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Effect of allopurinol on oxidative stress in obesity and liver content of free fatty acids

ABSTRACT
Oxidative stress appears as the key feature associated with dysfunction in adipose tissue and a major factor in the mechanisms of altered lipid metabolism in obesity. Cellular response of adipocytes in the conditions of oxidative stress results in maintaining systemic pro-inflammatory state, insulin resistance and increased accumulation of very long-chain saturated fatty acids (VLCSFAs) to the liver, which are lipotoxic and lead to further injury. Therefore, the therapeutic purposes of lowering the production of ROS, may have beneficial effects on obesity and its associated complications. The aim of the study was to determine the influence of allopurinol (xanthine oxidase inhibitors) on oxidative stress in adipose tissue and liver saturated fatty acids content in a model of fructose-induced obesity. We used a model of high-fructose diet (HFD) in male rats Wistar (16 weeks, 35% glucose-fructose corn syrup), divided into three groups: control; HFD; HFD and allopurinol administration (150 mg/kg in drinking water for 16 week). Analysis of fatty acids was performed by Gas Chromatograph with MS detector. Serum levels of glucose and uric acid (UA); weight, markers of oxidative stress- MDA (malondialdehyde), glutathione (GSH) and glutathione peroxidase (Gpx) in the retroperitoneal tissue were investigated. The results showed significantly elevated of VLCSFAs, retroperitoneal tissue/ body weight ratio, MDA, Gpx, glucose and UA levels in serum and decreased levels of glutathione in HFD rats compared to the control group. In the group treated with allopurinol the retroperitoneal tissue/ body weight ratio, the levels of MDA, Gpx, VLCSFAs, UA and glucose levels in serum were significantly reduced while glutathione levels were elevated in comparison with HFD rats. The inhibition of xanthine oxidase and UA by allopurinol prevents the development of oxidative changes in adipose tissue. This effect probably suppresses inflammation in adipose tissue, improves insulin sensitivity, reduce VLCSFAs levels and thereby prevent the further lipotoxic liver damage.

Key words: allopurinol, free fatty acids, adipose tissue, xanthine oxidase, oxidative stress.

Introduction
Obesity is a global public health problem commonly associated with metabolic diseases including insulin resistance, type 2 diabetes, cardiovascular disease and nonalcoholic fatty liver disease. Adipocyte dysfunction in obesity is a result of abnormal differentiation of adipocytes, which overload of lipids, leads to the development of hypoxia, oxidative stress and cell damage (Yun et al., 2002). Formation of ROS increased selectively in visceral fat of obese subjects and mice, accompanied by augmented expression of enzymes such as xanthine oxidase (XO), production of uric acid (UA) and decreased expression of antioxidative enzymes (Yamashita et al., 1986; Cheung et al., 2007). The conditions of oxidative stress, adipocyte cell response results in the dysregulated production of
adipocytokines, such as TNF-α, IL-6 and leptin, that cause inflammation and insulin resistance in adipose tissue (Surmi et al., 2008). Insulin resistance and adipocyte dysfunction induce disturbances in lipid metabolism regulation, leading to increased exports of free fatty acids (FFAs) to the liver and peripheral tissues and there results in a decreased insulin sensitivity and ectopic fat deposition. Particularly, FFAs from visceral adipose tissue is directly deposited into the portal vein, increasing the risk of fatty liver disease (Yoshii et al., 2006). Recently, it has been shown that excess serum very long-chain saturated fatty acids (VLCSFAs) (like palmitol or stearin acid) are hepatotoxic and can induce lipoapoptosis and liver damage (Malhi et al., 2006). These facts determines the visceral fat mass increase as a high risk factor for the metabolic complications of obesity (Yoshii et al., 2006). Therefore, the increased oxidative stress in accumulated fat should be an important target for the development of new therapies.

There is evidence that allopurinol reduces blood UA and oxidative stress, prevents the development of some of the characteristics of the metabolic syndrome such as hyperglycemia, hypertriglyceridemia, hyperuricemia and body weight gain in fructose fed rodents (Nakagawa et al., 2006). The influence of allopurinol on liver content of saturated FFAs has not yet been investigated.

In the present study, we suggest that obesity induce oxidative stress in adipose tissue may cause metabolic disorders affecting other organs and tissues by increasing circulating VLCSFAs.

The aim of the study was to determine the influence of allopurinol (xanthine oxidase inhibitors) on oxidative stress in adipose tissue and liver VLCSFAs content in a model of fructose-induced obesity.

Materials and Methods

Animal models

Male albino Wistar rats were housed in a 20+2°C room temperature and with a standard 12-h light/ dark cycle. They all received a standard diet and water ad libitum. The standard diet was composed of starch - 50%, protein - 20%, fat – 4.5%, 5% cellulose, standard vitamins and mineral mix. At the beginning of experiment the body weight of rat was 140-180 g. After acclimatization (two weeks) the animals were divided randomly into three groups: control group (C) - rats, maintained on plain water (n= 7); fructose group (HFD) - rats received 35% high-fructose corn syrup in drinking water for 16 weeks (n= 7) and treatment group (ALL) of HFD and allopurinol administration - 150 mg/kg in drinking water for 16 week. Food intake was recorded daily and their weight was monitored weekly. At the end of the experiments, the rats were killing with lethal dose of thiopental. All manipulations were performed at 4-8°C. Analysis was performed immediately after thawing of the samples. The experimental procedures were approved by the Home Office for Care and Use of Laboratory Animals and performed with a strong consideration for ethics of animal experimentation according to the International Guiding Principles for Animal Research approved in Bulgaria.

Blood and tissue collection

At the termination of the experiment blood was collected from the tail vein of rats after pre-narcosis with Thiopental at a dose of 15mg/kg i.p. Serum obtained (subject to rules of good practice Lab) used to determine some biochemical indicators on the same day, and froze the rest. Under general anesthesia with Thiopental 30mg/kg (rapid intravenous) conducted a laparotomy. We took different organs for research, preparation of homogenate, and then was quickly dissected and frozen. The weight of retroperitoneal fat were measured. Tissue extracts were prepared by homogenisation with buffer PBS-50mM, pH=7.4 (1 g tissue, 9 mL of buffer) inice using ateflon glass homogenizer (2000 rpm/3 min). Homogenates centrifuged in refrigerated centrifuge (4000 rpm/10 min). Supernatant was used for analysis after having identified the concentration of protein.

Biochemical Analyses

The blood concentrations of glucose and were determined by commercial kits (F. Hoffmann - La Roche Inc.) anduric acid (Folin reagent) in serum by spectrophotometric assays was measured.

Determination of total thiols

Tissue thiols were determined by the method of Ellman (Hu, 1994), based on the absorption of the colour complex between thiol groups and DTNB (5,5-bis-dithio/2-nitrobenzoic acid, DTNB) at 412 nm. Standard solutions of reduced glutathione (GSH) were used to calculate the concentration of thiol groups.

Determination of lipid peroxides

Membrane lipid peroxidation was assayed by MDA(malondialdehyde) measured by its thiobarbituric acid (TBA)
(Merk, Germany) reactivity in hepatic homogenates using the method detailed by Porter et al. (1976). Results were expressed in nmol MDA per g tissue and were determined using the extinction coefficient of MDA–TBA complex at 532 nm= 1.56 – 10^{-5} cm^{-1} M^{-1} solution.

**Determination of Glutathione peroxidase activity**

The activity of GSHPx was determined according to Iqbal et al. (2002) with modifications. The activity was assayed by following the oxidation of NADPH at 340 nm for 5 min (25°C) in the presence of GSH-Rd and GSH. The isolated lung mitochondria or liver homogenate (50 µL) was incubated with 25 mM potassium phosphate; 0.5 mM EDTA, pH 7.4; 0.5 mM NaNO_{2}; 0.3 mM NADPH; 0.64 U GSHRd; and 1 mM GSH10 (final volume of 140 µL). The reaction was started with 0.1 mM hydrogen peroxide. Values were corrected for non-enzymatic oxidation of GSH and NADPH by hydrogen peroxide. The results were expressed in units of GSHPx activity using a molar extinction coefficient of 6.22 × 10^{-3} µmol × cm^{-1} for NADPH. The unit was defined as the oxidation of 1 µmol of NADPH/min.

**Fatty Acid Analysis**

**Standards and reagents**

Fatty Acid Methyl Esters (F.A.M.E.) mix standard (Supelco F.A.M.E. Mix C4-C24), nonadecanoic acid and methyl ester nonadecanoic acid standards were purchased from Sigma-Aldrich™ (Buchs, Switzerland). All chemicals used were of analytical, HPLC, or GC grades (Sharlau, Gato Perez, Spain).

**Sample Preparation**

Each collected sample was immediately centrifuged at 4000×g for 10 min and transferred into a clean Eppendorf tube. The homogenate samples were stored at −20°C until analysis. Aliquots (200µL) of supernatant were spiked with internal standard (I.S.) working solution (25 µL C19:0 and 25 µL C19:0 methyl ester) and 2mL 0.4M KOH internal standard (I.S.) working solution (25 µL C19:0 methyl ester) and 2mL 0.4M KOH solution. The reaction was started with 0.1 mM hydrogen peroxide. Values were corrected for non-enzymatic oxidation of GSH and NADPH by hydrogen peroxide. The results were expressed in units of GSHPx activity using a molar extinction coefficient of 6.22 × 10^{-3} µmol × cm^{-1} for NADPH. The unit was defined as the oxidation of 1 µmol of NADPH/min.

30 s and the methyl ester NEFAs were obtained. Samples were evaporated to dryness under N2 gas. Hexane (100 µL) was added to each tube when analysis.

**Gas chromatography-mass spectrometry**

The gas chromatography analysis was performed by a model FOCUS Gas Chromatograph equipped with Polaris Q MS detector (Thermo Scientific, West Palm Beach, USA). The capillary column used was a TR-5 MS (Thermo Scientific, West Palm Beach, USA), 30 m length and 0.25 mm i.d. Helium was used as a carrier gas at a flow rate of 1 mL/minute. Peak identification was done by two parameters: retention time (RT) based on fatty acid methyl esters (FAME) mix standard, and mass spectra (ratio m/z) – compared to the internal Data Base (Thermo Sciences Mass Library; Thermo Corporation, Waltham, USA). FAMEs were quantified by the method of external standard. The FA content of C18:0 (Stearic acid); C20:0 (Arachidic acid); C22:0 (Behenic acid) and C24:0 (Lignoceric acid) was expressed as a percentage of total FAs content (BDS EN ISO 5508:2000).

**Statistical analyses**

All results were expressed as means ± SEM as indicated in the figures and table. Statistical significance of the studied parameters analyzed by Student’s t test. P values of less than 0.05 were regarded as significant. All test were 2-tailed. The statistical procedure was performed with GraphPad In Stat software.

**Results**

All results were expressed as means ± SEM as indicated in the figures and table. Statistical significance of the studied parameters analyzed by Student’s t test. P values of less than 0.05 were regarded as significant. All test were 2-tailed. The statistical procedure was performed with GraphPad In Stat software.

In the fructose fed rats and treated with allopurinol the level of parameters (excluding body weight) were significantly lower as compared to these of the untreated fructose-fed rats. The body weight were not significant decrease in ALL group compared with the HFD group (Table 1). We found marked oxidative stress in adipose tissue in the group of HFD, considering the significantly increased lipid peroxidation (MDA) and Gpx-activity, while the levels of GSH were significantly reduced as compared with the control group (Table 2.). MDA levels in adipose tissue was found to be significantly increased lipid peroxidation (MDA) and Gpx-activity, while the levels of GSH were significantly reduced compared with the control group (Table 2.). MDA levels in adipose tissue was found to be significantly increased lipid peroxidation (MDA) and Gpx-activity, while the levels of GSH were significantly reduced compared with the control group (Table 2.).
be higher by 45% in the HFD group than that of the control group. In fructose-fed rats and treatment with allopurinol were found significantly reduced levels of MDA and Gpx-activity and increased GSH compared with the HFD group (Table 2).

Discussion

Recently, fructose consumption has been suggested to be one of the environmental factors contributing to the development of insulin resistance, obesity, dislipidemia and other abnormalities of the metabolic syndrome (Basciano et al., 2005). The development of obesity and metabolic disturbances in fructose-fed rats is well documented in the literature and was also established in our previous experiments (Bratoeva et al. 2010; Ivanova et al., 2012). Our results show that administration of HFD for 16 weeks leads to the development of obesity, increased of retroperitoneal adipose tissue weight, hyperglycemia and elevated intrahepatic VLCSFAs.

Obesity is an enlargement of adipose tissue to store excess energy intake. It is assumed that the development of obesity in the consumption of fructose is the absence of satiety feeling, leading to hyperphagia, increased food intake, positive energy balance and increased body weight (Bray et al., 2004), which was supported by our results. Obesity and hypertrophy of visceral fat causes adipocyte dysfunction, in which are released from fat tissue proinflammatory cytokines such as TNF-α and IL-6, and the ROS (Furukawa et al., 2004).

Table 1. Markers of metabolic abnormalities measured in the control and experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>C</th>
<th>HFD</th>
<th>ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>initial</td>
<td>143.9 ± 10.6</td>
<td>162.0 ± 7.1</td>
<td>163.9 ± 14.1</td>
</tr>
<tr>
<td>final</td>
<td>250.0 ± 8.1</td>
<td>366.0 ± 21.5*</td>
<td>327.8 ± 7.6*</td>
</tr>
<tr>
<td>Retroperitoneal fat weight (g)</td>
<td>0.65 ± 0.04</td>
<td>1.77 ± 0.08**</td>
<td>1.48 ± 0.08#</td>
</tr>
<tr>
<td>Glucose/serum (mmol/L)</td>
<td>8.14 ± 0.31</td>
<td>10.72 ± 0.56*</td>
<td>8.967 ± 0.15#</td>
</tr>
</tbody>
</table>

Fatty acid/liver

| 18:0  | 1.35 ± 0.53 | 2.71 ± 0.28 | 2.05 ± 0.4 |
| 20:0  | 0.71 ± 0.09 | 2.41 ± 0.26 | 1.21 ± 0.3 |
| 22:0  | 0.74 ± 0.04 | 2.31 ± 0.4  | 1.34 ± 0.5  |
| 24:0  | 0.74 ± 0.08 | 2.37 ± 0.47 | 1.45 ± 0.5  |
| Total VLCSFAs | 0.88 ± 0.15 | 2.45 ± 0.08* | 1.51 ± 0.18** |

Mean levels±SEM; n=7; C- control group rats; HFD-fructose-drinking rats; ALL-fructose-fed rats treated with allopurinol. The weight of retroperitoneal adipose tissue is presented as a percentage of total body weight of the rats.

p < 0.05 - statistical significance between treated and control groups.

p < 0.01 - statistical significance between treated and control groups.

p < 0.05 - statistical significance between ALL and HFD groups.

p < 0.01 - statistical significance between ALL and HFD groups.

Table 2. Markers of oxidative stress and antioxidative capacity measured in the control and experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>C</th>
<th>HFD</th>
<th>ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric acid/serum (µmol/L)</td>
<td>80.94 ± 5.5</td>
<td>315.9 ± 48.2**</td>
<td>81.31 ± 6.28**</td>
</tr>
<tr>
<td>MDA/adipose tissue (mmol/g pr.)</td>
<td>0.046 ± 0.01</td>
<td>0.064 ± 0.02*</td>
<td>0.047 ± 0.004*</td>
</tr>
<tr>
<td>GSH/adipose tissue (mmol/g pr.)</td>
<td>149.9 ± 4.87</td>
<td>125.9 ± 1.56**</td>
<td>136.2 ± 9.00##</td>
</tr>
<tr>
<td>Gpx activity/adipose tissue (U/mL)</td>
<td>0.555 ± 0.03</td>
<td>0.626 ± 0.01**</td>
<td>0.563 ± 0.01##</td>
</tr>
</tbody>
</table>

Mean levels±SEM; n=7; C- control group rats; HFD-fructose-drinking rats; ALL-fructose-fed rats treated with allopurinol. 

p < 0.05 - statistical significance between treated and control groups.

p < 0.01 - statistical significance between treated and control groups.

p < 0.05 - statistical significance between ALL and HFD groups.

p < 0.01 - statistical significance between ALL and HFD groups.

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These products induce insulin resistance in adipose tissue and peripheral through activation of several kinases including IKK, JNK and inhibition of insulin receptor substrate - IRS (Hotamisligil et al., 1996; Aguirre et al., 2002). Our findings demonstrate that allopurinol significantly reduced the hyperglycemia and intrahepatic VLCSFAs in comparison with HFD group and a possible reason for this is retroperitoneal adipose tissue weight loss.

The imbalance between pro- and antioxidant factors has been shown to play an important role in mediating insulin resistance in hypertrophic adipose tissue (Konrad et al., 1999). In obese humans and mice it was found that GSH levels and antioxidant activity of superoxide dismutase, catalase and glutathione peroxidase in adipose tissue are reduced (Furukawa et al., 2004). Therefore, in obesity production of reactive free radicals is maintained at high levels that restrict antioxidant cell capacity. The availability of oxidative stress in this study was confirmed by both the high levels of MDA and Gpx-activity, and the significantly reduced levels of GSH in retroperitoneal adipose tissue in fructose fed rats. Altered activity of the Gpx is probably a compensatory response against oxidative stress (Kensler et al., 2007). Co-administration of allopurinol maintained normal Gpx-activity, restricted depletion of GSH and inhibited the MDA levels.

Several studies have shown that insulin resistance in adipose tissue leads to hyperglycemia, increasing adipocyte lipolysis and releasing of FFAs (Huang et al., 2009; Dela Peña et al., 2005). On the other hand, saturated FFA, exhibit a directly lipotoxicity on liver and β-pancreatic cells, leading to apoptosis and impaired insulin secretion. Donnelly et al. (2005) investigated the origin of saturated FFAs and found that visceral adipose tissue was contributing 82 % of the FFAs pool in the fasting condition and 62 % in the feeding condition. Consequently, the most of hepatic FFAs are the received from adipose tissue. It is well established that the development of Nonalcoholic fatty liver disease is closely linked to an excess flow of saturated FFAs arising from visceral adipose tissue. Chronic lipid supply exceeding the metabolic ability of the liver may induce oxidative liver damage (Wree et al., 2011). Mali et al. (2006) showed that VLCSFAs induce production of free radicals and JNK-dependent hepatocyte apoptosis. Indeed, our previous studies has shown that high-fructose fed rats causes a significant increase of lipid peroxidation and decrease of GSH in rat liver (Bratoeva et al., 2011). Therefore, treatment with allopurinol prevents oxidative stress in adipose tissue, the release of saturated FFAs and thus protects against liver damage caused by the action of VLCSFAs.

Fructose is unique among sugars in that it also results in a marked synthesis of uric acid and superoxide radicals (Johnson et al., 2007). It is known that the consumption of fructose induces the production of the UA in the liver by increased degradation of ATP to AMP by activating AMP-deaminase, result of which produces hydrogen peroxide. The results of our studies show significantly elevated serum levels of UA in fructose fed rats, which have been confirmed in other studies in experimental animal models and human studies (Beck-Nielsen et al., 1980; Thorburn et al., 1989). Tsushima et al. (2013) reported that the XO activity and uric acid production of adipose tissue is augmented in obesity. The free radicals generated during the oxidation process by xanthine oxidase induce lipid peroxidation, resulting in premature cell death (George et al., 2009), which is demonstrated by the high levels of MDA in the retroperitoneal adipose tissue. It is therefore possible that the beneficial effects of allopurinol may be attributed, in part, to the lowering of oxidants rather than an effect on uric acid per se (Nakagawa et al., 2006). In addition, the UA potently reduces endothelial NO levels bioavailability in skeletal muscles, which is known mechanism for inducing insulin resistance (Roy et al., 1998). Therefore, the application of allopurinol observed in our study reduces hyperglycemia by inhibiting production ROS and UA and increasing glucose uptake in skeletal muscles.

Conclusion

Fructose feeding rats negatively affected antioxidant capacity in the retroperitoneal adipose tissue. High serum levels of UA show increased XO-dependent production of ROS in parallel with metabolic changes. These results are consistent with results of other studies which indicated retroperitoneal adipose tissue weight gain, increased lipid peroxides and reduction of antioxidant enzymes, and hyperglycemia. The inhibition of xanthine oxidase and UA by allopurinol prevents the development of oxidative changes in adipose tissue and excessive release and hepatic uptake of VLCSFAs. This effect probably suppresses inflammation, improves insulin sensitivity, reduce VLCSFAs levels and thereby prevent the further lipotoxic liver damage.
References


