

LVV-Hemorphin-7 Lowers Blood Pressure in Spontaneously Hypertensive Rats: Radiotelemetry Study

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Summary

Cardiovascular effects of LVV-hemorphin-7, a member of the family of fragments from β -chain of human or bovine hemoglobin, were studied in conscious spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats by radiotelemetry. Intraperitoneal injection of hemorphin in a dose of 100 μ g/kg significantly decreased blood pressure in SHR, whereas negligible effect was seen in normotensive WKY rats. Blood pressure changes were accompanied by reduction of heart rate. In conclusion, a direct effect of LVV-hemorphin-7 on blood pressure was demonstrated in SHR. These biologically active peptides could be involved in blood pressure regulation especially in hypertensive rats, but the precise mechanism should be elucidated.

Key words

Hemorphin • Blood pressure • Heart rate • Hypertension • Rat

Introduction

Hemorphins are small peptides generated *in vitro* by enzymatic hydrolysis of hemoglobin or blood (Brantl *et al.* 1986, Piot *et al.* 1992, Ivanov *et al.* 1997). Their physiological functions are intensively discussed because they were found in a variety of mammalian tissues and fluids (Barkhudaryan *et al.* 1992, Glämsta *et al.* 1991, 1993, Nishimura and Hazato 1993, Moisan *et al.* 1998). Hemorphins are claimed to produce constriction of coronary vessels and platelet aggregation (Barkhudaryan *et al.* 1992), inhibit angiotensin-converting enzyme activity (Lantz *et al.* 1991), etc. The amino acid sequence

of the longest hemorphin, Leu-Val-Val-hemorphin-7 (LVV-H7), corresponds to fragment 32-41 of the β -chain of human or bovine hemoglobin. It can be noticed that all known hemorphins, whatever their source, originated from the same region of β -chain of hemoglobin. All these peptides exhibited affinity for opioid receptors (Glämsta *et al.* 1991, 1993, Nyberg *et al.* 1997).

LVV-hemorphin-7 was shown to bind to a specific receptor for angiotensin IV (3-8 fragment of angiotensin II). The receptor, termed AT₄, has been recently identified as insulin-regulated amino peptidase (insulin-responsive amino peptidase, IRAP, Albiston *et al.* 2001). IRAP is a membrane spanning zinc-dependent

metalloproteinase formerly known as oxytocinase or leucine amino peptidase (Rogi *et al.* 1996). Despite their unrelated structures, LVV-hemorphin-7 and angiotensin IV potently inhibit IRAP (Albiston *et al.* 2001). Similarly, LVV-H7 proved to be an effective inhibitor of angiotensin converting enzyme (Zhao *et al.* 1994, Lantz *et al.* 1991) and so did angiotensin IV (Fruitier-Arnaudin *et al.* 2002). Angiotensin IV and LVV-hemorphin are rather regulators of proteolytic enzymes than of hormone receptors. Moreover, LVV-H7 has a short-term pressor effect partially elicited by the activation of sympathetic nervous system by interacting with specific receptors functionally coupled with phenytoin-sensitive sodium channels (Moisan *et al.* 1998).

Similarly as hemorphins, some milk protein fragments behave as biologically active peptides. A tetrapeptide of lactoglobulin – α -lactorphin – was characterized by its weak interaction with μ opioid receptors (Teschmacher *et al.* 1997) and by ACE inhibiting activity (Antila *et al.* 1991). Recently, α -lactorphin was shown to lower dose-dependently blood pressure (BP) in conscious spontaneously hypertensive rats (SHR) (Nurminen *et al.* 2000). This effect of α -lactorphin in SHR was attributed to an improved vascular relaxation (Sipola *et al.* 2002).

The aim of our study was to find out if LVV-hemorphin-7 had similar effects on BP in adult conscious SHR as α -lactorphin. Hemorphins are present in rather high concentrations in blood plasma (pmols/ml, Nyberg *et al.* 1997), which are about thousand times higher than those of opioid peptides such as endorphins. Moreover, LVV-hemorphin-7 has an extreme stability in blood plasma (Nyberg *et al.* 1997) compared with short half-life of opioids and other BP regulating hormones. It means that hemorphins could act as long-living biologically active peptides, most probably inhibiting proteolytic enzymes such as angiotensin-converting enzyme, etc.

Methods

Animals

Adult male spontaneously hypertensive (SHR, n=5) and Wistar Kyoto (WKY, n=5) rats aged 12-16 weeks, were used in this study. The rats were housed under standard conditions (temperature 23 ± 1 °C, 12-h light-dark cycle), were fed standard laboratory chow and drank tap water *ad libitum*. The procedures and experimental protocol were approved by local ethics committee of the Institute of Physiology AS CR.

Surgical procedure

Animals were anesthetized with ether and telemetric transmitters (Data Sciences Inc., MN, U.S.A.) were implanted into the abdominal cavity and fixed to abdominal wall. The tip of the catheter connected to transmitter was inserted into the abdominal aorta *via* femoral artery. The rats were housed in individual cages and were allowed to recover for at least one week before the experiment.

Experimental protocol

For the registration of systolic (SBP) and diastolic (DBP) blood pressures as well as heart rate, values were collected using a computer-driven data acquisition system (Data Science Inc.). In each animal individual values were sampled for 10 s every 5 min. The cardiovascular parameters were measured for two hours before and four hours after the injection of saline or LVV-hemorphin-7. To test the influence of the injection on blood pressure changes, the animals received one day saline intraperitoneally in the dose of 1 ml/kg. Following day LVV-H7 was injected intraperitoneally in the dose of 100 μ g/kg (66 nmol/kg), which was an optimal dose chosen from the pilot study.

Hemorphin synthesis

LVV-hemorphin-7 synthesis was performed manually using the solid phase methodology (Barany and Merrifield 1980) with Boc/Bzl strategy. Protecting groups: Tos for Arg, Bzl for Thr, 2-Br-Z for Tyr, For for Trp. The first amino acid was attached to PAM resin. Threefold molar excess of protected Boc amino acid was used in the coupling step. At the end of the synthesis, the Boc group from leucine was cleaved off by 55 % TFA in DCM, the resin was washed with DCM, DMF, ether and the peptide-resin was dried. The detachment of the peptide from the resin and the side chain deprotection were carried out in liquid HF-anisole (9:1). Analytical HPLC showed purity >98 % of the main peak after preparative HPLC. FAB MS m/z: 1308,56 [M+H]⁺. Amino acid analysis: Leu 0.95, Val 2.01, Tyr 1.02, Pro 0.98, Trp 0.90, Thr 1.01, Glu 1.05, Arg 0.98, Phe 1.02.

Statistical analysis

The values were expressed as means \pm S.E.M. Differences between SHR and WKY rats were tested by Student's t test. Time-course of changes in blood pressure and heart rate was analyzed using a one-way analysis of variance (ANOVA) from Statistica package (StatSoft, Tulsa, USA). P<0.05 values were considered significant.

Results

There were no significant differences in body weight between SHR and WKY rats, whereas the baseline values of blood pressure (BP) were significantly higher and heart rate significantly lower in SHR in comparison with WKY rats (Table 1).

Table 1. Basal values of body weight (BW), systolic (SBP), diastolic (DBP) blood pressures as well as heart rate (HR) in spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) normotensive rats.

	WKY (n=5)	SHR (n=5)
BW (g)	299±11	294±6
SBP (mm Hg)	110±1	159±2**
DBP (mm Hg)	81±1	117±1**
HR (beats/min)	296±5	271±4**

Results are expressed as means ± S.E.M., ** p<0.001 when compared to Wistar-Kyoto control rats.

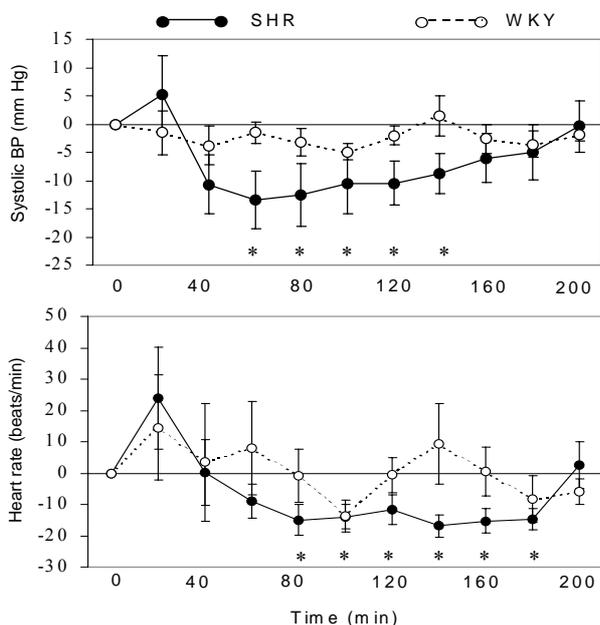


Fig 1. Time course of systolic blood pressure changes (upper panel) and heart rate changes (lower panel) after intraperitoneal injection of LVV-hemorphin-7 (100 µg/kg) in conscious SHR and WKY rats. Results are expressed as means ± S.E.M., * p<0.05 significantly different from basal values.

There were two phases in the time course of blood pressure response of SHR to 100 µg/kg of LVV-hemorphin-7 (Fig. 1, upper panel). After a short-lasting

small increase during the first 20 min there was a long-lasting BP decrease with maximum at 60-80 min. These BP changes were followed by the changes of heart rate the pattern of which was very similar to BP changes (Fig. 1, lower panel). The changes of blood pressure and heart rate in WKY were negligible.

Discussion

The results of our study indicated that LVV-hemorphin-7, a member of a family of hemoglobin fragments, decreased significantly blood pressure (BP) in conscious SHR measured by radiotelemetry. This is in a good agreement with the idea about the biological activity of these peptides. To our knowledge this is the first demonstration of long-lasting depressor activities of LVV-H7. Moisan *et al.* (1998) have demonstrated tachycardia and pressor effect of LVV-H7 after intravenous injection to anesthetized, vagotomized rats. However, both pressor effect and tachycardia elicited by LVV-H7 injection were short-lasting with half-time for recovery of 31±3 s and 73±15 s, respectively. Time-dependent BP changes in our SHR experiment had two phases: the increase of BP at the beginning and long-lasting BP decrease in the second phase. The initial BP increase in our study could be related to the pressor reaction seen by Moisan *et al.* (1998). Moreover, we can exclude that the initial BP increase would be the reaction on intraperitoneal injection because the administration of saline in the same volume did not have any effect in both SHR and WKY rats (data not shown). The discrepancy in the time of pressor effect of LVV-H7 between our and Moisan's study could be explained by different conditions and techniques of blood pressure monitoring.

The dose of hemorphin, which we have used in our study, was chosen according to our pilot study and its comparison with the study of Nurminen *et al.* (2000) who observed maximum BP reduction in SHR after 100 µg/kg of lactorphin. We were not able to make dose-response curves in all animals because of limited amount of hemorphin. Nevertheless, our dose was only the half of the hemorphin amount which produced maximal pressor effect in the study of Moisan *et al.* (1998).

All the experimental data lead to the conclusion that proteolytically degraded hemoglobin gives rise to biologically active peptides probably involved *in vivo* during pain, physical effort, inflammation and blood pressure regulation. The precise mechanisms by which the hemorphins could regulate blood pressure should be

further elucidated. The inhibition of angiotensin converting enzyme (ACE) could be one of the mechanisms. It has been shown that inhibitors of ACE (e.g. captopril, enalapril, etc.) have a proline residue in C-terminal of their amino acid sequence which is similar to that in hemorphins. Lantz *et al.* (1991) have demonstrated inhibitory effect of two synthesized hemorphins on ACE activity in lung extract. The most potent hemorphin appeared to be the LVV-hemorphin-6, which inhibited the ACE activity by 86 % compared to 98 % for bradykinin.

Recently we have published results covering immune modulation therapy based on the *in vitro* treatment of blood with ozone, ultraviolet light and elevated temperature (Tremblay *et al.* 2002). The treated blood was subsequently administered to the animals by intramuscular injection, which resulted in the considerable prevention of the kidney tissue destruction

and the reduction of animal death caused by renal ischemia. In the light of our present results it would be interesting to test if hemorphins are produced during *in vitro* blood treatment and if intramuscular injection of synthetic hemorphins could stimulate immune response and prevent the end-organ damage.

In conclusion, the long-lasting blood pressure decrease was seen in spontaneously hypertensive rats after a single intraperitoneal injection of LVV-hemorphin-7. The attenuation of renin-angiotensin system through the inhibition of ACE might be one of the cardiovascular effects of these opioid-active peptides.

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