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α-lactorphin and β-lactorphin improve arterial function in spontaneously hypertensive rats

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Abstract

α-lactorphin (Tyr-Gly-Leu-Phe) lowers blood pressure in conscious adult SHR. This tetrapeptide is originally released from milk protein α-lactalbumin by enzymatic hydrolysis. In order to evaluate the antihypertensive mechanisms of α-lactorphin, the effects of the tetrapeptide on vascular function were investigated in (30–35 weeks old) spontaneously hypertensive rats (SHR) with established hypertension and age-matched normotensive Wistar-Kyoto (WKY) rats *in vitro*. In addition, we studied the vascular effects of another structurally related tetrapeptide, β-lactorphin (Tyr-Leu-Leu-Phe), which originates from milk protein β-lactoglobulin. Endothelium-dependent relaxation to acetylcholine (ACh) was reduced in mesenteric arterial preparations of SHR as compared to those of WKY. In SHR, the ACh-induced relaxation was augmented by α-lactorphin or β-lactorphin. The role of nitric oxide (NO) is suggested, since this improvement was abolished by the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). Simultaneous potassium channel inhibitor tetraethylammonium (TEA) elicited no additional effect on the ACh-induced relaxation. The cyclooxygenase inhibitor diclofenac did not attenuate the augmented ACh relaxation induced by α-lactorphin or β-lactorphin, suggesting that endothelial vasodilatory prostanoids were not involved in the effect of the tetrapeptides. Endothelium-independent relaxation to the NO donor sodium nitroprusside (SNP) was augmented in mesenteric arterial preparations of SHR by simultaneous β-lactorphin. The tetrapeptides did not alter vascular responses in mesenteric arteries from WKY. In conclusion, both α-lactorphin and β-lactorphin improved vascular relaxation in adult SHR *in vitro*. The beneficial effect of α-lactorphin was directed towards endothelial function, whereas β-lactorphin also enhanced endothelium-independent relaxation.

Keywords: α-lactorphin; β-lactorphin; Vascular function; SHR; NO

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Introduction

Milk proteins are precursors of peptides, which possess various biochemical and physiological properties, including opioid activity, immunomodulatory and cardiovascular effects [1]. These biologically active peptide fragments are released from milk proteins in enzymatic hydrolysis either during gastrointestinal digestion or during fermentation of milk [2].

α-lactorphin (Tyr-Gly-Leu-Phe) and β-lactorphin (Tyr-Leu-Leu-Phe) are tetrapeptides, which correspond the amino acid sequences 50–53 of α-lactalbumin and 102–105 of β-lactoglobulin, respectively [3]. They can be released from the milk whey proteins in enzymatic proteolysis by pepsin and trypsin [4]. Structurally these tetrapeptides closely resemble endogenous opioid peptides, since the N-terminal amino acid residues in β-endorphin, enkephalins and dynorphin A are Tyr-Gly-Gly-Phe- [2]. In fact, both tetrapeptides bind to opioid receptors and show weak opioid activity *in vitro* [5]. α-Lactorphin inhibits the contraction of stimulated guinea pig ileum in a naloxone-sensitive manner [4]. *In vivo,* endogenous opioid peptides modulate cardiovascular variables [6–10], but so far the observed effects have been inconsistent, due to variety of experimental models and anaesthetics used. We have recently shown that subcutaneously administered α-lactorphin lowers blood pressure in conscious SHR with established hypertension [11]. The effect was not attributable to α-lactorphin's hydrolysis to its constituent amino acids tyrosine, glycine, leucine or phenylalanine, because the single amino acids failed to influence blood pressure. The depressor response to α-lactorphin was naloxone-sensitive, suggesting that opioid receptors may have been involved in this effect. However, the actual antihypertensive mechanism of the tetrapeptide is still unknown.

In order to evaluate blood pressure lowering mechanisms of α-lactorphin, the effects of this tetrapeptide on mesenteric arterial function were studied in adult SHR and age-matched normotensive WKY *in vitro* in mesenteric arterial preparations. We also investigated the vascular effects of another, structurally related tetrapeptide, β-lactorphin. We especially studied the role of endothelium-derived factors NO, prostanoids, and hyperpolarisation as the mediators of the effects of the tetrapeptides by pretreating the mesenteric arterial rings of SHR with the NOS inhibitor L-NAME, the potassium channel inhibitor TEA or the cyclooxygenase inhibitor diclofenac.

Methods

Experimental protocol

Male SHR and WKY were obtained from Harlan Ltd, IN, U.S.A. The animals were housed in a standard animal laboratory (illuminated from 6 a.m. to 6 p.m., room temperature 22–24 °C), and had free access to tap water and food pellets (R36, Lactamin, Stockholm, Sweden). The protocol of the study was approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland.

At the age of 30–35 weeks, the rats were anaesthetized with CO_2/O_2 (70/30%; AGA, Riihimäki, Finland) and decapitated. The superior mesenteric artery was carefully excised and cleaned of adherent connective tissue. Sections of 3-mm-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) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, glucose

11.1, $CaCl_2 \times 2H_2O$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \times 7H_2O$ 1.2 and aerated with O_2/CO_2 (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, U.S.A.). The presence of intact endothelium was confirmed by observing a relaxation response to 1 μM ACh in rings precontracted with 1 μM noradrenaline (NA). Only endothelium-intact preparations were used.

Mesenteric arterial responses in vitro

The effects of α-lactorphin or β-lactorphin on the cumulative relaxation response to ACh and SNP (from 1 nM to 10 μM) were determined. After a 15 min incubation with either of the tetrapeptides (0.1 mM) or vehicle, the mesenteric artery preparations were precontracted with NA (1 μM) before administration of ACh or SNP. The duration of incubation and concentration of the tetrapeptides were chosen on the basis of a small-scale pilot study. The relaxing compounds were added only after the precontraction or a 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 next response curve.

Cumulative response to ACh were also elicited in the presence of 0.1 mM L-NAME (the inhibitor of NOS), L-NAME in combination with 1 mM TEA (a non-specific inhibitor of potassium channels), and in the presence of 3 μM diclofenac (the inhibitor of cyclooxygenase). L-NAME, TEA and diclofenac, in doses used in this experiment, have been shown to inhibit NOS, potassium channels and cyclooxygenase, respectively [12–14].

Drugs

α-lactorphin (Tyr-Gly-Leu-Phe) and β-lactorphin (Tyr-Leu-Leu-Phe) were synthesized and supplied by the Agrifood Research Finland (Jokioinen, Finland). The solid phase peptide synthesis by the Fmoc method was carried out on a semi-automatic peptide synthetizer (Nova-Syn Gem, Calbiochem-Novabiochem, Switzerland) according to the instructions of the manufacturer. Fmoc amino acid esters and resins (Fmoc-Phe-Novasyn KA 125) were purchased from Novabiochem. Cleavage reaction was carried out for 1.5 h at room temperature in a mixture containing 95% (v/v) trifluoroacetic acid and 5% water. After filtering to remove the resin, the cleavage mixture was evaporated to dryness and extracted with diethylether. The residue was dissolved in 10% acetic acid and lyophilized. Reversed-phase HPLC was used to purify the peptides.

Acetylcholine chloride, diclofenac, N^G-nitro-L-arginine methyl ester hydrochloride, noradrenaline bitartrate, sodium nitroprusside dihydrate and tetraethylammonium tetrahydrate were purchased from Sigma Chemical Co, St. Louis, MO, U.S.A. All solutions were freshly prepared before use and protected from light.

Data presentation and statistical analysis of results

The relaxation responses are presented as a percentage of precontraction level. ANOVA for repeated measurements was applied for data consisting of observations at successive time points. One-way

ANOVA followed by the Tukey's test was used when carrying out pairwise comparisons between the test groups. All results are expressed as mean ± SEM. Differences were considered significant when $P < 0.05$.

Results

Endothelium-dependent relaxation evoked by ACh was significantly depressed in mesenteric resistance arteries of SHR as compared to those of WKY. The maximal ACh relaxation was 32 ± 8 % in SHR and 93 ± 2 % in WKY ($P < 0.001$). In preparations obtained from SHR, the response to ACh was improved by α-lactorphin or β-lactorphin ($P < 0.05$, to 49 ± 8% and 61 ± 8% of precontraction level, respectively, Fig. 1A), whereas in preparations from WKY this effect was not observed (90 ± 6% and 93 ± 2% of precontraction level, Fig. 1B). α-Lactorphin or β-lactorphin did not affect the vascular tone of quiescent mesenteric arterial preparations of SHR or WKY.

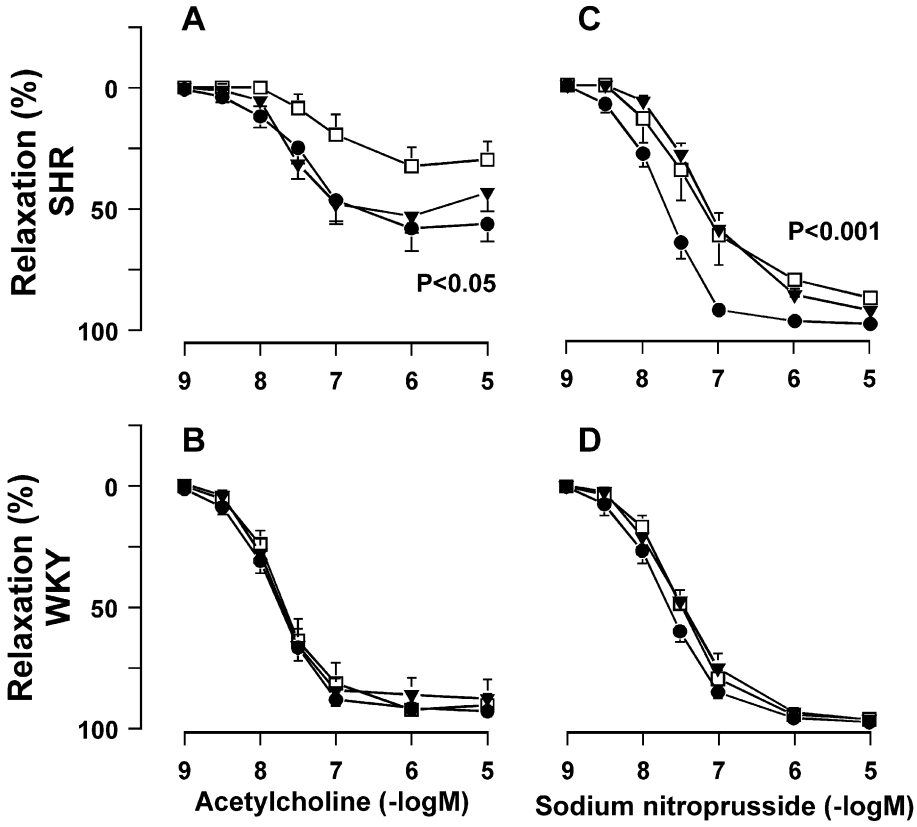

Fig. 1. Effect of α-lactorphin (0.1 mM; ▼), β-Lactorphin (0.1 mM; ●) and vehicle (□) on the ACh and SNP relaxations in NA-precontracted mesenteric arterial preparations of SHR (1A, 1C) and WKY (1B, 1D). Data are mean ± SEM (n = 5–7 in each group). $P < 0.05$, $P < 0.001$ ANOVA for repeated measurements.

Endothelium-independent relaxation to SNP in mesenteric arterial rings did not differ statistically significantly between SHR and WKY (Fig. 1C and 1D). In SHR, the SNP-induced relaxation was improved in the presence of β-lactorphin ($P < 0.001$, Fig. 1C). However, the maximal relaxation response to SNP was not affected by β-lactorphin (Fig. 1C). The endothelium-independent relaxation was not affected by α-lactorphin. In WKY, the tetrapeptides had no effect on the relaxation induced by SNP (Fig. 1D).

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, in the presence of L-NAME combined with the potassium channel inhibitor TEA, and in the presence of the cyclooxygenase inhibitor diclofenac. L-NAME abolished the improvement in the ACh relaxation induced by α-lactorphin or β-lactorphin in mesenteric arterial preparations of SHR (Fig. 2A). The addition of TEA to the organ bath simultaneously with L-NAME did not elicit further dilation (Fig. 2B). Cyclooxygenase inhibition with diclofenac did not reduce the improvement in ACh relaxation induced by α-lactorphin or β-lactorphin (Fig. 2C). However, diclofenac augmented the response to ACh in the arterial preparations of SHR (Fig. 2C). L-NAME, diclofenac or TEA themselves had no direct relaxant or contractile effect on the vascular tone.

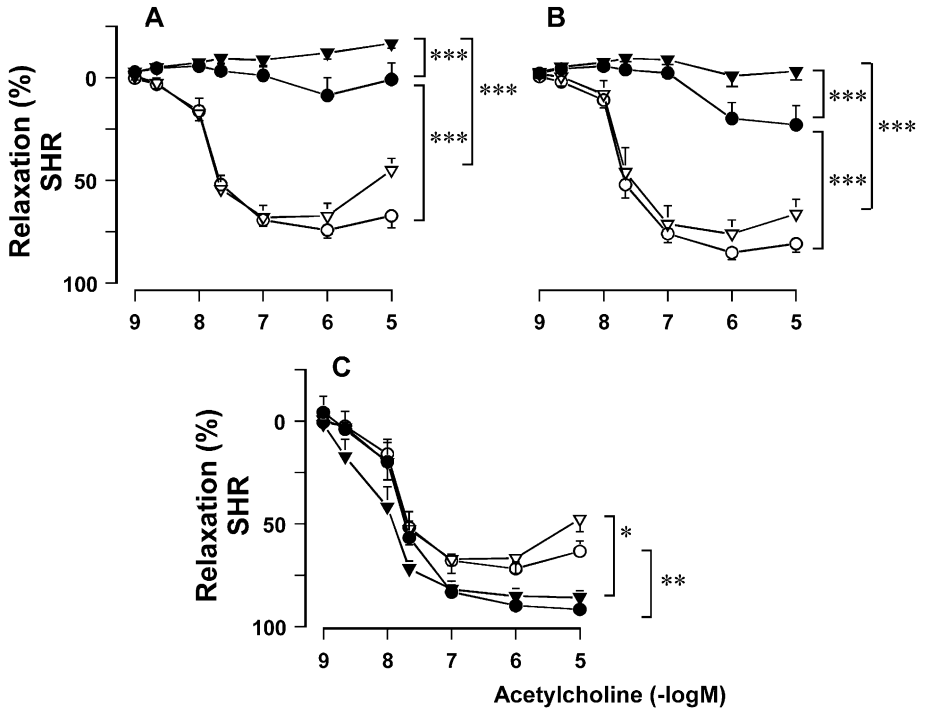

Fig. 2. Relaxations to ACh in NA-precontracted mesenteric arterial preparations of SHR incubated with α-lactorphin (0.1 mM; ∇) or β-lactorphin (0.1 mM; ○). The relaxation responses were studied in the presence of 0.1 mM L-NAME (2A), in the presence of 0.1 mM L-NAME in combination with 1 mM TEA (2B), and in the presence of 3 μM diclofenac (2C) (solid symbols). Data are mean ± SEM (n = 5–7 in each group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ ANOVA for repeated measurements.

Discussion

We investigated the vascular effects of α-lactorphin and β-lactorphin in mesenteric arterial preparations from adult SHR with established hypertension and normotensive age-matched WKY. Especially, we aimed at evaluating the role of endothelium-derived factors (NO, prostanoids, hyperpolarisation) in the effects of these tetrapeptides, since endothelium-dependent vasodilation, as assessed by relaxation response to ACh, is impaired in different types of hypertension [15,16]. In agreement with this finding, ACh-induced relaxation was reduced in mesenteric resistance arteries obtained from adult SHR when compared to age-matched normotensive WKY. α-Lactorphin and β-lactorphin, tetrapeptides originally released from milk proteins, improved the endothelium-dependent relaxation in SHR. This effect was not observed in the arteries of WKY, in which the relaxation to ACh was already maximal.

Vasodilation is reduced in endothelial dysfunction, presumably because the synthesis and/or release of NO is decreased. During the development of hypertension, a decline in the activity and expression of endothelial NOS has been shown in the SHR aorta [17]. In addition, a reduced release of NO from endothelial cells of hypertensive rats has been described [18]. The significance of endothelial NO in the relaxation response to ACh is further confirmed by the diminished relaxation in the presence of NOS inhibition [19]. In the present study, the improved relaxation response to ACh by α-lactorphin or β-lactorphin in SHR was abolished by the NOS inhibitor L-NAME. Thus, it seems likely that the effect of the tetrapeptides was mediated, at least partly, by NO. Whether the augmented relaxation response in the presence of the tetrapeptides was due to increased synthesis or release of NO or decreased degradation of the compound remains to be clarified.

Besides NO, endothelium-dependent hyperpolarization causes vasorelaxation, which is mediated by a putative endothelium-derived hyperpolarizing factor (EDHF) [20]. EDHF availability is also suggested to be impaired in SHR [21,22]. The chemical nature of EDHF is still unknown, but it seems to act as a potassium channel opener [23], and can be inhibited by potassium channel blockers [24]. EDHF-mediated vasodilatation can be observed after inhibition of endothelial NOS [25]. In the present study, the possible role of EDHF in the effect of α-lactorphin or β-lactorphin was investigated by adding the non-specific potassium channel inhibitor TEA to the organ bath simultaneously with L-NAME. No additional effect by TEA was observed, which suggests that EDHF did not play a major role in the vascular effects of α-lactorphin or β-lactorphin in our experimental setting.

Endothelial cyclooxygenase produces several prostanoids, which may also be involved in vascular dysfunction associated with hypertension [26]. The inhibition of cyclooxygenase has been reported to enhance endothelium-mediated vasodilation in SHR [27]. In our study, cyclooxygenase inhibition by diclofenac did not diminish the improved endothelium-dependent relaxation in the presence of α-lactorphin or β-lactorphin, suggesting that vasodilatory prostanoids, such as prostacyclin, were not involved. On the contrary, the ACh relaxation tended to be somewhat more pronounced by diclofenac. Thus, vasoconstrictive prostanoids may have a role in the worsened ACh relaxation in mesenteric arterial rings of SHR as compared to those of WKY. Release of vasoconstrictive cyclooxygenase products, e.g. prostaglandin $F_{2\alpha}$, prostaglandin H_2 or thromboxane A_2, has been proposed to be increased in endothelial dysfunction in SHR [28–30].

The relaxation response to the NO donor SNP was similar in mesenteric arteries of SHR and WKY, indicating that the responsiveness of vascular smooth muscle to NO was not impaired in

SHR. β-Lactorphin moderately enhanced the endothelium-independent relaxation to SNP in SHR arteries. This suggests an enhanced sensitivity of arterial smooth muscle to NO by the tetrapeptide. In previous studies, the relaxation to SNP was enhanced after high-calcium diet in deoxycorticosterone-NaCl hypertension [31] and after high-potassium or high-calcium diet in SHR [32]. The augmented vascular smooth muscle relaxation to exogenous NO was speculated to have reflected an improvement in general vascular relaxation properties, e.g. regulation of intracellular calcium [31]. Our 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 smooth muscle to relax in response to NO-donors, whereas α-lactorphin does not seem to enhance the sensitivity of the smooth muscle to NO. The improvement in the SNP relaxation induced by β-lactorphin but not by α-lactorphin is also suggested to underlie the observation that the ACh-relaxation was slightly less attenuated when L-NAME or L-NAME and TEA were co-administrated in the presence of β-lactorphin than in the presence of α-lactorphin. This difference might be due to vascular smooth muscle-derived relaxing factor, on which β-lactorphin may have influenced more beneficially than α-lactorphin.

We have recently shown that α-lactorphin lowers blood pressure in SHR via naloxone-sensitive mechanism [11], suggesting that opioid receptors are involved in the antihypertensive action of the peptide. Both α-lactorphin and β-lactorphin bind to opioid receptor in radioreceptor assay [5]. It has been proposed that opioid receptor stimulation in the vascular endothelium releases NO [33,34]. Moreover, endogenous opioid peptides, endomorphins, have been shown to decrease hindquarter perfusion pressure in rats in a NO-dependent manner, since the vasodilator responses to endomorphins were attenuated by L-NAME [7,8]. Endogenous opioid methionine enkephalin has also elicited a NO-dependent pial artery dilation, which was blocked by the endothelial NOS antagonist N-iminoethyl-L-ornitine (L-NIO) [9]. Recently it has been observed that nociceptin, another endogenous opioid peptide, dilates systemic vascular bed through a NO-dependent pathway [10]. Thus, the improvement of the endothelium-dependent relaxation induced by α-lactorphin or β-lactorphin could partially be related to opioid receptors in the endothelium.

Previous studies have observed alterations in opioid receptor binding in the brain of hypertensive rats [35]. In addition, difference in the sensitivity to endogenous opioid peptides has been demonstrated between SHR and WKY [36,37]. Thus, it might be possible that the difference between SHR and WKY in the sensitivity to lactorphins may be a consequence of high BP state.

Other vasorelaxant opioid peptides than α-lactorphin or β-lactorphin have also been found in milk. Casein-derived casoxin D (Tyr-Val-Pro-Phe-Pro-Pro-Phe) [38] and casomokinin L (Tyr-Pro-Phe-Pro-Pro-Leu) [39] relaxed canine mesenteric artery strips in endothelium-dependent manner. The relaxation induced by casoxin D was not diminished by a NOS inhibitor but was antagonised by a cyclooxygenase inhibitor [38]. On the contrary, the casomokinin L-induced relaxation was partly blocked by a NOS inhibitor [39]. L-NAME-sensitive vasodilation in mesenteric arterial rings of SHR has also been induced by a peptide (Arg-Ala-Asp-His-Pro-Phe) derived from egg yolk protein ovalbumin [40]. Thus, peptides released from milk proteins or other foods may influence vascular function *in vitro* via several mechanisms.

In conclusion, α-lactorphin or β-lactorphin improved the impaired vascular function in mesenteric arterial rings of adult SHR. In the improved endothelium-dependent relaxation, NO seemed to be involved, whereas vasodilatory prostanoids did not have a role.

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