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THE EFFECT OF DIETARY-INDUCED OBESITY ON LIPID PEROXIDATION, ANTIOXIDANT ENZYMES AND TOTAL PLASMA ANTIOXIDANT CAPACITY

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The aim of this study was to examine the effect of dietary-induced obesity on some parameters of oxidative stress and antioxidant defence. The studies were performed in adult male Wistar rats. Control group received normal laboratory chow (62% calories as carbohydrates, 26% protein and 12% fat). High-calorie high-fat group (HCHF) was fed standard chow supplemented with lard (48% calories as carbohydrates, 20% as protein and 32% as fat) and high-calorie normal-fat group (HCNF) received standard chow and liquid diet containing sucrose, glucose, whole milk powder and soybean powder (60% carbohydrates, 26% protein, 14% fat). After 8 weeks body weight of HCHF and HCNF-fed rats was higher than body weight of controls by 9.3% and 15.2%, respectively. Plasma concentration of thiobarbituric acid-reactive substances (TBARS) increased in these groups by 43% and 52%, respectively. The activity of superoxide dismutase (SOD) decreased in HCHF group by 47.5% and in HCNF group by 21.1%. Glutathione peroxidase (GPx) activity in the blood tended to increase in both experimental groups but this was not significant. Plasma total antioxidant status (TAS) measuring the combined free radicals scavenging ability of nonenzymatic antioxidants was lower in HCHF and in HCNF group compared to control (−8.8% and −9%, respectively). The major observed lipid abnormalities were hypertriglyceridemia in HCHF group and decreased HDL-cholesterol in HCNF group. TBARS correlated negatively with SOD ($r = -0.84$, $p < 0.001$) and with TAS ($r = -0.47$, $p < 0.05$). These results indicate that obesity leads to oxidative stress which can contribute to obesity-associated diseases such as atherosclerosis, diabetes mellitus and arterial hypertension.

Key words: *obesity, oxidative stress, free radicals, antioxidant enzymes.*

INTRODUCTION

Obesity is a risk factor of atherosclerosis. This risk is attributed in part to hyperlipidemia, arterial hypertension and non insulin-dependent diabetes mellitus often accompanying obesity, however, obesity is also an independent risk factor (1—3). This direct atherogenic effect of obesity may be associated

with several mechanisms: 1) insulin resistance/hyperinsulinemia (4, 5); 2) disturbed balance between coagulation and fibrinolysis cascade due to overproduction of plasminogen activator inhibitor-1 (6) and tissue factor (7) by adipose tissue; 3) excess of proinflammatory cytokines secreted by adipose tissue, such as tumor necrosis factor-α (TNF-α) (8, 9) and interleukin-6 (10); 4) stimulation of renin-angiotensin system (11) which participates in atherogenesis (12).

Interestingly, little is known about the effect of obesity on oxidant--antioxidant balance Reactive oxygen species and lipid peroxidation play an essential role in the pathogenesis of atherosclerosis. Oxidized low density lipoproteins (LDLs) are taken up by macrophages through scavenger receptors leading to intracellular cholesterol accumulation and formation of foam cells. Oxidatively modified LDLs are chemotactic for monocytes and vascular smooth muscle cells and induce their proliferation, partially by stimulating expression of adhesion molecules, cytokines and growth factors. In addition, oxy-LDLs are cytotoxic for endothelial cells and induce humoral as well as cell-mediated immune response (13—15). Moreover, free radicals play an important role in the pathogenesis of other obesity-associated complications such as diabetes mellitus, endothelial dysfunction, arterial hypertension and heart failure (16).

The purpose of this study was to examine the effect of obesity induced by high-calorie diet on lipid peroxidation, antioxidant enzymes activity and total antioxidant potential of plasma in the rat.

MATERIALS AND METHODS

Induction of obesity

The study was carried out on adult (3 month-old), male Wistar rats weighing initially 351.4 ± 18.1 g (mean ± SD). The protocol of animal studies was reviewed and approved by Animal Care Committee of University School of Medicine in Lublin. Obesity was induced by offering the animals two types of "cafeteria diet" as described by Kahn and Pedersen (17). After the 2-week baseline period the animals were randomized into three groups (10 rats each). Control group was fed standard rat chow *ad libitum*. One experimental group received high calorie high fat diet (HCHF) consisting of standard rat chow supplemented with lard. Another group received high calorie diet with percentage fat content similar to control group (high calorie normal fat group, HCNF). This group was fed standard chow combined 1 : 1 (w/w) with the liquid diet. The liquid diet contained sucrose (12.5 g/100 g), glucose (12.5 g/100 g), whole milk powder (12.5 g/100 g) and soybean powder (12.5 g/100 g) suspended in tap water (50 g/100 g). The energy content of diets was calculated from the declarations given by the manufacturers and is presented in Tab. 1. All diets contained also the same amounts of vitamin mixture (2 g/100 g), mineral mixture (5 g/100 g), D,L-methionine (0.3 g/100 g) and choline chloride (0.2 g/100 g). Vitamin mixture used by us (Polfamix F, Polfa Kutno, Poland) contained vitamin E (3.4 g/kg) but not other antioxidant vitamins. Neither vitamin mixture nor mineral mixture (MM, Polfa Kutno, Poland) contained any significant amounts of selenium. The average daily energy intake was measured weekly by weighing the amount of chow served and correcting for wastes.. Body weight of animals was controlled every

2 weeks. All the groups were fed their respective diets for 8 weeks. The animals had free access to tap water throughout the experiment.

After 8 weeks the animals were anaesthetized with pentobarbital (50 mg/kg i.p.). Then blood was obtained from abdominal aorta and collected in heparinized tubes (for SOD, GPx and TAS) and EDTA-containing tubes (for TBARS, and plasma lipids). The animals were killed by the lethal dose of pentobarbital. Antioxidant enzymes, plasma lipids and haemoglobin were measured immediately and plasma for TBARS and TAS was frozen and stored at −25°C.

TBARS assay

The concentration of thiobarbituric acid-reactive substances (TBARS) was assayed by the method of Ledwożyw *et al.* (18). The blood was centrifuged at 3000 × g for 10 min. 0.5 ml of plasma was mixed with 2.5 ml of 1.22 M tricholoroacetic acid in 0.6 M HCl and allowed to stand for 15 min. Then 1.5 ml of thiobarbituric acid (46 mM in 80 mM NaOH) was added and the sample was heated for 30 min in a boiling water bath. After cooling to a room temperature 4 ml of n-butanol was added and the sample was shaken vigorously for 3 min and centrifuged for 10 min at 1500 × g. The organic layer was removed and its absorbance was measured at 532 nm. Standard curve was prepared using malonyldialdehyde bis-dimethylacetal.

Superoxide dismutase (SOD) assay

SOD activity was measured in erythrocytes by the spectrophotometric method using RANSOD kit (Randox Laboratories Ltd., UK), based on the method of McCord and Fridovich (19). In this method xanthine and xanthine oxidase are used to generate superoxide anion (O_2^-) which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD inhibits the formation of the formazan dye, and its activity is measured as percent inhibition and compared with a calibration curve prepared with purified SOD contained in the kit. Briefly, 0.5 ml of blood was centrifuged for 10 min at 3000 × g, plasma was aspirated and erythrocytes were washed four times with 3 ml of physiological saline and centrifuged for 10 min at 3000 × g after each wash. Then 2 ml of cold deionized water was added to induce hemolysis. The lysate was diluted 50 fold with 10 mM phosphate buffer (pH 7.0). 0.05 ml of the diluted sample was mixed with 1.7 ml of substrate (0.05 mM xanthine + 0.025 mM INT in 40 mM CAPS buffer containing 0.94 mM EDTA, pH 10.2) and with 0.25 ml of xanthine oxidase (80 U/l). The sample was incubated at 37°C and its absorbance against air was read at 505 nm after 30 and 150 seconds. One unit of SOD is the amount of enzyme that inhibits the rate of the formazan dye formation by 50%. The intraassay coefficient of variation was 4.2%. The assay was linear from 30 to 60% inhibition. SOD activity was expressed in units/g haemoglobin. Haemoglobin concentration was determined by the cyanmethaemoglobin method (20).

Glutathione peroxidase (GPx) assay

Blood GPx was assayed by the modified method of Paglia and Valentine (21) using RANSEL kit (Randox Laboratories Ltd., UK). In this method GPx catalyzes the oxidation of glutathione (GSH) to the oxidized form (GSSG) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, GSSG is converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. Decrease in NADPH is measured by monitoring the absorbance at 340 nm. 0.05 ml of blood was diluted to 2 ml with diluent contained in the kit and 0.05 ml of this sample was mixed with 2.5 ml of solution containing 4 mM glutathione, 0.5 U/l glutathione reductase, 0.34 mM NADPH, 0.05 M phosphate buffer (pH 7.2) and 4.3 mmol/l EDTA. Subsequently 0.1 ml of 4.86 mM cumene hydroperoxide was added. The sample was incubated at 37°C and absorbance

against air was monitored for 3 minutes. Finally, the enzyme activity was expressed in U/g haemoglobin.

Total antioxidant status (TAS)

Total antioxidant capacity of plasma was assessed by the method described by Miller *et al.* (22, 23) using Total Antioxidant Status — TAS kit (Randox Laboratories Ltd., UK). In this method 2,2'-azino-di-3-ethylbenzothiazoline-6-sulphonate (ABTS) is incubated with peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation ($ABTS^+$) which absorbance is measured at 600 nm. The ability of antioxidants contained in the sample to inhibit this reaction is measured and compared to standard — 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), therefore this method is also called Trolox equivalent antioxidant capacity (TEAC). In brief, 20 µl of plasma was mixed with 1 ml of chromogen (6.1 µmol/l metmyoglobin +610 µmol/l ABTS) and 200 µl of 1250 µmol/l H_2O_2 was added. The sample was incubated at 37°C and absorbance at 600 nm was read after 3 minutes. The results are expressed in mmol Trolox/l.. Unlike measuring the concentration of individual antioxidants, this method also takes into account the interactions among them. Plasma albumin, uric acid and SH-group containing low-molecular weight compounds such as glutathione are the main known contributors to plasma TAS assay (24).

Plasma lipids

Plasma triglycerides (TG) were assayed by peroxidase-coupled method (25), total plasma cholesterol (TC) by the enzymatic method (26) and HDL-cholesterol (HDL-C) by the enzymatic method after precipitation of other lipoproteins with $MgCL_2$ and dextran sulphate (27). VLDL+LDL cholesterol was calculated from the above data using a modified Friedewald formula: $VLDL\text{-}C + LDL\text{-}C = TC - (HDL\text{-}C + 0.16 \times TG)$ (28).

Reagents

Vitamin mixture (Polfamix F) and mineral mixture (MM) were obtained from Polfa Kutno, Poland. D,L-methionine, choline chloride, and 2-thiobarbituric acid were purchased from Sigma-Aldrich.

Statistics

The data are presented as mean ± SD. Statistical analysis was performed by ANOVA followed by Duncan's multiple range test for comparisons of different means. Linear regression was used to assess the relation between variables. To be considered statistically significant, we required $P < 0.05$ (95% confidence limits).

RESULTS

The energy intake during baseline period in all groups averaged 101 ± 5 kcal/rat/day. Rats who remained on the control diet did not significantly alter their intake over subsequent 8-week period. In contrast, both types of cafeteria diet prompted the animals to increased food intake. The average energy intake in both experimental groups was similar (*Tab. 1*).

Table 1. Composition of diets and average energy intake.

	Control group	High calorie high fat group	High calorie normal fat group
Carbohydrates (% of energy)	62	48	60
Protein (% of energy)	26	20	26
Fat (% of energy)	12	32	14
Total energy itake (kcal/rat/day)	101 ± 5	130 ± 8	132 ± 7

Body weight of all groups of animals increased progressively throughout the experimental period. After 8 weeks control animals weighted 388 ± 19.8 g which was significantly higher than at the beginning of the experiment ($+10.5\%$, $p < 0.05$). The average body weight of rats fed high fat diet and high calorie normal fat diet increased during 8 weeks to 424.7 ± 35 g and 447.7 ± 35.4 g, respectively, and was significantly higher than terminal body weight of controls ($+9.3\%$, $p < 0.05$ and $+15.2\%$, $p < 0.001$, respectively). These data indicate that our experimental protocol induced moderate obesity. The difference in body weight between both high-calorie groups was not significant.

The concentration of TBARS in plasma was higher in obese animals than in controls. HCHF and HCNF diets caused elevation of TBARS by 43% and 52%, respectively (*Fig. 1*). The difference between both obese groups was not significant. In contrast to TBARS, SOD activity decreased in both

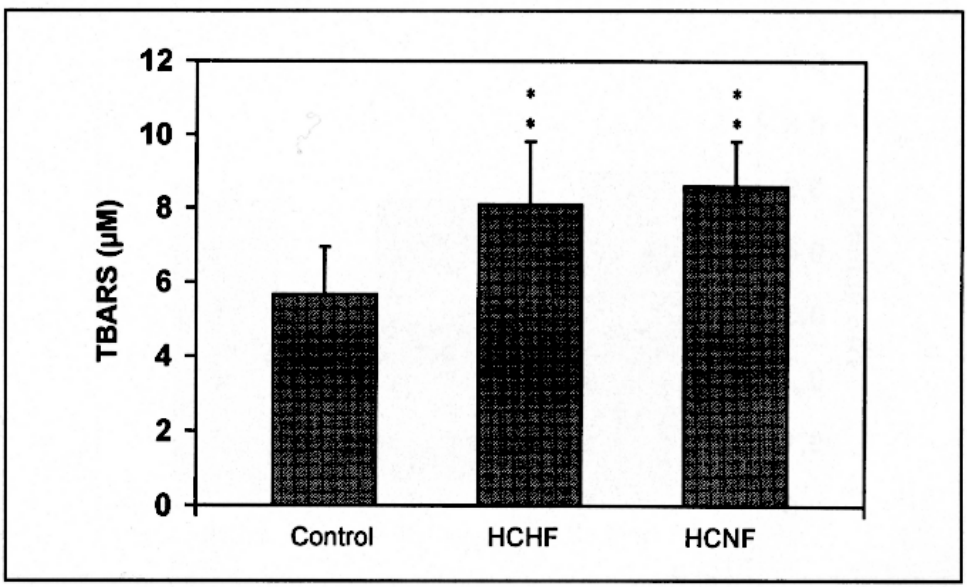

Fig. 1. Plasma concentration of thiobarbituric acid-reactive substances (TBARS). HCHF-high calorie high fat group, HCNF-high calorie normal fat group. ** $p < 0.01$ (compared to control by ANOVA and Duncan's test). $n = 10$ in each group.

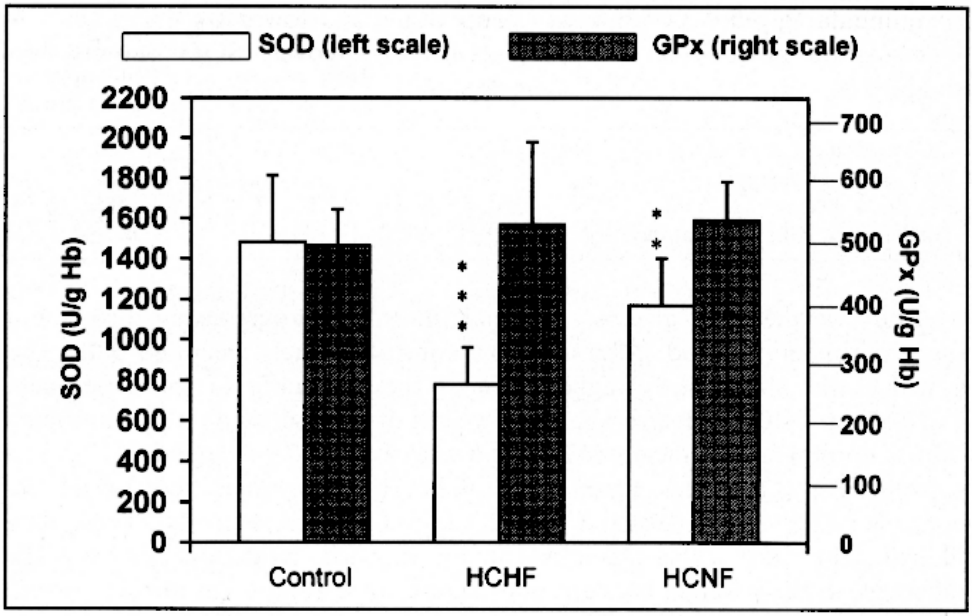

Fig. 2. The activity of antioxidant enzymes: superoxide dismutase (SOD, left scale) and glutathione peroxidase (GPx, right scale) in the blood. HCHF-high calorie high fat group, HCNF-high calorie normal fat group. ** $p < 0.01$; *** $p < 0.001$ (compared to control by ANOVA and Duncan's test). n = 10 in each group.

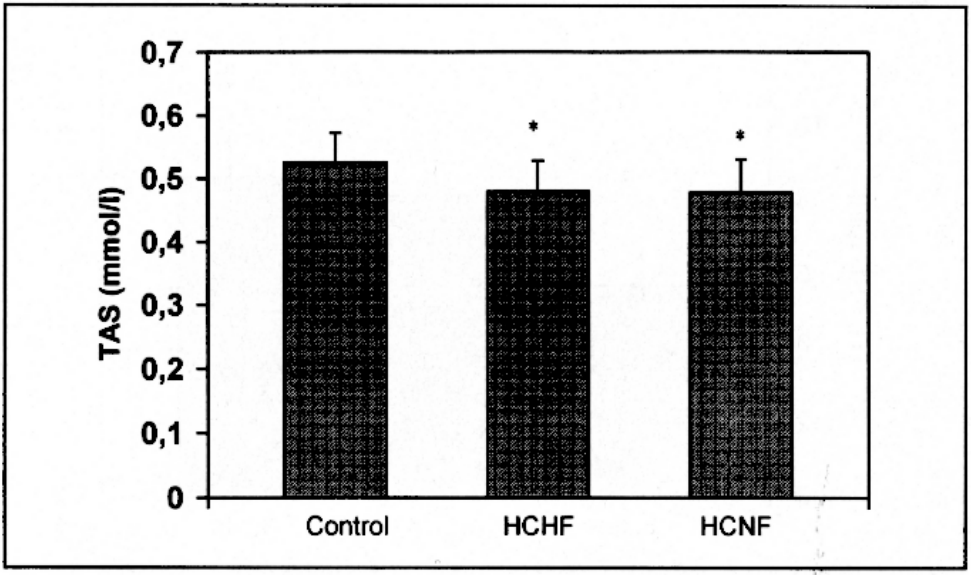

Fig. 3. Plasma total antioxidant status (TAS). HCHF-high calorie high fat group, HCNF-high calorie normal fat group. * $p < 0.05$ (compared to control by ANOVA and Duncan's test). n = 10 in each group.

experimental groups. In high fat group SOD was lower by 47.5% and in high calorie normal-fat group by 21.1%. Moreover, SOD activity was significantly lower in HCHF group than in HCNF group (−23.5%, $p < 0.01$). Although we observed the tendency to elevation of GPx activity in both experimental groups, this phenomenon was not significant (*Fig. 2*). Total antioxidant status decreased slightly but significantly both in high fat and in high calorie normal fat group by 8.8% and 9%, respectively (*Fig. 3*).

Plasma triglycerides increased significantly only in high fat group. Plasma obtained from two rats in this group was lipemic and triglycerides concentration in these samples exceeded 160 mg/dl. When all the animals from this group were taken into account plasma TG was 54% higher than in controls. If these two lipemic samples were excluded from the analysis, mean plasma TG concentration was elevated by 31.5%, which was still significant (*Tab. 2*). Total plasma cholesterol in HCNF group was lower than in controls (−20.8%) whereas in high fat group was not changed. The concentration of cholesterol contained in HDL fraction was also lower in HCNF group (−21.6%) but not changed in HCHF group. The mean LDL+VLDL cholesterol tended to increase in high fat group (+56.3%) and to decrease in high calorie normal fat group (−50%) but due to high variability these differences did not reach the level of significance. The average atherogenic ratio (VLDL+LDL divided by HDL) was almost doubled in HCHF group whereas in HCNF group tended to decrease but again, this was not significant (*Tab. 2*). The TBARS/TG ratio increased significantly in HCNF group (36.9±6.2 nmol/mg vs. 29.3±3.1 nmol/kg in controls, $p < 0.05$) but not in HCHF group (27.3±4.8 nmol/mg). Plasma glucose concentration did not differ among control and experimental groups (data not shown). Total plasma protein, haemoglobin concentration and platelet count was similar in all groups (data not shown).

Table 2. Plasma lipids concentration in different groups of rats.

	Control group	High calorie high fat group	High calorie normal fat group
Triglycerides	49.2±12.7	64.7±15.8 *	50.9±13.4
Total cholesterol	79.8±19.4	83.0±10.7	62.5±4.9 *
HDL-cholesterol	66.2±13.0	64.9±8.29	51.9±4.7 **
VLDL+LDL cholesterol	4.8±3.6	7.5±4.6	2.4±2.0
(VLDL+LDL)/HDL	0.062±0.052	0.122±0.077	0.046±0.041

* $p < 0.05$; ** $p < 0.01$ (compared to control by ANOVA and Duncan's test). n = 10 in control and high calorie normal fat group, n = 8 in high fat group (two lipemic plama samples with TG level > 160 mg/dl were excluded from this group).

When data from all three groups were analyzed together, TBARS demonstrated strong inverse correlation with SOD ($r = -0.84$, $p < 0.001$) and moderate inverse correlation with TAS ($r = -0.47$, $p < 0.05$) but not with GPx. The correlation between TBARS and TG was not significant ($r = 0.15$, $p = 0.54$). TBARS tended to correlate negatively with total plasma cholesterol, HDL-cholesterol and VLDL+LDL cholesterol, but neither of these relationships reached the level of significance. SOD activity was positively correlated with HDL-cholesterol ($r = 0.58$, $p = 0.018$). Body weight correlated significantly only with plasma triglycerides ($r = 0.54$, $p = 0.031$) but not with either other lipid parameters or oxidant-antioxidant parameters.

DISCUSSION

Measurement of TBARS concentration, although non-specific, is widely used as an indicator of lipid peroxidation process and indirectly, of oxidative stress. Increased TBARS together with alterations of antioxidant mechanisms reported in this study indicates that oxidative stress is associated with dietary-induced obesity, at least in the rat. Interestingly, in our study the difference of body weight between control and experimental groups was rather moderate whereas changes in TBARS and SOD activity were very pronounced. TBARS, GPx and TAS values were similar in both obese groups irrespectively of administered diet whereas SOD activity was more severely affected in HCHF group.

Only few previous studies addressed the problem of oxidative stress in obesity. Congenitally obese (ob/ob) mice are characterized by increased concentration of lipid peroxidation products in plasma, liver and brain and increased susceptibility of hepatic microsomal fraction to undergo peroxidation in vitro (29). They also demonstrate increased brain and blood glutathione peroxidase and decreased GPx, glutathione reductase and catalase activity in the liver (30). Cu,Zn-SOD in liver and kidney and hepatic glutathione transferase activity are lower in ob/ob mice than in their lean littermates (31). Increased concentration of lipid peroxidation products and stimulation of manganese SOD activity has been described in myocardium of Zucker fatty rats (32). These data were obtained in specific models of obesity: leptin deficiency (ob/ob mice) or defective leptin receptor (Zucker fatty rats). However, together with our findings obtained in animals made obese by cafeteria diet they support the hypothesis that oxidative stress is associated with obesity itself irrespectively of underlying cause.

The cause of increased free radicals formation and consequently of enhanced lipid peroxidation in obesity can not be deduced from our study. Malonyldialdehyde (MDA), the main component of plasma TBARS, originates

from several sources: 1) peroxidation of plasma lipids, 2) blood platelets, 3) peroxidation of lipids in endothelial and other cells (33). Therefore the mechanism of increased TBARS in obesity may be multifactorial. First, high calorie diet may stimulate mitochondrial oxidative metabolism and increase leakage of electrons from mitochondrial respiratory chain (34). The opposite, i.e. decreased mitochondrial free radicals formation and improvement of antioxidant defence was observed following caloric restriction (35). Second, free radical cascade may be triggered by activated phagocytes which generate oxygen free radicals during respiratory burst (36). Adipocytes secrete TNF-α — a potent stimulator of phagocytes respiratory metabolism (37) and the expression of this cytokine increases in obesity (8). Adipose tissue is also the source of interleukin-6 (IL-6) — another proinflammatory cytokine (10). Plasma concentration of both TNF-α and IL-6 increases in obese rodents and humans (9, 38, 39). Consequently it is conceivable to assume that obesity is associated with chronic low-level inflammation (40). Indeed, increased adhesiveness and superoxide production by granulocytes have been demonstrated in obese humans (41).

Altered plasma lipid profile could also contribute to increased lipid peroxidation. Both experimental groups have demonstrated different lipid abnormalities. In HCHF group we have observed hypertriglyceridemia and, although non-significant, tendency to elevation of VLDL + LDL cholesterol. Interestingly, the amount of cholesterol contained in HDL fraction did not change in this group, whereas it decreased significantly in HCNF group. In contrast to HCHF group, plasma triglycerides were normal and VLDL + LDL cholesterol tended to decrease in HCNF-fed animals. At present we can not explain why HDL fraction behaved differently in both experimental groups. However, lack of detrimental effect of HCHF diet on HDL cholesterol should not suggest that this type of diet is more beneficial than high-calorie diet with normal fat content. In particular, plasma triglycerides and VLDL + LDL cholesterol changed in an unfavourable manner in HCHF-fed animals. SOD activity was also more profoundly affected in HCHF than in HCNF group. In addition, we have assayed only total HDL cholesterol without analysis of its subfractions. Some HDL particles, which contain apoprotein E, can bind to LDL receptors as well as to scavenger class B type I receptors and may be atherogenic (42). The concentration of HDL with apo-E increases following high-fat diet and could be elevated in HCHF group.

Hypertriglyceridemia observed by us in HCHF group could contribute significantly to oxidant-antioxidant imbalance. It may increase lipid peroxidation simply by an excessive availability of substrates - fatty acids contained in plasma triglycerides, since TBARS/TG ratio in this group did not differ from control.. Plasma lipid peroxides concentration is significantly higher in patients with hyperlipidemia (43, 44). Moreover, hypertriglyceridemia has

been demonstrated to increase oxidative metabolism and release of superoxide anion by polymorphonuclear leukocytes (45) and monocytes (46, 47). Increased concentration of lipid peroxidation products was described in sucrose-fed rats (48) and fructose-fed rats (49) — two models of hypertriglyceridemia not accompanied by obesity.

Hypercholesterolemia is also associated with elevated plasma lipid peroxides concentration (43, 50). Dietary-induced hypercholesterolemia in the rabbit stimulates superoxide anion production by leukocytes (50) and by endothelial cells (51). Urinary excretion of isoprostanes — the marker of in vivo lipid peroxidation is increased in patients with hypercholesterolemia (52). However, these results should not be extrapolated to rats which, unlike rabbits and humans, have very low plasma LDL concentration. Dietary-induced hypercholesterolemia in the rat is not associated with changes in MDA level either in plasma or in liver homogenates (53, 54). In contrast to our results, hypercholesterolemia in the rat increased erythrocytes SOD and decreased GPx activity (54). In addition, TBARS tended to correlate negatively with VLDL+LDL cholesterol in our study. Thus it is unlikely that hypercholesterolemia contributed significantly to oxidative stress in HCHF-group.

In HCNF group TBARS/TG ratio clearly increased indicating that lipid peroxidation resulted from causes other than availability of substrate. This group was also characterized by lowering of total and HDL cholesterol. Negative correlation between TBARS and HDL together with known antioxidant properties of this lipoprotein fraction (55) suggest that decreased HDL could contribute to enhanced lipid peroxidation. However, since changes in TBARS, antioxidant enzymes and TAS are similar and of similar magnitude in both obese groups, it seems that in general obesity itself is more important in inducing oxidative stress than lipid abnormalities, which were clearly different in both groups.

One of sources of plasma MDA may be platelet arachidonate metabolism (56). The aggregability of platelets is increased in hyperlipidemia and obesity (43, 52, 57, 58). Although platelet count was normal in our model of obesity we can not exclude that platelet hyperactivity contributed substantially to increased lipid peroxidation.

We have observed a negative correlation between plasma TBARS level and SOD activity. Decreased SOD may be secondary to enzyme inactivation by oxygen free radicals but this mechanism probably can not solely explain lower dismutase activity because the level of TBARS was similar in both experimental groups whereas SOD was more affected in high fat group. Alternatively TBARS may arise from primarily impaired SOD scavenging activity.

In conclusion, this study demonstrates that dietary-induced obesity results in oxidative stress, as evidenced by increased plasma level of lipid peroxidation

products, exhaustion of nonenzymatic antioxidants and changes in the activity of antioxidant enzymes. Oxidative stress can contribute to such complications as atherosclerosis, arterial hypertension, endothelial dysfunction and diabetes mellitus, which often accompany obesity.

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