Brain, Liver, and Serum Salusin-alpha and -beta Alterations in Sprague-Dawley Rats with or without Metabolic Syndrome

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Background: This metabolic syndrome (MetS) study was designed to investigate changes in expression of the neuropeptides salusin-α (Sal-α) and salusin-β (Sal-β) in brain and liver tissue in response to obesity and related changes induced by high-fructose diet and explored how these changes were reflected in the circulating levels of Sal-α and Sal-β, as well as revealing how the lipid profile and concentrations of glucose and uric acid were altered.

Material/Methods: The study included 14 Sprague-Dawley rats. The control group was fed ad libitum on standard rat pellets, while the intervention group was given water with 10% fructose in addition to the standard rat pellet for 3 months. Sal-α and Sal-β concentrations in the serum and tissue supernatants were measured by ELISA, and immunohistochemical staining was used to demonstrate expression of the hormones in brain and liver.

Results: Sal-α and Sal-β levels in both the serum and the brain and liver tissue supernatants were lower in the MetS group than the control group. Sal-α and Sal-β were shown by immunohistochemistry to be produced in the brain epithelium, the supraoptic nucleus of the hypothalamus, and the liver hepatocytes.

Conclusions: The decrease in Sal-α and Sal-β might be involved in the etiopathology of the metabolic syndrome induced by fructose.

MeSH Keywords: 14-3-3 Proteins • Liver Failure, Acute • Neuroendocrine Secretory Protein 7B2

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**Background**

Metabolic syndrome (MetS) is a serious public health problem that has become increasingly common around the world [1]. The prevalence of MetS ranges between 3% and 37%, depending on how the study groups are defined. Although it is considered to comprise, a combination of cardiometabolic risk factors, including glucose intolerance, dyslipidemia, abdominal obesity, non-alcoholic fatty liver (NAFL), insulin resistance (IR), and hypertension, its etiology is not certain. Recently, MetS has been associated with peptide hormones that are synthesized in a multitude of tissues [1]. For instance, it was reported that ghrelin levels fell [2] while leptin levels increased [3], and the synthesis of other adipokine hormones either decreased or increased in MetS [2]. Two new neuropeptide hormones synthesized from preprosalusin, the 28-amino-acid Salusin-α (Sal-α) and the 20 amino-acid Salusin-β (Sal-β), were discovered about 10 years ago; they affect the cardiovascular system [4,5]. These bioactive peptides are synthesized in a host of tissues, including the small intestine, stomach, adrenal medulla, thymus, lymph nodes, spleen, bone marrow, saphena, salivary glands, lungs, skeletal muscle, testes, heart, and adrenal cortex [5]. It has also been demonstrated that Sal-α and Sal-β are synthesized in the aorta, left internal mammary artery (LIMA), and saphena [6]. Salusins are present in many biological fluids such as urine and blood [5]. The major function of these 2 peptides is to exert a systemic hypotensive effect, lowering the arterial pressure [5,6]. Sal-α also has a mitogenic effect on the vascular smooth muscle cells (VSMC) of both humans and rats. Administration of Sal-α inhibits foam cell formation, thereby reducing atherosclerotic plaques [6]. However, it has also been reported that Sal-β accelerates the formation of macrophage foam cells [5].

It is well-known that obesity is a critical factor in the development of MetS. The functional impairment of the brain, caused by excessive feeding, produces an abnormal physiological and homeostatic response and increases the amount of fatty tissue. Abnormal values resulting from dysfunctions in several organs, including the liver and brain, due to MetS disturb homeostasis [2]. The excess glucose taken up as a result of liver dysfunction is converted to fatty acids and lipids, and this conversion brings about NAFL [2]. Elevated transformation of fatty acids to triglycerides (TG) and very low-density lipoprotein cholesterol (VLDL-C) in the liver causes heart disease and chronic diabetes. As the foregoing discussion shows, the 2 major organs likely to be most affected by MetS are the brain and liver. Although salusins are known to be synthesized in the brain and liver in rats [5], no human or animal study has explored the changes of these 2 peptides in those tissues due to MetS.

Therefore, our principal aims in this study were: a) to examine by ELISA how Sal-α and Sal-β level changes in the serum of rats in which MetS has been induced by a fructose diet, and whether the 2 are related; b) to examine by ELISA how Sal-α and Sal-β level changes in the brain and liver of these rats and whether the 2 are related; c) to explore by immunohistochemical (IHC) staining the localization of Sal-α and Sal-β level in the brain and liver of these rats; and d) to examine whether Sal-α and Sal-β level in the serum of these rats is related to fasting plasma glucose (FPG) and the lipid profile [high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), VLDL-C, total cholesterol (TC), and triglyceride (TG)].

**Material and Methods**

**Experimental animals and experimental protocol**

After approval by the Ethics Board of Firat University Medical School pursuant to resolution number 147 of the Board on 29 December 2011, the study was started in the laboratories of the Experimental Research Unit of Firat University (FUDAM). Fourteen Sprague-Dawley male rats, 35–40 days old and weighing 159–180 grams, were randomly allocated to 2 groups (n: 7) – control (C) and MetS. After a 1-week adaptation period, the lengths and weights of the rats were measured and their basal body mass index (BMI) values were recorded. The body weights of the animals were determined in a closed plastic container from which the tare was deducted. The distance from the nose tip to the tail tip was the measure of animal length. The BMI values used to identify obesity in the rats were BMI=0.72 g/cm² for the control group and BMI ≥1.00 g/cm² for the obese group [7].

To meet the water needs of the animals, the rats in the control group were provided with normal water, while the rats in the MetS group received 10% fructose solution ad libitum. The diagnosis of MetS in this study was based on Adult Treatment Panel III (ATP III) criteria, according to which the presence of any of 3 parameters is diagnostic for MetS [8]. Although it was previously claimed that a fructose-rich diet caused the development of MetS within 2 months, recent research indicates that the process takes 3 months [2]. In the present study, glucose (measured using a glucometer), HDL-C, and BMI values were checked at the end of the 3-month diet program to determine if MetS had developed. The data presented here represent the means of 6 independent measurements.

**Preparation and storage of samples**

At the end of the 12-week diet program initiated after the adaptation period, the rats were sacrificed following a 1-night fast. Blood samples were collected into 2 different tubes – they were divided equally between plain biochemistry tubes and biochemistry tubes containing 500 Kallikrein Inhibitor Unit (KIU) aprotinin [9] and 10 μl Tween-20 [5]. The samples were
then centrifuged at 4000 rpm for 5 min. The sera obtained were stored at ~80°C pending analysis. Biochemical parameters were analyzed in the serum samples that did not contain aprotinin, while Sal-α and Sal-β peptides were measured in the samples with aprotinin and Tween-20.

Brain and liver tissues were divided into 2 groups, 1 to determine Sal-α and Sal-β concentrations and 1 for IHC staining. The samples for IHC staining were immediately put into 10% formaldehyde. Samples of 50–100 mg, weighed to identify the tissue concentrations of peptides, were put into Eppendorf tubes that were kept in boiling water for 5 min to prevent proteolysis of Sal-α and Sal-β [10]. The boiled tissue samples were then transferred to plain biochemistry tubes containing 5–10 mL 5% w/v phosphate buffered saline (PBS). After homogenization with a glass rod, the tissues were centrifuged at 4000 rpm for 10 min at +4°C. The supernatants were placed in Eppendorf tubes containing 500 KIU aprotinin and 10 μL Tween-20 and stored at ~80°C pending analysis. The amounts of Sal-α [cat no: E91892] and Sal-β [cat no: E2026, USCN Life Science Inc., Wuhan] in the tissue supernatants and serum samples were measured in the same experimental series using commercial ELISA kits. The validity of the assays for the tissues in question was tested (recovery, range, sensitivity, linearity, intra-assay, and inter-assay). All the details of these tests are available. It was found that salusin concentrations could be measured with the same sensitivity (pg/mg tissue) in the tissue supernatants as in the serum samples (pg/mL).

**Biochemical parameters**

The fasting serum glucose (FSG) levels in the rats were measured using a glucometer immediately after the animals were sacrificed. Other biochemical parameters in the samples stored at ~80°C without aprotinin were analyzed in the Medical Biochemistry Laboratory. LDL-C values were calculated according to the Friedewald formula [11]. Levels of alanine amino transferase (ALT), gamma-glutamyl transferase (GGT), total protein (TP), HDL-C, TG, TC, and FSG were measured using a Konelab 60 autoanalyzer. Fasting serum insulin (FSI) was determined by previously described [12] using an Immulite 2000 autoanalyzer (Siemens, Los Angeles, USA) with the kits recommended by the manufacturing company.

**Immunohistochemistry of tissue salusin**

Salusin-alpha and -beta expression in the tissue samples was determined using the Avidin-Biotin Peroxidase Complex (ABC) method suggested by Hsu et al. [13] with a minor modification [7]. Primary antibody for alpha salusin [cat no: H-010-67] and salusin beta [cat no: H-010-68] was from Phoenix Pharmaceuticals. Immunohistochemical staining was assessed according to the intensity and extent of staining: 0 = no staining; +1 = slight staining; + 2 = moderate staining; and +3 = intense staining.

**Table 1. Serum levels of biochemical parameters.**

| Parameters       | Control (n:7) | MetS (n:7) |
|-----------------|--------------|------------|
| ALT (IU/L)      | 51.33±3.30   | 65.00±3.16** |
| FSG (mg/dl)     | 91.33±6.19   | 188.67±9.67** |
| FSI (μIU/mL)    | 0.25±0.09    | 1.08±0.09*  |
| GGT (μIU/L)     | 4.47±0.34    | 5.05±0.29** |
| HDL-C (mg/dl)   | 42.67±6.02   | 32.50±6.41* |
| LDL-C (mg/dl)   | 90.00±4.05   | 133.67±6.28* |
| TG (mg/dl)      | 127.33±5.13  | 130.83±8.18* |
| TC (mg/dl)      | 154.67±8.14  | 195.00±18.98** |
| TP (g/dl)       | 6.50±0.47    | 7.23±0.29*  |
| UA (mg/dl)      | 6.80±3.30    | 9.90±2.83   |
| VLDL-C (mg/dl)  | 24.50±3.27   | 28.50±5.43  |

ALT – alanine aminotransferase; FSG – fasting serum glucose; FSI – fasting serum insulin; GGT – gamma glutamyltransferase; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; MetS – metabolic syndrome; UA – uric acid; TG – triglycerides; TC – total cholesterol; TP – total protein; VLDL-C – very low density lipoprotein cholesterol.

*p<0.05; **p<0.01.

**Statistical analyses**

The parameters examined in the control and MetS groups were recorded as mean ± standard deviation and analyzed using SPSS 21.00. To determine statistical significance, the Wilcoxon rank test was used for repeated measurements and the Mann-Whitney U test was used to compare data between groups. A Spearman correlation test was used to identify correlations within groups. Values of p≤0.05 were accepted as statistically significant.

**Results**

The BMI values at the beginning of the diet program were 0.55±0.2 g/cm² in the control group and 1.00±0.02 g/cm² in the MetS group, with no statistically significant difference between the groups (p>0.05). The post-diet BMI values were 0.72±0.2 g/cm² in the control group and 0.57±0.2 g/cm² in the MetS group, with no statistically significant difference between the groups (p>0.05). The post-diet BMI values were 0.72±0.2 g/cm² in the control group and 1.00±0.02 g/cm² in the MetS group, with a statistically significant difference (p<0.01).

In comparison to the control group, the MetS group had significantly elevated ALT, FSG, GGT, LDL-C, TC (p<0.01), FSI, TG, and TP (p<0.05). The MetS group had significantly lower HDL-C levels (p<0.05) compared with the control group. The VLDL-C levels were also higher in the MetS than the control group, but this increase was not statistically significant (p>0.05) (Table 1).
The MetS group had lower Sal-α and Sal-β levels (p<0.05) in the serum and in the brain and liver (Figure 1) than the control group (p>0.05). Although the decreases in Sal-α levels in the serum and brain tissue were not statistically significant (p>0.05), the decrease in the liver tissue was significant (p<0.05). The decreases in Sal-β in the serum and liver tissues were statistically significant (p<0.05), but the fall in Sal-β levels in the brain was not (p>0.05).

All the cell nuclei in the brain and liver samples stained blue with Mayer hematoxylin (contrast staining). The IHC study demonstrated that Sal-α and Sal-β were expressed in the cytoplasm of immunopositive cells and were concentrated in the apical sections of the epithelial cells surrounding the tissues. Depending on the hormone level, specific parts of the immunopositive cells were stained in shades of red. Photographs depicting Sal-α and Sal-β level in the brain (Figure 2) and liver (Figure 3) samples from the control and MetS group rats are shown.

Figure 1. Changes in serum, brain and liver Sal-α and Sal-β peptide levels. a: p<0.05. Serum Sal-β control vs. Serum Sal-β MetS (a) Liver Sal-α control vs. Liver Sal-α MetS (a) Liver Sal-β control vs. Liver Sal-β MetS (a).

Figure 2. Immunohistochemistry of Sal-α and Sal-β peptides in brain [A1: Brain (negative); A2: Brain control Sal-β; A3: Brain MetS Sal-β; B1: Brain (negative); B2: Brain control Sal-α; B3: Brain MetS Sal-α]. Arrows show Sal-α and Sal-β immunoreactivity. Mag. 400×.
presented. The density of Sal-α staining in the control group brain and liver was strongly positive, but much less so in the MetS group. Statistical comparison of the IHC staining of Sal-β level showed that the staining intensity in the brain and liver was strongly positive in the control group, but less strongly positive in the MetS group.

A multitude of changes were established in the parameters studied in the serum, as well as in the brain and liver samples from control and MetS group rats. These correlations, identified by the Pearson test, were classified on the basis of study group and type of correlation and are presented in Table 2.

Discussion

As MetS affects the entire endocrine system, it causes physiological and biochemical abnormalities. Although the etiology of the disease is yet to be clarified, several studies implicate peptide hormones in its etiopathology [14]. We designed this study to investigate MetS-related changes in the levels of the recently discovered salusins in the blood, the brain (which controls much of the endocrine system), and the liver (the site of many biochemical processes), demonstrating that salusins are involved in the regulation of blood pressure. The study also addressed changes in liver enzymes. ALT activity in the rats with MetS was 26.63% higher than in controls. This elevation in ALT agrees with the results of previous MetS studies [15]. In addition, the GGT level was 12.97% higher in the MetS group than the controls. Despite being consistent with the results of many previous studies [16], this observation is incompatible with the results reported by Donati et al. [17]. Fructose has been reported to cause inflammation, and GGT could have been elevated for that reason [18].

Concerning the lipid profile, we observed that all parameters (LDL-C, TG, TC, and VLDL-C), with the exception of HDL-C, were higher in the MetS group than the control group. The increases in the lipid profile are compatible with the results of previous MetS studies of LDL-C [15], TC [19], and VLDL-C [15]. These increases can be explained as follows: fructose is converted first to fructose 1-phosphate [20] and is then converted by aldolase to trioses, which enhance fatty acid synthesis and the formation of triacylglycerols [21]. We found that protein levels were 11.23% higher in the MetS than the control rats. An extensive literature search revealed only 1 study reporting increased protein levels [22]. It is believed that elevated protein levels are associated with the diet, because high-sugar diets (fructose in this study) can cause inflammation that increases levels of a specific protein called C-reactive protein [23]. Since,
Table 2. Correlations between the control and metabolic syndrome groups.

| Group | Relationship | r    | p    |
|-------|--------------|------|------|
| Control | BMI (4-week) – Liver Sal-α | +0.850 | 0.032 |
| Control | BMI (4-week) – Liver Sal-β | +0.878 | 0.021 |
| Control | HDL-C – Liver Sal-α | +0.843 | 0.035 |
| Control | Liver Sal-α – Liver Sal-β | +0.960 | 0.002 |
| Control | LDL-C – VLDL-C | +0.830 | 0.041 |
| Control | TC – Brain Sal-β | +0.816 | 0.048 |
| Control | FSI – Liver Sal-β | –0.964 | 0.036 |
| Control | GGT – Serum Sal-β | –0.832 | 0.040 |
| Control | HDL-C – TG | –0.935 | 0.006 |
| Control | TG – UA | –0.905 | 0.035 |
| MetS | BMI (4-week) – Liver Sal-β | +0.856 | 0.030 |
| MetS | BMI (4-week) – FSI | –0.987 | 0.002 |
| MetS | FSG – Liver Sal-α | +0.885 | 0.019 |
| MetS | HDL-C – VLDL-C | –0.956 | 0.003 |
| MetS | TC – Serum Sal-α | +0.895 | 0.016 |
| MetS | LDL-C – Brain Sal-β | –0.877 | 0.022 |

FSG – fasting serum glucose; FSI – fasting serum insulin; GGT – gamma glutamyltransferase; HDL-C – high-density lipoprotein cholesterol; LDL-C – Low-density lipoprotein cholesterol; Sal-α – Salusin-α; Sal-β – Salusin-β; TG – triglycerides; TC – total cholesterol; TP – total protein; BMI – body mass index; VLDL-C – very low density lipoprotein cholesterol; UA – uric acid.

The present study shows that the amount of insulin increased by 432% in the rats with MetS. We think that increased insulin is associated with the obesity observed in MetS, as it has been reported that insulin resistance increases with obesity [31]. Increased insulin resistance causes insulin to accumulate in the circulation instead of being used by the tissues. Insulin that cannot be used by the tissues does not allow cellular uptake of glucose, resulting in increased blood glucose levels [32]. It is well known that hyperglycemia activates serine kinase cascades [33], causing the phosphorylation of insulin receptor substrates (IRS) (which have tyrosine kinase activity) and other proteins. IRS-1 and IRS-2 lead to increased serine phosphorylation and a decrease in tyrosine phosphorylation [34]. Consequently, reduced activity in signal molecules such as phosphotyrosylinositol-3 (IP-3) mitigates the effect of insulin, which in turn causes the development of insulin resistance [31].

As already noted, peptide hormones are involved in the etiopathology of MetS. Since one of the criteria for MetS is hypertension, we examined how Sal-α and Sal-β level, causing severe hypertension and bradycardia, changed in response to MetS. The levels of Sal-α and Sal-β in the sera of rats with induced MetS were lower than those in the control group; however, the decrease was statistically significant for Sal-β but not Sal-α. When the serum concentrations of Sal-α and Sal-β were compared, the Sal-β values in both normal and MetS rats were 2–2.5 times higher than Sal-α. Previous studies have also reported that Sal-β levels were higher than Sal-α [35]. Likewise, human studies have demonstrated 5–6 times higher Sal-β levels [6].

We explored the changes in Sal-α and Sal-β concentrations in response to MetS by IHC and in brain homogenates. Sal-α level in the brains of MetS rats were statistically insignificantly lower (11%) than in the control group, while Sal-β level was 24% lower. Why Sal-α and Sal-β level in the kidney increases with MetS, and why Sal-α and Sal-β level in the brain and liver decreases with MetS, remain unknown and constitute an important future research topic.

In the present study, we determined where Sal-α and Sal-β were localized in the liver tissue by IHC and used ELISA to explore the change in their synthesis. IHC staining demonstrated that Sal-α and Sal-β were mainly produced by the hepatocytes and to a lesser extent by Kupffer cells, and that their amounts per mg/tissue decreased in MetS. This observation was consistent with the intensity of immunoreactivity. Not only was it confirmed in several studies that Kupffer and hepatocellular cells in the liver synthesize peptide hormones [34], but our study also established that the relevant cells had the characteristics of their endocrine counterparts. Since the 26% decrease in Sal-α and the 23% decrease in Sal-β in the liver due to MetS was reflected in the salusin levels in the circulation, the biological effects of Sal-β have presumably been impaired. We also

it has been reported that plasma CRP levels shown positive and significant correlations with body fat mass [24]. Here, it was therefore assumed that in MetS there are high concentrations of total protein, which are directly due to increased adipose tissue mass because adipose tissue produces and releases a vast array of protein cytokines, chemokines, acute-phase proteins, complement-like factors, and adhesion molecules [25].

Uric acid levels in the serum were 45.58% higher in the MetS group than the controls. This increase could have been caused by fructose, which has been reported to have such an effect [26] in both humans [27] and rodents [28]. Elevated uric acid causes hyperinsulinemia, hypertriglyceridemia, hypertension, and insulin resistance [29]. A study of U.S. adults demonstrated that sugar consumption and uric acid concentrations were correlated. The National Health and Nutrition Examination Survey reported that uric acid concentrations were directly proportional to systolic blood pressure in those who consumed sweetened carbonated drinks [30].
found that the livers of both control and MetS rats produced more Sal-α and Sal-β than the brain, and that the liver was a main site of Sal-β synthesis. The liver produces more salusins per mg tissue than the brain, human aorta, LIMA, and saphenae, but less than rat kidney tissues with and without MetS.

Theoretically, fructose should have different effects on salusin synthesis in brain and liver, as the fructose transporter glucose transporter-5 (GLUT5) is found in the liver but not the brain. However, in the present study we found no remarkable difference between these tissues in the effects of fructose on salusin synthesis. We believe this can be explained by the decrease in the synthesis of GLUT5 receptors due to MetS, because MetS has already been reported to inhibit GLUT5 receptors [36]. Therefore, it can be argued that the presence or absence of GLUT5 transporter in these tissues is irrelevant, as the inhibition of GLUT5 receptors in the liver could cause it to respond to fructose in the same way as brain tissue [36].

Conclusions

The epithelial cell layer of the brain was demonstrated by IHC to synthesize both Sal-α and Sal-β. Sal-α and Sal-β synthesis decreased in response to MetS, and this decrease was reflected in the salusin levels in the circulation. Likewise, it was demonstrated that Kupffer and hepatocellular cells in the liver synthesize both Sal-α and Sal-β. The Sal-α and Sal-β levels in the liver fell due to MetS, like the levels in brain tissue, and the decrease was also reflected in circulating Sal-α and Sal-β levels. Correlations were found between the BMIs of the rats and several other parameters. Since the circulating concentrations of Sal-α and Sal-β fall in MetS, we think that this parameter can be added to the ATP-III diagnostic criteria (glucose, HDL-C, hypertension, TG and BMI) for MetS and could be useful in the diagnosis and monitoring of the disease.

Conflict of interest

The authors declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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