i>et al.</i> 1991
	β -Lactorphin (Tyr-Leu-Leu-Phe)	β -Lactoglobulin (f102–105)	Inhibits/stimulates contraction in guinea pig ileum assay, binds to opioid receptor in radioreceptor assay	Yoshikawa <i>et al.</i> 1986; Antila <i>et al.</i> 1991
ACE INHIBITION	IPP (Ile-Pro-Pro)	β -Casein (f74–76) κ -Casein (f108–110)	Inhibits ACE in spectrophotometric assay	Nakamura <i>et al.</i> 1995a
	VPP (Val-Pro-Pro)	β -Casein (f84–86)	Inhibits ACE in spectrophotometric assay	Nakamura <i>et al.</i> 1995a
	α -Lactorphin	α -Lactalbumin (f50–53)	Inhibits ACE in spectrophotometric assay	Mullally <i>et al.</i> 1996
	β -Lactorphin	β -Lactoglobulin (f102–105)	Inhibits ACE in spectrophotometric assay	Mullally <i>et al.</i> 1996
MINERAL-BINDING PROPERTY	Caseinophosphopeptides (- Ser(P)-Ser(P)-Ser(P)- Glu-Glu -)	α_{s1} -Casein (f59–79), α_{s2} -Casein (f46–70), β -Casein (f33–48)	Increases solubility of calcium, enhances absorption of calcium	Berrocal <i>et al.</i> 1989; Gagnaire <i>et al.</i> 1996
ANTITHROMBOTIC EFFECT	Casoplatelin (Met-Ala-Ile-Pro-Pro-Lys-Lys- Asn-Gln-Asp-Lys)	κ -Casein (f106–116)	Inhibits aggregation of ADP-activated platelets and binding of fibrinogen γ -chain to receptor at platelet surface	Jollés <i>et al.</i> 1986
ANTIMICROBIAL ACTIVITY	Casocidin-1	α_{s2} -Casein (f165–203)	Inhibits growth of <i>E. coli</i> and <i>Staphylococcus carnosus</i>	Zucht <i>et al.</i> 1995
IMMUNO- MODULATORY ACTIVITY	Tyr-Gly	α -Lactalbumin (f50–51)	Enhances proliferation of human peripheral blood lymphocytes	Kayser & Meisel 1996
	Thr-Thr-Met-Pro-Leu-Trp	α_{s1} -Casein (f194–199)	Stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages	Migliore-Samour <i>et al.</i> 1989
	Pro-Gly-Pro-Ile-Pro-Asn, Leu-Leu-Tyr	β -Casein (f63–68), (f191–193)	<i>in vitro</i>	

Several milk casein-derived peptides are able to lower blood pressure (Table 4). Various peptides that consist of 6–12 amino acid residues have been reported to possess antihypertensive effects in SHR after a single oral administration (Karaki *et al.* 1990; Fujita *et al.* 1996; Maeno *et al.* 1996). Likewise, isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) have dose-dependently lowered blood pressure after a single oral administration in SHR (Nakamura *et al.* 1995b). The effect of the tripeptides lasted for eight hours. No effect on blood pressure was observed in normotensive WKY (Nakamura *et al.* 1995b). Milk whey protein-derived peptides have also been shown to influence blood pressure. A small dipeptide Tyr-Pro isolated from the whey of a yoghurt-like fermented product lowered SBP in SHR for eight hours after a single oral administration (Yamamoto *et al.* 1999).

An acute antihypertensive effect in SHR has also been observed after a single oral administration of a sour milk product containing the tripeptides IPP and VPP (Calpis[®]) (Nakamura *et al.* 1995b). In addition, long-term feeding of SHR with a diet enriched with Calpis[®] -powder has attenuated development of hypertension in young prehypertensive SHR (Nakamura *et al.* 1996).

The design of some of the studies investigating the acute effects of milk protein-derived peptides does, however, raise questions. The number of rats has sometimes been quite small (n=3) (Karaki *et al.* 1990; Abubakar *et al.* 1999). Another problem is that the baseline SBP level has not always been reported for the peptide groups and the control group separately (Nakamura *et al.* 1995b; Fujita *et al.* 1996; Maeno *et al.* 1996; Yamamoto *et al.* 1999). Thus, interpretation of these results is somewhat difficult.

In humans, the antihypertensive effect of milk protein-derived peptides has yet to be demonstrated. However, some studies have investigated the effect of fermented milk products containing IPP and VPP on blood pressure. In a small controlled randomized clinical trial, daily intake of a fermented milk product (Calpis[®]) for eight weeks (95 ml/d) lowered blood pressure in mildly hypertensive patients (n=30) (Hata *et al.* 1996). Most of the patients were taking

Table 4. ACE inhibitory activity of selected milk protein-derived antihypertensive peptides.

Peptide	Antihypertensive oral dose in SHR	Maximal decrease in SBP, mean±SEM	ACE-inhibition (IC ₅₀)*	Reference
Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	100 mg/kg (72 µmol/kg)	34±13 mmHg (n=3)	59 µM	Karaki <i>et al.</i> 1990
Ala-Val-Pro-Tyr-Pro-Gln-Arg	100 mg/kg (121 µmol/kg)	10±1 mmHg (n=3)	15 µM	Karaki <i>et al.</i> 1990
Thr-Thr-Met-Pro-Leu-Trp	100 mg/kg (134 µmol/kg)	14±4 mmHg (n=3)	16 µM	Karaki <i>et al.</i> 1990
Ile-Pro-Pro	5 mg/kg (15 µmol/kg)	18±4 mmHg (n=4-6)	5 µM	Nakamura <i>et al.</i> 1995b
Val-Pro-Pro	5 mg/kg (16 µmol/kg)	20±2 mmHg (n=4-6)	9 µM	Nakamura <i>et al.</i> 1995b
Lys-Val-Leu-Pro-Val-Pro-Gln	2 mg/kg (3 µmol/kg)	32±6 mmHg (n=5)	1000 µM	Maeno <i>et al.</i> 1996
Lys-Val-Leu-Pro-Val-Pro	1 mg/kg (1.5 µmol/kg)	32±1 mmHg (n=?)	5 µM	Maeno <i>et al.</i> 1996
Tyr-Pro-Phe-Pro-Pro-Leu	10 mg/kg (14 µmol/kg)	24±? mmHg (n=5)	?	Fujita <i>et al.</i> 1996
Ile-Pro-Ala	8 mg/kg (24 µmol/kg)	31±? mmHg (n=3)	141 µM	Abubakar <i>et al.</i> 1999
Tyr-Pro	10 mg/kg (36 µmol/kg)	32±7 mmHg (n=5)	720 µM	Yamamoto <i>et al.</i> 1999

*IC₅₀, Inhibitory concentration 50%, the concentration at which 50% of enzyme activity is inhibited; ACE, angiotensin-converting enzyme; SHR, spontaneously hypertensive rat; SBP, systolic blood pressure; n, number of animals; ?, not reported

antihypertensive medication. As compared with baseline (156/89 mmHg), SBP decreased by 14 mmHg and DBP by 7 mmHg. In another clinical pilot study, an eight-week intake of a different sour milk product (150 ml/d) lowered SBP and DBP in mildly hypertensive patients (n=17) (baseline blood pressure 148/94 mmHg) (Seppo *et al.* 2002). These patients were not taking antihypertensive medication. In addition, one small clinical study has investigated the effect of fermented milk supplemented with whey protein concentrate on blood pressure in healthy normotensive men (n=20) (Kawase *et al.* 2000). SBP was reported to be slightly lowered in the group receiving the fermented milk for eight weeks (400 ml/d) as compared with that group receiving a skim milk-based control fluid.

The biologically active peptide fragments may be released from milk proteins in enzymatic proteolysis by digestive enzymes in the gastrointestinal tract. *In vitro*, enzymes such as trypsin, pepsin and chymotrypsin have been used to release peptides from their parent proteins (Meisel & Bockelmann 1999). Biologically active peptides are also generated during milk fermentation by enzymes produced by various lactic acid bacteria, e.g. *Lactobacillus helveticus*, *Lactococcus lactis* subsp. *cremoris* FT4 and *Lactobacillus delbrueckii* subspecies *bulgaricus* SS1 (Nakamura *et al.* 1995a; Gobbetti *et al.* 2000). Once liberated from proteins, these peptides may influence different physiological functions.

2.3 POSSIBLE MECHANISMS BY WHICH MILK PROTEIN-DERIVED PEPTIDES INFLUENCE BLOOD PRESSURE

2.3.1 ACE inhibitory activity

The most often studied mechanism underlying the blood pressure-lowering effect of milk protein-derived peptides is inhibition of the activity of ACE (Yamamoto 1997; Takano 1998). Inhibition of the renin-angiotensin system with ACE inhibitors or with angiotensin receptor antagonists is an effective means to

lower elevated blood pressure in hypertensive patients (for review, see Fyhrquist *et al.* 1995) or prevent the development of genetic hypertension in SHR (Freslon & Giudicelli 1983; Richer *et al.* 1991).

ACE is an enzyme, which catalyses the conversion of decapeptide angiotensin I into octapeptide angiotensin II, the effector compound of the renin-angiotensin system (for review, see Brown & Vaughan 1998). Angiotensin II has a central role in the regulation of blood pressure and vascular structure. Reducing the levels of angiotensin II by ACE inhibition results in decreased vasoconstriction, a decrease in blood pressure as well as diminished sympathetic activity and aldosterone secretion (for review, see Fyhrquist *et al.* 1995).

ACE is identical to kininase II, an enzyme that rapidly inactivates bradykinin. Bradykinin is a nonapeptide that also participates in blood pressure regulation. Increased bradykinin levels following ACE inhibition lead to indirect vasodilatation by increased production of nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) in vascular endothelium (for review, see Mombouli & Vanhoutte 1995). Thus, the increased availability of bradykinin due to ACE inhibition may be partly responsible for the advantageous effects of ACE inhibitors on blood pressure (for review, see Mombouli & Vanhoutte 1995).

The importance of the renin-angiotensin system in blood pressure regulation is indisputable. In addition to the well-established circulating renin-angiotensin system, components of the system are localized in several tissues, e.g. the heart, vascular wall, kidney, adrenal gland and brain (for review, see Stroth & Unger 1999).

Several milk protein-derived peptides have been shown to inhibit the activity of ACE (Table 4) (for review, see FitzGerald & Meisel 2000). The most potent ACE inhibitory activities have been measured for tripeptides IPP and VPP, which have also been reported to lower blood pressure in SHR (Nakamura *et al.* 1995b). The potency of the ACE inhibitory effect of milk protein-derived

peptides, such as IPP and VPP is, however, considerably lower than that reported for ACE inhibitory drugs. For instance, IC₅₀ for captopril is within the nanomolar range (Wyvratt & Patchett 1985), whereas the IC₅₀ values for IPP and VPP are one thousand-fold higher (Table 4). Therefore, the blood pressure-lowering effect of the milk peptides does not correlate with the ACE inhibitory activity, and other mechanisms by which they lower blood pressure seem likely.

2.3.2 Opioid-like activity

Several milk protein-derived peptides that possess opioid-like activities have been identified (for reviews, see Teschemacher *et al.* 1997; Meisel & FitzGerald 2000). Opioids are substances that elicit effects similar to morphine, such as sedation and antinociception. The effects of opioids are mainly mediated through opioid receptors (μ , δ , κ) in the central nervous system. Opioid receptors have also been found in peripheral tissues related to cardiovascular regulation, including the vascular endothelium (Cadet *et al.* 2000), vascular smooth muscle (Saeed *et al.* 2000), sympathetic nerves (Hughes *et al.* 1977) and adrenal glands (Viveros *et al.* 1979). In fact, endogenous opioid peptides, such as endorphins and enkephalins, may be involved in blood pressure regulation (for review, see Sirén & Feuerstein 1992). The endogenous opioid system has been suggested to play an adaptive role in cardiovascular control during stress situations (for review, see Sirén & Feuerstein 1992). Endogenous opioids have further been proposed to have a role in the pathogenesis of hypertension in SHR (Levin *et al.* 1986). Difference in the sensitivity to endogenous opioid peptides has been demonstrated between SHR and WKY (Wong & Ingenito 1995; Tsuda *et al.* 2000). In patients with essential hypertension, plasma β -endorphin levels are higher than in normotensive subjects (Guasti *et al.* 1996; Saadjian *et al.* 2000), although reduced plasma levels of β -endorphin and leu-enkephalin have also been reported in the former group (Zheng *et al.* 1995). Thus, endogenous opioids may be involved in the pathogenesis of hypertension.

β -Endorphin lowers blood pressure in conscious and anaesthetized rats after peripheral or central administration (Bolme *et al.* 1978; Lemaire *et al.* 1978; Petty *et al.* 1982; Sitsen *et al.* 1982; Levin *et al.* 1986; Unal *et al.* 1997). The cardiovascular effects of enkephalins (leu-enkephalin, met-enkephalin) seem to be strongly influenced by anaesthesia. In conscious animals, centrally or peripherally administered enkephalins have raised blood pressure (Schaz *et al.* 1980; Sander *et al.* 1982). In contrast, a depressor response has most often been observed in anaesthetized animals (Schaz *et al.* 1980; Sander *et al.* 1982; Clark *et al.* 1988; Rhee & Park 1995). In some studies, however, the effect of met-enkephalin on blood pressure has been negligible, regardless of anaesthesia (Laubie *et al.* 1977; Bellett *et al.* 1980). Newly discovered endogenous opioid peptides, endomorphins (Zadina *et al.* 1997), have also decreased systemic arterial pressure in anaesthetized rats after peripheral administration (Champion *et al.* 1997). However, the effect of these peptides on blood pressure in conscious animals remains unclear.

As mentioned above, several milk protein-derived peptides that elicit opioid-like activities have been found (Table 5) (for reviews, see Teschemacher *et al.* 1997; Meisel & FitzGerald 2000). Agonistic properties have been demonstrated in radioreceptor studies and in isolated organ preparations such as guinea pig ileum and mouse vas deferens (Chiba & Yoshikawa 1986). Milk caseins are the usual sources of peptides with opioid-like properties. The opioid peptides derived from α -casein are named α -exorphins, whereas the opioid peptides originating from β -caseins are called β -casomorphins. Peptides that originate from κ -casein are called casoxins. In addition, whey proteins, e.g. α -lactalbumin and β -lactoglobulin, contain sequences of opioid peptides in their primary structures (for reviews, see Teschemacher *et al.* 1997; Meisel & FitzGerald 2000).

α -Lactorphin is a tetrapeptide (Tyr-Gly-Leu-Phe) found in the primary structure of bovine milk whey protein α -lactalbumin (f50–53). β -Lactorphin (Tyr-Leu-Leu-Phe) originates from another whey protein, β -lactoglobulin (f102–105). These tetrapeptides are released from the milk proteins by enzymatic digestion with

TABLE 5. Endogenous opioid peptides and milk protein-derived peptides with opioid-like activity.

Peptide	Amino acid sequence	Precursor/ Source
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	Proenkephalin A
Met-enkephalin	Tyr-Gly-Gly-Phe-Met	Proenkephalin A
β -Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Ile-Ile-Lys-Asn-Val-His-Lys-Gly-Gln	Proopiomelanocortin
Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH	Prodynorphin
Nociceptin/ Orphanin FQ	H ₂ N-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-COOH	Pronociceptin
Endomorphin 1	Tyr-Pro-Trp-Phe-NH ₂	unknown
Endomorphin 2	Tyr-Pro-Phe-Phe-NH ₂	unknown
.....		
β -Casomorphin-11	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu	β -Casein
β -Casomorphin-7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	β -Casein
β -Casomorphin-5	Tyr-Pro-Phe-Pro-Gly	β -Casein
β -Casomorphin-4	Tyr-Pro-Phe-Pro	β -Casein
α_{s1} -Exorphin	Arg-Tyr-Leu-Gly-Tyr-Leu-Glu	α_{s1} -Casein
α_{s1} -Exorphin	Arg-Tyr-Leu-Gly-Tyr-Leu	α_{s1} -Casein
Casoxin A	Tyr-Pro-Ser-Tyr-Gly-Leu-Asn-Tyr	κ -Casein
Casoxin B	Tyr-Pro-Tyr-Tyr	κ -Casein
Casoxin C	Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg	κ -Casein
Casoxin D	Tyr-Val-Pro-Phe-Pro-Pro-Phe	α_{s1} -Casein
α -Lactorphin	Tyr-Gly-Leu-Phe	α -Lactalbumin
β -Lactorphin	Tyr-Leu-Leu-Phe	β -Lactoglobulin

pepsin and trypsin (Antila *et al.* 1991). They bind to opioid receptors at micromolar concentrations *in vitro* (Yoshikawa *et al.* 1986; Antila *et al.* 1991). In addition, the structures of α -lactorphin and β -lactorphin closely resemble the N-

terminal amino acid residues of many endogenous opioid peptides which share a tetrapeptide sequence Tyr-Gly-Gly-Phe- at their N-termini (Table 5) (for review, see Dhawan *et al.* 1996).

In summary, the involvement of the opioid system in cardiovascular regulation is complex due to the existence of numerous endogenous opioid peptides and multiple opioid receptors. Moreover, variation in species, anaesthesia, route of administration, stressed vs. resting animals, etc., have produced contradictory results in studies attempting to clarify the role of endogenous opioids in cardiovascular regulation. Nevertheless, increasing amount of evidence suggests that both endogenous and exogenous opioid receptor ligands may affect blood pressure.

2.3.3 Influence on arterial tone

Milk protein-derived peptides may also influence blood pressure by affecting arterial tone. The arterial tone is maintained by vascular endothelium, which lines all blood vessels. The vascular endothelium responds to various physical, chemical and hormonal signals and to haemodynamic changes by releasing vasorelaxing substances, such as nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), and vasoconstricting factors like angiotensin II and endothelin-1 (for review, see Aleixandre & Lopez-Miranda 1999; Mombouli & Vanhoutte 1999) (Figure 1).

NO is constantly released in small amounts by the endothelial cells, e.g. in response to shear stress, acetylcholine (ACh) and bradykinin (for reviews, see Marín & Rodríguez-Martínez 1997; Vallance & Chan 2001). In the endothelium, NO is synthesized from L-arginine by the constitutive endothelial NO synthase (NOS) isoenzyme. Endothelial NOS, like the other NOS isoenzymes (neuronal and inducible NOS), is competitively inhibited by L-arginine analogues such as N^G-nitro-L-arginine methyl ester (L-NAME) (for review, see Hobbs *et al.* 1999). Release of NO causes vasodilation by activating soluble guanylate cyclase,

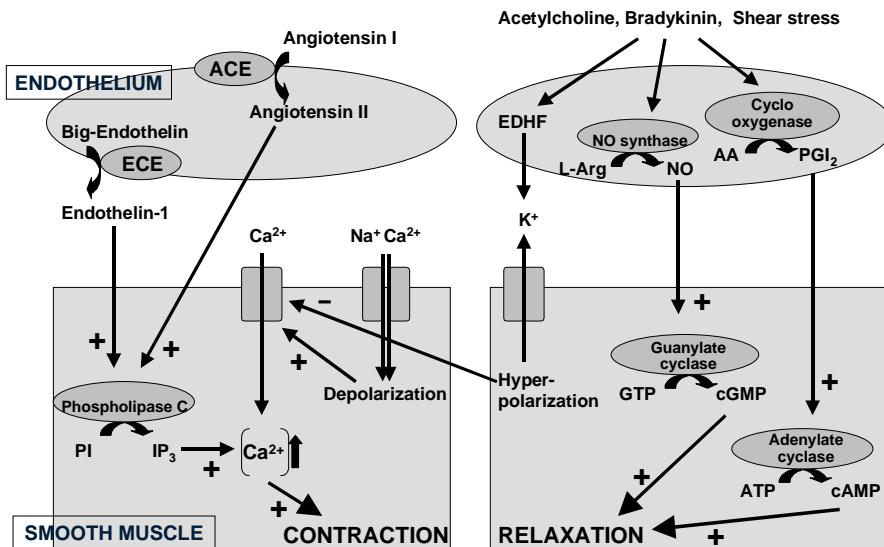


Figure 1. Endothelium-derived vasoconstricting and vasorelaxing factors (modified from Mombouli & Vanhoutte 1999).

Angiotensin II and endothelin-1 stimulate phospholipase C, leading to inositol triphosphate (IP_3) -production and release of intracellular calcium, and to contraction of vascular smooth muscle. Depolarization, on the other hand, causes vasoconstriction by increasing calcium influx into the cell. Acetylcholine, bradykinin and shear stress stimulate endothelial nitric oxide (NO) synthase to produce NO, which then diffuses into smooth muscle cells and causes vasodilation via increased production of cyclic guanosine monophosphate (cGMP). They also stimulate the production of endothelium-derived hyperpolarizing factor (EDHF), which induces hyperpolarization of the smooth muscle membrane and thereby inhibits calcium influx. Endothelial cyclooxygenase produces prostacyclin (PGI_2), relaxing vascular smooth muscle via increased production of cyclic adenosine monophosphate (cAMP). AA, arachidonic acid; ACE, angiotensin-converting enzyme; ATP, adenosine triphosphate; ECE, endothelin-converting enzyme; GTP, guanosine triphosphate; PI, phosphoinositol.

which produces the intracellular messenger, cyclic guanosine monophosphate (cGMP) (for review, see Marín & Rodríguez-Martínez 1997). Endothelium-derived NO contributes to the overall regulation of arterial blood pressure by relaxing vascular smooth muscle.

Prostanoids are produced from arachidonic acid by cyclooxygenase (COX) isoforms 1 and 2. The majority of tissues constitutively express COX-1, whereas inducible COX-2 is expressed mainly after inflammatory or mitogenic stimuli. COX isoenzymes are inhibited by COX inhibitors such as non-steroidal anti-inflammatory drugs (for review, see Vane *et al.* 1998). Prostacyclin is a vasodilatory prostanoid that is produced in endothelial cells. Endothelium also produces other vasodilatory prostanoids, e.g. prostaglandin E₂. In addition, endothelial COX produces vasoconstrictive prostanoids such as prostaglandin F_{2α}, prostaglandin H₂ or thromboxane A₂. Under normal circumstances, however, the influence of the small amounts of vasoconstrictor prostanoids released by endothelial cells is masked by the production of prostacyclin and other endothelium-derived vasodilatory substances (for review, see Mombouli & Vanhoutte 1999).

The endothelium-dependent relaxation of the vascular wall cannot be fully explained by the release of NO and prostacyclin since a degree of a relaxation can be achieved in the presence of inhibitors of NOS and COX (for review, see Félétou & Vanhoutte 1999). The additional relaxing factor EDHF causes smooth muscle relaxation by increasing the membrane potential of muscle cells. Hyperpolarization then inhibits calcium entry into the cell via calcium channels. While the nature of EDHF remains obscure, it seems to activate calcium-activated potassium channels in vascular smooth muscle cells (Oltman *et al.* 1998; Fisslthaler *et al.* 1999). Possible candidates for EDHF include metabolites of arachidonic acid, such as epoxyeicosatrienoic acids, and their dihydroxy-eicosatrienoic acid metabolites (Campbell *et al.* 1996; Oltman *et al.* 1998; Fisslthaler *et al.* 1999), K⁺ itself (Edwards *et al.* 1998) or the electrical couplings via gap junctions (Brandes *et al.* 2000). Induction of a specific endothelial cytochrome P450 isoenzyme has enhanced the formation of certain epoxy-eicosatrienoic acids as well as EDHF-mediated hyperpolarization and relaxation, and has thus been proposed to act as an EDHF synthase (Fisslthaler *et al.* 1999).

Endothelial dysfunction is a common finding in experimental models of genetic hypertension and in human essential hypertension (for reviews, see Harrison 1997; Alexandre & Lopez-Miranda 1999). The dysfunction may be defined as an imbalance between the synthesis, release and effect of factors synthesized by the endothelial cells that relax or contract the vascular smooth muscle (for reviews, see Harrison 1997; Alexandre & Lopez-Miranda 1999). Endothelial dysfunction is often associated with impaired function of the NO pathway (for reviews, see Harrison 1997; Marín & Rodríguez-Martinez 1997; Boulanger 1999). Whether the reduction in endothelium-dependent vasodilation is due to reduced release, enhanced breakdown or reduced response to NO is unclear. In any case, treatments that increase NO bioavailability may restore endothelial function and have beneficial effects on blood pressure and hypertension-related vascular injury (for review, see Vallance & Chan 2001). In contrast, inhibition of NO synthesis with NOS inhibitors, such as L-NAME, diminishes endothelium-dependent relaxation of isolated arteries, decreases blood flow *in vivo* and induces pronounced and sustained hypertension (for review, see Vapaatalo *et al.* 2000). In addition, release of vasoconstrictory prostanoids has been proposed to be increased in endothelial dysfunction in SHR (Matrougui *et al.* 1997; Kagota *et al.* 1999; Zhou *et al.* 1999). Moreover, inhibition of COX normalizes endothelial dysfunction in patients with essential hypertension and in SHR (Takase *et al.* 1994; Taddei *et al.* 1997). The endothelium-dependent hyperpolarization mediated by EDHF is also suggested to be impaired in SHR (Fujii *et al.* 1992).

Functions other than vasodilation may also be affected in endothelial dysfunction. For instance, the expression of adhesion molecules that can interact with platelets and leucocytes is increased in damaged endothelial cells. Likewise, the propensity of vascular smooth muscle cells to proliferate or migrate becomes enhanced due to increased release of growth promoters from the dysfunctional endothelial cells (for review, see Haller 1997).

In addition to endothelium-derived substances, various other factors, including endogenous opioid peptides, can influence arterial tone. Endomorphins and

met-enkephalin have been shown to possess vasodilatory activity, which has been attenuated by L-NAME (Champion & Kadowitz 1998; Huggins *et al.* 2000). The presence of opioid receptors in the endothelium has also been demonstrated (Cadet *et al.* 2000). Consequently, opioid receptor stimulation in the vascular endothelium has been proposed to release NO (Stefano *et al.* 1995, 1998).

Some milk-derived peptides have been shown to have vasodilatory effects *in vitro*. Casomokinin L (Tyr-Pro-Phe-Pro-Pro-Leu), a derivative of α -casein-derived peptide casoxin D (Tyr-Val-Phe-Pro-Pro-Phe), relaxed canine mesenteric arteries (Fujita *et al.* 1996). The effect was NO-dependent since the relaxation induced by casomokinin L was partly inhibited by L-NAME. The relaxation induced by casoxin D was not inhibited by the NO synthase inhibitor but by the COX inhibitor indomethacin, suggesting that vasodilatory prostanoids were involved in the action of this peptide (Yoshikawa *et al.* 1994). Vasodilatory peptides have also been identified in foods other than milk. An ovalbumin-derived hexapeptide (Arg-Ala-Asp-His-Pro-Phe) exerts a dose-related and NO-dependent vasodilation in mesenteric arterial preparations of SHR (Matoba *et al.* 1999). A single oral administration of this peptide has lowered SBP in adult SHR (Matoba *et al.* 1999). In addition, peptic digests of certain food proteins have inhibited ECE activity *in vitro* (Okitsu *et al.* 1995).

2.3.4 Mineral binding properties

Another mechanism by which milk protein-derived peptides may influence blood pressure is by enhancing calcium absorption via casein-derived phosphopeptides (for review, see Scholz-Ahrens & Schrezenmeir 2000; Vegarud *et al.* 2000). These peptides may also affect the absorption of other minerals, e.g. iron and zinc. Although there is evidence of improved calcium absorption by casein-derived phosphopeptides *in vitro* (Kitts *et al.* 1992; Yuan & Kitts 1994), *in vivo* studies have produced conflicting results. Calcium absorption in rats has been enhanced in some studies following the addition of

casein phosphopeptides to the diet (Hansen *et al.* 1996; Tsuchita *et al.* 2001), whereas other studies have not detected any improvement (Brommage *et al.* 1991; Kopra *et al.* 1992; Bennett *et al.* 2000). As mentioned previously, the advantageous effect of dietary calcium on blood pressure in humans has been observed in several epidemiological and intervention studies (for reviews, see Geleijnse & Grobbee 2000; Miller *et al.* 2000). Casein-derived phosphopeptides may therefore indirectly influence blood pressure by increasing calcium absorption.

3 AIMS OF THE STUDY

Epidemiological and clinical data imply that milk and milk products have beneficial effects on blood pressure. The effect may be due to calcium or other electrolytes in milk. During recent years, however, experimental studies have shown that various milk protein-derived peptides possess antihypertensive properties. The present study investigated the effects of various milk products and milk protein-derived peptides on blood pressure, development of hypertension, and arterial function using SHR as the experimental model of hypertension.

The specific aims of the study were:

- To investigate the acute effects of milk protein-derived peptides α -lactorphin and β -lactorphin on blood pressure in hypertensive SHR and normotensive WKY.
- To investigate the role of opioid mechanisms and ACE inhibitory activity in the antihypertensive effect of α -lactorphin in SHR.
- To examine the effects of α -lactorphin and β -lactorphin on arterial function *in vitro* and to evaluate the importance of various endothelium-derived factors (NO, prostanoids, EDHF) in the vascular actions of the peptides.
- To evaluate the effect of long-term intake of milk peptides IPP and VPP, and various milk products containing these tripeptides on the development of hypertension in young prehypertensive SHR.

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS

SHR, WKY and Wistar rats were used in the studies as presented in Table 6. The rats were purchased from Harlan Ltd, UK, Harlan Ltd, IN, USA, or Laboratory Animal Centre, University of Helsinki, Finland. Rats were housed 4-5 to a cage in a standard experimental animal laboratory (illuminated from 6 a.m. to 6 p.m., room temperature 22–24°C, relative humidity 40±5%) and had free access to food pellets (R36, Lactamin, Stockholm, Sweden) and drinking fluid. During the radiotelemetry experiments the animals were housed individually after the implantation of a telemetric device.

The study protocols were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, or by the Provincial State Office of Southern Finland.

TABLE 6. Experimental animals

Experiment	Strain	Number	Gender	Age Weight
Acute experiments (Study I and unpublished data)	SHR	28	Male	18–26 weeks
	WKY	9		250–490 g
Long-term experiments (Studies III, IV and unpublished data)	SHR	129	Male	6–8 weeks 120–180 g at baseline
Studies on arterial function (Study II)	SHR	27	Male	30–35 weeks
	WKY	10		
Functional bioassay of ACE inhibitory activity (Study IV)	Wistar	18	Female	220–240 g

4.2 TREATMENTS

4.2.1 Acute experiments (Study I, unpublished data)

In the acute experiments, adult SHR received subcutaneous (s.c.) injections of saline or α -lactorphin (Tyr-Gly-Leu-Phe) (1 μ g/kg–1 mg/kg) or β -lactorphin (Tyr-Leu-Leu-Phe) (1–100 μ g/kg). α -Lactorphin was also given to WKY (100 μ g/kg–1 mg/kg s.c.). In addition, constituent amino acids of the peptides were given to SHR at the doses that could maximally be released from α -lactorphin or β -lactorphin after 100 μ g/kg (L-tyrosine 35 μ g/kg, L-glycine 15 μ g/kg, L-leucine 25 μ g/kg, L-phenylalanine 30 μ g/kg s.c.). Captopril (1 μ g/kg–10 mg/kg s.c.) was also given to SHR.

Pretreatment with an opioid receptor antagonist naloxone (0.3, 1 and 3 mg/kg s.c.), a bradykinin B₂-receptor antagonist HOE140 (250 μ g/kg s.c.) or saline (1 ml/kg s.c.) were given to SHR 30 min before the administration of α -lactorphin (100 μ g/kg s.c.). Doses of naloxone and HOE140 were selected on the basis of previous studies. Naloxone (1 mg/kg s.c.) has been shown to antagonize the antinociceptive effect of morphine at 30 min in rats (Kontinen & Kalso 1995). HOE140 has been demonstrated to inhibit the depressor response to exogenous bradykinin at a comparable dose as that used in the present study (Berkenboom *et al.* 1995).

4.2.2 Long-term experiments (Studies III, IV, unpublished data)

Blood pressure- and body weight-matched young prehypertensive SHR (6–8 weeks) were divided into groups (n=8–11 per group) to receive different drinking fluids *ad libitum* for 12–14 weeks:

Study III:

- 1) A control group receiving tap water
- 2) A group receiving skim milk (Valio Ltd, Helsinki, Finland)
- 3) A group receiving the whey of fermented milk A containing 14–16 mg/l IPP and 20–26 mg/l VPP (Valio Ltd, R&D, Helsinki, Finland)
- 4) A group receiving fermented milk B containing 6–8 mg/l IPP and 10–12 mg/l VPP (Calpis[®]).

Study IV:

- 1) A control group receiving tap water
- 2) A group receiving IPP and VPP dissolved in tap water
- 3) A group receiving a sour milk product containing 16–18 mg/l IPP and 16–18 mg/l VPP (Valio Ltd, R&D, Helsinki, Finland). This product is referred to as fermented milk C.

In addition, the effect of long-term oral intake of α -lactalbumin or a peptic hydrolysate of α -lactalbumin on the development of hypertension was investigated in young prehypertensive SHR (unpublished data). The doses of α -lactalbumin or peptic hydrolysate of α -lactalbumin (255 mg/kg) were chosen to contain the amount of α -lactorphin that lowered blood pressure in adult SHR (100 μ g/kg s.c.). The oral bioavailability was estimated to be 1%. The calculations were based on theoretical release of 39 mg of α -lactorphin from 1 g of α -lactalbumin (Meisel 1998).

4.3 MEASUREMENT OF BLOOD PRESSURE

4.3.1 Radiotelemetry (Study I, unpublished data)

For the direct radiotelemetric registration of blood pressure and heart rate, data were collected with a computer-driven data acquisition system (Data Sciences Inc, MN, USA). Baseline cardiovascular parameters were measured for one hour before the injections in the acute experiments and for three days before

the beginning of the treatment period in the long-term experiment. For implantation of the telemetric transmitter devices, the rats were anaesthetised with fentanyl-fluanisone (0.5 ml/kg intraperitoneally (i.p.) solution containing fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml) and midazolam (0.5 mg/kg i.p.). The tip of the catheter connected to the transmitter was inserted into the abdominal aorta below the renal artery, and the transmitter was fixed to the abdominal wall. After the operation, buprenorphin (0.1 mg/kg s.c.) was given to relieve pain for 3 days. Ampicillin (1 g/kg i.p.) was given during the surgical procedure and 2 days afterwards. The rats were allowed post-operative recovery of at least one week before the experiments.

4.3.2 Tail-cuff method (Studies III, IV)

In the long-term experiments investigating the effect of milk products or milk protein-derived peptides on the development of hypertension, SBP was measured weekly using a tail-cuff blood pressure analyser (IITC Life Science, Model 179, Woodland Hills, CA, USA). Before the measurement, the rats were kept at 30°C for 30 min to enable pulsations of the tail artery to be detected. When three consecutive measurements were obtained without disturbance of the signal, the arithmetic mean was recorded as the SBP.

4.4 ARTERIAL FUNCTION

4.4.1 Arterial preparations (Studies II, IV, unpublished data)

In the studies examining the effects of milk protein-derived peptides on arterial function, the superior mesenteric artery was carefully excised and cleaned of adherent connective tissue. Sections of 3 mm in length were cut 5 mm distally from the mesenteric artery-aorta junction. The rings were placed between stainless steel hooks and mounted in an organ bath chamber in Krebs-Ringer buffer (pH 7.4) and aerated with O₂/CO₂ (96%/4%). The rings were initially

equilibrated for 45 min at 37°C with a resting tension of 1.0 g. The force of contraction was measured with an isometric force displacement transducer and registered with a polygraph (FTO3 transducer, Model 7P122E Polygraph; Grass Instrument Co, Quincy, MA, USA). Acetylcholine (ACh; 1 μ M) -induced relaxation after noradrenaline (NA; 1 μ M) precontraction was used to test the presence or absence of endothelium.

4.4.2 Arterial responses in the presence of α -lactorphin or β -lactorphin (Study II)

The effects of α -lactorphin or β -lactorphin on the cumulative relaxation response to ACh (1 nM–10 μ M) and sodium nitroprusside (SNP) (1 nM–10 μ M) were determined. After a 15-min incubation with the tetrapeptides (0.1 mM) or vehicle, the mesenteric artery preparations were precontracted with NA (1 μ M) before administration of ACh or SNP. ACh and SNP were added only after the precontraction or the previous level of relaxation was stable. After the maximal relaxation response was reached, rings were rinsed with Krebs-Ringer buffer and allowed at least a 30-min recovery period at the resting tension before the next response curve. Cumulative responses to ACh were also elicited in the presence of 0.1 mM L-NAME (a non-selective inhibitor of NOS), L-NAME in combination with 1 mM tetraethylammonium tetrahydrate (TEA; a non-selective inhibitor of potassium channels), and 3 μ M diclofenac (a non-selective inhibitor of cyclooxygenase).

4.4.3 Arterial responses after long-term intake of milk products (unpublished data)

After the long-term intake of various milk products (Study III), cumulative relaxation responses to ACh and SNP (1 nM–10 μ M) were examined after NA precontraction (1 μ M). Cumulative concentration-response curves were determined for NA (1 nM–0.1 mM) and potassium chloride (KCl, 20–125 mM).

In the experiments with KCl, NaCl was replaced with KCl on an equimolar basis to maintain a constant osmolality.

4.4.4 Functional bioassay of ACE inhibitory activity (Study IV)

The arterial rings were prepared as described in the section 4.4.1. The ACE inhibitory activity of IPP and VPP (0.1–3.3 mM) and captopril (10 µM) was assayed *in vitro* by preincubating mesenteric artery preparations with test substances for 15 min and measuring the response to a single administration of 0.1 µM angiotensin I or 0.1 µM angiotensin II. Angiotensins were administered only once to avoid tachyphylaxis (Khairallah *et al.* 1996).

4.5 COLLECTION OF SAMPLES

During the last week of the long-term experiments the rats were housed individually in metabolic cages (Studies III, IV). The consumption of freely accessible feed and drinking fluid was measured, and the estimated intake of electrolytes was estimated. Urine was collected over a 24-h period, urine volumes were measured and the samples were stored at -80°C until the biochemical determinations were performed.

At the end of experiments (Studies III, IV), the animals were rendered unconscious with CO₂/O₂ (70%/30%; AGA, Riihimäki, Finland) and decapitated. Blood samples were taken into chilled tubes in ice using EDTA as an anticoagulant for plasma renin activity measurements (Study III).

4.6 BIOCHEMICAL DETERMINATIONS

The peptide contents of the fermented milk products and feed were determined by the modified method of Masuda *et al.* (1996), in which the peptide fraction

was collected by gel filtration chromatography (Superdex Peptide HR 10/30, Amersham Pharmacia Biotech, UK) and analysed by reversed phase high performance liquid chromatography (HPLC) at 214 nm (Novapak C18, Waters Alliance HPLC, UV, USA). The electrolyte compositions of the fermented milk products were determined by atomic absorption spectrophotometry (Philips PU 9400X atomic absorption spectrophotometer, flame detection, UK) (Studies III, IV).

Plasma renin activity (Study III) was determined by radioimmunoassay (Medix Angiotensin I test[®], Medix Biochemica, Kauniainen, Finland). Urine sodium and potassium concentrations were measured by flame photometer using an ion-selective electrode compensator (human serum pool, IL model 943, Instrumentarium Laboratory, Milan, Italy) (Burtis *et al.* 1975). Urine calcium and magnesium were determined by flame atomic absorption spectrometry (Cali *et al.* 1973) (Studies III, IV).

The ACE-inhibitory activity was measured by a spectrophotometric assay (Cushman & Cheung 1971, with modifications of Nakamura *et al.* 1995a) (Study I). The method is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (Hip-His-Leu) catalysed by ACE. After a 30-min incubation at 37°C, the hippuric acid was extracted with ethyl acetate and measured spectrophotometrically (Perkin Elmer Lambda Bio UV/VIS Spectrometer, Germany).

4.7 COMPOUNDS

α -Lactorphin (Tyr-Gly-Leu-Phe) and β -lactorphin (Tyr-Leu-Leu-Phe) were synthesized and supplied by the Agrifood Research Finland, Food Research (Jokioinen, Finland). The solid-phase peptide synthesis by the 9-fluorenylmethoxycarbonyl chemistry (Fmoc method) was carried out on a semi-automatic peptide synthesizer (Nova-Syn Gem, Calbiochem-Novabiochem, Switzerland). Reversed phase HPLC was used to purify the peptides.

Ile-Pro-Pro and Val-Pro-Pro were obtained commercially (Peninsula Laboratories Europe Ltd, St. Helens, UK).

α -Lactalbumin was isolated from a bovine whey protein concentrate. α -Lactalbumin was separated from β -lactoglobulin using pH adjustment, heat treatment and centrifugation (Tupasela *et al.* 1997). Total protein was analysed by the Kjeldahl method, and the purity of individual protein fractions was assessed by ion exchange chromatography (Humprey & Newsome 1984). Peptic hydrolysate of α -lactalbumin was produced by dissolving the freeze-dried protein in hydrogen chloride and hydrolysing it with pepsin. After hydrolysis, the pH of the mixture was adjusted by adding sodium hydroxide. Finally the mixture was cooled and freeze-dried.

The fermented milk products A (Study III) and C (Study IV) were manufactured from skim milk that was heat-treated (110°C/10 min) and inoculated with 10% of *Lactobacillus helveticus* (LBK16H strain). The milk was fermented for 24 h at 37°C. Fermented milk B (Calpis[®]) was obtained commercially from The Calpis Food Industry Co. Ltd, Tokyo, Japan (Study III). The starter culture for fermented milk B has been reported to contain *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura *et al.* 1995a). Energy, nutrient and electrolyte contents in the milk products and feed are presented in Table 7.

In addition, the following compounds were used: acetylcholine chloride, angiotensin I acetate, angiotensin II acetate, captopril, diclofenac, L-glycine hydrochloride, L-leucine, N^G-nitro-L-arginine methyl ester hydrochloride, noradrenaline bitartrate, L-phenylalanine, sodium nitroprusside dihydrate, tetraethylammonium tetrahydrate, L-tyrosine hydrochloride (Sigma Chemical Co, MO, USA), ampicillin (A-Pen[®], Orion, Finland), buprenorphin (Temgesic[®], Reckitt & Colman, UK), fentanyl-fluanisone (Hypnorm[®], Janssen Pharmaceutica, Belgium), HOE140 (icatibant; D-Arg⁰[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]bradykinin; Hoechst AG, Germany), midazolam (Dormicum[®], Hoffmann - La Roche, Switzerland) and naloxone hydrochloride (Endo Laboratories, NY, USA).

All compounds given to animals subcutaneously or intraperitoneally were dissolved in physiological saline (0.9% NaCl). Orally administered Ile-Pro-Pro and Val-Pro-Pro were dissolved in tap water. Compounds used in the *in vitro* experiments were dissolved in distilled water, except for α -lactorphin and β -lactorphin, which were dissolved in Krebs-Ringer buffer, and TEA, which was dissolved in dimethylsulfoxide (DMSO) (96%). All solutions were freshly prepared before use and protected from light.

4.8 STATISTICAL ANALYSIS

The values are expressed as means \pm SEM. One-way analysis of variance (ANOVA) followed by Tukey's test was used when carrying out pairwise comparisons between the treatment groups. Comparisons to baseline were performed by Student's *t* test (Study I). Mann-Whitney *U* test was used to compare the differences between SHR and WKY (Study I). Data for relaxation responses, presented as a percentage of precontraction level (Study II), and for SBP (Studies III, IV) were analysed by two-way ANOVA with repeated measures for overall treatment effect. $P < 0.05$ was considered statistically significant.

Table 7. Contents of energy, nutrients, electrolytes and tripeptides IPP and VPP in different drinking fluids and feed in the long-term experiments (Studies III, IV).

	Water	IPP & VPP	Skim milk	Fermented milk A	Fermented milk B	Fermented milk C	Feed
Study	III, IV	IV	III	III	III	IV	III, IV
Energy kcal/100g	0	0	34	36	48	43	301
Protein g/100g	0	0	3.4	0.7	1.8	2.4	18.5
Fat g/100g	0	0	0.08	0.07	0.04	0.49	4
Carbohydrate g/100g	0	0	4.9	7.9	10	7.2	56
Sodium mg/100g	0.6	0.6	44	88	66	26	275
Potassium mg/100g	0.2	0.2	160	320	94	150	600
Calcium mg/100g	2.1	2.1	120	440	66	330	980
Magnesium mg/100g	0.2	0.2	12	21	6.6	33	200
IPP mg/l	0	16–30	UDL	14–16	6–8	16–18	0
VPP mg/l	0	16–30	UDL	20–26	10–12	16–18	0

UDL, under detection limit

5 RESULTS

5.1 EFFECTS OF α -LACTORPHIN AND β -LACTORPHIN ON BLOOD PRESSURE (Study I, unpublished data)

α -Lactorphin (s.c.) dose-dependently decreased blood pressure in conscious SHR (Study I). The lowest effective dose was 10 $\mu\text{g}/\text{kg}$. Maximal reductions in SBP (23 ± 4 mmHg from the baseline value of 166 ± 5 mmHg) and DBP (17 ± 4 mmHg from baseline of 111 ± 4 mmHg) were obtained after 100 $\mu\text{g}/\text{kg}$ of α -lactorphin; no further reductions were observed at a higher dose of 1 mg/kg. The decrease in blood pressure after 100 $\mu\text{g}/\text{kg}$ of α -lactorphin was maximal within 50–100 min and returned to baseline within 200 min. Heart rate was not significantly influenced by α -lactorphin.

Maximal reductions in SBP and DBP by α -lactorphin (100 $\mu\text{g}/\text{kg}$ s.c.) in normotensive WKY were 16 ± 3 mmHg and 12 ± 3 mmHg, respectively (from baseline values of 128 ± 3 mmHg and 86 ± 4 mmHg) (Study I). The depressor effect was not enhanced with a larger dose of α -lactorphin (1 mg/kg). Although the responses to α -lactorphin were somewhat weaker in WKY than in SHR, no significant differences were observed in the magnitude of blood pressure decreases between the rat strains.

β -Lactorphin (1–100 $\mu\text{g}/\text{kg}$ s.c.) decreased blood pressure in conscious SHR (Table 8). β -Lactorphin at a dose of 100 $\mu\text{g}/\text{kg}$ lowered SBP maximally by 17 ± 1 mmHg and DBP by 13 ± 2 mmHg (from baseline values of 167 ± 3 mmHg and 114 ± 4 mmHg, respectively). Due to the weak solubility of β -lactorphin, effects of doses larger than 100 $\mu\text{g}/\text{kg}$ could not be investigated.

The single constituent amino acids of 100 $\mu\text{g}/\text{kg}$ α -lactorphin or β -lactorphin (L-tyrosine, L-glycine, L-leucine, L-phenylalanine) did not significantly affect blood pressure after subcutaneous administration in SHR at the maximal doses released from the peptides (Study I).

TABLE 8. Effect of β -lactorphin (s.c.) on blood pressure (BP) in SHR (unpublished data).

Treatment	Baseline (mmHg)		Maximal change in BP (mmHg)			
	SBP	DBP	SBP	Δ	DBP	Δ
Saline 1 ml/kg	165 \pm 4	115 \pm 3	170 \pm 7	6 \pm 8	114 \pm 5	-1 \pm 5
β -Lactorphin 1 μ g/kg	167 \pm 6	118 \pm 3	155 \pm 7	-13 \pm 6	107 \pm 6 [#]	-11 \pm 4
β -Lactorphin 10 μ g/kg	164 \pm 2	114 \pm 3	150 \pm 4 [#]	-15 \pm 4	101 \pm 2 ^{###}	-13 \pm 1
β -Lactorphin 100 μ g/kg	167 \pm 3	114 \pm 4	150 \pm 3 ^{###}	-17 \pm 1 [*]	102 \pm 4 ^{###}	-13 \pm 2

Values are presented as mean \pm SEM, n=6–8.

* P <0.05 vs. saline, [#] P <0.05, ^{##} P <0.01, ^{###} P <0.001 vs. baseline.

The opioid receptor antagonist naloxone did not affect blood pressure in SHR at doses of 0.3 and 1 mg/kg s.c., whereas a significant decrease in both SBP and DBP was observed after 3 mg/kg (Study I). Pretreatment with naloxone dose-dependently did, however, antagonize the decrease in blood pressure induced by α -lactorphin (100 μ g/kg). After a large dose of naloxone (3 mg/kg), subsequent administration of α -lactorphin increased SBP by 28 \pm 6 mmHg (P <0.001) and DBP by 24 \pm 5 mmHg (P <0.01).

The ACE inhibitor captopril lowered SBP by 24 \pm 4 mmHg and DBP by 16 \pm 4 mmHg at a dose of 10 mg/kg s.c. in SHR, whereas lower doses of captopril (1 μ g/kg–1 mg/kg s.c.) did not significantly influence blood pressure (Study I).

Bradykinin B₂-receptor antagonist HOE140 (250 μ g/kg s.c.) did not have a significant effect on blood pressure (Study I). Furthermore, pretreatment with HOE140 failed to significantly influence the blood pressure response to α -lactorphin (100 μ g/kg s.c.).

The concentrations of α -lactorphin and captopril needed to inhibit 50% of ACE activity (IC₅₀) were measured spectrophotometrically. The IC₅₀ value obtained for α -lactorphin was 1 260 μ mol/l (630 mg/l) and for captopril 0.007 μ mol/l (1.5 μ g/l). These values are in accordance with those reported previously (Mullally *et al.* 1996).

5.2 EFFECT OF α -LACTALBUMIN ON DEVELOPMENT OF HYPERTENSION (unpublished data)

Long-term oral intake of α -lactalbumin (255 mg/kg) did not influence the development of hypertension in young SHR measured by radiotelemetry (Figure 2). SBP rose gradually during the experiment in all groups (Figure 2). In the group that received peptic hydrolysate of α -lactalbumin (255 mg/kg), blood pressure increased slightly but not significantly as compared with the water group. After the 12-week treatment period, SBP and DBP values in the water group were 166 ± 3 mmHg and 125 ± 5 mmHg, in the group receiving α -lactalbumin 172 ± 4 mmHg and 130 ± 6 mmHg, and in the group receiving peptic hydrolysate of α -lactalbumin 185 ± 4 mmHg and 139 ± 4 mmHg, respectively.

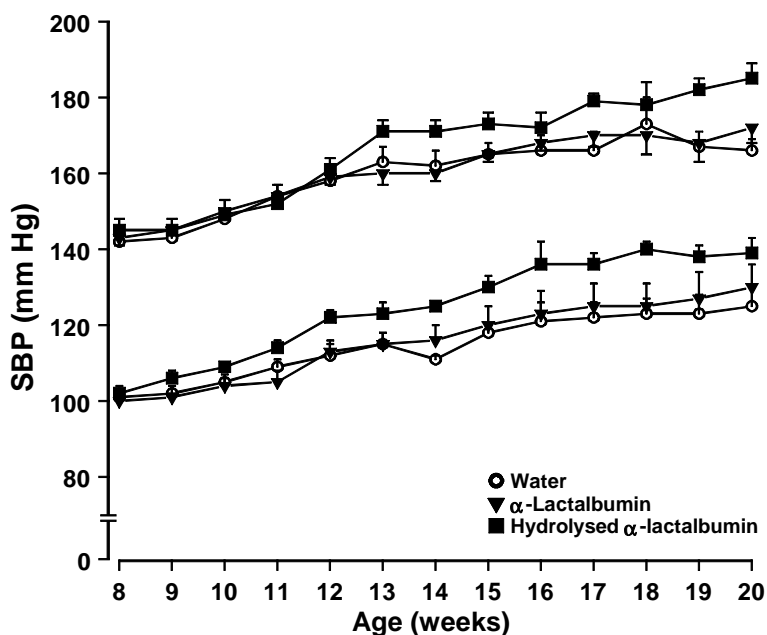


Figure 2. Long-term intake of α -lactalbumin or peptic hydrolysate of α -lactalbumin showed no influence on development of hypertension in young SHR as measured by radiotelemetry. The baseline SBP and DBP values were 142/101 mmHg in the water group, 143/100 mmHg in the group receiving α -lactalbumin and 145/102 mmHg in the group receiving the peptic hydrolysate of α -lactalbumin (unpublished data).

5.3 EFFECTS OF α -LACTORPHIN AND β -LACTORPHIN ON ARTERIAL FUNCTION (Study II)

Endothelium-dependent relaxation evoked by ACh was significantly depressed in mesenteric arteries of adult SHR as compared with the relaxation response in vessels of normotensive WKY (Study II). In SHR, maximal relaxation to ACh ($32\pm 8\%$ of the precontraction level) was observed at $1\ \mu\text{M}$ ACh. A higher concentration of ACh elicited a slight contraction in the arterial preparations. In WKY, maximal relaxation to ACh ($93\pm 2\%$ of the precontraction level) was measured at $10\ \mu\text{M}$ ACh.

In arterial preparations obtained from SHR, the relaxation response to ACh was improved by preincubation with α -lactorphin or β -lactorphin (to $49\pm 8\%$ and $61\pm 8\%$ of the precontraction level, respectively). In arterial preparations from WKY, this effect of α -lactorphin or β -lactorphin was not observed ($90\pm 6\%$ and $93\pm 2\%$ of precontraction level) (Study II). α -Lactorphin or β -lactorphin had no effect on the vascular tone of quiescent mesenteric arterial preparations of SHR or WKY.

To study the influence of different endothelium-derived factors on the effect of α -lactorphin or β -lactorphin in SHR, the cumulative response curve to ACh was elicited in the presence of the NOS inhibitor L-NAME, L-NAME combined with the potassium channel inhibitor TEA, and cyclooxygenase inhibitor diclofenac (Study II). L-NAME abolished the improvement in the ACh relaxation induced by α -lactorphin or β -lactorphin in mesenteric arterial preparations of SHR. The simultaneous addition of TEA to the organ bath did not elicit further dilation. Cyclooxygenase inhibition with diclofenac did not reduce the improvement in ACh relaxation induced by α -lactorphin or β -lactorphin. In fact, diclofenac tended to augment the response to ACh in the arterial preparations of SHR. Neither L-NAME, diclofenac nor TEA had a direct relaxant or contractile effect on vascular tone.

Endothelium-independent relaxation to SNP in mesenteric arterial rings did not differ between SHR and WKY. In SHR, the SNP-induced relaxation dose-response curve was shifted to the left in the presence of β -lactorphin (Study II). However, the maximal relaxation response to SNP was unaffected by the tetrapeptide. Endothelium-independent relaxation was also unaffected by α -lactorphin. In WKY, the tetrapeptides had no effect on the relaxation induced by SNP.

5.4 EFFECTS OF MILK PRODUCTS AND MILK-DERIVED TRIPEPTIDES IPP and VPP ON DEVELOPMENT OF HYPERTENSION IN SHR (Studies III, IV)

5.4.1 Blood pressure

In long-term studies investigating the effect of IPP and VPP on development of hypertension in young prehypertensive SHR, SBP rose gradually during the experiments. After 10 weeks of treatment, the blood pressure persisted at a stable hypertensive level in all groups in both experiments (Studies III, IV).

In the groups receiving tripeptides IPP and VPP (Study IV) or fermented milk products containing the tripeptides (Studies III, IV), the development of hypertension was attenuated as compared with the groups receiving water. After the treatment period, SBP was 12 mmHg lower in the group receiving tripeptides IPP and VPP (181 ± 2 mmHg) than in the water group (193 ± 1 mmHg) (Study IV). In the groups receiving fermented milk containing IPP and VPP, SBP levels were significantly lower than in water groups. In Study III, SBP values after the 12-week treatment period were 176 ± 1 mmHg in the group receiving the whey of fermented milk A, 186 ± 1 mmHg in the group receiving fermented milk B and 197 ± 1 mmHg in the water group. At the end of Study IV, SBP was 176 ± 1 mmHg in the group receiving fermented milk C and 193 ± 1 mmHg in the water group. Skim milk had no effect on the development of hypertension in young SHR (Study III).

In Study IV, SBP was monitored four weeks after treatment withdrawal to examine whether the attenuation of hypertension development was due to the treatments. In the groups receiving the tripeptides or fermented milk C, the SBP rose gradually after the treatment withdrawal reaching the level of the control group within the four weeks.

5.4.2 Arterial function (unpublished data)

The effect of long-term intake of milk products on arterial function was determined. Maximal relaxation responses to ACh and SNP and contraction forces to NA and KCl are presented in Table 9. The maximal relaxation responses were observed at 1 μ M ACh and 10 μ M SNP. The maximal contractions were measured at 0.1 mM NA and 125 mM KCl.

TABLE 9. Maximal relaxation responses and contraction forces after long-term intake of milk products in SHR (unpublished data)

Treatment	Maximal relaxation (%)		Maximal contraction (g)	
	ACh	SNP	NA	KCl
Water	24 \pm 5	89 \pm 2	1.96 \pm 0.1	1.92 \pm 0.2
Skim milk	33 \pm 3	91 \pm 2	1.94 \pm 0.1	1.91 \pm 0.1
Fermented milk A	36 \pm 5	97 \pm 1*	1.87 \pm 0.1	1.63 \pm 0.1
Fermented milk B	40 \pm 3	93 \pm 1	1.80 \pm 0.2	1.55 \pm 0.1

Values are mean \pm SEM, n=6–10. * P <0.05 vs. water. ACh, acetylcholine; SNP, sodium nitroprusside; NA, noradrenaline; KCl, potassium chloride.

5.4.3 ACE inhibitory activity (Study IV)

In the functional bioassay evaluating the ACE inhibitory activity of IPP and VPP, angiotensin I (0.1 μ M) contracted mesenteric arterial preparations similarly to angiotensin II (0.1 μ M). The angiotensin I-induced contraction was abolished by

preincubation with the ACE inhibitor captopril (10 μ M). IPP also dose-dependently inhibited the angiotensin I-induced contraction (Figure 3); the smallest effective concentration was 1 mM. The largest concentration of IPP (3.3 mM) abolished the contraction response to angiotensin I, while the angiotensin II-induced contraction remained unaffected. VPP of up to 3.3 mM had no effect on the angiotensin I contraction in this experimental setting.

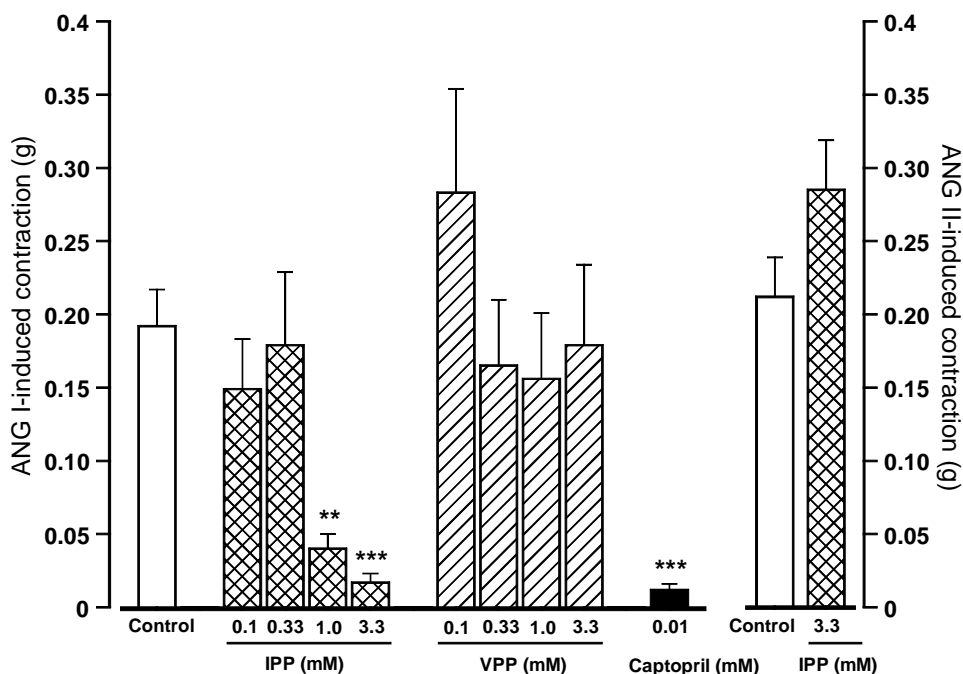


Figure 3. Effects of IPP and VPP on angiotensin I- and angiotensin II-induced contractions in mesenteric artery preparations of Wistar rats compared with the effects of captopril and control. Values are mean \pm SEM (n=6–8 in each group). ** P <0.01, *** P <0.001 vs. control. ANG I, angiotensin I; ANG II, angiotensin II. Control, open bars; IPP, cross-hatched bars; VPP, hatched bars; captopril, solid bar.

5.4.4 Body weight gain, consumption of drinking fluid and feed, estimated intake of electrolytes and tripeptides IPP and VPP (Studies III, IV)

In the groups receiving fermented milk products A and B (Study III), the intake of IPP and VPP varied due to different peptide contents in the milk products and different consumption of each product. The approximate calculated intake of IPP in the groups receiving fermented milk products A and B was 0.4 mg/day and 0.2 mg/day, respectively, whereas the corresponding intakes of VPP were 0.6 mg/day and 0.3 mg/day.

The intakes of IPP and VPP in the group receiving fermented milk C (Study IV) were calculated based on the daily consumption of the product. In the group receiving IPP and VPP, the intakes of the tripeptides were adjusted to correspond to the intakes in the group receiving fermented milk C. The intakes of IPP and VPP in both groups were 2.5–3.5 mg/kg/day (Study IV).

Intake of IPP and VPP had no effect on body weight gain, consumption of drinking fluid or feed, or the estimated intake of electrolytes (Study IV).

The consumption of milk products varied depending on the product. In the groups that consumed large amounts of the milk product, such as the skim milk (Study III) or fermented milk C (Study IV), the consumption of feed was low. Thus, the daily energy intakes between the study groups did not differ. Body weight gain was slightly attenuated in the groups receiving fermented milk products A and B (Study III). Due to the different compositions of the milk products, the estimated intake of electrolytes and nutrients differs between the groups (Table 10).

5.4.5 Urinary volume and electrolyte excretion (Studies III, IV)

Intake of the tripeptides IPP and VPP had no effect on urinary volume or urinary excretion of electrolytes (Study IV).

In the groups receiving milk products, the urinary volume and the urinary excretion of sodium, potassium, magnesium and calcium were closely related to the estimated intakes of these electrolytes (Table 10). Due to differences in the electrolyte compositions between the milk products, the intakes of electrolytes, and thus, the urinary excretions of electrolytes, differ between the groups (Table 10).

5.4.6 Plasma renin activity (Study III)

Plasma renin activity was increased in groups receiving fermented milk products A and B (4.4 ± 0.5 and 4.6 ± 0.5 ng angiotensin I/ml/h, respectively) as compared with the groups receiving water (1.4 ± 0.3 ng angiotensin I/ml/h) and skim milk (2.6 ± 0.4 ng angiotensin I/ml/h).

Table 10. Body weight gain, consumption of feed and drinking fluid, estimated intake of energy, nutrients and electrolytes, urine volume and urinary excretion of electrolytes in long-term experiments III and IV.

	Water	Skim milk	Fermented milk A	Fermented milk B	Water	Peptides	Fermented milk C
Study	III	III	III	III	IV	IV	IV
Body weight, g baseline	134±1	132±1	134±2	132±2	138±4	138±5	137±5
Body weight, g end of experiment	342±4	356±4	310±9 ^{†§}	294±7 ^{†§}	333±8	334±5	324±10
Feed intake, g/d	22±1	16±1 [†]	17±1	14±2 [†]	17±1	17±1	6±1*
Drink intake, g/d	32±3	55±3 [†]	27±1 [§]	25±1 [§]	33±2	31±3	64±2*
Estimated intake of							
Sodium mg/d	62±3	69±4	71±4	56±6	48±3	46±2	34±2*
Potassium mg/d	134±7	185±10 [†]	190±8 [†]	109±13 [§]	104±6	99±4	132±5*
Calcium mg/d	220±12	224±13	287±13 ^{†§}	156±22 ^{†§}	171±9	162±7	270±9*
Magnesium mg/d	45±2	39±2	40±3	30±4 [†]	35±2	33±1	33±1
Urine							
Volume ml/d	11±2	19±2 [†]	4±1 [†]	8±1	10±1	12±2	35±2*
Sodium mg/d	27±2	33±2	27±2	30±2	26±1	23±1	22±2
Potassium mg/d	76±5	122±4 [†]	85±4 [§]	63±4 [§]	56±8	53±9	114±4*
Calcium mg/d	0.7±0.1	1.6±0.2	2.3±0.5 [†]	1.7±0.3	0.6±0.1	0.7±0.1	9.7±0.6*
Magnesium mg/d	3.6±0.5	2.1±0.7	5.2±0.5 [§]	4.6±0.7 [§]	3.6±0.4	3.8±0.4	11±0.3*

[†] $P < 0.05$ vs. water (Study III)

[§] $P < 0.05$ vs. skim milk (Study III)

* $P < 0.05$ vs. water and IPP & VPP (Study IV)

6 DISCUSSION

Epidemiological evidence implies that consumption of milk and dairy products is inversely associated with blood pressure and the risk for hypertension. Therefore, this study investigated the effect of milk products and milk protein-derived peptides on blood pressure, development of hypertension and arterial function using SHR as the experimental model for hypertension. Mechanisms underlying the effects of milk products and peptides were also studied.

6.1 METHODOLOGICAL ASPECTS

SHR is the most widely studied animal model for human essential hypertension. SHR is an inbred rat strain that readily develops hypertension and its complications with increasing age. Despite the frequent use of SHR, this strain has occasionally been criticized as being an inappropriate model for human essential hypertension (for review, see Zicha & Kunes 1999). Indisputedly, the comparison of human essential hypertension with genetic hypertension of the rat is complex because the species differ in their genetic defects and gene-environment interactions. Nevertheless, the basic principles regarding development of hypertension in rats and humans are surprisingly similar (for review, see Zicha & Kunes 1999). Normotensive WKY are generally used as a control for SHR, even though some of the physiological and biochemical differences between the strains are likely to be unrelated to hypertension (Lindpaintner *et al.* 1992). These differences are due to the selective breeding of SHR, in which the desired phenotype (spontaneous hypertension) in the selected individuals has coincidentally been accompanied by other genetic characteristics, which have, therefore, also become fixed in the hypertensive inbred strain. To date, however, WKY are considered an appropriate normotensive control rat strain for SHR.

In the present study, blood pressure was measured either directly by radiotelemetry or indirectly by the tail-cuff method. Radiotelemetric

measurements provide the possibility of obtaining continuous, high-fidelity recordings of blood pressure for relatively long periods of time. In addition, the method allows blood pressure monitoring in conscious, freely moving animals without the need for restraints or anaesthesia. The main limitation of this method is the drift in telemeter settings and sensitivity. A recalibration of the telemeter device immediately before implantation is therefore essential to ensure the accuracy of blood pressure measurements (for review, see van Vliet *et al.* 2000). Maintenance of the method is also relatively expensive.

When a study design requires monitoring of a large number of animals over a long time period, the non-invasive tail-cuff method is commonly chosen, as in the present study. The indirect tail-cuff recording of blood pressure shows good correlation with direct recordings using intra-arterial catheters in rats (Bunag & Butterfield 1982). It may, however, overestimate blood pressure levels (Bazil *et al.* 1993). The inevitable use of restraints produces a stress artefact manifesting as elevated plasma catecholamines and cortisol (Kvetnansky *et al.* 1977). In addition, to obtain reliable SBP values via the tail-cuff method, the animals require warming prior to actual measurement. This obviously causes further stressful stimuli and may influence blood pressure (Kenney *et al.* 1995). Another limitation of the tail-cuff method is that it reliably produces only SBP values.

The arteries of SHR and patients with essential hypertension have alterations in the structure and function caused by sustained hypertension (Hollenberg 2000). Some degree of vascular structural alterations have already been observed in young 3- to 4-week-old SHR even when blood pressure is similar to that in age-matched WKY (Rizzoni *et al.* 1994; Dickhout & Lee 1997). However, structural changes in the superior mesenteric artery have become evident only in older SHR (16- to 28-week-old) with established hypertension (Lee *et al.* 1983; Lee 1987). The structural alterations of blood vessels manifest functionally as impaired endothelium-dependent vasodilator responsiveness. In young SHR, the relaxation response to ACh is similar to that in WKY, whereas in older SHR

with established hypertension as in our study, it is impaired (Dohi *et al.* 1990; Fujii *et al.* 1993; Rizzoni *et al.* 1994).

The major complications of hypertension result from the affliction of relatively large arteries (for review, see Safar *et al.* 1998). The effects of different antihypertensive interventions on the function of conduit arteries are therefore of importance. In this study, the main superior mesenteric artery was used as a model of a conduit artery. It is highly suitable for organ bath studies because it produces a stable precontraction, and its diameter (1-1.5 mm) allows preparation without a microscope. The mesenteric arteries of normotensive WKY were used as a model of arteries with normal functional structure.

6.2 EFFECTS OF α -LACTORPHIN AND β -LACTORPHIN ON BLOOD PRESSURE AND ARTERIAL FUNCTION

α -Lactorphin and β -lactorphin, originally derived from milk whey proteins α -lactalbumin and β -lactoglobulin, respectively, lowered blood pressure in adult SHR. The antihypertensive effect of the tetrapeptides was not attributable to the constitutive amino acids L-tyrosine, L-glycine, L-leucine or L-phenylalanine.

Both α -lactorphin and β -lactorphin have been reported to bind to opioid receptors in a radioreceptor assay (Yoshikawa *et al.* 1986; Antila *et al.* 1991). In addition, the structures of α -lactorphin and β -lactorphin closely resemble the amino terminal amino acid sequence of most endogenous opioid peptides (Tyr-Gly-Gly-Phe-) (Table 5). In this study, opioid receptor antagonist naloxone dose-dependently inhibited the antihypertensive action of α -lactorphin, suggesting that opioid receptors were involved in the blood pressure-lowering effect of α -lactorphin. Naloxone is a non-selective opioid antagonist that mainly blocks the effect of opioids on μ -receptors. At large doses, however, it also inhibits the effects mediated via δ - and κ -opioid receptors (Chang & Cuatrecasas 1979; Leslie 1987). Any subtype of opioid receptors may, therefore, be responsible for the depressor effect of α -lactorphin.

Pretreatment with naloxone not only antagonized the decrease in blood pressure induced by α -lactorphin, but a large dose of naloxone reversed it into a pressor response. An increase in blood pressure during the presence of naloxone has been observed with other antihypertensive agents, such as alpha-methyldopa and clonidine (Farsang *et al.* 1984; Kunos *et al.* 1984). The pressor effect has been suggested to be due to noradrenergic vasoconstriction since it was associated with increased plasma concentration of catecholamines (Farsang *et al.* 1984). Furthermore, the effect was blocked by α_1 -adrenoceptor antagonist prazosin (Kunos *et al.* 1984). In anaesthetized rats, large doses of naloxone have also been shown to potentiate the pressor response to catecholamines (Feria *et al.* 1990). However, no experimental evidence exists that α -lactorphin could release catecholamines, and the mechanism of the increase in blood pressure by α -lactorphin after naloxone pretreatment remains undetermined.

Many of the known effects of opioids (e.g. sedation, analgesia) are mediated via the central nervous system. The level (central vs. peripheral) of the depressor response to α -lactorphin was not determined in this study. However, a peripheral site of action is feasible because it is unlikely that α -lactorphin as a tetrapeptide could easily cross the blood-brain barrier. Endogenous opioids and opioid receptors are present in some peripheral tissues, including the sympathetic ganglia, adrenal medulla and vascular endothelium (Hughes *et al.* 1977; Viveros *et al.* 1979; Cadet *et al.* 2000). In rats, intravenous administration of morphine has elicited a depressor response that depended on peripheral opioid receptor activation (Randich *et al.* 1993). In addition, peripherally administered endogenous opioid peptides, such as endomorphins, have lowered blood pressure in rats (Champion *et al.* 1997).

α -Lactorphin and β -lactorphin improved endothelium-dependent relaxation to ACh of mesenteric arteries of adult SHR with established hypertension *in vitro*. NOS inhibitor L-NAME abolished the improvement, suggesting an involvement of NO in this action. Endogenous opioid peptides, endomorphins and met-enkephalin have also produced vasorelaxation in rats in a NO-dependent

manner (Champion & Kadowitz 1998, 1999; Wilderman & Armstead 1998). Opioid receptor stimulation in the vascular endothelium has been proposed to release NO (Stefano *et al.* 1998).

The COX inhibitor diclofenac did not influence the improved relaxation response to ACh in the presence of α -lactorphin and β -lactorphin. This suggests that vasodilatory prostanoids were not involved. The vasodilator response elicited by endomorphins has also not been mediated by vasodilatory prostanoids (Champion & Kadowitz 1998; Champion *et al.* 1998). The relaxation response to ACh in the presence of the tetrapeptides tended to enhance after diclofenac pretreatment in our study. Production of vasoconstrictory prostanoids, such as prostaglandin $F_{2\alpha}$, prostaglandin H_2 or thromboxane A_2 , is increased in adult SHR (Matrougui *et al.* 1997; Kagota *et al.* 1999; Zhou *et al.* 1999), which may explain this finding.

The effect of TEA on ACh-induced relaxation of mesenteric arterial preparations was negligible in the presence of α -lactorphin and β -lactorphin. TEA is a non-selective potassium channel blocker that inhibits the EDHF-induced hyperpolarization and the subsequent smooth muscle relaxation. When administered to the organ bath simultaneously with L-NAME, TEA elicited no additional effect. This suggests that EDHF did not play a major role in the vascular effects of α -lactorphin or β -lactorphin in our experimental setting. Likewise, opening of the potassium channels was presumably not involved in the vasodilatory action of endomorphins (Champion & Kadowitz 1998; Champion *et al.* 1998).

β -Lactorphin improved the endothelium-independent relaxation response to SNP in SHR, whereas α -lactorphin had no effect on SNP-induced relaxation. This finding suggests that the improvement in the vascular function induced by β -lactorphin may be mediated not only by the endothelium, but that vascular smooth muscle may also be directly influenced. β -Lactorphin may increase the ability of vascular smooth muscle to relax in response to NO-donors, whereas α -lactorphin does not seem to enhance sensitivity of the smooth muscle to NO.

α -Lactorphin and β -lactorphin have been shown to possess ACE inhibitory activity *in vitro* (Mullally *et al.* 1996). This was also observed in the present study. However, as compared with captopril, whose IC₅₀ value was within nanomolar range, the ACE inhibitory activity of the tetrapeptides was very weak, with IC₅₀ values at millimolar level. In contrast, a comparable decrease in blood pressure was seen with 200-fold lower doses of α -lactorphin than of captopril. Since ACE is also involved in the breakdown of bradykinin, increased bradykinin levels are suggested to have a role in the antihypertensive action of ACE inhibitors (Brown & Vaughan 1998). Because blocking of bradykinin B₂-receptors with HOE140 did not attenuate the α -lactorphin-induced decrease in blood pressure, the involvement of bradykinin in the effect of α -lactorphin can, therefore, be ruled out.

Taken together, the data suggest that the antihypertensive effect and the improvement of the endothelium-dependent relaxation in SHR induced by α -lactorphin or β -lactorphin could be related to opioid receptors in the endothelium. Inhibition of ACE activity does not seem to be the mechanism of the depressor response to α -lactorphin or β -lactorphin.

α -Lactorphin is a peptide fragment, which contains the amino acid residues 50-53 of milk whey protein α -lactalbumin (Findlay & Brew 1972). The tetrapeptide was originally released from α -lactalbumin by *in vitro* proteolysis with pepsin and trypsin (Antila *et al.* 1991). Since α -lactorphin was able to lower blood pressure, it was of interest to investigate whether α -lactalbumin or a peptic hydrolysate of α -lactalbumin could also influence blood pressure in SHR.

The dose of α -lactalbumin or peptic hydrolysate of α -lactalbumin was calculated to contain the amount of α -lactorphin that elicited an antihypertensive effect in adult SHR. Bovine milk contains 0.9 g/l of α -lactalbumin (for review, see Hambræus 1985), whereas the maximum yield of α -lactorphin from 1 g of α -lactalbumin has been estimated as 39 mg (Meisel 1998). Thus, one litre of

milk can be calculated to maximally yield 35 mg of α -lactorphin. Oral bioavailability of α -lactorphin was assumed to be low, and only 1% of ingested α -lactorphin was estimated to be absorbed from the intestine.

Long-term intake of α -lactalbumin failed to influence the development of hypertension in young prehypertensive SHR in the present experimental setting. This failure could have been due to the inability of α -lactorphin to influence hypertension development. In addition, the tetrapeptide may have been released from α -lactalbumin in amount insufficient to elicit an effect on blood pressure. Inadequate absorption of α -lactorphin from the intestine is another possible explanation for the lack of effect. The effect of long-term intake of β -lactoglobulin, the source of β -lactorphin in milk, on the development of hypertension was not investigated since β -lactoglobulin is a major allergen of milk (for review, see Wal 1998). Therefore, implications of this protein in non-pharmacological treatment of hypertension would be scarce. This does not, however, rule out the possible use of β -lactorphin in the non-pharmacological treatment of hypertension because hydrolysis of β -lactoglobulin markedly reduces its allergenicity (Halcken & Høst 1997).

6.3 EFFECTS OF IPP, VPP AND MILK PRODUCTS ON DEVELOPMENT OF HYPERTENSION

Long-term oral administration of IPP and VPP attenuated the development of hypertension in young prehypertensive SHR. Hypertension development was also attenuated after long-term intake of fermented milk products containing these tripeptides. After treatment withdrawal, the SBP rose to the level of the control group, confirming that the antihypertensive effect was due to the treatments.

Various long-term antihypertensive treatments, such as ACE inhibitors, calcium channel blockers and beta-blockers, have improved relaxation responses in SHR (Tolvanen *et al.* 1996; Mervaala *et al.* 1998). In our study, arterial function

was evaluated *in vitro* after long-term oral intake of fermented milk products. Distinct improvement of arterial function was not observed, although endothelium-independent relaxation of mesenteric arteries was enhanced slightly.

Skim milk had no effect on the development of hypertension or arterial function, suggesting that a factor present in the fermented milk products but absent in the skim milk might be responsible for the effect. Possible factors are peptides IPP and VPP, which have been shown to possess antihypertensive properties in SHR (Nakamura *et al.* 1995b, 1996). A fermented milk product containing these tripeptides (Calpis[®]) has been reported to attenuate the development of hypertension in SHR (Nakamura *et al.* 1996). As compared with Calpis[®], which was fermented milk B in our study, the antihypertensive effect of the whey of another fermented milk containing IPP and VPP, i.e. product A, turned out to be stronger. One possible explanation for this is that fermented milk A contained twice the amount of IPP and VPP present in Calpis[®]. IPP and VPP have been reported to dose-dependently lower blood pressure in SHR after acute oral administration (Nakamura *et al.* 1995b). Intake of fermented milk products containing IPP and VPP has also lowered blood pressure in mildly hypertensive patients in two small clinical studies (Hata *et al.* 1996; Seppo *et al.* 2002). The fermented milk product A seemed to attenuate the development of hypertension slightly more than the product C, but comparisons are difficult for the obvious reason that the effects of these products were not investigated in the same study. If the effects of these two products differed, one possible underlying factor could be the apparent difference between the products: fermented milk A contained nearly exclusively the whey fraction, whereas product C also contained the casein fraction. Some evidence implies that whey-based products may influence blood pressure advantageously (Wu *et al.* 1998; Kawase *et al.* 2000).

The mechanism underlying the blood pressure-lowering effect of IPP and VPP has been proposed to be ACE inhibition (Nakamura *et al.* 1995a, 1995b; Takano 1998). These tripeptides have been reported to possess ACE inhibitory

activity in a spectrophotometric assay (Nakamura *et al.* 1995a). In our functional assay of ACE inhibitory activity, IPP and captopril inhibited the angiotensin I-induced contraction of mesenteric arteries *in vitro*. The compounds had no effect on the angiotensin II-induced contraction, and therefore, the involvement of angiotensin receptors is unlikely. The alleviation of hypertension development in SHR that received IPP and VPP from fermented milk products was accompanied by elevated plasma renin activity, thus providing indirect evidence of ACE inhibition. Treatment with ACE inhibitors raises plasma renin activity due to the lack of negative feedback induced by angiotensin II (Brunner *et al.* 1993). In agreement with our findings, ACE activity in the aorta of SHR has been shown to be reduced after long-term intake of Calpis® (Nakamura *et al.* 1996).

Some factors, however, oppose the hypothesis that ACE inhibition is the mechanism underlying the effect of IPP and VPP. IC₅₀ values determined for the tripeptides have been 1000-fold larger than that for captopril, which points to the significantly weaker ACE inhibitory activity of the tripeptides. In addition, the ACE inhibitory potency of IPP was much weaker than that of captopril in the functional assay since millimolar concentrations of IPP were needed to inhibit the angiotensin I-induced contraction of rat mesenteric artery, whereas captopril inhibited the contraction at a 300-fold lower concentration. The ACE inhibitory activity of VPP in spectrophotometric assays is not supported by our findings; in the functional bioassay for ACE inhibitory activity, VPP failed to show any potential for inhibiting contraction induced by angiotensin I.

To summarize, some evidence does lend support to the hypothesis that ACE inhibition is involved in the antihypertensive effect of IPP and VPP. Some additional mechanisms, however, are likely to be found in fermented milk products that contain the tripeptides since fermented milk C was more effective in alleviating the development of hypertension than similar amounts of IPP and VPP alone. This could be due to several factors. Other peptides with effects on blood pressure may have been produced during the enzymatic hydrolysis of

milk proteins. Peptides may also absorb better from a milk hydrolysate than from a water solution, as has been shown in the case of amino acids (Rérat *et al.* 1988). In addition, certain cell wall polysaccharide fractions of lactic acid bacteria used in the starter cultures of fermented milk products have been suggested to lower blood pressure in SHR (Furushiro *et al.* 1993; Nakajima *et al.* 1995).

Another element underlying the antihypertensive effect of fermented milk products may have been the electrolytes that these products contain. Intakes of calcium and potassium have been reported to attenuate the development of hypertension in SHR (Wuorela *et al.* 1992; Wu *et al.* 1998; Civantos *et al.* 1999). The role of calcium cannot be excluded because the amount of calcium in the fermented milks A and C was higher than in skim milk, nearly reaching the level reported to influence development of hypertension (Pörsti *et al.* 1990). However, an antihypertensive factor other than calcium is suggested since the development of hypertension was attenuated in the group receiving Calpis[®], despite the intake of calcium in this group being lower than in the skim milk group. The intake of potassium was comparable in the groups receiving fermented milk A and skim milk, but since skim milk failed to affect hypertension development, potassium was presumably not a notable factor. As compared with other milk products, the sodium content of fermented milk C was low and the sodium-potassium ratio for intake or excretion was lowest in SHR receiving product C, which may have resulted in greater attenuation of hypertension development. In this model of essential hypertension, moderate intake of sodium has produced less pronounced hypertension development than a high-sodium diet (Mervaala *et al.* 1992). However, the antihypertensive effect of IPP and VPP is not likely to be mediated via natriuresis or diuresis since long-term intake of these tripeptides did not affect urinary excretion of sodium or urine volume. In conclusion, despite the antihypertensive effect of IPP and VPP in SHR after long-term intake of fermented milk products, factors such as calcium may have a role as well.

An important issue regarding the possible usefulness of milk protein-derived peptides in non-pharmacological treatment of hypertension is whether their bioavailability after oral administration is sufficient to elicit systemic effects on blood pressure. Dipeptides and tripeptides can absorb from the gastrointestinal tract, whereas the intestinal absorption of tetrapeptides is more controversial (for reviews, see Silk *et al.* 1981, 1985; Webb *et al.* 1992). In addition to the size of the peptide, the amino acid structure seems to be of importance. For example, a Pro-Pro bond has been proposed to possess a high degree of resistance to any mammalian proteolytic enzyme (for review, see Vanhoof *et al.* 1995). In our long-term experiment, oral intake of IPP and VPP attenuated the development of hypertension in SHR. These tripeptides have been detected from the abdominal aorta of SHR after a single oral administration of a sour milk product containing the tripeptides (Masuda *et al.* 1996). Consequently, these milk protein-derived tripeptides might be absorbed from the intestine in sufficient amounts to elicit effects on blood pressure.

Taken together, this experimental study using SHR as the model of essential hypertension found that short-chain milk protein-derived peptides lowered blood pressure, attenuated the development of hypertension and improved arterial function by mechanisms related to opioid receptor stimulation or ACE inhibition. Electrolyte composition of the fermented milk products may have provided additional benefit.

7 SUMMARY AND CONCLUSIONS

This study investigated the effects of various milk products and milk protein-derived peptides on blood pressure, development of hypertension, and arterial function using SHR as the experimental model for hypertension.

The main findings are as follows:

- α -Lactorphin and β -lactorphin, tetrapeptides originally derived from whey proteins, lowered blood pressure in hypertensive SHR and in normotensive WKY.
- The antihypertensive effect of α -lactorphin was related to opioid receptor stimulation. ACE inhibition did not seem to be involved.
- α -Lactorphin and β -lactorphin improved arterial function in SHR in an endothelium-dependent manner *in vitro*. NO played a central role in this effect, whereas vasodilatory prostanoids and hyperpolarization appeared not to be implicated.
- Long-term intake of IPP and VPP or fermented milk products containing these tripeptides attenuated the development of hypertension in young SHR. Skim milk and α -lactalbumin had no influence on hypertension development.

In conclusion, in an experimental model of hypertension, certain fermented milk products and milk protein-derived peptides lowered blood pressure, attenuated the development of hypertension and improved arterial function. These findings encourage further investigation as to whether milk peptides could be beneficial in non-pharmacological treatment of essential hypertension.

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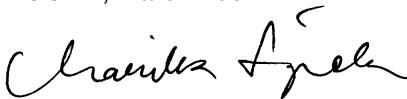
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Helsinki, March 2002

A handwritten signature in black ink, appearing to read 'Marika Sipola', written in a cursive style.

Marika Sipola

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