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Running head: Decreased hemorphin levels in obesity

M. Maraninchi*, D. Feron*, I. Fruitier-Arnaudin*, A. Bégu-Le Corroller¹, J.P. Nogueira¹, J. Mancini³, R. Valéro¹, J. M. Piot² and B. Vialettes¹.

1- APHM, La Timone Hospital, Department of Nutrition, Metabolic Diseases and Endocrinology, Marseille, France; Aix-Marseille Univ, UMR 1062 INSERM / 1260 INRA (NORT), Marseille, France.
2- University of la Rochelle, UMR 7266 CNRS-URL (LIENSS), AMES team, Pole of Science and Technology, La Rochelle, France.
3- APHM, La Timone Hospital, Biostatistics Research Unit, Marseille, France; Aix-Marseille Univ, UMR 912 (SESSTIM), Marseille, France.

* These authors contributed equally to the work.

Correspondence to:
Pr. Bernard Vialettes, Service de Nutrition, Maladies métaboliques et Endocrinologie, Hôpital La Timone, 264 Rue Saint Pierre, 13005, Marseille, France; Phone number: (33)491387572; Fax number: (33)491386599; E-mail: bernard.vialettes@ap-hm.fr.

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Abstract
Hemorphin peptides exhibit biological activities that interfere with the endorphin system, the inflammatory response, and blood-pressure control. VV-hemorphin-7 and LVV-hemorphin-7 peptides exert a hypotensive effect, in particular, by inhibiting the renin-angiotensin system (RAS). Furthermore, levels of circulating hemorphin-7 peptides have been found to be decreased in diseases such as type 1 and type 2 diabetes.

Because type 2 diabetes and obesity share common features, such as insulin-resistance, microinflammation, high glomerular-filtration rate (GFR), and cardiovascular risk, we evaluated serum VV-hemorphin-7 like immunoreactivity (VVH7-i.r.) levels, using an ELISA method, on a group of 54 obese subjects without diabetes or hypertension, compared to a group of 33 healthy normal-weight subjects.

Circulating VVH7-i.r. levels were significantly decreased in the obese group compared to the control group (1.98 ± 0.19 vs. 4.86 ± 0.54 µM, respectively, P < 0.01), and a significant negative correlation between VVH7-i.r. and diastolic blood pressure (DBP) was found in obese patients (r = -0.35, P = 0.011). There was no significant correlation between VVH7-i.r. level and insulin-resistance, metabolic syndrome, or GFR.

The decreased serum hemorphin-7 found in obese subjects, as in diabetes, may contribute to the development of hypertension and to the cardiovascular risk associated with these metabolic diseases.
Introduction

Hemorphins are small peptides generated by enzymatic cleavage from β-globin chains during catabolism of human hemoglobin. These peptides exhibit biological activities that interfere with the endorphin system, the inflammatory response, blood-pressure control, and cognitive function (1).

VV-hemorphin-7 and LVV-hemorphin-7 globin fragments peptides have been described to exert a hypotensive effect by specifically inhibiting the angiotensin-converting enzyme (ACE) (2, 3) and mimicking angiotensin IV cellular actions (4). We have previously shown decreased serum VVH7-i.r. levels in different type 1 and type 2 diabetic populations (5). Furthermore, a negative correlation between serum VVH7-i.r. level and diastolic blood pressure (DBP) has been found in non-albuminuric type 1 diabetic patients, suggesting that decreased VVH7-i.r. may influence vascular and renal complications in diabetes (5).

Unfortunately, the mechanisms involved in the reduction of VVH7-i.r. concentration in diabetes remain unexplained. Some hypotheses, such as interference of the glycation phenomenon at the cleavage site of the hemoglobin β chain (6), or dysfunction of enzymes involved in hemorphin production (cathepsin D) or catabolism (dipeptidyl peptidase IV [DPP-IV] and ACE) have been ruled out (5).

Because obesity and type 2 diabetes share insulin-resistance, high glomerular-filtration rate (GFR), microinflammation, and high cardiovascular risk, we investigated whether obesity could also exhibit such hemorphin abnormalities.
Subjects and methods

Subjects

The obese group was composed of 54 adult obese subjects (body-mass index [BMI] ≥30 kg/m²) attending our outpatient clinic Nutrition department. Obese subjects were selected with an absence of diabetes (diagnostic criteria for type 2 diabetes as defined by the American Diabetes Association) and were not being treated for hypertension. The control group was composed of 33 healthy adult normal-weight subjects (18.5 ≤ BMI <25 kg/m²) issued from the local Blood Donor Center Bank. Subjects with chronic hemolysis, attested by low haptoglobin levels, were excluded.

Blood samples were collected after an overnight fast. Sera were immediately separated by centrifugation (3500 rpm, 15 min, 4°C) and stored at -80°C. Systolic and diastolic blood pressure (SBP and DBP) were measured in the sitting position after 15 min of rest using a Dynamap monitor (Dinamap pro 1000, GE Healthcare, Freiburg, Germany). Mean blood pressure (MBP) was calculated as the result of the following equation: DBP + 1/3 (SBP-DBP).

Insulin-resistance was estimated using the quantitative insulin-sensitivity check index (QUICKI) (7). Metabolic syndrome was defined by the International Diabetes Federation (IDF) criteria. GFR was estimated using the Modification of Diet in Renal Disease (MDRD) equation (8).

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Marseille. Written informed consent was obtained from all patients.

VVH7-i.r. serum level measurement by competitive antigen ELISA
Serum VVH7-i.r. was measured by an ELISA method as previously described (5). A 96-well ELISA plate was coated overnight at 4°C with the antigen VVH7 (Altergen, Strasbourg, France) at a final concentration of $2.6 \times 10^{-9}$M. Then, the wells were blocked by a solution of PBS-BSA 1% for 2 h. Rabbit polyclonal antibodies, raised against the C-part of VVH7 (diluted 1:10 000), and anti-rabbit IgG peroxidase conjugate secondary antibody (diluted 1:20 000) were used sequentially. Thereafter, tetramethyl-benzidine liquid substrate was added and the reaction was stopped with $\text{H}_2\text{SO}_4$ 0.5 M. The absorbance was read at 450 nm.

In order to determine serum VVH7-i.r. concentrations, a standard range of synthetic VVH7 was made, which included concentrations from $10^{-13}$ to $10^{-5}$M. The ratio between the absorbance at 450 nm in the presence ($B$) and absence ($B_{max}$) of VVH7, against a logarithmic plot concentration of VVH7, displayed a typical calibration graph. The IC50 (i.e. the concentration of peptides able to inhibit 50% of coated antigen-antibody binding) was $6.87 \times 10^{-7}$M.

**Laboratory analyses**

Plasma glucose was assayed using the hexokinase oxidase method (Beckman Coulter, Galway, Ireland) and plasma insulin levels were assessed using an electrochemiluminescence method (Roche Diagnostic, Mannheim, Germany). Total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, and triglycerides were determined using enzymatic methods (CHOD-PAP, HDL-c plus, and GPOPAP, respectively, Roche, Grenoble, France). Plasma creatinine was assessed by the Jaffé method (Beckman Coulter, Galway, Ireland). Haptoglobin and lactic-dehydrogenase activity were measured by the nephelometric method and the ultraviolet (UV) test, respectively (Beckman Coulter, Galway, Ireland).
Statistical analyses

Results were expressed as means ± SEM. Non-parametric statistical tests were used (Mann-Whitney test and Spearman’s rank correlation test). A $P$ value $<0.05$ was considered significant for all the analyses.

Results

The main characteristics of the obese population are described in Supplementary Table 1.

Serum VVH7-i.r. was significantly decreased in the obese group compared to the control group (1.98 ± 0.19 vs. 4.86 ± 0.54 µM, respectively, $P < 0.01$) (Figure 1A). Age (35.85 ± 1.60 vs. 45.3 ± 2.07 years, respectively) and gender (44 women and 10 men vs. 18 women and 15 men, respectively) did not influence VVH7-i.r. levels in either group.

In the obese group, we found a significant negative correlation between VVH7-i.r. level and DBP ($r = -0.35$, $P = 0.011$) (Figure 1B) but not with SBP ($P = 0.29$) or MBP ($P = 0.07$). We found no significant correlation between VVH7-i.r. levels and BMI ($P = 0.10$) or adipose tissue distribution (waist circumference [W], $P = 0.34$; hip circumference [H], $P = 0.06$; W/H ratio, $P = 0.89$; or waist-to-height ratio, $P = 0.28$). VVH7-i.r. levels showed no significant difference between the obese subgroup that exhibited metabolic syndrome (40% prevalence of the total obese population) and the obese subgroup without this phenotype (1.83 ± 0.32 vs. 2.03 ± 0.26 µM, respectively, $P = 0.43$). Insulin sensitivity, assessed by fasting insulin levels ($P = 0.45$) and QUICKI ($P = 0.33$), GFR ($P = 0.28$) or circulating lipid parameters, such as triglycerides ($P = 0.45$), total cholesterol ($P = 0.52$), HDL- ($P = 0.10$), and LDL-cholesterol ($P = 0.73$), were not related to VVH7-i.r. serum concentrations in our obese population.
Discussion

In the present study, we have shown, for the first time, a significant decrease of serum hemorphin-7 in obese subjects compared to healthy control subjects, which is similar to the reduction already described in subjects with type 1 and 2 diabetes. Only a few data are available on hemorphin-7 peptides in human pathological situations. Levels of circulating hemorphin-7 peptides have also been found to be decreased in breast cancer (9), but increased in patients with an abdominal aortic aneurysm (10). Our study shows a significant negative correlation between VVH7-i.r. and DBP in the obese population, which is similar to that described previously in non-albuminuric type 1 diabetic patients (5), and reinforces the hypothesis that an abnormal decrease in VVH7-i.r. indicates susceptibility to the development of hypertension and cardiovascular disease.

Hemorphins are known to interfere with the renin-angiotensin system (RAS). In vitro, VV- and LVV-hemorphin-7 peptides exert competitive inhibition on ACE activity (3). Hemorphins are also a ligand of the insulin-regulated amino peptidase (IRAP)/angiotensin-4 receptor (AT4R) and reproduce angiotensin IV cellular actions that induce vasodilatation (2, 4). Moreover, LVV-hemorphin-7 injections in hypertensive rats reduce blood pressure and heart rate (11). These converging observations suggest that hemorphin-7 peptides could represent natural hypotensive substances. One could postulate that the decrease of circulating VVH7-i.r. in obese and diabetic patients contributes to increased DBP by interfering with the RAS.

The mechanisms of hypertension in obesity are complex. Sympathetic nervous activation, impaired pressure-regulated natriuresis, hormonal and adipocytokine anomalies, and endothelial dysfunction have been implicated. Activation of RAS seems to play a central role. RAS is
activated in obesity despite plasma volume expansion and sodium retention. In addition to renal overproduction of renin, adipose tissue produces angiotensinogen, which could also increase blood pressure.

The decrease of hemorphin-7 serum levels in obese and diabetic patients is a new mechanism and may be involved in some of the pathways leading to hypertension. The hypothesis that insulin-resistance is shared by both diseases and could be the common denominator has failed. Whatever the marker is for insulin-resistance, it is not related to the VVH7-i.r. values. Another hypothesis is that high GFR, observed in both diseases (12), could produce renal escape of hemorphins into the urine, thus leading to its decrease in serum. Again, the absence of a correlation between VVH7-i.r. level and creatinine clearance has eliminated this hypothesis. Low-grade inflammation status, which occurs in both diabetes and obesity, could represent another hypothesis that remains to be explored, but requires a larger population study. The association of reduced hemorphin status with very disparate diseases such as diabetes, obesity and breast cancer, could suggest that mechanisms shared by these diseases as endothelial dysfunction and angiogenesis could lead to the hemorphin anomaly. Indeed, Perrot-Applanat et al. has shown that the NF-κB gene signature is highly similar between TNFα stimulated endothelial cells and breast tumor biopsies (13). It is possible that TNFα activation is the common denominator governing these processes and the decrease of hemorphin in these apparently unrelated diseases. We have also hypothesized in the past that hemorphin decrease in blood could be related to lysosomial and/or extralysosomal protease hyperactivities. Indeed, such anomalies of either cathepsin S or 20S proteasome have been described in obesity (14) and breast cancer (15) respectively.
In conclusion, decreased serum hemorphin-7 is a common feature in metabolic diseases such as diabetes and obesity, and could contribute to the risk of future development of hypertension and, potentially, of cardiovascular and renal complications.

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Disclosure
The authors declare no conflict of interest.

Supplementary Material is available at www.nature.com/obesity.
References


Figure legends

Figure 1: Decrease of circulating VV-hemorphin-7 like immunoreactivity (VVH7-i.r.) levels and negative correlation between VVH7-i.r. and diastolic blood pressure (DBP) in obese subjects.

(A) Distribution of VVH7-i.r. in the serum of control and obese subjects, using a box-plot representation. The median is indicated by a thick black line inside the box. The top and bottom of the box are the upper and lower quartiles, and indicate the inter-quartile range (IQR). The whiskers indicate the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile. Outliers (between 1.5 and 3 times the IQR) are marked with a circle and extreme values (more than 3 times the IQR) with an asterisk.

(B) Correlation between VVH7-i.r. serum levels and DBP in the obese group. Spearman correlation coefficient was determined to assess the significant association between parameters (r = -0.351, P = 0.011).
Figure 1

A: Box plot showing VHH7-Ir. levels in control and obese subjects. The p-value is less than 0.001.

B: Scatter plot showing diastolic blood pressure in relation to VHH7-Ir. concentration.