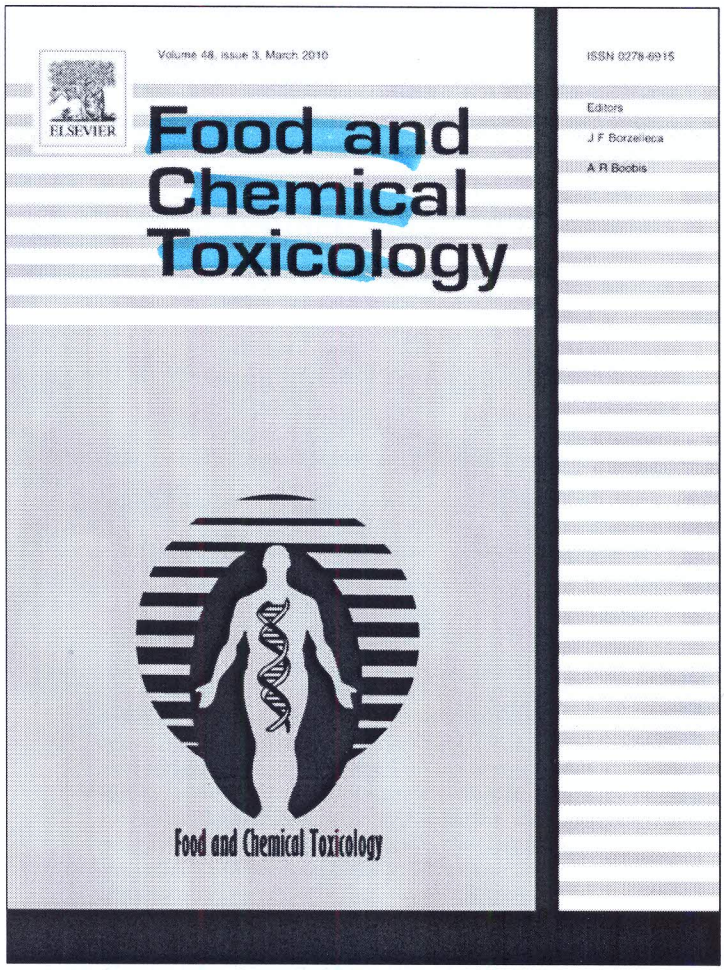

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Chronic high fat, high cholesterol supplementation decreases 18 kDa Translocator Protein binding capacity in association with increased oxidative stress in rat liver and aorta

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ABSTRACT

It is well known that high fat and high cholesterol levels present a contributing factor to pathologies including fatty liver and atherosclerosis. Oxidative stress is also considered to play a role in these pathologies. The 18 kDa Translocator Protein (TSPO), formerly known as the peripheral-type benzodiazepine receptor, is known to be involved in cholesterol metabolism, oxidative stress, and cardiovascular pathology. We applied a high fat high cholesterol atherogenic (HFHC) diet to rats to study correlations between cardiovascular and liver pathology, oxidative stress, and TSPO expression in the liver and the cardiovascular system. This study corroborates the presence of increased oxidative stress markers and decreased anti-oxidants in liver and aorta. In addition, it appeared that induction of oxidative stress in the liver and aorta by atherogenic HFHC diet was accompanied by a reduction in TSPO binding density in both these tissues. Our data suggest that involvement of TSPO in oxidative stress and ROS generation, as reported in other studies, may also take part in atherogenesis as induced by HFHC diet. Presently, it is not clear whether this TSPO response is compensatory for the stress induced by HFHC diet or is a participant in the induction of oxidative stress.

1. Introduction

A large number of cardiovascular deaths (75%) can be attributed to atherosclerosis, a progressive inflammatory and degenerative disease that affects large and medium sized arteries, including deposition of fibrotic and lipid-containing plaques (Costopoulos et al., 2008). Known causes for atherosclerosis include congenital hyperlipoproteinemias, metabolical hyperlipidemia, gender, age, arterial hypertension, and diabetes (Bergeron and Havel, 1997). Risk factors include the impact of lipid peroxides present as metabolites (Esterbauer et al., 1985). Regarding the pathogenesis of age-dependent degenerative disorders such as atherosclerosis, various studies have shown the involvement of oxidative stress (Janero, 1990; Feillet-Coudray et al., 2009). The development of atherosclerosis includes accumulation of fat in the liver, followed by excessive ROS production by mitochondria in the liver, resulting in lipid peroxidation (Oliveira et al., 2006; Matsuzawa et al., 2007). Free-radical generation appears to be a common event in humans and experimental animals with fatty liver regardless of the underlying etiology (Domenicali et al., 2005). It was recently shown that patients with nonalcoholic fatty liver disease (NAFLD) have endothelial dysfunction potentially responsible for cardiovascular disease in long term and that enhanced oxidative stress, inflammation and abnormal lipoprotein metabolism, could account for the proatherogenic processes (Villanova et al., 2005; Lizardi-Cervera and Aquilar-Zapata, 2009).

In general, an increase in the generation of reactive oxygen species (ROS) as it occurs with oxidative stress can lead to an inflammatory response in the cardiovascular system, including damage to endothelial cells and oxidative damage to cellular macromolecules (Plaa and Witschi, 1976; Muller, 2005). For example, advanced oxidation protein products (AOPPs) that have been

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correlated with atherosclerosis, can be involved in the proliferation of vascular smooth muscle cells (Liu et al., 2006; Li et al., 2007). Oxidative stress marker levels are mostly analyzed in the plasma, for example in patients with chronic inflammatory disease and in various animal models (Himmelfarb et al., 2000; Li et al., 2007). However, an important consideration should be that the relevant oxidative processes primarily take place in the blood vessel wall intimae (Stenvinkel et al., 2003, Woods et al., 2003; Stocker and Keany, 2004) and in the liver (Kirsch et al., 2003; Kitade et al., 2006). Interestingly, the pharmacokinetic analysis of clearance of protein oxidation products by Iwao et al. (2006) showed that scavenger receptors are expressed most in sinusoidal endothelial cells and Kupffer cells in the liver, suggesting that the liver is the main route for plasma clearance of AOPPs in relation to potential development of atherosclerosis.

It is well known that mitochondria are a main source of cellular ROS (Lenaz, 1998; Murphy, 2009). Therefore, we hypothesize that mitochondrial activity in the liver and aorta may play important roles in the development of atherosclerosis, including enhanced generation of ROS correlated with impaired mitochondrial cholesterol metabolism. Recent studies by us and others suggest that the mitochondrial 18 kDa Translocator Protein (TSPO), also known as peripheral-type benzodiazepine receptor (PBR), may be involved in several aspects of cardiovascular disease (Veenman and Gavish, 2006). The TSPO is ubiquitously and inhomogeneously expressed within and among tissues throughout the body, including the cardiovascular system and the liver (Gavish et al., 1999; Veenman and Gavish, 2006). In the cardiovascular lumen, TSPO are present in platelets, erythrocytes, lymphocytes, and mononuclear cells (Canat et al., 1993; Maeda et al., 1998; Veenman and Gavish, 2006). In the walls of the cardiovascular system, TSPO can be found in the endothelium, the striated cardiac muscle, the vascular smooth muscles, and the mast cells (Taniguchi et al., 1980; French and Matlib, 1988; Stoebner et al., 1999; Veenman and Gavish, 2006). In the liver, TSPO is found in mitochondria of hepatocytes, as well as in other type liver cells such as hepatic stellate cells and parenchymal cells (Woods et al., 1996; Fischer et al., 2001). It is well established that TSPO's primary intracellular location is the outer mitochondrial membrane (O'Beirne et al., 1990; Krueger and Papadopoulos, 1990; Veenman et al., 2007). Various studies have indicated that mitochondrial TSPO is involved in the regulation of cholesterol transport into mitochondria in relation to bile production and steroidogenesis, in oxidative stress and apoptosis, in inflammatory and immune responses, and in stress responses (Gavish et al., 1992; Knudsen et al., 1993; Papadopoulos et al., 2006; Veenman and Gavish, 2006; Veenman et al., 2007; Dimitrova-Shumkovska et al., 2010). These data suggest that the TSPO may be involved in cardiovascular diseases, including oxidative stress related to atherosclerosis. Recently, we found that the TSPO appears to be an active participant in the generation of ROS and maintenance of the mitochondrial membrane potential (Veenman et al., 2008; Kugler et al., 2008; Shoukrun et al., 2008; Zeno et al., 2009). On the other hand, ROS may participate in the regulation of TSPO function (Delavoie et al., 2003). In the liver, the TSPO was found in colocalization with a ROS chelator, the mitochondrial manganese-dependent superoxide dismutase (SOD) (Fischer et al., 2001). It has been suggested that TSPO's known interactions with the mitochondrial respiration chain and related electron transport and be part of TSPO's ability to generate ROS (Veenman et al., 2010a,b).

To substantiate the postulation that TSPO activation in association with oxidative stress in the liver may be a contributing factor to atherosclerosis, we applied a high fat, high cholesterol (HFHC) diet to rats. Various studies have shown that such a diet can induce steatohepatitis and atherosclerosis with hyperlipidemia and oxidative stress in rodents (Paigen et al., 1987, Paigen, 1995; Laine et al., 2004; Lorkowska et al., 2006; Matsuzawa et al., 2007; Feillet-Coudray et al., 2009). Thus these studies present a solid model to study TSPO involvement in oxidative stress related to these aspects of cardiovascular disease. We measured effects of HFHC diet on aorta and liver morphology i.e. pathology, as well as on TSPO binding characteristics in these organs. We also measured lipid levels in the plasma potentially related to the induction atherosclerosis in this model. To establish potential correlations between changes in TSPO binding characteristics and oxidative stress in the target organs, we measured parameters of oxidative stress, including lipid peroxidation, protein carbonylation, and expression of ROS chelators, in the liver, plasma, and aorta.

2. Material and methods

2.1. Research animals and materials

Wistar rats were obtained from the Military Medical Academy (VMA, Belgrade, Republic of Serbia) and maintained and bred in our own animal facilities at the Department of Physiology and Biochemistry of the Faculty of Natural Sciences and Mathematics, Skopje, Republic of Macedonia. All procedures with the animals were in accordance with National Institutes of Health (USA) guidelines for the care and use of experimental animals (NIH Publication No. 85-23, revised 1996), and the experimental protocol was reviewed and approved by the local ethics committee. Rat feed was from Filpaso (Skopje, Republic of Macedonia). [^{3}H]PK 11195 was obtained from New England Nuclear (Boston, MA) and PK 11195 from Sigma-Aldridge (Rehovot, Israel). Enzyme-colorimetric test kits were from Human Diagnostics (Wiesbaden, Germany). The SOD determination kit RA20408 was from Fluka-Biochemika (Steinheim, Germany). The glutathione assay kit and glutathione reductase assay kit were from Sigma–Aldrich (Steinheim, Germany). Trichloracetic acid (TCA), 2-thiobarbituric acid (TBA), trifluoroacetic acid (TFA), 1,1,3,3 tetraethoxypropane (TEP), 5,5′-dithio2-nitrobenzoic acid (DTNB), T-Chloramine, Phosphate buffer solution (1 M) were from Sigma–Aldrich (Steinheim, Germany). 2,4 dinitrophenylhydrazine (DNPH), Folin–Ciocalteu reagent, Guanidine chloride, ascorbic acid and ammonium iron (II) sulphate ($(NH4)_2$ Fe $(SO_4)_2$ were from Merck (Darmstadt, Germany). Standard chemicals and consumables were obtained from various commercial sources.

2.2. High fat high cholesterol (HFHC) atherogenic treatment

Male *Wistar* rats, 22 weeks old at the start of the experiment, were used. The rats were kept on a regular dark/light cycle, and received water and regular or supplemented chow ad libitum. Prior to the experimental procedures, the rats were fed a standard commercial pellet feed (Filpaso, 52.11). The components of the commercial feed as listed by the manufacturer were: crude fat, minimum 5.7%; crude proteins, minimum 18%; carbohydrates, minimum 60%; fiber, maximum 4.5%; ash, maximum 8%. Before application of the HFHC diet, the rats were randomized into two groups: (1) control rats (*C-rats*) receiving mentioned commercial pellet feed throughout the experiment ($n = 18$); (2) experimental rats (*HFHC-rats*) receiving normal chow supplemented with 3% cholesterol, 7.5% cocoa butter, 0.5% sodium cholate, 20% butter fat salt free (custom tailored HFHC diet, a modification of the original ICN Atherogenic diet for mice and rats; Research Diets Cat. No.900865, supplied by Filpaso), 10–15 g per day/per animal for a period of 18 weeks ($n = 20$). As this atherogenic diet is characterized by high fat, high cholesterol content, the acronym for this group of rats is *HFHC-rats*.

2.3. Tissue collection

For sacrifice by exsanguinations, the animals were heavily anesthetized with a ketamine:xylazine mixture (90 mg/kg, ip and 10 mg/kg, ip, respectively) applied after an overnight fast of 12 h. For plasma separation, blood samples from the exsanguinations were collected into tubes with anticoagulant solution (heparin). Separated plasma was stored at −80 °C for 6–8 weeks until further analysis. Tissue samples were removed immediately, flash frozen, weighted, and stored at −80 °C until further analysis. The samples of blood, liver, heart, aorta, and testes were harvested between 10 and 12 AM. Tissue homogenates (liver, aorta, heart, and testis) were prepared for our various assays. For assays of the oxidative stress markers: [thiobarbituric acid reactive substances (TBARs), glutathione (GSH), protein carbonyls (PC) superoxide dismutase activity (SOD), glutathione reductase (GSSG-Red)], tissue homogenates were prepared in 1.12% KCl at +4 °C. For advanced oxidation protein products (AOPPs), tissue homogenates were prepared in 50 mM PBS at +4 °C. For these homogenates we used an Ultrasonic Homogenizer (Cole – Parmer Instrument, Chicago, IL). For TSPO binding assays, tissue homogenates were prepared in 50 mM PBS on ice with a Kinematika Polytron (Luzerne, Switzerland), as described previously (Awad and Gavish, 1987).

2.4. Histology

Aorta and liver were subjected to histological analysis. The aortas were dissected transversely into 5 mm segments beginning at the aortic arch and continuing into the abdominal aorta in all animals. After removal and dissection, the aorta segments were immediately fixed in 10% buffered formalin, and processed for embedding in paraffin. From the prepared microtome sections (3–5 μM), five sections of each segment were randomly chosen for histological analysis. In this way, 20 aortic slice samples along the length of the aorta from each animal were microscopically investigated. The mean areas of foamy cells formation in the aorta were identified and their square areas were quantified utilizing a light microscope connected to a video camera (Nikon-Eclipse E600, Program Lucia 4.21, Nikon, Düsseldorf, Germany).

Paraffin sections (3–5 μM) from blocked liver (1 × 0.5 × 0.5 cm) of 10 animals each from the HFHC diet regimen group and the control group were cut with a microtome. Histological sections of all cases were stained with haematoxylin and eosin. The sections were studied for the occurrence of pathomorphological changes (pale-steatotic lesions). In the C-rats no pale-steatotic lesions were detected. Lipid deposits in the liver sections of the HFHC rats were quantified (Nikon-Eclipse E600, Program Lucia 4.21).

2.5. Routine plasma analysis

Plasma of rats taken at day 0, at week 15, and at week 18 of the HFHC diet was obtained by exsanguination. The blood samples were collected into tubes, and centrifuged using 1450g at 4 °C for 10 min. Various plasma parameters [albumins (Alb), total proteins (Tot Proteins), creatinin (Creat), urea, creatin kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanin aminotransferase (ALT) and uric acid (UA)] were measured by an Clinical Chemistry Analyzer System (AU 400, Olympus, Tokyo, Japan) using routine clinical chemical assays. Lipoprotein levels in the collected rats' plasma were determined by means of diagnostic kits (enzyme-colorimetric test, Human diagnostics, Wiesbaden, Germany).

2.6. Protein quantification

General protein content of tissue homogenates for carbonylization assays was measured according to Levine et al. (1990). For [^{3}H]PK 11195 binding assays and specific assays for oxidative stress, general protein content in the tissues in question were measured according to Lowry et al. (1951).

2.7. Determination of lipid and protein oxidation in plasma and tissues

2.7.1. Lipid peroxidation

Lipid peroxidation products (LPO) from liver, aorta, heart and testis were measured as thiobarbituric acid reactive substances (TBARs) in 2.5% (w/v in 1.12% KCl) of tissue homogenates and from 250 μL of undiluted heparinised plasma as described previously (Ohkawa et al., 1978; Draper and Hadley, 1990). Steady-state LPO (performed in the presence of the anti-oxidant butylated hydroxytoluene (BHT), to ensure that only LPO products present in the liver at the time of collection were measured), as well as LPO generated *in vitro* with ferro-ascorbate were assayed (Stroev and Makarova, 1989). The results of the TBARs assays were presented as nanomoles of TBARs per gram of tissue, and μM TBARs/L blood plasma, calculated from the absorbance at 532 nm (Cintra, UV/Vis Spectrometer, GBC, Melbourne, Australia) using 1,1,3,3-tetraethoxipropane (TEP) as external standard.

2.7.2. Protein carbonyls

Briefly, 500 μL of a 10 mM solution of dinitrophenylhydrazine (DNPH) was added to the 500 μL of liver and aorta homogenates (2.5% w/v in 1.12% KCl), and incubated for 60 min in the dark at room temperature with vortexing every 10 min. Subsequently, the solution was precipitated with the use of an equal quantity of 20% trichloroacetate (TCA). The precipitate was pelletted by centrifugation (12,000*g*, +4 °C for 5 min) and was washed three times with an ethanol: ethyl acetate solution (1:1, v/v) to remove free DNPH and lipid contaminants. Samples were then resuspended in 1 mL of 6 M guanidine at 37 °C (GdmCL, dissolved in 20 mM phosphate buffer, adjusted to pH 2.3 with trifluoroacetic acid, TFA). The carbonyl content was calculated from the absorbance measurement at 340 nm (Cintra, UV/Vis GBS, Spectrophotometer) with the use of a molar absorption coefficient of 22,000 M^{-1} cm^{-1}. The procedure for carbonyl determination in undiluted plasma was as described for the tissue homogenates, with the difference that 100 μL plasma was submitted to 100 μL DNPH for derivatisation for 1 h, and precipitated with 100 μL ice cold TCA.

2.7.3. Advanced oxidation protein products

Levels of advanced oxidation protein products (AOPPs) were determined in liver homogenates and diluted plasma according to Witko-Sarsat et al. (1996). Briefly, AOPPs were measured spectrophotometrically and calibrated with chloramine-T solutions that in the presence of potassium iodide absorb at 340 nm. Briefly, 1 mL of 2.5% tissue homogenate diluted 1/20 in 50 mM PBS (containing BHT, 100 μM and EDTA, 1 mM) was placed in quartz cuvettes and 50 μL of 1.16 M potassium iodide was added, followed by 200 μL of acetic acid after 2 min. In quartz cuvettes, used as blanks, 50 μL of 1.16 M potassium iodide was added to 1 mL of PBS followed by 200 μL of acetic acid. AOPPs concentrations were expressed as nanomoles per mg protein of analyzed tissue.

AOPPs in circulating blood were determined in plasma-diluted 1:5 in PBS. To exclude interference of turbidity of lipids on light absorption, the diluted samples were centrifuged (10,000g, 1 h, 4 °C). The 200 μL of plasma samples below the lipid layer were used for measurement in which 10 μL of 1.16 M potassium iodide was added followed by 20 μL of acetic acid. In quartz cuvettes, used as blanks, 10 μL of 1.16 M potassium iodide was added to 200 μL of PBS followed by 20 μL of acetic acid. The chloramine-T absorbance at 340 nm being linear within the range of 0–100 μmol/liter, AOPPs concentrations were expressed as nanomoles per mg protein of analyzed tissue, or as μM/L plasma.

2.8. Determination of anti-oxidant activities in liver and plasma

2.8.1. Tissue anti-oxidant analysis

Liver superoxide dismutase activity (SOD) (EC 1.15.1.1) activity was assayed according to Winterbourn et al. (1975), with a SOD determination kit (RA20408, Fluka, Biochemika, Steinheim, Germany), according to which one unit of SOD activity is defined as the amount of enzyme activity that causes 50% inhibition of the reduction of tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). SOD is capable to inhibit the formation of a water soluble formazan dye typically formed due to reduction of WST-1 by super oxide anions. SOD activity in liver tissue was determined and expressed as units of SOD per milligram protein according to the instructions of the manufacturer (Fluka, Biochemika). Liver glutathione reductase (GSSG-Red; EC 1.6.4.2) activity was assayed with a glutathione reductase assay kit (GRSA 114K4000, Sigma–Aldrich, Steinheim, Germany) according to previously described methods (García-Alfonso et al., 1993). The glutathione reductase activity was expressed as nanomoles of NADPH oxidized to NADP per minute per milligram protein, according to the instructions of the manufacturer.

Liver glutathione (GSH) content was measured with a glutathione assay kit (CS0260, Sigma–Aldrich, Steinheim, Germany) according to methods described previously (Akerboom and Sies, 1981). Flash frozen tissue ground to form a fine powder was treated with 5% trichloroacetic acid (TCA) followed by centrifugation at 10,000*g*, 4 °C, for 10 min. The supernatant was assayed spectrophotometrically for acid-soluble glutathione at 412 nm using 5,5′- dithiobis (2-nitrobenzoic acid) (DTNB) to TNB according to the instructions of the manufacturer.

Free sulfhydryl (SH) groups as an index of redox status in the plasma were measured (Hu, 1993). Briefly, 1 mL of buffer containing 0.1 M/L tris(hydroxymethyl)aminomethane, 10 mM EDTA, pH 8.2, and 50 μL plasma were added to cuvettes, followed by 50 μL of 10 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in methanol. Blanks were run for each sample in parallel, with the exception that there was no DTNB in methanol. Following incubation for 15 min at room temperature, sample absorbance was read spectrophotometrically at 412 nm. The concentration of sulfohydryl groups was determined using the TNB molar absorption coefficient of 14,100 M^{-1} cm^{-1}, and the results are presented as μM/L in plasma.

2.9. TSPO binding characteristics

Maximal binding capacity (B_{max}) and equilibrium dissociation constant (K_d) of the binding of the TSPO specific ligand, [^{3}H]PK 11195, in whole cell membrane homogenates from the liver, aorta, heart, brain, kidney, and testis, were determined as previously described (Awad and Gavish, 1987). Briefly, [^{3}H]PK 11195 binding to membrane homogenates was assayed in 50 mM potassium phosphate buffer, pH 7.4, at 4 °C in a final volume of 500 μL. The reaction mixture contained 400 μL of the membrane homogenate in question (≈100 μg protein) and 25 μL of [^{3}H]PK 11195 solution (0.2–6 nM final concentrations) in the absence (total binding) or presence (nonspecific binding) of 75 μL unlabeled PK 11195 (10 μM final concentration). After incubation for 60 min at 4 °C, the samples were vacuum filtered through Whatman GF/C filters, washed three times with 4 mL of 50 mM phosphate buffer and placed in vials containing 4 mL of Opti-Fluor. Radioactivity was counted after 12 h with a 1600CA Tri-Carb liquid scintillation analyzer (Packard, Meriden, CT). Scatchard analysis of [^{3}H]PK 11195 binding was done to determine the B_{max} and K_d values.

2.10. Statistical analysis

Data are presented as means ± SD. Significance was determined using analysis of variance and post hoc analysis as appropriate, while for dependent groups paired samples *t*-test was used. The significance of association was determined by two-tailed *t*-test. STATISTICA 5.0 (Stat Soft Inc., Tulsa, USA) was used. $P < 0.05$ was considered indicative for significant differences. Pearson's correlation coefficient was used as a measure of linear association between two variables.

3. Results

By observing the rats in their cages, atherogenic HFHC diet appeared to be well tolerated by the rats. Reflecting the higher caloric intake, weight gain was considerably higher in the animals fed the HFHC diet (+73%, Table 1). Hyperlipidemia compared to control levels was established in rats fed the HFHC diet for 18 weeks as confirmed by the higher serum levels of total cholesterol (+94%), HDL cholesterol (+28%), n-HDL (LDL) cholesterol (+148%), and triglycerides (+89%) (Table 1). Over the 18 week period of the HFHC diet, levels of total cholesterol (TC) as well as triacylglycerides (TAG) showed significant increases of around 2-fold at weeks 10, 14, and 18 (Fig. 1). This was associated with increased ratios of TC:HDL from 2.5 (day 0) to 3.7 for week 18 (not shown). A high positive correlation between TC and TAG increases was observed due to the HFHC diet was observed ($r = 0, 9228$).

To have further indications regarding the effects of HFHC diet we measured a number of stress parameters in blood plasma which typically are used to determine the potential occurrence of shock or injury. After 15 weeks of HFHC diet first decreases in CK and decreased uric acid levels were observed (Table 2). After 18 weeks of HFHC diet rats showed significant decreases in urea, creatinine, total protein, and albumin levels (Table 2), and further increases in creatine kinase (CK) and uric acid levels (Table 2).

We observed histological abnormalities manifested at the endothelium of aorta and by pathology of liver tissue from rats fed the HFHC diet for 18 weeks (Fig. 2). In the endothelium of the aorta wall of the HFHC rats, we observed the appearance of foamy cells (20–25% of the lumen circumference, visible among 6 animals from 10 analyzed). Larger abnormalities like thin plaque lesions with inflammatory cells activity were visible among 3 of the 6 HFHC rats (Fig. 2b and c). All the control aortas remained negative for these changes (Fig. 2a). Morphologically, the liver was bright yellow, soft and greasy in all the observed HFHC rats, indicative of fatty liver. Microscopically, severe intracellular accumulation of fatty vacuoles was abundant in rats for 18 weeks on HFHC diet (Fig. 2e), which remained completely absent in control rats (Fig. 2d). Oxidative stress parameters measured in plasma also appeared to be affected in HFHC rats (Table 3). The level of free SH groups were significantly decreased in the plasma of HFHC rats compared to the control rats (−49%). Furthermore, significant increases in protein oxidation products and lipid peroxidation products were observed in plasma of the HFHC rats compared to control rats (+194% for AOPPs; +31% for protein carbonyls; +44% for lipid peroxides).

Our various assays also confirmed oxidative stress levels in the liver and aorta. In particular, HFHC diet appeared to affect TBARs production in the liver in comparison to controls, both with steady-state levels (i.e. otherwise untreated liver homogenates) and with *in vitro* ferro-ascorbate induced TBARs production. The "steady-state" levels of lipid peroxides showed that the HFHC diet induced a significant increase in TBARs production in the liver in comparison with the control group (+44%, Fig. 3A). In the aorta, TBARs levels were also significantly increased (+63%) in comparison to control (Table 4). TBARs production, measured with the same type of incubation, did not significantly change in heart and slightly, but significantly decreased in testis tissue (−16%). When homogenates were exposed to oxidative stress induced with the peroxidation initiator, Fe^{2+}–ascorbic acid, peroxidation of lipids was significantly increased in liver tissue (+98%), but not in heart and testis (Fig. 3B), suggesting reduced anti-oxidant activity in the liver.

As shown in Fig. 4, AOPPs content in the liver, as a marker of protein oxidation was significantly increased in the HFHC group as compared to the control group (+237%). Similarly, in the aorta, as shown in Table 4, AOPP levels increased by 236% in HFHC rats compared to control rats. Measurement of protein carbonyl (PC) residues indicative of oxidative protein damage in the liver suggested a slight increase of 14% compared to control ($p = 0.05$) (Fig. 4). Regarding protein carbonyls (PC) in the aorta an increase of 35% compared to control was found in the HFHC group.

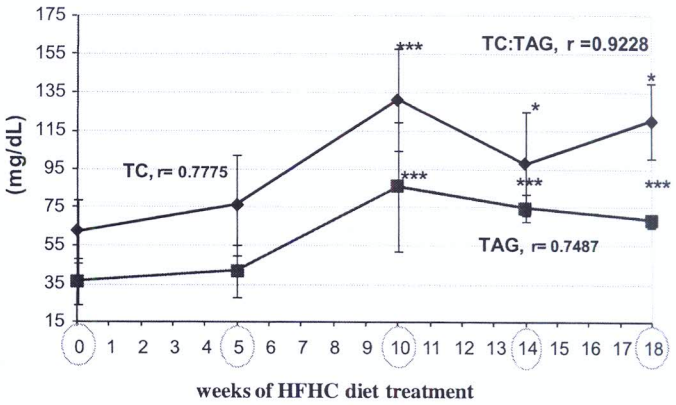


Fig. 1. Time-course of total cholesterol (TC) and triacylglycerides (TAG) fluxes in the plasma of rats treated with HFHC diet for a period of 18 weeks. Significant changes compared with baseline (at day 0), $^{*}p < 0.05$; $^{***}p < 0.001$.

Table 2
Plasma levels of stress related parameters in HFHC rats compared to control rats.

Variables	Before the experiment (week 0) Control	15 week Atherogenic diet	End of the experiment (18 week) Atherogenic diet
Glucose, mM/L	4 ± 0.7	6 ± 0.4	5 ± 1.6
Urea, mM/L	7 ± 2	6 ± 0.8	**4 ± 0.6***
Creatinin, μM/L	74 ± 14	64 ± 4	**45 ± 4****
AST, U/L	126 ± 18	141 ± 16	138 ± 38
ALT, U/L	57 ± 6	74 ± 13.5	54 ± 12
CK, U/L	322 ± 140	**568 ± 202***	**742 ± 423***
LDH, U/L	1263 ± 445	1326 ± 664	1266 ± 498
tot.Proteins, g/L	76 ± 4	79 ± 3	**62 ± 8****
Albumins, g/L	33 ± 2	32 ± 3	**24 ± 3.4*****
Uric acid, μM/L	113 ± 31	**62 ± 11*****	**152 ± 69***

Values are means ± SD, with *t*-test for dependent samples applied. Significant changes compared to control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Parameters presenting significant effects of atherogenic HFHC diet are presented in **bold**.

Table 1
Body weight and blood lipoprotein levels of HFHC rats compared to controls.

18 weeks treatment		
Parameters	Control	HFHC
Body weight, g	292 ± 31 ($n = 18$)	**505 ± 61***** ($n = 20$)
TC, mg/dL	62 ± 17 ($n = 18$)	**120 ± 20*** ($n = 20$)
n-HDL-C, mg/dL	36 ± 12 ($n = 18$)	**89 ± 23***** ($n = 20$)
HDL-C, mg/dL	25 ± 4 ($n = 18$)	**32 ± 1*** ($n = 20$)
TAG, mg/dL	36 ± 12 ($n = 18$)	**68 ± 3***** ($n = 20$)
TC:HDL-C	2.5	3.7

Values are means ± SD, with *t*-test for dependent samples applied. Significant changes (**bold**) compared to control: $^{*}p < 0.05$; $^{***}p < 0.001$. Abbreviations: TC, total cholesterol; HDL-C, HDL cholesterol; n-HDL-C, non-HDL cholesterol; TAG, triacylglycerides. TC:HDL-C shows the correlation between HDL-C and total cholesterol. To convert the mean values of TC, n-HDL-C, and HDL to mM/L, divide by 38.65. To convert TAG to mM/L, divide by 88.54.

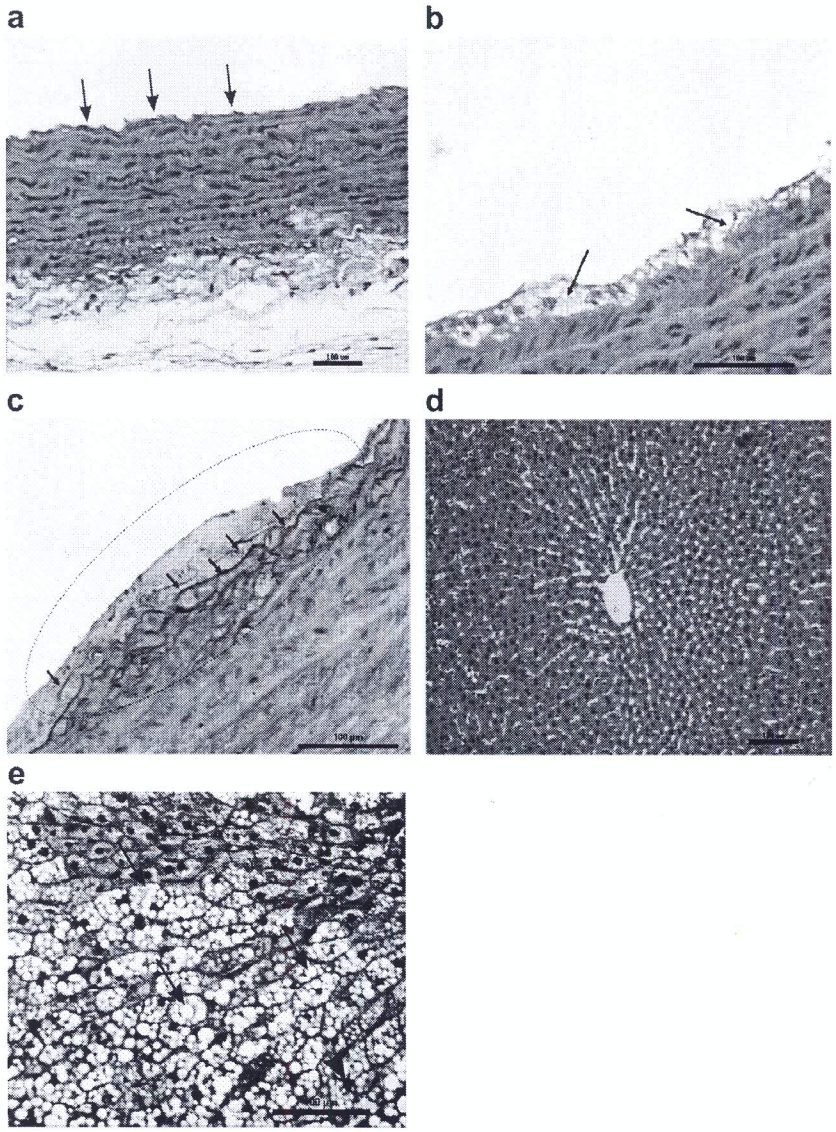


Fig. 2. Representative photomicrographs of microtome sections of the aorta and liver. (a) Aorta of the control group, the arrows indicate the normal endothelial cells. (b) Visible subendothelial changes (arrows) that indicate the beginning of arterial plaque formation in aorta of rat exposed to HFHC diet for 18 weeks. (c) Inflammatory activity of underlying media (arrows); visible components of fibrinoid tissue (encircled). (d) Normal liver tissue with hepatocytes showing no signs of intracellular accumulation of fatty vacuoles. (e) Severe intracellular accumulation of fatty vacuoles visible as clear vacuoles within parenchyma cells of the liver of HFHC rats. In some parenchyma cells, the visible heterochromatic nucleus (arrows) is present in the squeezed rim of cytoplasm around the fat vacuole. Morphologically the liver was bright yellow, soft and greasy. (H&E staining for all micrographs.) Scale bars are 100 μm as indicated.

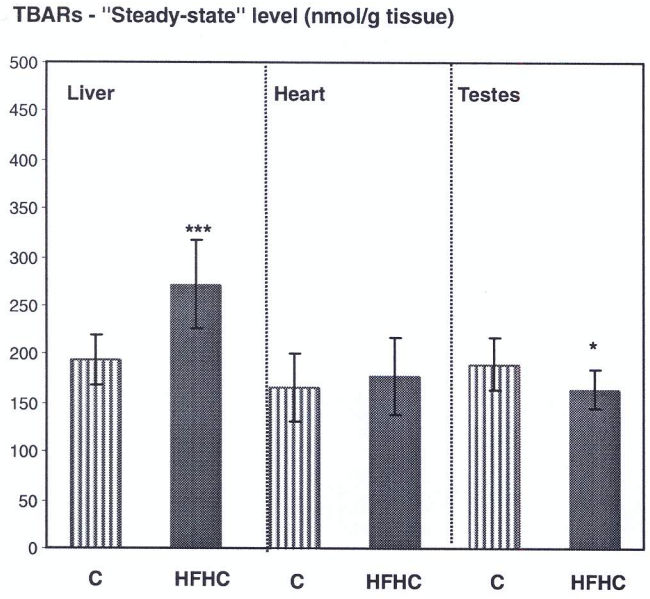
Table 3
Effects of HFHC diets for 18 weeks on plasma oxidative stress parameters in rats.

18 weeks treatment		
Parameters	Control	HFHC
AOPP, μM/L	77 ± 35 (n = 12)	**226 ± 82***** (n = 14)
Carbonyls, nmol/mg	0.16 ± 0.04 (n = 12)	**0.2 ± 0.05*** (n = 14)
Free SH groups, μM/L	106 ± 40 (n = 12)	**54 ± 19***** (n = 12)
TBARS, μM/L	7.5 ± 1.5 (n = 14)	**10.8 ± 2.7*** (n = 14)

*$p < 0.05$; ***$p < 0.001$ versus control (t-test for independent samples).

Concerning anti-oxidant activities in liver tissue, Fig. 5 shows significantly reduced activity of superoxide dismutase (SOD) in HFHC rats compared to control rats (−62%). Fig. 5 also shows that there is a significant interaction between glutathione level and glutathione reductase activity in liver tissue. The analyzed results indicated that the glutathione (GSH) content in liver tissue of HFHC diet exposed animals was significantly decreased (−65%), with simultaneous significant enhancement achieved in activity of glutathione reductase (+134%), as compared to control rats.

Binding assays with the TSPO specific ligand [^{3}H]PK 11195 were done to determine potential effects of HFHC diet on TSPO binding characteristics in rat liver and aorta as organs involved in atherosclerosis. We also examined heart, kidney, brain, and testis to determine whether the effects seen regarding TSPO binding characteristics were specific for the liver and aorta as a consequence of the HFHC diet. To our knowledge, TSPO binding characteristics of the aorta of rats have not been described before, neither in treated nor in untreated rats. In the present study TSPO binding characteristics of the aorta of untreated control rats were as follows: B_{max} = 4100 ± 1370 fmol/mg and K_d = 1.2 ± 0.4 nM (Table 5). The B_{max} and K_d values for TSPO in the liver, heart, kidney, brain, and testes of control rats (Table 5) were in the range of previous described results (Gavish et al., 1999). Regarding the effect of the HFHC diet, we found that the B_{max} for TSPO in liver of HFHC rats was significantly lower than that of control rats (−38%, Table 5).



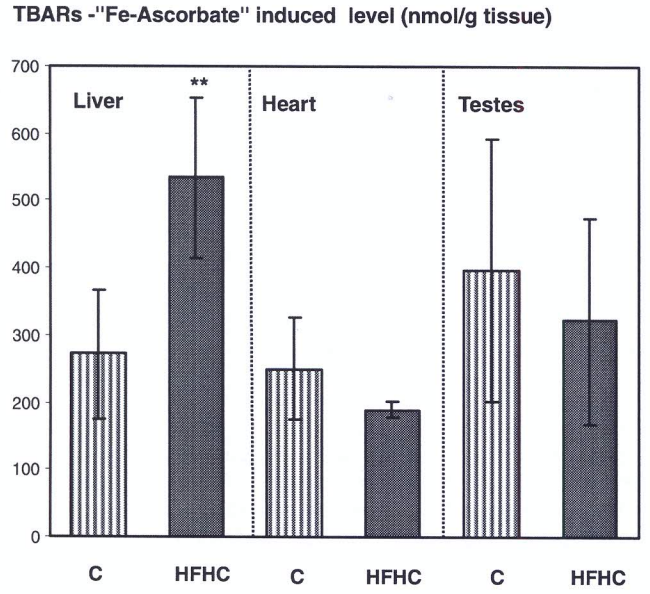


Fig. 3. TBARs formation in liver, heart and testes homogenates of rats subjected to atherogenic HFHC diet in comparison to control rats. (A) This histogram represents TBARs production in tissue sample (nmol/g tissue) without additional incubation of the probes with Fe^{2+}–ascorbic acid (steady-state reaction). (B) This histogram represents TBARs production (nmol/g tissue) after incubation with 4×10^{-5} M Fe^{2+}–2.6 mM ascorbic acid (stimulated lipid peroxidation). Significant changes in TBARS concentration compared with control group, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 4
Effects of HFHC diets for 18 weeks on aorta oxidative stress parameters in rats.

Variables/aorta	Control	HFHC
TBARs, nmol/mg	1.22 ± 0.2 (n = 8)	**2.0 ± 0.4***** (n = 10)
AOPP, nmol/mg	12.5 ± 4.5 (n = 8)	**42.0 ± 26.0**** (n = 10)
PC, pmol/mg	63.5 ± 24.0 (n = 8)	**86.2 ± 26.7*** (n = 10)

Mann–Whitney, non-parametric test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Significant changes compared to control marked in **bold**.

HFHC diet also resulted in a significant decrease of the B_{max} for TSPO in aorta compared to control (−30%) (Table 5). In contrast to liver and aorta, a significant enhancement in the B_{max} of TSPO

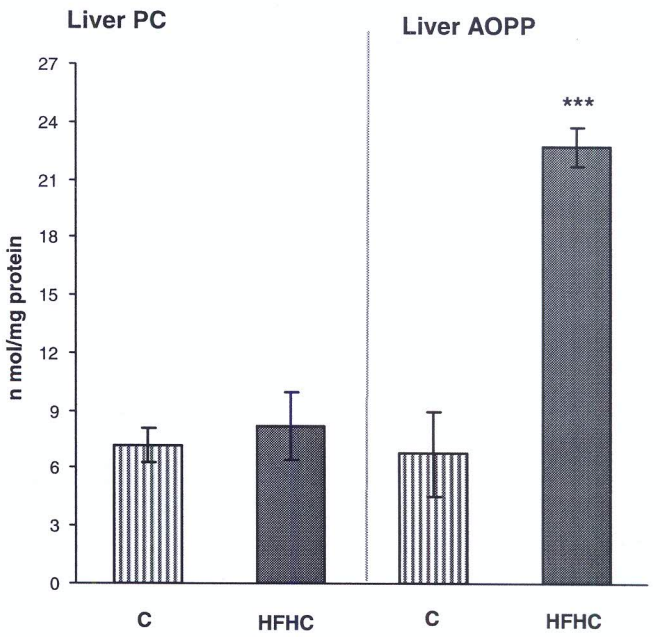


Fig. 4. Protein carbonyls (PC) and advanced oxidation protein products (AOPPs) in liver of HFHC rats compared to control rats (C). Significant changes compared to control, $^{***}p < 0.001$.

(+37%) determined with [^{3}H]PK 11195 binding was observed in testis tissue due to the HFHC diet, compared to control (Table 5). [^{3}H]PK 11195 binding levels in heart, kidney, and brain appeared not to be affected by the HFHC diet (Table 5). For all tissues, both in the HFHC and control group, K_d values determined with [^{3}H]PK 11195 binding were in the nM range (0.8–1.9 nM). Representative examples of Scatchard analysis in liver, aorta, and testis are shown in Fig. 6.

4. Discussion

We used a high fat, high cholesterol (HFHC) diet applied to rats to determine relations between systemic hypercholesterolemia, atherogenic pathology and oxidative stress in the liver and aorta, in correlation with modulations in TSPO binding characteristics in these organs. Atherogenic diets (containing cholesterol, saturated fat, cocoa butter, and chocolate) have been in use for over 20 years by several investigators to study atherosclerosis in various vertebrates, including rodents (Faggiotto et al., 1984; Dimitrova, 2002; Vergnes et al., 2003; Kitade et al., 2006). As rat presents a resistant animal model for provoking atherosclerosis, relatively long time-courses are required to induce even moderate hypercholesterolemia and triglyceridemia (Nakamura et al., 1989; Lorkowska et al., 2006). Therefore, we applied an increased cholesterol percentage in our custom tailored diet (3% in our study, compared to 1–2% in other studies (Paigen, 1995; Lorkowska et al., 2006; Pisulewski et al., 2006), during a time period of 18 weeks.

In our study, this HFHC diet with 3% cholesterol resulted in considerable obesity combined with moderate morphological changes in the endothelial tissue in the aorta of rat. Previous studies applying 1–2% cholesterol diets did not affect the endothelium, although sometimes increased lipid loading density of the adventitial vasa vasorum was observed, possibly causative for the outflow of cholesterol from the vessel wall (Pisulewski et al., 2006; Lorkowska et al., 2006). The endothelial changes in our HFHC rats were in correlation with changes in plasma activity of CK and uric acid, which may contribute to tissue damage due to inflammation (Laine et al., 2004). The above parameters are also associated with high plasma

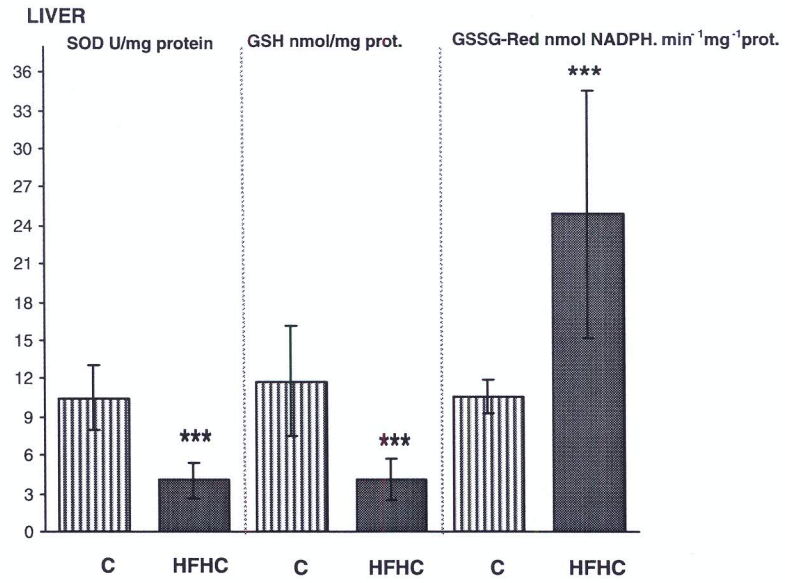


Fig. 5. Anti-oxidant parameters in liver of HFHC rats, in comparison to control rats. Significant changes in glutathione content (GSH), superoxide dismutase (SOD) and activity of glutathione reductase (GSSG-Red) compared to control, ***$p < 0.001$.

Table 5
Effect of HFHC diet on TSPO binding characteristics in various tissues.

Tissue	C – Control			High fat high chol. diet – HFHC		
	n	B_{max} (fmol/mg)	K_d (nmol)	n	B_{max} (fmol/mg)	K_d (nmol)
Aorta	10	4100 ± 1400	1.2 ± 0.4	11	**2870 ± 1300****	1.53 ± 0.8
Heart	14	4390 ± 1800	1.8 ± 0.9	9	4480 ± 1100	1.7 ± 0.6
Liver	10	2180 ± 800	1.7 ± 0.6	7	**1380 ± 500***	**0.8 ± 0.2*****
Kidney	7	4270 ± 900	1.9 ± 0.9	7	3580 ± 900	1.3 ± 0.4
Brain	7	300 ± 100	1.5 ± 0.4	7	320 ± 100	**0.7 ± 0.4****
Testis	14	3001 ± 400	0.8 ± 0.3	9	**4240 ± 800*****	0.9 ± 0.3

Average B_{max} values (fmol/mg protein) of [^{3}H]PK 11195 binding to TSPO of HFHC treated rats tissues versus Control. Mann–Whitney, non-parametric test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Significant changes compared to control marked in **bold**.

cholesterol levels and cardiovascular disorders in humans (Meisinger et al., 2008. We also observed steatohepatitis in our HFHC rats, as previously described by other studies (Kirsch et al., 2003; Lorkowska et al., 2006). Abnormal liver chemistries accompanying fatty liver have been associated with abdominal obesity, hyperlipidemia, and arterial hypertension (Laine et al., 2004). Our HFHC fed rats also showed obesity and hyperlipidemia. In parallel, we observed increased blood urea, decreased levels of total proteins and decreased albumin in the plasma that may be either related to impaired protein synthesis in the liver, or due to increased catabolism, as a consequence of the HFHC diet (Harrison et al., 1994). Thus, our HFHC paradigm with enhanced fat and cholesterol levels applied for 18 weeks was effective in inducing obesity and various parameters indicative of fatty liver, hyperlipidemia, and atherogenic symptoms.

We analyzed several oxidative stress parameters in plasma, liver, and aorta in this paradigm, such as TBARs (which are indicative of lipid oxidation), AOPP and PC (which are indicative of protein oxidation), and SH groups (which are indicative of anti-oxidant activity) (Kirsch et al., 2003; Domenicali et al., 2005; Matsuzawa et al., 2007; Sena et al., 2008). In our HFHC rats, we found increases in the levels oxidative stress parameters and decreases in the indicators for anti-oxidant activity. Imbalance between production and scavenging of superoxide anions is known to result in increased oxidative stress and inflammation in the cardiovascular system (Wu et al., 2004; Oliveira et al., 2006). Plasma SH, hepatic GSH and SOD levels were reduced in our rats fed the HFHC diet, indicative of reduced anti-oxidant activity (Nguyen-Khoa et al., 2001; Yang et al., 2004; Didion and Faraci, 2005; Mari et al., 2006). AOPP and TBARs levels were enhanced in the plasma, aorta, and liver, of HFHC rats, in agreement with previous observations (Kirsch et al., 2003; Carmiel-Haggai et al., 2005; Matsuzawa et al., 2007). In the liver, Fe-ascorbate induced TABRs levels were also enhanced, giving further indication of impaired anti-oxidant capability of the liver. In accordance with a study on rats with choline deficiency-induced fatty liver, from Domenicali et al. (2005), PC levels were also enhanced in plasma and aorta, but not in liver. In mice, however, atherogenic diet can lead to enhanced PC levels in the liver (Matsuzawa et al., 2007). Alteration in liver function can range from mild elevations of liver enzymes – via enhanced production of oxygen-derived free radicals by redox cycling resulting in depletion of intracellular reduced glutathione – to decreased mitochondrial membrane potential, initiation of lipid peroxidation, and ultimately cell death (Bironaite and Ollinger, 1997; Stadlbauer et al., 2005). Enhanced oxidative stress, inflammation, and abnormal lipoprotein metabolism may account for the pro-atherogenic effect of fatty liver disease (Lizardi-Cervera and Aquilar-Zapata, 2009). In particular the high AOPP levels we found in the liver, plasma, and aorta, as induced by the HFHC diet, may present factors reflecting liver pathology and atherogenesis (Watanabe et al., 2004; Oettl et al., 2008). The parameters for enhanced oxidative stress we detected in the plasma of our HFHC rats may correlate with inflammatory processes, including the

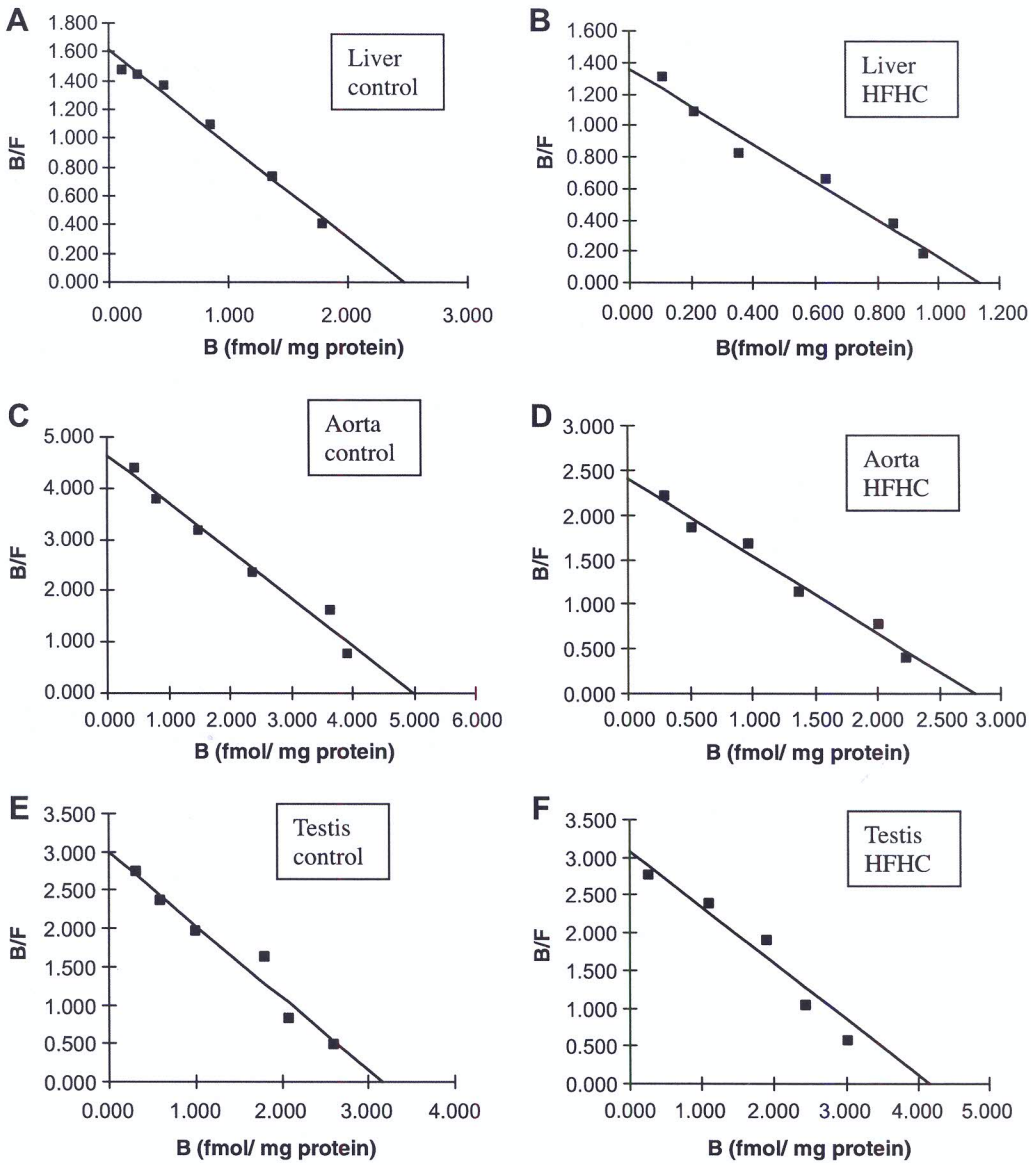


Fig. 6. Changes in TSPO binding characteristics due to the HFHC diet. Representative Scatchard plots of saturation curves of [^{3}H]PK 11195 binding to membrane homogenates of liver (A and B), aorta (C and D), and testes (E and F), respectively, of control rats (A,C,E) and rats exposed to HFHC-diet for 18 weeks (B,D,F). B: bound; B/F: bound over free.

atherogenic effects determined in our study (Witko-Sarsat et al., 1998; Liu et al., 2006). In general, the involvement of oxidized proteins in atherosclerosis has been studied less than oxidized lipids. However, protein oxidation products have been found in the extracellular matrix of human and animal atherosclerotic plaques (Woods et al., 2003; Liu et al., 2006). The study by Liu et al. (2006) was the first to our knowledge, which provided *in vivo* evidence for a causal relationship between chronic AOPPs accumulation and atherosclerosis. This study suggested that increases in plasma AOPP, particularly in the hypercholesterolemic environment, accelerate atherosclerosis progression (Liu et al. (2006)). Interestingly, a study from Wong et al. (2008) showed that protein carbonyls are not merely damage-causing but, can also serve as a second messenger for signal transduction in vascular smooth muscle cells. In our study, we also found very strong effects of HFHC diet on AOPP levels in aorta, plasma, and liver, typically 3-fold increases compared to rats fed with control diet. Thus apart from showing liver pathology and indications of developing atherosclerosis, the rats fed with our HFHC diet for 18 weeks also showed oxidative stress characteristics in liver, aorta, and plasma, and verily not only lipid peroxidation, but also protein oxidation, providing further evidence that oxidative stress is part and parcel of atherosclerosis.

It has been suggested that the mitochondrial protein, TSPO, which is present in various components of the cardiovascular system, including heart and blood vessels, where it has been detected in mast cells, smooth muscular cells, and dermal vascular endothelial cells, may play a role in cardiovascular disorders (Veenman and Gavish, 2006). The TSPO is involved in various mechanisms that also have been found to play a role in atherosclerosis, including oxidative stress, ROS generation, inflammation, immune responses, apoptosis, and mitochondrial cholesterol transport (Ruff et al., 1985; Myers et al., 1991; Bessler et al., 1997; Papadopoulos et al., 1997,2006; Kelly-Hershkovitz et al., 1998; Bono et al., 1999; Veenman et al., 2004,2007,2008,2010a,b; Levin et al., 2005; Veenman and Gavish, 2000,2006; Kugler et al., 2008; Shoukrun et al., 2008; Zeno et al., 2009; Dimitrova-Shumkovska et al., 2010). In the liver, which is considered to play an important role in oxidative stress related to cardiovascular diseases, the TSPO has been detected in mitochondrial and nonmitochondrial

locations of liver cells (Hirsch et al., 1989; Bovolin et al., 1990; O'Beirne et al., 1990). In hepatocytes TSPO appears to be localized to mitochondria, whereas in nonhepatocytes TSPO's location appears not to be mitochondrial, but possibly in biliary epithelial cell plasma-membranes (Woods et al., 1996). In addition, TSPO are found in hepatic stellate cells and parenchymal cells (Fischer et al., 2001). To our knowledge, the binding characteristics of TSPO in the aorta of rats have not been reported before.

To establish a factual correlation between atherogenic challenges and TSPO binding characteristics, we assayed TSPO binding characteristics in the liver and the aorta of our HFHC rats, in comparison to normal fed rats. In addition, we also determined TSPO binding characteristics in a number of steroidogenic and nonsteroidogenic tissues to see whether effects on TSPO levels due to HFHC diet may be specific for liver and aorta. It appeared that the enhancement of oxidative stress in the liver and aorta due to our applied atherogenic HFHC diet is accompanied by reductions in TSPO binding density in these organs. In the liver also a significant increase in TSPO binding affinity could be observed. As TSPO may be modulated by ROS, and can modulate ROS generation itself (Courtiere et al., 1995; Papadopoulos et al., 1997, 2006; Stoebner et al., 2001; Delavoie et al., 2003; Veenman et al., 2007, 2008; Zeno et al., 2009), it may be that the oxidative stress detected in liver and aorta of our HFHC rats may be directly associated by a reduction in TSPO binding in these organs. Previous *in vitro* studies of rat liver also showed that particular forms of oxidative stress can reduce TSPO binding density (Courtiere et al., 1995). For example, 0.001 mM $Fe[^{2+}]$ in combination with 1 mM ascorbate reduced TSPO binding density by half (Courtiere et al., 1995). Vice versa, it has been suggested that the TSPO can reduce the production of free radicals induced by UV (Stoebner et al., 2001). Interestingly, it was also shown that in response to UV irradiation-induced ROS covalent TSPO polymers were formed in Leydig and breast cancer cells *in vitro* and *in vivo*, resulting in increased binding affinity of the TSPO (Delavoie et al., 2003). Other evidence for TSPO's participation in oxidative processes is indicated in a study by Carayon et al. (1996), where a correlation between the levels of TSPO expression and the resistance to H_2O_2 toxicity was demonstrated in hematopoietic cell lines. It may be that the reduction in TSPO binding density we found in the present study may impair such protective, anti-oxidative functions of the TSPO. Alternatively, the reduced levels of TSPO may be a compensatory response to the challenges posed by the HFHC diet, i.e. a reduction of ROS generation by the TSPO and protection against the triggering of cell death, as for example also found in knockdown of TSPO by genetic manipulation in various studies (Levin et al., 2005; Shoukrun et al., 2008; Zeno et al., 2009). It may be that the enhanced levels of AOPP found in liver and aorta may be a contributing factor to the reduced levels of TSPO in these organs. As an alternative explanation, regulation of TSPO binding density can take place via modulations of gene expression (Giatzakis and Papadopoulos, 2004). With various studies we have shown that steroids and stress are able to regulate TSPO binding density (for reviews see for example Veenman and Gavish, 2006; Veenman et al., 2007; Mazurika et al., 2009). Thus, enhanced general stress levels and modulations in hormonal levels may also affect TSPO levels. In our HFHC rats we found enhanced stress levels and enhanced levels of TSPO binding in the testis, indicating that this steroidogenic organ is affected by the atherogenic diet we applied.

The reductions in TSPO binding density found in the aorta of rats subjected to HFHC diet may potentially also be correlated with enhanced local oxidative stress. Indeed, we found that in the aorta and the plasma the levels of the markers indicating oxidative stress and endothelial damage were consistently elevated. Others have also suggested a role for TSPO in vascular inflammatory responses, for example in vascular permeability caused by carrageenin (Lazzarini et al., 2001; Faure et al., 2002; Laitinen et al., 2009). Presently, it is not known which components of the vascular wall, i.e. mast cells, smooth muscular, or dermal vascular endothelial cells, would be important for the potential correlation between TSPO expression and atherosclerosis (Stoebner et al., 1999; Morgan et al., 2004; Veenman and Gavish, 2006). Similarly, we as yet also do not know in which liver cells TSPO plays a role in the oxidative stress detected in our study.

In our present study, TSPO density in the heart did not appear to be affected by the HFHC diet, while in the testes TSPO density was increased. The increase in TSPO density in the testes possibly may be due to TSPO responses to altered hormonal levels as a consequence of the stress induced by the HFHC diet. For example, increases in testosterone levels can induce a decrease in TSPO levels in the testes (Amiri et al., 1991; Veenman et al., 2007). Regarding other tissues, the binding analysis done on HFHC diet group showed no differences in binding levels in kidney, heart and brain homogenates compared to control, even though a significant increase in TSPO binding affinity in the brain could be observed (Table 5). Thus it appears that the decreases in TSPO binding density we see in liver and aorta of our rats as a consequence of the HFHC diet are specific for the aorta and liver.

As the present study shows that HFHC diet causes histopathological changes and oxidative stress in correlation with reduced TSPO binding density in aorta and liver, additional questions are coming up. Indeed it would be interesting to find out whether modulation of TSPO responses by TSPO ligands could counteract or enhance the effects of HFHC. Similarly, it would be interesting to study in this paradigm the effects of hormones, as they are known to affect TSPO expression. For example, it is known that testosterone levels are reduced in humans as well as rats as a consequence of a fattening diet and obesity (Cano et al., 2008; Macdonald et al., 2009). As reduced testosterone levels are correlated with reduced TSPO levels in various tissues (Weizman et al., 1992), this may present part of the mechanism whereby the HFHC diet and obesity of the rats of our study may lead to reduced TSPO levels in aorta and liver. Alternatively, when enhanced oxidative stress in our paradigm contributes to changes in TSPO levels in various tissues, it would be interesting to study TSPO homomer polymerization, as for example found by Delavoie et al. (2003). The appearance of TSPO multimers would suggest whether changes in TSPO binding capacity and affinity in some of the tissues studied may be due to the oxidative stress caused by the HFHC diet.

In conclusion, with the high fat, high cholesterol atherogenic (HFHC) diet applied to the rats of our study, we observed simultaneous increases in oxidative stress parameters in liver, plasma, and aorta wall, as well as liver pathology and the development of focal inflammatory activity in aortic tissue. This was correlated with decreases in TSPO binding density in the liver and aorta. Such decreases were not observed in other organs. Thus, these effects of HFHC on TSPO binding density appear to be rather specific for the liver and aorta. We consider that the TSPO response in liver and aorta may be compensatory for the oxidative stress induced by HFHC diet. Alternatively, the TSPO response may either be part of the oxidative stress mechanisms, or result from it. Our study suggests that TSPO may present a target for novel therapies to reduce the risk of atherosclerosis, in particular its component of oxidative stress.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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