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Relationship of angiotensinase and vasopressinase enzymatic activities between hypothalamus and plasma in aged rats by high-fat diet

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Graphical Abstract
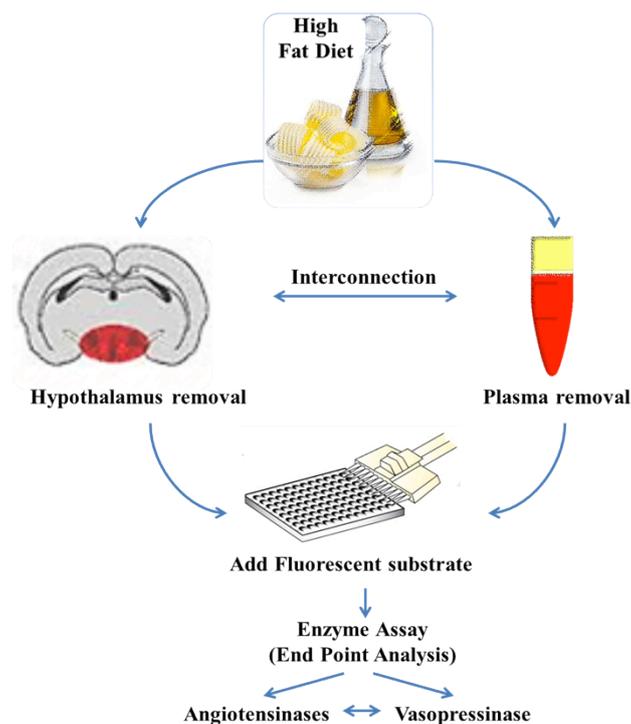
Abstract: High-fat diets are associated with the development of hypertension. However, a high intake of monounsaturated fat has been proposed to be a dietary factor that can decrease the incidence of hypertension. The renin-angiotensin system (RAS) and vasopressin interact to regulate blood pressure at central and peripheral level. In this study, we investigated the effect of different degrees of dietary fatty acid saturation in the control of RAS and vasopressin on brain-blood. To improve our understanding of their interaction and their relationship, we analyzed angiotensin- and vasopressin-metabolizing activities in hypothalamus and plasma, collected from old Wistar rats fed for 24 weeks with diets enriched with extra virgin olive oil (monounsaturated fat) or butter plus cholesterol (saturated fat) compared with a standard diet. We found no angiotensinase and vasopressinase activities in hypothalamus and plasma, with no significant correlations between enzymatic activities in either region. Therefore our results do not support the beneficial influence of extra virgin olive oil to regulate blood pressure on the central or systemic levels. Furthermore, substrates downstream of vasopressin and the RAS may be similarly unaffected, and given the previously described potential of the Mediterranean diet as a tool in the treatment of hypertension, further studies need to be done in order to clarify the therapeutic mechanism.

Keywords: angiotensinases, vasopressinase, renin-angiotensin system, brain-blood connection, dietary fat

Introduction

Dietary fat intake determines the fatty acid composition of cell membranes and plays a well-recognized role in cardiovascular risk and the development of cardiometabolic disease[1]. Moreover, diet composition may also influence brain activity as the dietary fat modifies the brain membrane fluidity[2,3]. A high intake of monounsaturated fat has been proposed to decrease the incidence of hypertension[4-6]. In addition, increasing saturation of dietary fat resulted in increasing plasma total cholesterol concentration[7] and systolic and diastolic blood pressures[8]. The systemic and the local renin-angiotensin system (RAS), and vasopressin in brain[9], interact together to regulate blood pressure (BP) by various endocrine and autonomic mechanisms[10]. The hypothalamic stimulatory effect of angiotensin (Ang) III (Ang III) on vasopressin release has been clearly demonstrated[11]. The hypothalamus is known to integrate behavioral, endocrine, neuroendocrine, and autonomic responses (including those that regulate cardiovascular function) to maintain homeostasis[12]. We hypothesized that the direct and/or indirect relationship between the hypothalamus and plasma, both of which regulate BP, may be mediated to some extent by angiotensinase and vasopressinase enzymatic activities and the metabolism of their corresponding peptidergic substrates.

In order to analyze the link between RAS and vasopressin in the control of BP, as well as the relationship between the hypothalamus and plasma, we have studied the metabolism of (A) Ang I to Ang 2-10, (B) Ang II to Ang III, (C) Ang III to Ang IV and (D) the metabolism of vasopressin by measuring the activities of (A) aspartyl aminopeptidase (AspAP), (B) glutamyl aminopeptidase (GluAP), (C) alanyl aminopeptidase (AlaAP), arginyl aminopeptidase (ArgAP), and (D) cystinyl aminopeptidase (CysAP), respectively[13,14]. Additionally, we observed the activities of angiotensinase and vasopressinase in plasma and their soluble and membrane-bound fractions from the anterior hypothalamus. In our model, aged male Wistar rats were fed with high-fat diets (HFD) supplemented with different degrees of dietary fatty acid saturation, such as olive oil rich in monounsaturated fatty acids (MUFAs), or butter plus cholesterol rich in saturated fatty acids (SAFAs). Enzymatic activities were determined by fluorimetry using arylamide derivatives as substrates, to determine the effect of dietary fat on RAS, vasopressin, and ultimately blood pressure regulation.

Experimental Methods

Animals and treatments

17-week-old male Wistar rats were purchased from Harlan Ibérica (Barcelona, Spain). The animals were allowed free access to food and water during 24 weeks and were maintained on a 12 hours light/dark cycle in a controlled temperature (20-25ºC) and humidity (50 ± 5%) environment.

Mean body weight was ∼495 g at beginning of the study. Experimental procedures for animal use and care were in accordance with European Communities Council Directive 2010/63/UE and Spanish regulation RD 53/2013. Rats were randomly assigned into three groups (5-6 animals per group) as follows. In standard diet (S) group, rats were fed with a commercial diet for experimental control. In HFD groups, one group of mice (VOO) was fed with a diet supplemented with 20% monounsaturated fat (virgin olive oil, VOO), and the last group (Bch) was fed a diet supplemented with 20% saturated fat (butter) plus 0.1% cholesterol . The HFD groups were isocaloric. Food composition in different groups and nutritive value are shown in supplemental tables (**Appendix 1 and 2**). Twenty four weeks after the feeding period, a sample of blood was collected for peptidase activities assay, then the animals were sacrificed under equithensin (2ml/kg body weight) and perfused with saline solution through the left cardiac ventricle. The blood was centrifuged for 10 min at 2,000 g to obtain the plasma, which was stored at -20ºC. Hypothalamus tissue was also collected and dissected as previously described[10,15]. Briefly, the brain was quickly removed (less than 60 s) and cooled in dry ice. The hypothalamus (pooled left and right) was dissected according to the stereotaxic Paxinos and Watson atlas[16]. The selected area was between 7.7 mm and 3.7 mm anterior to the interaural line.

Sample preparation for enzyme activity assay

Samples from plasma were directly used for the peptidase activity assay. Samples from the hypothalamus were quickly removed and frozen in dry ice. To obtain the soluble fraction, tissue samples were homogenized in ten volumes of hypoosmolar medium (10 mM HCl-Tris buffer, pH 7.4) and ultracentrifugated (100,000 g for 30 min at 4ºC). The resulting supernatants were used to measure soluble (sol) angiotensinase and vasopressinase activities and protein content, assayed in triplicate. To solubilize membrane proteins, pellets were rehomogenized in HCl-Tris buffer (pH 7.4) plus Triton X-100 (1%). After centrifugation (100,000 g for 30 min at 4ºC), supernatants were used to measure membrane-bound (mb) activity and proteins, also in triplicate. To ensure the complete recovery of activity, detergent was removed from the medium by adding to the samples adsorbent polymeric Biobeads SM-2 (100 mg/ml) purchased from Bio-Rad (Richmond, VA, USA) and shaking for 2 h at 4ºC[17].

Peptidase activities assay

Sol and mb angiotensinase and vasopressinase activities were determined fluorometrically using the arylamide derivatives aspartyl-, glutamyl-, alanyl-, arginyl-, and cystinyl-β-naphthylamide (-β-NNap) as substrates according to the method of Ramírez[18]. Briefly, for AlaAP and ArgAP assay, 10 µL of each supernatant were incubated for 30 min at 37ºC with 100 µL of substrate solution (100 µM Alanyl- or Arginyl-β-NNap), 1.5 mM albumin seric bovine (BSA), and 0.65 mM dithiothreitol (DTT) in 50 mM phosphate buffer pH

7.4). For AspAP assay, 10 µL of each supernatant were incubated for 30 min at 37ºC with 100 µL of substrate solution (100 µM Aspartyl-β-NNap), 1.5 mM albumin seric bovine (BSA), and $3 \cdot 10^{-8}$ mM $CaCl_2$ in 50 mM Tris-HCl buffer pH 7.4). For GluAP assay, 10 µL of each supernatant were incubated for 30 min at 37ºC with 100 µL of substrate solution (100 µM α-Glutamyl-β-NNap), 1.5 mM albumin seric bovine (BSA), 0.65 mM DTT, and 50 mM $MnCl_2$ in 50 mM Tris-HCl buffer pH 7.4). Finally, for CysAP assay, 10 µL of each supernatant were incubated for 30 min at 37ºC with 100 µL of substrate solution (100 µM Cystinyl-β-NNap), 1.5 mM albumin seric bovine (BSA) and 0.65 mM DTT in 50 mM Tris-HCl buffer pH 6.0). All the reactions were stopped by adding 100 µl of 0.1 M acetate buffer (pH 4.2). The amount of β-NNap released as a result of enzymatic activity was measured fluorometrically at 412 nm emission wavelength with 345 nm excitation wavelength. Activities of specific peptidases were expressed as pmol of β-NNap hydrolyzed per minute and per milligram of protein. Fluorogenic assays were linear with respect to time of hydrolysis and protein concentration. Protein concentration was determined colorimetrically according to Bradford method[19] with BSA as standard. All chemical products were supplied by Sigma (St. Louis, MO USA).

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Tukey post-hoc test for multiple comparisons. Relations between variables were determined by Pearson's coefficients of correlation analysis. Statistical significance was evaluated with Sigmaplot v.11 software (Systat Software, Inc., San Jose, CA, USA). A P-value below 0.05 was considered to be statistically significant. All results were expressed as mean ± standard error.

Results

Results are represented in Figure 1-6. In spite of the importance of MUFAs to maintain normal total body weight **(Figure 1A)**, plasma triglycerides (data not shown) and cholesterol levels **(Figure 1B)** and, fundamentally, BP **(Figure 2)**, there were no significant differences in systemic angiotensinase (AlaAP, ArgAP, AspAP, GluAP) and vasopressinase (CysAP) activities in plasma **(Figure 3 and 4)**. Likewise, there were no significant differences in either soluble nor membrane bound angiotensinase **(Figure 5)** and vasopressinase **(Figure 6)** activities in hypothalamus.

Therefore, the type of fat added to the diet – monounsaturated or saturated – did not seem relevant in the metabolic control of central and systemic RAS and vasopressin. Besides, significant correlations were not observed between fractions of angiotensinase and vasopressinase activities from hypothalamus with their homologs in plasma.

Additionally, the hydrolysis of amino acid naphthylamides by aminopeptidases from hypothalamus and plasma also did not show significant correlation with plasma cholesterol levels.
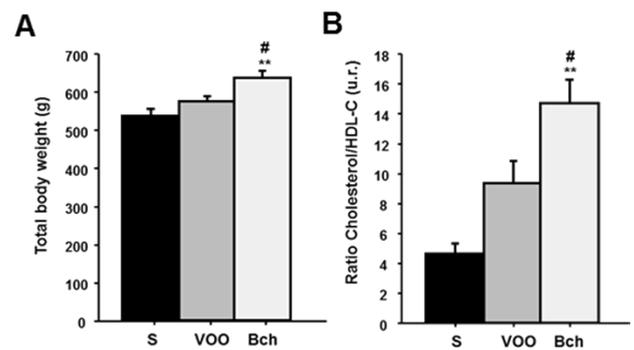

Figure 1. A. Total body weight after feeding period. **B.** Blood cholesterol ratio between total cholesterol and high density lipoprotein-cholesterol plasma fraction (HDL-C) after feeding period. A higher ratio means a higher risk of heart disease. ** ($p < 0.001$) versus standard diet (S), # ($p < 0.05$) versus virgin olive oil diet (VOO).
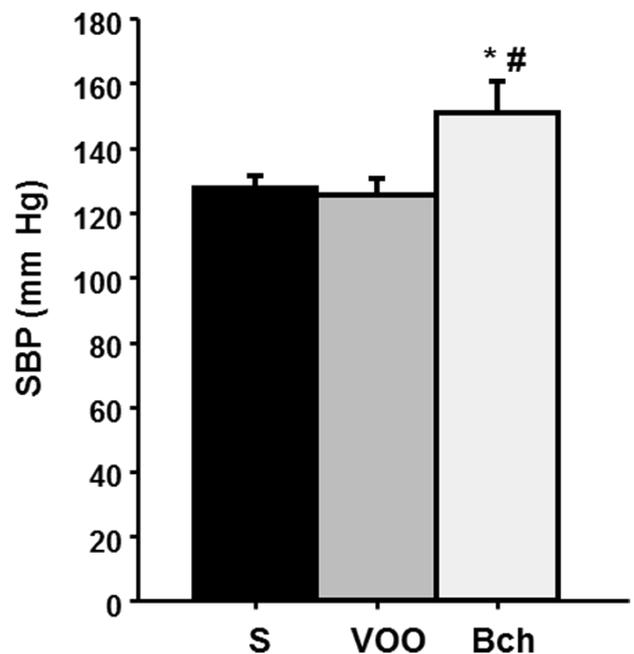

Figure 2. Systolic blood pressure (SBP) after feeding period. * ($p < 0.05$) versus standard diet (S), # ($p < 0.05$) versus virgin olive oil diet (VOO).
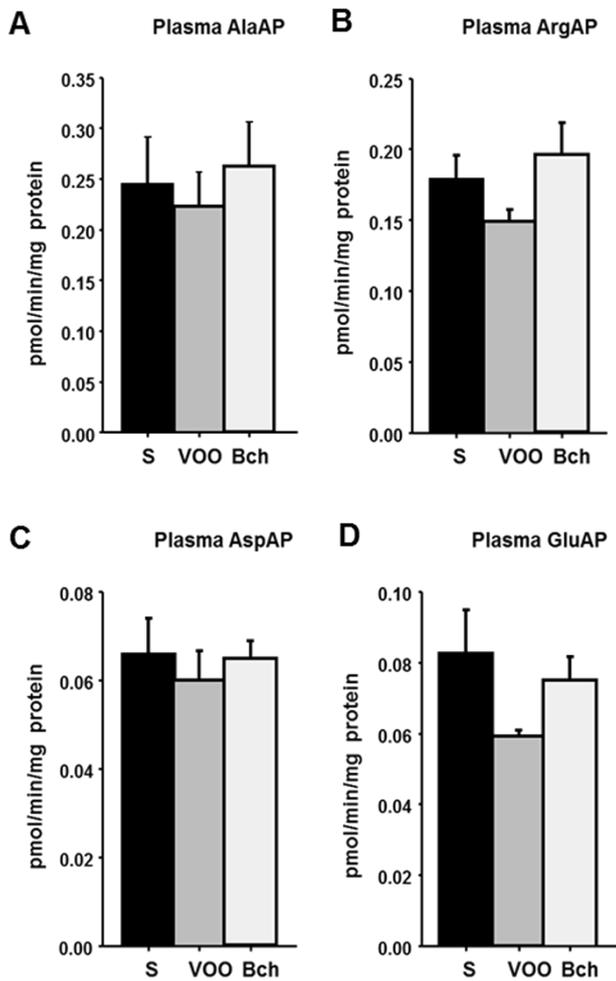
Figure 3. Systemic angiotensinase activities. No significant differences were found in plasma (**A**) AlaAP, (**B**) ArgAP, (**C**) AspAP, and (**D**) GluAP between dietary groups after the feeding period.
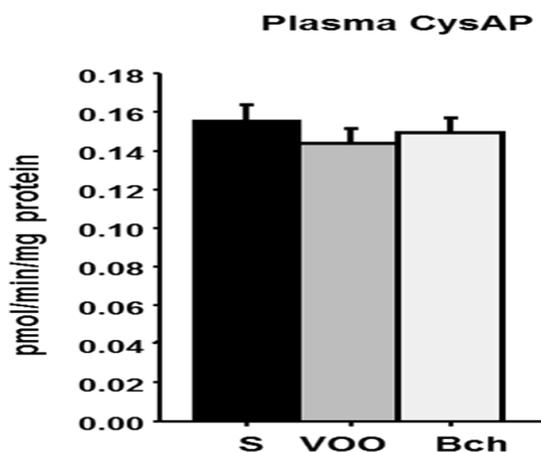

Figure 4. Systemic vasopressinase activity. No significant differences were found in plasma CysAP between dietary groups after the feeding period.

Discussion

In this study we analyzed the potential role dietary fatty acid saturation in the control of RAS and vasopressin on brain-blood connection. After 24 weeks of treatment with diets supplemented with monounsaturated fat (olive oil, rich in MUFAs) or saturated fat (butter plus cholesterol, rich in SAFAs), our results did not show diet-specific differences in angiotensinase and vasopressinase activities in either plasma or the hypothalamus. The two evaluated diets did not significantly change aminopeptidase (AP) activity as compared to the standard diet, and likewise there were no significant correlations between metabolic activities in the brain and blood, suggesting that the fatty acid composition of the diet might not have influence on the function of the hypothalamus-plasma connection.

Previous studies have also determined no changes in global AP/neuropeptidase (e.g.: AspAP and GluAP) activity in frontal cortex[3,20] in adult male rats whose diets were supplemented with fatty acids with varying degrees of saturation, such as fish oil (rich in polyunsaturated fatty acids, PUFAs), olive oil (rich on monounsaturated fatty acids, MUFAs), and coconut oil (rich in saturated fatty acids, SAFAs). The authors proposed that the types of lipids in the diet affect the fluidity of the membrane, with increase or loss of membrane-associated enzymes[20]. They observed that the diet composition affects fatty acid distribution in the brain. The change in fluidity may also affect the tertiary structure of the enzyme embedded in the membrane. This may affect the binding of the enzyme with its substrate[2,20], which may explain the positive or negative correlation between fatty acid content and membrane-bound and soluble fraction of the angiontensin- and vasopressin-degrading enzymes observed in the previous work; however it was not possible to clarify it in this current work.
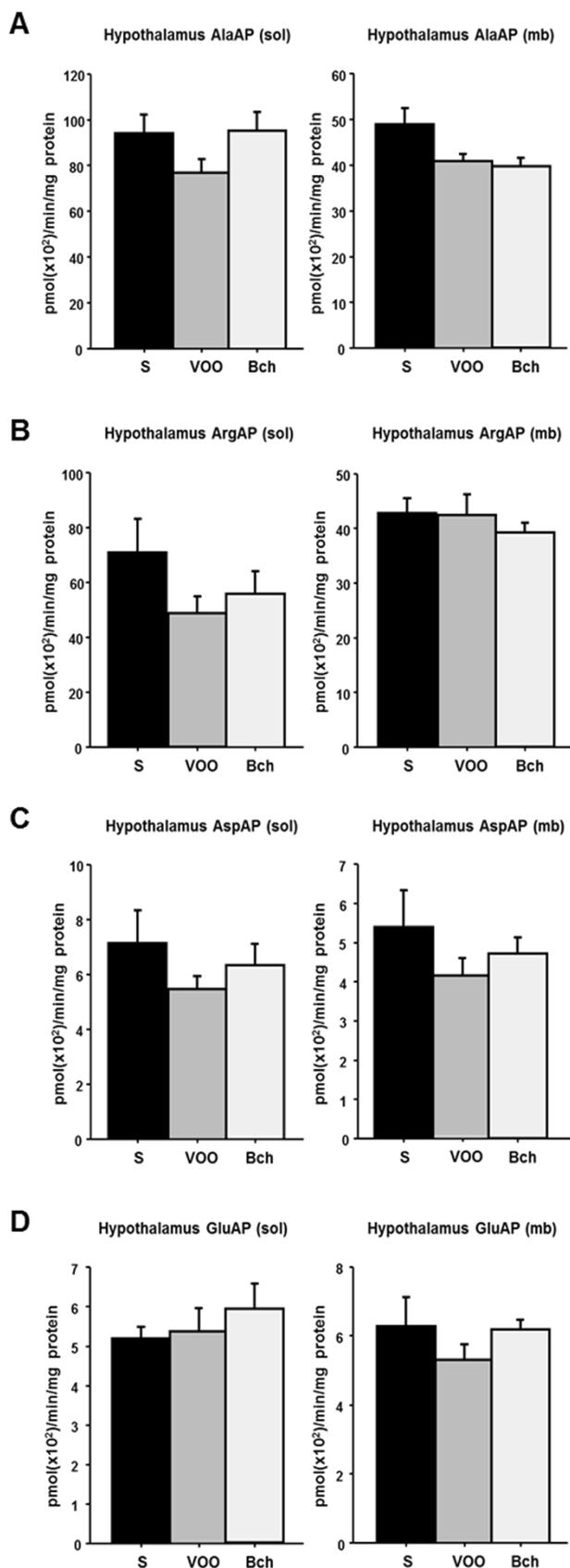
Figure 5. Soluble (sol) and membrane-bound (mb) fractions of central angiotensinase activities. No significant differences were found in hypothalamus (**A**) AlaAP, (**B**) ArgAP, (**C**) AspAP, and (**D**) GluAP between dietary groups after the feeding period.
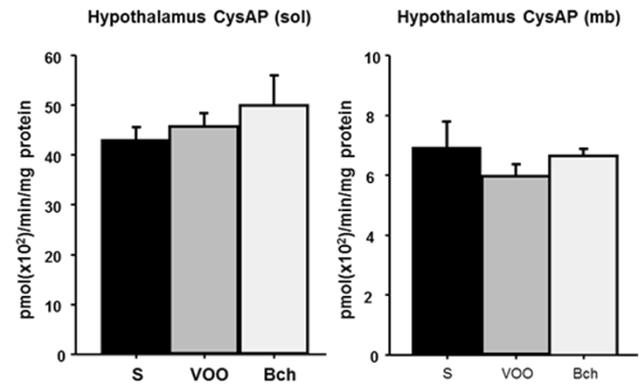

Figure 6. Soluble (sol) and membrane-bound (mb) fractions of central vasopressinase activity. No significant differences were found in hypothalamus CysAP between dietary groups after feeding period.

Many studies reveal that a HFD can modify BP by dysregulation of RAS and their regulatory enzymes: the effect of the diet on peripheral enzymatic activity was clearly demonstrated[21,22]. Our results showed a decrease of BP that correlated with MUFAs, although that physiologic response was not linked to enzyme activities in hypothalamus and plasma. Other works[23,24] described significant changes in their angiotensinase and vasopressinase activities in central and visceral tissues such as pituitary gland[25], heart[25], aorta[25], adrenal gland[25], kidney[25], liver[26], and testis[27], due to fat saturation in the diet, but seemingly did not investigate enzyme activity in the hypothalamus and plasma. The Bch diet was the only treatment which determined increase in body weight[28], high serum triglyceride and cholesterol levels[28], systolic blood pressure[28], serum nitrates and nitrites[28], and hepatic inducible nitric oxide (NO) synthase (iNOS) expression[28], however, these metabolic changes produced by a diet supplemented with saturated fat did not show an expected hypothalamus-plasma imbalance on the RAS-vasopressin nexus. No significant correlations were observed between the AP values and lipid profile; nevertheless, previous results have suggested that cholesterol influences serum AP activities[29,30], including the enzymes of study, raising the possibility that these compounds create a biochemical environment that regulates the activity of these enzymes. Therefore, the present results may be partially or indirectly due to an increase in plasma total cholesterol, as a result of increasing saturation of dietary fat.

Other authors[15,31-33] showed that an increase in AspAP and GluAP suggest a heightened metabolism of Ang II, which leads to an increase in Ang III formation. Therefore, if both angiotensinases are modified according to the degree of saturated fat in the diet, their substrates, such as Ang I and Ang II, and their metabolic products, such as Ang III and des-Asp-Ang I, may also be modified. Consequently, their roles in the control of BP and other physiologic functions may be similarly affected. It has been demonstrated that the fat saturation of the diet also influences other enzymes, such as dipeptidyl peptidase-IV (DPP-IV)[34,35] and gamma-

glutamyl transpeptidase (GGT)[25,36,37]. Taken together, these results suggest that dietary fat saturation has a wide range of effects on various enzyme systems, despite not having been demonstrated in the hypothalamus and plasma.

It is well established that the RAS over-activity is connected to the hypertension produced by saturated fat. Hypertension models have demonstrated a concomitant reduction of AspAP and therefore reduced formation of Ang 2-10, which has been suggested to counteract Ang II[31]. Thus, the reduction of Ang 2-10, together with a higher release of vasopressin due to the increased availability of Ang III, may contribute to the higher BP in L-NAME treated spontaneously hypertensive rats (SHR)[38]. Despite this, it is known that VOO treatment of SHR delays the decrease of systolic BP, and also presents with decreased levels of NO and 8-isiprostatones assayed in urine[21]. Coupled with these results, a highly significant down-regulation in AspAP and GluAP stimulates a higher formation of angiotensin 2-10 in the renal cortex[21], as well as, a higher availability of Ang II in the renal medulla of animals fed a VOO diet than in animals fed a standard diet[21].

Our results showed that rats in the Bch group, but not the VOO group, had increased BP[28]; however, the lack of changes in hypothalamus and plasma activities in rats treated with HFD suggested no involvement of hypothalamus/plasma Ang III and Ang IV and determined unaltered vasopressin. On the other hand, previous work observed hypercholesterolemia and increased body weight, as well as significantly increased serum ArgAP[22] and decreased GluAP[24], in rats fed with a diet supplemented with VOO as compared to the standard diet. Likewise, sol AlaAP and ArgAP activities were increased in the brain[31]. These findings show that a diet supplemented with olive oil modifies certain AP activities in brain and serum, and these results may reflect functional modifications in susceptible endogenous substrates.

Oxytocin, together with vasopressin, is the principal substrate of CysAP. The Zorad group showed that obesity is associated with reduced plasma oxytocin due to increased peptide degradation by liver and adipose tissue rather than changes in hormone synthesis[39]. The use of polyunsaturated fatty acid, such as fish oil, demonstrates higher levels of CysAP activity in mice than in those that were fed diets containing saturated oils (lard or coconut)[40]. Our previous results also highlighted an increase mb CysAP in the liver with VOO[26], but no evidence of changes in vasopressinase activity was found in the hypothalamus and plasma. Oxytocinase/vasopressinase inhibition has been suggested as a candidate approach in the therapy of obesity[39,41-43].

Conclusion

All enzymatic activities found in hypothalamus and plasma showed no significant differences in our three experimental diet groups, which might indicate that if membrane changes occur, they could not significantly be involved in angiotensin-/vasopressin-degrading activities. Therefore, we propose that the absence of correlations between these

enzymes and the type of fatty acids indicates that they might not be involved in the modulation of RAS and cognitive functions and, consequently, in the onset of hypertension and possible neurodegenerative disorders as causes of this disorder. Finally, our results do not support the beneficial influence of virgin olive oil on central and systemic cardiovascular function, despite previous evidence that the Mediterranean diet plays a major beneficial role in lowering BP. The present observation should be taken into account in strategies for the prevention of such diseases and as future diagnostic tools.

Acknowledgments

Appendix (Supplemental Tables)

Appendix 1: Food composition in three different diets.

CHOW COMPOSITION			
Ingredients (g/Kg)	S	VOO	Bch
Casein	-	162	162
Methionine	-	3	3
Sucrose	-	288	288
Starch	-	160	160
Maltodextrin	-	100	100
Virgin olive oil	-		
Butter	-	200	234
Cholesterol	-		10
Mineral-Vitamin Correcting	-	43	43
Choline chloride	-	4	4
Fiber (Cellulose)	-	40	40
TOTAL	-	1000	1000
LIPID PROFILE (%)			
Saturate	25	13.5	50.5
Monounsaturated	21	73.7	23.4
Polyunsaturated	54	8.4	3

Appendix 2: Nutritive value of different diets.

NUTRITIOUS VALUE OF THE DIET (%)						
Diet type	S		VOO		Bch	
	g	Kcal	g	Kcal	g	Kcal
Proteina	16.5	20	16.5	14	16.5	14
Carbohydrates	60	72	55	48	55	48
Fats	3	8	20	38	20	38
Total energy (Kcal/g)	3.410		4.740		4.740	

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