An excessive consumption of a diet rich in saturated fatty acids is a key factor in pathogenesis of cardiovascular diseases which are strictly connected with leptin imbalance in the vessels. However, whether vitamin E supplementation would influence leptin expression in aortic layers is still unknown. For 3 or 6 weeks male Wistar rats were fed a high-fat (20% w/w) diet with lard as dietary fat source with or without vitamin E supplementation (50 mg/100 g of diet). The 6-week intake of an atherogenic diet increased total cholesterol (TC) and high density lipoprotein cholesterol (HDL) plasma levels simultaneously lowering TC/HDL ratio (ANOVA p≤0.0001 for all three parameters). After longer period of feeding it was stated that leptin expression in all three aortic layers was enhanced (ANOVA p≤0.0001 for endothelium, tunica media and adventitia, respectively) with decreased leptin plasma concentration (ANOVA p≤0.0001). After both periods of feeding vitamin E supplementation caused an increase in plasma HDL content and a decrease of TC/HDL ratio. In the 3-week experiment vitamin E addition caused a decrease in leptin plasma levels (Fisher’s test, 3L versus 3LE p≤0.002) and an increase in leptin expression in all three aortic layers (Fisher’s test, 3L versus 3LE p≤0.005, p≤0.01 and p≤0.05 respectively for endothelium, tunica media and adventitia). The contradictory results were observed in the 6-week experiment in which vitamin supplementation decreased leptin expression in the aortic endothelium (Fisher’s test, 6L versus 6LE p≤0.001) with lack of changes in the other two layers of the aorta and plasma. The study showed that vitamin E supplementation influenced leptin expression in aortic layers in rats fed atherogenic diet differently depending on the length of feeding period. It may suggest that vitamin E consumption plays an important role in the control of leptin status in the endothelium.

**Key words:** atherogenic diet, aorta, leptin, vitamin E, peroxynitrite, prostaglandin E₂, cyclooxygenase

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**INTRODUCTION**

One of the main factors influencing pathological changes in blood vessels is nutrition. A high-fat diet rich in saturated fatty acids and cholesterol is known to cause not only obesity but also atherosclerosis and coronary insufficiency (1). Leptin, which is dependent on nutritional status of organism, is stated to be a link between obesity and cardiovascular disease (CVD) (2). This 167-amino acid protein is produced mainly by white adipose tissue. The most potent form of leptin receptor (OB-Rb) was identified not only in the endothelial (3) and vascular smooth muscle cells (4) but also in atherosclerotic lesions (5). Leptin plays an important role in modulation of endothelium function via regulation of nitric oxide (NO) production and endothelium-derived hyperpolarizing factor mechanisms (6). On the other hand, it can also exhibit strong atherogenic properties. Under pathological conditions leptin causes NO/ONOO⁻ (peroxynitrite) imbalance characteristic for endothelium dysfunctions (7). It has been shown that leptin promotes a vascular smooth muscles cells migration and proliferation participating in the formation of a atherosclerotic plaque (8-9).
poorly understood. Consumption of vitamin E positively affects the function of the cardiovascular system. It is well documented that vitamin E inhibits platelet aggregation (13) and secretion of proinflammatory cytokines (interleukine-1β) (14-15). On the other hand these processes can be promoted by leptin (16). These contradictory actions of leptin and vitamin E suggest that interactions between these two factors in the aortic layers are worth to be examined.

Based on these findings, our study was designed to evaluate changes in leptin expression in aortic wall layers (endothelium, tunica media and adventitia) with respect to supplementation with vitamin E and period of feeding with atherogenic diet.

**METHODS AND MATERIALS**

**Animals, diets and experimental design**

The study was approved by the Third Local Animal Care and Use Committee in Warsaw (Poland).

The experiment was conducted on 24 male adult Wistar rats (supplied by The Kielanowski Institute of Animal Physiology and Nutrition in Jablonna, Poland) with an initial body weight of 292.8 ± 8.7 g. Animals were kept individually in polypropylene cages under stable conditions (temperature 22°C; humidity 50%; 12:12 light:dark cycle). They were given free access to food and water.

During first week of adaptation, animals were fed a standard rodents’ feed Labofeed H (Andrzej Morawski Feed Production Plant, Kcynia, Poland). Subsequently, in each time-group (3- and 6-week group), animals were divided into two groups (6 animals in each group) fed high-fat (20% w/w), semi-synthetic diets with lard as a dietary fat source, with or without vitamin E (50 mg/100 g of diet) (Table 1). Vitamin E was added as all racemic α-tocopheryl acetate (Medana Pharma S.A., Poland). The fatty acids profile of used lard was determined with AOCS Ce 2-66 method by Industrial Chemistry Research Institute in Warsaw. The main fatty acids were: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3) - 24.33 g, 14.37 g, 45.63 g, 8.43 g and 0.64 g per 100 g of product, respectively.

The groups of animals were fed experimental diets for 3 and 6 weeks. Each rat’s body weight was monitored once each week. The food intake was counted by each day measurement of unconsumed diet through the whole experiment. Body weight gain was counted as: (final body weight - initial body weight)/number of days (21 or 42, respectively to the period of feeding).

After the proper feeding period rats were anesthetized via the peritoneum thiopental administration and bled by cardiac puncture. Blood collected to tubes with heparin was centrifuged (20 minutes, 3000 rpm, 4°C) and the obtained plasma was stored at −20°C until further analysis. The aorta was removed and fixed in buffered 10 % formalin for 24 hours.

**Plasma lipid profile colorimetric assays**

Concentrations of plasma total cholesterol (TC), triacylglycerols (TAG) and high-density lipoprotein cholesterol

<table>
<thead>
<tr>
<th>Table 1. Composition of diets (g/100 g diet).</th>
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<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Wheat starch</td>
</tr>
<tr>
<td>Casein</td>
</tr>
<tr>
<td>Fat - Lard</td>
</tr>
<tr>
<td>Potato starch</td>
</tr>
<tr>
<td>Mineral mixture</td>
</tr>
<tr>
<td>Vitamin mixture</td>
</tr>
<tr>
<td>DL-Methionine</td>
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<tr>
<td>Vitamin E</td>
</tr>
</tbody>
</table>

1Mineral mix composition (in 100 g of mix): CaHPO4 - 73.5 g, K2HPO4 - 8.1 g, K2SO4 - 6.8 g, NaCl - 3.06 g, CaCO3 - 2.1 g, Na2HPO4 - 2.14 g, MgO - 2.5 g, MnCO3 - 421 mg, CuCO3 - 33 mg, KJ - 720 µg, C3H4(OH)(COO)2Fe - 706 mg, C3H4(OH)(COOH)Fe - 558 mg, ZnCO3 - 81 mg.

2Vitamin mix composition (in 100 g of mix): nicotinic acid - 400 mg, calcium pantothenate - 400 mg, pyridoxine-HCl - 50 mg, thiamin-HCl - 50 mg, riboflavin - 80 mg, folic acid - 20 mg, biotin - 4 mg, vitamin B12 - 0.3 mg, vitamin E - 1000 mg, vitamin A - 20000 IU, vitamin D3 - 20000 IU, vitamin K - 50 mg, PABA - 1 g, inositol - 1 g.

3Vitamin E naturally appearing in lard and used in vitamin mixture was not counted into added 50 mg/100 g of diet. According to Kunachowicz et al., 2005 (17), content of vitamin E in lard is 1.2 mg/100g of product and this amount was taken into consideration when calculation of total vitamin E intake was made as well as vitamin E content in vitamin mixture.

Fig. 1. Leptin expression in the aortic wall layers (microscope photo, x400). E - endothelium; M - tunica media; A - adventitia; 0–III - intensity of immunohistochemical reactions.
(HDL) were measured using enzymatic-colorimetric methods. Kits containing ready to use liquid reagents were purchased from PTH Hydrex (Warsaw, Poland). The analyses were held according to kits manuals (TC - cat. no. HXB104; HDL - cat. no. HXB106; TAG - cat. no. SL17628).

Leptin radioimmunological assay

Plasma leptin concentration was measured using rat leptin RIA kit (cat. no. RL-83K, LINCO Research, USA). The intra- and inter-assay precision was 2.4% and 4.8%, respectively. The sensitivity of the test was 0.5 ng/ml. The assay was conducted according to the kit manual.

Leptin immunohistochemical assay in aortic layers

Aorta specimens were analyzed immunohistochemically using the rabbit polyclonal antibody Anti-OB (AB-20) from Santa Cruz Biotechnology Inc. (cat. no. sc-842). Three-micrometer sections of formalin-fixed, paraffin-embedded aorta specimens were deparaffinized with xylene in two changes of 10 minutes each, and rehydrated using graded alcohols (99.8%, 96% and 70%) twice for 5 minutes each. Antigens were retrieved by heating the sections in 0.01 M citrate buffer (pH 6.0) for 20 minutes at 700 W in a microwave oven. Afterwards, specimens were cooled, rinsed with distilled water, kept in phosphate buffered saline (PBS, pH 7.4) for 5 minutes and then incubated for 15 minutes in 10% bovine serum albumin to block non specific tissue reactivity.

The sections were then incubated with the primary antibody Anti-OB (AB-20) in a 1:1000 dilution overnight at 4°C. After incubation with the primary antibody, the slides were incubated with biotinylated secondary goat anti-rabbit IgG (1:1500, cat. no. 830/, Immunotech, Marseille) for 30 minutes. Endogenous peroxidase activity was inactivated by 5-minute incubation with 3% H2O2, and the sections were then incubated with a peroxidase-conjugated streptavidin (1:500, cat. no. 309/, Immunotech, Marseille) for 45 minutes. The complex was detected with 3.3'-diaminobenzidine (Sigma-Aldrich, USA) as a chromogen, whereby the sections were lightly counterstained with hematoxylin and mounted in DPX mounting medium (BDH, International, England).

Normal rabbit IgG of the same concentration as the primary antibody was used as a negative control.

Leptin localization was identified in one 3-micrometer section per animal, separately in endothelium (E), tunica media (M) and adventitia (A).

Results were calculated according to Ball et al. (18). One hundred cells per each layer of aorta were assigned to an intensity category of 0 (absent), 1 (weak), 2 (moderate), or 3 (strong) (intensity of each reaction is presented on Fig. 1). The percentage of cells in each intensity category was determined as N0, N1, N2, and N3, respectively. A weighted average (ID score) was then calculated as:

$$\text{ID} = \frac{(N_0^0 + N_1^1 + N_2^2 + N_3^3)}{100}$$

The ID score therefore ranged from 0 (absent staining in all cells) to a maximum of 3 (100% cells having a staining intensity of 3).

Statistical analysis

Statistical analysis was performed with use of STATISTICA v. 10.00 (StatSoft Polska Sp. z o.o., Cracow, Poland). Results of the simple regression and a two-way analysis of variance (ANOVA) followed by post hoc Fisher’s test were considered statistically significant at p≤0.05. All data are presented as means ± standard deviation.

Table 2. Body weight gain (g/d), intake of diet (g/d/100 g fbw) and vitamin E (mg/d/100 g fbw), plasma lipid profile: TC, HDL and TAG concentrations (mg/dl) and TC/HDL ratio.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Group 3L</th>
<th>3LE</th>
<th>6L</th>
<th>6LE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight Gain</td>
<td>5.10 ± 0.23B</td>
<td>4.71 ± 0.37B</td>
<td>4.11 ± 0.37A</td>
<td>6.30 ± 0.26C</td>
</tr>
<tr>
<td>after 21 (g/d)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Final Body Weight Gain</td>
<td>5.10 ± 0.23B</td>
<td>4.71 ± 0.37B</td>
<td>1.55 ± 0.62A</td>
<td>1.60 ± 0.12A</td>
</tr>
<tr>
<td>after 21 or 42 days (g/d)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Intake of Diet (g/d/100 g fbw)</td>
<td>6.087 ± 0.414B</td>
<td>5.984 ± 0.589B</td>
<td>5.302 ± 0.385A</td>
<td>5.306 ± 0.198A</td>
</tr>
<tr>
<td>Vitamin E (mg/d/100 g fbw)</td>
<td>0.616 ± 0.042A</td>
<td>3.597 ± 0.354C</td>
<td>0.537 ± 0.039A</td>
<td>3.190 ± 0.119B</td>
</tr>
<tr>
<td>of TC (mg/dl)</td>
<td>74.50 ± 8.58B</td>
<td>63.40 ± 5.03A</td>
<td>100.50 ± 6.03B</td>
<td>95.50 ± 5.13C</td>
</tr>
<tr>
<td>HDL</td>
<td>21.50 ± 2.64B</td>
<td>26.40 ± 2.07B</td>
<td>61.00 ± 3.16C</td>
<td>66.33 ± 4.80D</td>
</tr>
<tr>
<td>TAG</td>
<td>102.00 ± 8.68B</td>
<td>117.75 ± 2.75C</td>
<td>93.83 ± 5.34A</td>
<td>95.00 ± 3.23AB</td>
</tr>
<tr>
<td>TC/HDL ratio</td>
<td>3.48 ± 0.32B</td>
<td>2.40 ± 0.10B</td>
<td>1.65 ± 0.12A</td>
<td>1.44 ± 0.11A</td>
</tr>
</tbody>
</table>

1All values are means ± standard deviation.
2fbw - final body weight, TC - total cholesterol, HDL - high-density lipoprotein cholesterol, TAG - triacylglycerols; 3L and 6L - groups fed basic diet for 3 and 6 weeks, respectively; 3LE and 6LE - groups fed basic diet with additional vitamin E (50 mg/100 g of diet) for 3 and 6 weeks, respectively; A,B,C,D - indicate values significantly different in rows (ANOVA with Fisher's post-hock test; p≤0.05); n=6 for each group.
increased TC and HDL content (ANOVA, p≤0.0001 for both parameters), however decreased TAG content and TC/HDL ratio (ANOVA, p≤0.0001 for both parameters). Plasma total cholesterol concentration was lower when vitamin E was added but only in the 3-week experiment (Fisher’s test, 3L versus 3LE: p≤0.02) (Table 2). The vitamin E supplementation caused 

increase in HDL cholesterol content in plasma after both periods of feeding (Fisher’s test, 3L versus 3LE: p≤0.05 and 6L versus 6LE: p≤0.02). This was linked to lower TC/HDL ratio observed after both periods (Fisher’s test, 3L versus 3LE: p≤0.0001 and 6L versus 6LE: p≤0.05). TAG concentration was higher in plasma of rats fed diet with additional vitamin E in the
and positively correlated with the body weight gain ($p < 0.05$, respectively for endothelium, tunica media and adventitia) we observed an increase of leptin expression in endothelium (Fisher’s test, 6L versus 6LE: $p < 0.005$, $p < 0.01$ and $p < 0.05$, respectively for endothelium, tunica media and adventitia) (Fig. 2). The observed increase was the highest in the tunica media (9-fold), while in the endothelium and adventitia the increase was more than 4-fold. The opposite effect was observed in the endothelium after longer period of feeding when the vitamin E addition caused the 27% decrease in leptin expression in endothelium (Fisher’s test, 6L versus 6LE: $p < 0.001$). No influence of vitamin E on leptin expression was found in tunica media and adventitia after 6 weeks.

DISCUSSION

One of the main factors promoting pathological changes in blood vessels is an improperly balanced diet. A high-fat diet rich in saturated fatty acids and cholesterol is known to cause atherosclerosis and coronary insufficiency. On the other hand, consumption of vitamins with antioxidative properties, such as vitamin E, positively affects the function of the cardiovascular system. Leptin, which is influenced by nutritional status of organism, has its receptors in the vessels. Whether a high-fat diet supplemented with vitamin E will change leptin expression in individual layers of the aorta wall (endothelium, tunica media and adventitia) was in our interest. All of the animals were fed high-fat (20% w/w) diets, as such an amount highly exceeds rats’ nutritional needs for fat according to American Institute of Nutrition (19). Lard was used as a dietary fat source as it is a good source of saturated fatty acids and because it is recommended to be eliminated from diet for people with CVD (20). We expected that on such diets animals would have put on weight and became obese. After 3 weeks we observed such a tendency, but after this period the animals which were aimed to follow by elongated period of feeding with high-fat diet based on grape seed oil or flaxseed oil was required to observe the leptin resistance (10). Not only did the period of feeding influence leptin content in aortic layers. But the obtained results showed that the diet supplemented with vitamin E for 3 weeks increased leptin protein expression in all three aortic layers. It is difficult to judge whether such an increase of leptin expression can exert positive or negative effect on vessels function. Beltowski et al. (31) suggested that in physiological conditions leptin acts as vasodilating mediator and in pathological states it exhibits vasoconstrictory activity. Leptin is stated to increase endothelial NO synthase (eNOS) activity and NO production by Akt/eNOS-dependent mechanism has been already shown as the pathway via vitamin E induces expression of other adipokine - adiponectin - in adipocytes (29). Besides, Zeigerer et al. (30) observed that the use of PPARγ agonist increased leptin gene expression while forming intracellular pool of leptin in adipocytes.

In all three aortic layers (endothelium, tunica media and adventitia) we observed an increase of leptin expression followed by elongated period of feeding with the atherogenic diet. As we discussed in our previous studies this may be connected with an intensified leptin production and secretion from perivascular adipose tissue or with an increased sensitivity of endothelium to circulating with blood leptin. We also observed that such an increase depended on the type of dietary fat administrated with diet (flaxseed oil versus grapes seed oil) (10). Not only did the period of feeding influence leptin content in aortic layers. But the obtained results showed that the diet supplemented with vitamin E for 3 weeks increased leptin protein expression in all three aortic layers. It is difficult to judge whether such an increase of leptin expression can exert positive or negative effect on vessels function. Beltowski et al. (31) suggested that in physiological conditions leptin acts as vasodilating mediator and in pathological states it exhibits vasoconstrictory activity. Leptin is stated to increase endothelial NO synthase (eNOS) activity and NO production by Akt/eNOS-dependent mechanism has been already shown as the pathway via vitamin E induces expression of other adipokine - adiponectin - in adipocytes (29). Besides, Zeigerer et al. (30) observed that the use of PPARγ agonist increased leptin gene expression while forming intracellular pool of leptin in adipocytes.

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effect on leptin content in aortic layers. In endothelium vitamin E supplementation caused a decrease of leptin expression with lack of changes in tunica media and adventitia.

These different effects induced by vitamin E on leptin expression can be explained by its contradictory effects on prostaglandin E2 (PGE2) production which stimulates leptin expression (36). The prostacyclins (for example PGI2 and PGE2) have been now widely examined in case of atherosclerosis as they are said to be an important modulator of vascular functions (37). Wu et al. (38) found that vitamin E in dose-dependent manner increased the production of both PGI2 and PGE2 in the human aortic endothelial cells by stimulation of cytosolic expression of phospholipase A2 (PLA2) and arachidonic acid (AA) release from membrane phospholipids. In the same study vitamin E inhibited cyclooxygenase (COX) activity which should have exerted an opposed effect on PGE2 production. That can suggest that vitamin E acts on PGE2 synthesis in a dose-dependent way. On the other hand, in their earlier experiments Wu et al. (39) observed that vitamin E decreased the age-dependent increase in PGE2 synthesis. However, such effect was stated only in old mice in which vitamin E preferentially inhibited COX activity. Such non-antioxidant effect of vitamin E was also observed in other human tissues and cells. Modulating COX-2 activity in duodenal smooth muscles vitamin E influenced the amplitude and frequency of their contractions (40). Later it was reported that in macrophages vitamin E decreased the COX-2 activity by affecting the production of peroxynitrite (ONOO- ) that is a product of NO and superoxide anion radical (41).

These different effects of vitamin E on PGE2 synthesis could be dependent upon oxidative status of organism (Fig. 3). When the organism is young or when the atherogenic diet consumption is not excessive, vitamin E stimulates PLA2 activity and PGE2 production. Then leptin expression is increased and NO synthesis is well balanced. However, when organism is old or when the atherogenic diet is consumed for a longer period, oxidative stress is enhanced directing vitamin E to act as antioxidant and to neutralize overly produced ONOO- inhibiting COX-2 activity. The PGE2 synthesis is decreased leading to alteration in leptin expression. Therefore, vitamin E seems to be an important modulator of leptin expression in aortic walls. Inhibiting ONOO- production vitamin E might not only decrease leptin synthesis but can also adversely affect its production due to an antioxidant/ superoxide activity, platelet aggregation and arterial thrombogenesis. J Am Coll Cardiol 1999; 34: 1208-1215.


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Received: October 7, 2013
Accepted: January 21, 2014

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