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Serum Visfatin Levels in an Atherosclerotic Animal Model Treated with Rosuvastatin Combined with Physical Exercise

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Abstract: Visfatin is a relatively novel adipokine which gained ground in current research during the last years and the research on its contribution in the pathogenesis of atherosclerosis is ongoing. This study aimed to investigate the effects of combined rosuvastatin and exercise treatment on serum visfatin levels and on other biomarkers related to lipid metabolism and inflammation in mice receiving an atherogenic diet. Thirty five C57BL/6 male mice were assigned to four groups, group A: control, group B: atherogenic fed animals, group C: atherogenic animals along with exercise-training on treadmill and group D: rosuvastatin treatment (0.3 mg kg^{-1} body weight/day per os) along with exercise training. The duration of the study was 12 weeks. Rosuvastatin treatment in combination to physical exercise resulted in improved serum lipid levels and reducedserum visfatin, sirtuin-1, adiponectin and HMW adiponectin levels in comparison to the non-treated animals. Rosuvastatin treatment of mice combined with exercise effectively reduces serum visfatin levels in atherogenic fed mice. This reduction is directly related to a concurrent reduction of serum total adiponectin and HMW adiponectin levels.

Key words: Visfatin, adiponectin, rosuvastatin, atherosclerosis, reduces

INTRODUCTION

Atherosclerosis is a progressive multifactorial disease of the arteries which results serious morbidity and mortality during adulthood. Atherosclerosis is considered to be a chronic inflammatory disease which is induced by the accumulation of leucocytes and macrophages in the atherotic plaque (Tuttolomondo *et al*., 2012). It comprises two distinct major conditions, Ischemic Heart Disease (IHD) and Cerebrovascular Disease (CVD). IHD and CVD are the first causes of death worldwide with an estimated prevalence of 247.9 deaths/100,000 persons in 2013 which represent 84.5% of all reasons of cardiovascular deaths and 28.2% of all-cause mortality. The economic burden of coronary atherosclerotic disease for hospitalization only in the US is 10.4 billion dollars. Several factors contribute to the pathogenesis of the disease including diabetes, dyslipoproteinemia, tobacco smoking, obesity, advanced age, male sex, blood hypercoagulability and genetic predisposition.

Several factors have been implicated during the atherosclerotic process including cytokines (IL-6, IL-8, TNF-a and MCP-1), adhesion molecules, (E selectin, ICAM 1 and VCAM 1) and inflammatory markers (hsCRP, SAA and calprotectin) (Larsson *et al*., 2005). An additional marker involved in the pathways of atheroma formation is adiponectin, the most recognized adipokine which is directly related to the development of cardiovascular disease and the metabolic syndrome (Tian *et al*., 2009, 2012; Komatsu *et al*., 2012). Adiponectin secretion seems to be enhanced by sirtuin-1, a cytokine which enhances the metabolic efficiency of adipose tissue (Banks *et al*., 2008). Sirtuin-1 improves glucose tolerance and decreases hepatic glucose production. Its levels seem, however, to be down-regulated in obese humans and rodents (Qiao and Shao 2006; Dos Sentos Costa *et al*., 2010).

Visfatin is a novel lipokine which was firstly described by Fukuhara *et al*. (2005). It binds at a distinct site of the insulin receptor (other than that of insulin) resulting to hypoglycemia (Adeghate, 2008). Its expression is regulated by hypoxia, inflammation and hyperglycemia (Bae *et al*., 2006; Dedoussis *et al*., 2009; Alexiadou *et al*., 2012). It seems to be related to systemic hypertension, vascular tone and atherosclerotic disease (Dahl *et al*., 2007; Wang *et al*., 2009; Hsu *et al*., 2015; Liakos *et al*., 2015).

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Statin therapy has been implemented in the prevention of major cardiovascular events in hyperlipidemic patients. It has been also associated with a favorable decrease of the progression of common carotid artery intima-media thickness (Huang *et al*., 2013). Previous studies have suggested that statin therapy might decrease serum levels of visfatin (Kadoglou *et al*., 2012; Petreanu *et al*., 2014). However, evidence remains scarce and conflicting in this field.

A wide range of mechanisms may explain the beneficial properties of exercise on cardiovascular health. Among them, the increase in serum HDL levels, the up regulation of atheroprotective genes such as PON1 and SRB1, the enhanced production of anti-inflammatory cytokines as well as of antioxidant enzymes have been previously described (Bowles and Laughlin, 2011).

The purpose of the present experimental study is to investigate the impact of rosuvastatin therapy in combination with treadmill exercise on serum visfatin levels in an Atherosclerotic Model in mice. Furthermore, we aim to associate serum visfatin levels to serum adiponectin and sirtuin-1 levels.

MATERIALS AND METHODS

Animals study design: The 35, 10 week old C57BL/6 male mice were obtained from BSRC "Al. Fleming" (Vari, Greece) and left to be acclimatised for 1 week before the experiment start. The animals were housed under conditions of controlled temperature (23±2°C) and humidity (60%) with 12 h light/dark cycle. All possible precautions were taken to avoid animal suffering at each stage of the experiment. The experimental protocol was approved by the Veterinary Directorate of Athens Prefecture and by the Ethics Committee of the Medicine School of the National and Kapodistrian University of Athens according to the EU legislation regarding the use of animals in biomedical science.

Mice had free access to food and water throughout the study (12 weeks): The mice were randomized into four experimental groups as follows: A: animals received normal chow diet (4RF25, Mucedola, Milan, Italy, 3.48% fat); B: animals received an atherogenic diet (15.8% fat, 1.25% cholesterol. 0.5% sodium cholate) (Harlan Teklad, TD.90221, Cocoa Butter Diet with 75% Purina, Mouse Chow #5015) for 12 weeks; C: animals received the atherogenic diet for the same period along with exercise on a treadmill at a speed of 10-15 m min^{-1} with a gradually increasing duration of 30 min, 5 days $week^{-1}$ for 12 weeks following an exercise program as describe in Table 1; D: as in the C group in combination with rosuvastatin daily administration. Rosuvastatin was administrated p.o. in drinking water in a concentration tested to result in a daily uptake of 0.3 mg kg^{-1} body weight. Rosuvastatin was provided as tablets (Crestor; tablets, 5 mg) and was firstly diluted in PBS (pH 7.4). Afterwards, this solution was mixed with the respective quantity of water.

Table 1: Exercise program followed by the mice in groups C and D

Weeks	Exercise duration (min)	Treadmill speed (m min^{-1})
1	10	10
2	20	10
3	30	12
4-12	30	15

Blood collection-serum and plasma measurements: Blood samples of mice were collected at the beginning of the study (T0) and at the end of the experimental period (T1) (at 9:00 a.m., after a 12 h fast) using capillary tubes introduced into the medial retro-orbital venous plexus under light ether anaesthesia. Serum glucose levels were analyzed by GOD-PAP Method while serum concentrations of total, LDL, HDL cholesterol and triglycerides were determined as previously described (Korou *et al*., 2010).

At the end of the study, serum visfatin levels were measured with an immunometric assay using a commercially available kit (USNC Life Science Inc., Wuhan, China). In addition, serum total adiponectin levels were determined using a commercial ELISA kit (USNC Life Science Inc., Wuhan, China). High molecular weight adiponectin levels were also measured using a sandwich ELISA kit (AKMAN-011). Moreover, the levels of serum sirtuin-1 levels weredetermined with the use of an ELISA kit (USNC Life Science Inc., Wuhan, China).

Statistical analysis: The results were expressed as median (interquartile range). Box plots were prepared for serum visfatin, serum sirtuin-1, serum adiponectin and serum HMW adiponectin levels and the Pearson's correlation coefficient method followed (or Spearman occasionally) were used in order to find out the linear correlation between these markers. The normality of the distributions was assessed by the Kolmogorov-Smirnov test. One sample t-test was followed in order to find the corresponding relationship between the studied parameters.

RESULTS AND DISCUSSION

No significant differences in serum glucose and lipid levels were observed at baseline between groups. At the end of the study, serum glucose levels were decreased in atherogenic diet fed animals (group B) when compared to

control animals (group A) ($p<0.001$). Serum total cholesterol levels were higher in B group in comparison to controls ($p = 0.005$). These levels were lower in rosuvastatin treated mice (group D) compared to those of animals of group B ($p = 0.003$) while total cholesterol levels in group D were higher compared to group C ($p = 0.033$). LDL cholesterol levels were increased in the atherogenic fed animals in group B compared to both control (group A) and rosuvastatin treated (group D) animals at the end of the experimental period ($p = 0.004$ and $p = 0.001$, respectively). Moreover, LDL levels were higher in group C compared to group D ($p = 0.036$). Serum HDL cholesterol levels were lower in atherogenic group B in comparison to control animals ($p = 0.001$) whereas, these levels were increased in the exercise or the exercise/rosuvastatin treated groups when compared to the non treated animals receiving atherogenic diet ($p = 0.002$ and $p = 0.008$, respectively). Serum triglyceride levels were lower in atherogenic fed animals (group B) compared to controls ($p = 0.004$) (Table 2).

Body weight levels in the rosuvastatin treated group were higher in comparison to group B at the end of the experimental period ($p = 0.001$).

Serum visfatin levels in the animals receiving the atherogenic diet (group B) correlated significantly and positively with serum adiponectin ($r = 0.714$, $p = 0.047$) and HMW adiponectin levels ($r = 0.833$, $p<0.010$). Serum adiponectin and HMW adiponectin levels were significantly increased in the atherogenic group B as compared to the control animals ($p = 0.012$, $p = 0.001$, respectively for serum adiponectin and HMW adiponectin levels) (Fig. 1).

Serum visfatin, adiponectin as well as HMW adiponectin levels were lower in the animals under physical exercise treatment (group C) compared to the non-treated animals (group B) ($p = 0.002$, $p<0.001$, $p<0.001$ for serum visfatin, adponectin and HMW adiponectin levels, respectively). In addition, serum visfatin, sirtuin-1, adiponectin and HMW adiponectin levels were lower in animals of group D compared to the atherogenic group B ($p = 0.002$, $p<0.001$, $p<0.001$, $p<0.001$ for serum visfatin, sirtuin-1, adponectin and HMW adiponectin levels, respectively). Finally, when comparing the rosuvastatin treated group with group C, serum sirtuin-1 and serum adiponectin levels differed between groups and the group D presented the lower values ($p<0.001$, $p = 0.003$, respectively) (Fig. 1).

Atherosclerosis is a chronic, complex, progressive disease which starts during childhood and results various cardiovascular diseases including cerebrovascular and ischemic heart disease. The contribution of adipose tissue and lipokines in the pathogenesis of these diseases has gained interest during the last decade. Since, the discovery of leptin several adipokines have been described in the field including adiponectin, sirtuin and several others. Visfatin is a relatively novel adipokine which gained ground in current research during the last years. The purpose of the present study was to investigate visfatin serum levels in an atherogenic experimental model and to associate it with serum sirtuin-1 and adiponectin.

In our study, mice fed with an atherogenic diet developed had significantly increased serum cholesterol (89.72% compared to controls) which was accompanied by a decrease in serum HDL (45.83% compared to

Table 2: Body weight measurements and serum glucose, total cholesterol, LDL cholesterol, HDL cholesterol and Triglycerides at baseline (T0) and at the end of the experimental period (T1)

	Measurements (median (interquartile range))			
Parameters	Group A	Group B	Group C	Group D
Body weight (g)				
T0	27.00 (4.50)	30.00 (4.00)	28.00 (2.00)	24.00 (2.00)
T1	29.00 (4.50)	28.00 (6.00)[d]	22.00 (7.00)	22.00 (4.00)[b]
Serum glucose (mg dL^{-1})				
T0	140.50 (53.50)	129.00 (44.00)	118.00 (47.00)	201.00 (87.50)
T1	209.50 (114.50)[b]	135.00 (44.00)[a]	133.00 (62.00)	141.00 (47.50)
Serum total cholesterol (mg dL^{-1})				
T0	78.00 (14.50)	79.00 (9.00)	81.00 (6.50)	70.00 (31.50)
T1	82.50 (28.50)[b]	445.00 (330.00)[a, d]	621.00 (409.00)[d]	311.00 (185.00)[b, c]
Serum LDL-cholesterol (mg dL^{-1})				
T0	29.60 (6.10)	36.10 (6.00)	32.20 (9.90)	32.00 (35.60)
T1	38.80 (16.30)[b]	416.40 (341.40)[a, d]	512.20 (396.80)[d]	278.60 (161.45)[b, c]
Serum HDL-cholesterol (mg dL^{-1})				
T0	38.00 (10.00)	36.60 (3.90)	34.00 (4.50)	29.00 (5.50)
T1	37.00 (6.00)[b]	16.00 (14.00)[a, c, d]	31.00 (11.00)[b]	29.00 (5.00)[b]
Serum triglycerides (mg dL^{-1})				
T0	69.50 (28.75)	32.00 (12.00)	62.00 (30.50)	51.00 (26.50)
T1	41.50 (32.00)[b]	35.00 (18.00)[a]	92.00 (81.00)	31.00 (20.50)

[a]Significantly different from A group; [b]Significantly different from B group; [c]Significantly different from C group; [d]Significantly different from D group

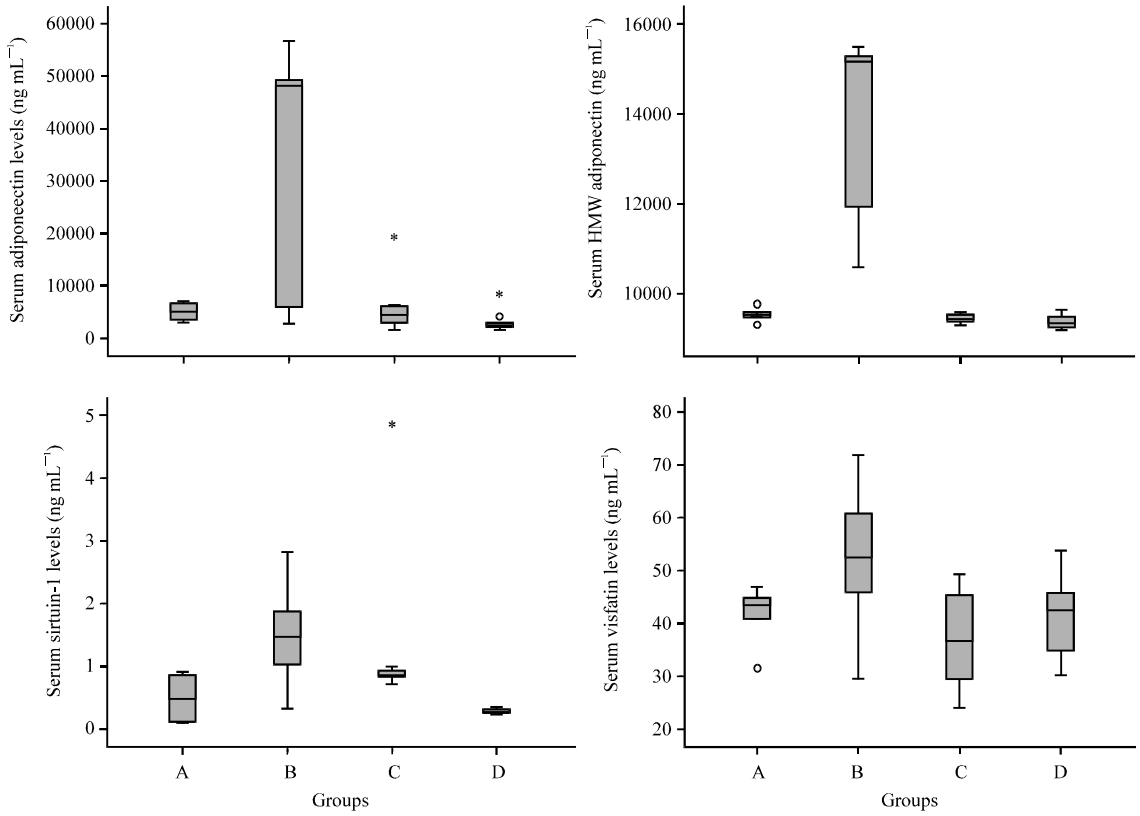

Fig. 1: Box plots of ELISA results. All units are measured in ng/mL

controls). The triglycerides were 28.39% lower compared to those of the control group. This finding can be explained by the inhibition of 7a-hydroxylase by chocolate which can be found in atherogenic diet (Shi *et al*., 2002; Li *et al*., 2008). We also noticed a relative decrease in body weight (45.27%). Exercise increased the HDL cholesterol (31.17%) and was also associated with a mild decrease in body weight (12.73%). Rosuvastatin plus exercise significantly reduced the LDL cholesterol (34.9% compared to controls) and the body weight (19.2%) and at the same time increased the levels of HDL 36.78%).

All examined lipokines (visfatin, sirtuin-1, total adiponectin and HMW adiponectin) were increased among mice fed the atherogenic diet (compared to controls). This observation reached, however, statistical significance only in the case of total adiponectin (84.69%) and HMW adiponectin (31.18%). Exercise reduced all four factors; however, statistical significance was observed only in visfatin, (29.37%), total adiponectin (81.80%) and HMW adiponectin (31.84%). Natural exercise in combination with rosuvastatin led to significant reduction of all four factors. When we compared groups C vs. D we observed significantly reduced levels of sirtuin-1 (79.04%) and total adiponectin (46.8%) among mice treated with rosuvastatin plus exercise. As previously mentioned, serum visfatin levels in the animals receiving the atherogenic diet (group B) correlated significantly and positively with serum adiponectin and HMW adiponectin levels.

Previous studies suggest that statin therapy might actually decrease visfatin levels, however, these results are not always unanimous. For instance, simvastatin treatment in hyperlipidemic patients reduces visfatin but increases adiponectin according to Petreanu *et al*. (2014). Atorvastatin also seems to reduce visfatin levels in diabetic patients, whereas rosuvastatin seems to effectively reduce visfatin levels in hyperlipidemic patients (Kostapanos *et al*., 2008; Kadoglou *et al*., 2012). This was not, however, the case with non-diabetic patients who suffered from metabolic syndrome (Pfutzner *et al*., 2007).

The findings of our study partially explain the beneficial effect of statin therapy in mice offered an atherogenic diet. Visfatin up-regulation has been previously linked to acceleration in the course of atherosclerosis (Wan *et al*., 2014). Concurrently, it increases the rate of ischemic cerebrovascular disease (Kong *et al*., 2014). In a cellular basis, visfatin seems to

induce cholesterol accumulation in macrophages, thus accelerating the process of atherosclerosis (Zhou *et al*., 2013). Dahl *et al*. (2007) suggested that it should be seen as an inflammatory regulator which contributes to the destabilization of atherosclerotic plaques by enhancing matrix metalloproteinase 9 activity in THP-1 monocytes and TNF-a and IL-18 in peripheral mononuclear cells.

CONCLUSION

According to the results of our study rosuvastatin treatment of mice fed with an atherogenic diet effectively reduces serum visfatin levels. This reduction accompanies and is directly related to a concurrent reduction of serum total adiponectin and HMW adiponectin levels. These findings suggest that visfatin might be an important moderator of atherosclerotic disease progression which deserves further investigation in future clinical studies.

IMPLICATIONS

Visfatin is a promising lipokine which seems to regulate the process of atherosclerotic plaque formation. However, its correlation with disease progression at a cellular level does not justify its use in current clinical practice. Future studies should aim to directly correlate visfatin levels after statin therapy to ultrasonographic and pathology findings of atherosclerotic lesions. This way we will be able to understand whether it might actually become a useful marker of disease progression which could serve as a screening tool.

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