Serum PON1 activity and oxidative stress in non-alcoholic fatty liver disease

Nonalkolik karaciğer yağlanması hastalığında oksidatif stres ve PON1 aktivitesi

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Abstract

Background: Recent studies have shown that non-alcoholic fatty liver disease (NAFLD) is associated with increased carotid intima-media thickness as a reliable marker of early atherosclerosis, but possible direct relationships between NAFLD and carotid artery atherosclerosis is unknown. Therefore, the aim of this study was to investigate serum paraoxonase and arylesterase activities (PON1) along with lipid hydroperoxide (LOOH) levels.

Methods: We studied 32 consecutive patients with biopsy proven NAFLD and 28 healthy controls. Serum basal/salt-stimulated paraoxonase and arylesterase activities were detected spectrophotometrically. LOOH levels were measured by the FOX–2 assay.

Results: Serum basal/salt-stimulated paraoxonase and arylesterase activities were significantly lower in NAFLD than controls ($p<0.05$, $p<0.05$, $p<0.05$; respectively), while LOOH levels were significantly higher ($p<0.05$).

Conclusions: These results suggest that NAFLD are associated with increased oxidative stress and decreased PON1 activity. Thus, low serum PON1 activity could contribute to, in part, pathogenesis of carotid arterial atherosclerosis in patients with NAFLD.

Keywords: PON1 activity, lipid hydroperoxide, atherosclerosis, non-alcoholic fatty liver disease

Özet

Amaç: Yapılan son çalışmalar erken atherosklerozun güvenilir bir belirtisi olan artmış karotis intima-media kalınlığı ile nonalkolik karaciğer yağlanması hastalığı arasında bir ilişki olduğunu ortaya koymuştur. Ancak, karotis arter aterosklerozu ile non-alkolik karaciğer yağlanması hastalığı arasında direk bir ilişki olup olmadığı bilinmemektedir. Bu nedenledir ki, bizler lipid hidroperoksit ile serum paraoksanaz ve arilesteraz aktivitelerini (PON1) araştırmayı amaç edindik.

Materyal ve metod: Çalışmamıza karaciğer biopsileri alınmış 32 hasta ve 28 sağlıklı ve gönüllü kontrol grubu dahil edildi. Serum bazal ve tuz ile uyarılmış paraoksanaz ve arilesteraz aktiviteleri spektrofotometrik olarak ölçülürken, lipid hidroperoksit seviyeleri ise FOX-2 metodu ile çalışıldı.

Bulgular: Lipid hidroperoksit seviyeleri control grubu ile karşılaştırıldığında hasta grubunda daha yüksek iken ($p<0.05$), serum bazal ve tuz ile uyarılmış paraoksanaz ve arilesteraz aktiviteleri daha düşük idi ($p<0.05$, $p<0.05$, $p<0.05$; sırasıyla).

Sonuç: Bu çalışmadaki bulgularımıza göre non-alkolik karaciğer yağlanması olan hastalarda oksidatif stresin artığı ve PON1 aktivitesinin azaldığını tespit ettik. Böylece, düşük PON1 aktivitesi nonalkolik karaciğer yağlanması olan hastalardaki carotis arter atherosklerozunun patogenezinde etkili olabileceğini tespit ettik.

Anahtar kelimeler: PON1 aktivitesi, lipid hidroperoksit, ateroskleroz, nonalkolik karaciğer yağlanması

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinicalpathological condition characterized by a necroinflammatory disorder with the fatty infiltration of hepatocytes. This term was first used by Ludwig et al. (1). NAFLD is increasingly recognized as the most prevalent chronic liver diseases in Western countries (2). This condition may be severe and is characterized by a wide spectrum of pathological lesions, ranging from pure steatosis to steatohepatitis, which can progress to liver cirrhosis and hepatocellular carcinoma in a minority of the patients (3).

It has been recently reported that NAFLD is strongly associated with several atherosclerotic risk factors such as dyslipidemia, hypertension, diabetes, central obesity (4, 5). It is hypothesized that NAFLD is not only a marker of cardiovascular disease but may also be involved in development of disease (6). An association between NAFLD, carotid arterial remodeling and cardiovascular disease has been shown in recent studies (4, 7, 8). The arterial endothelium is a target for the atherosclerotic process. NAFLD is associted with systemic endothelial dysfunction (9), a marker of early generalized atherosclerosis. Also, it has been shown that oxidative stress may play an important role in the pathogenesis of NAFLD (10). Endothelial damage and increased oxidative stress and decreasing antioxidant enzymes in patients with NAFLD may result in prooxidation environment in the sub endothelial region. Also, low density lipoprotein particles, which are thought to be more atherogenic, could be increased in NAFLD patients (11). These species have been implicated in atherogenic process (12).

It is well known that serum PON1 activity is generally considered to vary in response to the consumption of PON1 for the prevention of oxidation (13). In fact, the enzyme serum PON1 has an important role in prevention of atherosclerosis (14). PON1 is an antioxidant enzyme associated with high-density lipoprotein (HDL) and plays a key role in the protection of LDL and HDL from oxidation by hydrolyzing activated phospholipids and lipid peroxide products (15). PON1 destroys LDL lipid peroxides accumulation on LDL and arteries in vitro and ex vivo (16). In addition, serum PON1 activity contributes to the antiatherogenic effect of HDL, and its activity has been shown to be inversely associated with oxidative stress in serum (17). Furthermore, several studies have shown that low serum PON1 activity constitutes a risk factor for atherosclerotic disease such as coronary artery disease (18), hypercholesterolemia (19), type 2 diabetes (20) and renal failure (21) that is under increased oxidative stress. In a recent article, Pasqualini et al. reported that PON1 plays a key role in regulation of endothelial function as PON1 activity modulates endothelial functions (22).

Ultrasonographically measured carotid intima-media thickness, as a reliable index of subclinical atherosclerosis (23), is markedly increased in patients with NAFLD (8, 24). In only few studies, PON1 activity has been suggested to be increased in patients with NAFLD (25, 26). However, although an association between NAFLD and increased carotid intima-media thickness has been described in the literature, there is no known about serum PON1 activity and its relationship with carotid intima-media thickness as a reliable marker of early atherosclerosis in patients with NAFLD. We hypothesized that PON1 activity may influence carotid intima-media thickness by protecting against atherosclerosis. Therefore, the aim of this study was to investigate serum paraoxonase and arylesterase activities along with lipid hydroperoxide (LOOH) levels.

Methods

SubjectsA total of 32 consecutive patients with biopsy proven NAFLD and 28 healthy controls were enrolled in the present study. The study protocol was carried out in accordance with the Helsinki Declaration as revised in 1989 and approved by the local research committee for ethics. All subjects were informed about the study protocol and the written consent was obtained from each study paticipant.

Initial evaluation

The diagnosis of NAFLD in subjects with chronic hypertransaminasemia (>6 months) was based on the following criteria; exclusion of any other putative cause of chronic liver disease, evidence of bright liver at ultrasound scan, and liver biopsy.

All patients with NAFLD included in the present study underwent a percutaneous true-cut liver biopsy under ultrasonic guidance. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin, masson-trichrome, and reticulin silver statin. Necroimflamatory grading and fibrosis scoring was made based on a modification of the scoring system proposed by Brunt *et al*. (2). All grading and staging was performed by a single pathologist without knowledge of the patients clinical or laboratory data.

None of the healthy controls had any known disease and none was taking any medications. Routine biochemical findings of the healthy controls subjects were also within the normal range. In all healthy controls an abdominal ultrasound was performed to exclude bright liver. All controls had alanine-aminotransferase and aspartate-aminotransferase within normal range, were negative for hepatitis C virus, hepatitis B virus, and human immunodeficiency virus infections or history of liver disease.

Height and weight were measured for the calculation of the body mass index (BMI = weight/height^2 (kg/m^2). Systolic and diastolic blood pressure was measured. Hypertension was defined as a systolic blood pressure of 140 mmHg or more, a diastolic blood pressure of 90 mmHg or more, or self-reported use of anti-hypertensive medications.

Exclusion criteria

Exclusion criteria included recent gastrointestinal by-pass surgery, smoking habit, pregnancy, serum total bilirubin level higher than 2 mg/dL, usage of estrogens, tamoxifen and glucocorticoids, rheumatoid arthritis, renal diseases, cancer, systemic or local infection, and history of excess alcohol ingestion, averaging more than 30 gm/day (3 drinks per day) in the previous 10 years, or history of alcohol intake averaging greater than 10 gm/day (1drink per day: 7 drinks per week) in the previous 1 year.

Blood Sample Collection

Blood samples were obtained fallowing an overnight fasting state. Samples were withdrawn from a cubital vein into blood tubes and immediately stored on ice at 4°C. The serum was then separated from the cells by centrifugation at 3000 rpm for 10 min and they were stored until analyzing at -80°C.

Measurement of paraoxonase and arylesterase activities

Paraoxonase and arylesterase activities were measured using paraoxon and phenyl acetate substrates. The rate of paraoxon hydrolysis (diethyl-*p*-nitrophenylphosphate) was measured by monitoring the increase of absorbance at 412 nm at 37^{0}C. The amount of generated *p*-nitrophenol was calculated from the molar absorptivity coefficient at pH 8, which was 17,000 M^{-1} cm^{-1} (27). Paraoxonase activity was expressed as U/L serum. Phenyl acetate was used as a substrate to measure the arylesterase activity. Enzymatic activity was calculated from the molar absorptivity coefficient of the produced phenol, 1310 M^{-1} cm^{-1}. One unit of arylesterase activity was defined as 1 µmol phenol generated/min under the above conditions and expressed as U/L serum (28). Paraoxonase phenotype distribution was determined by a double substrate method that measures the ratio of paraoxonase activity (with 1M NaCl in the assay) to arylesterase activity, using phenylacetate (27).

Measurement of LOOH levels

Serum LOOH levels were measured with FOX-2 assay. The principle of the assay depends on the oxidation of ferrous ion to ferric ion via various oxidants and the produced ferric ion is measured with xylenol orange. LOOH's are reduced by triphenyl phosphine (TPP), which is a specific reductant for lipids. The difference between with and without TPP pretreatment gives LOOH levels (29).

Other parameters

The levels of triglyceride, total cholesterol, HDL-Cholesterol, LDL-Cholesterol, glucose, AST and ALT parameters were determined by using commercially available assay kits (Abbott®) in an autoanalyzer (Germany, Aeroset, Abbott®).

Statistical analysis

Data were presented as mean ± SD. Qualitative variables were assessed by Chi-square test. Continious variables were compared using Student t test. Pearson correlation test was used to find out the correlation of LOOH levels, paraoxonase and arylesterase activities with liver biopsy specimens' histological findings. A *p* value of less than 0.05 was regarded as significant.

Results

The demographic and clinical data of study population are shown in Table 1. There were no statistically significant differences between two groups in regard to age, gender, and BMI (p> 0.05 for all). Serum triglyceride, total cholesterol, LDL-Cholesterol, glucose, AST and ALT levels were significantly higher in patients with NAFLD than in controls (p<0.05 for all), while HDL- Cholesterol levels were significantly lower (p<0.05).

Necroimflamatory grades and fibrosis scores of the 32 subjects with NAFLD were as follows:

-Grade 1 (mild) necroimflamatory changes, in 12 subjects; grade 2 necroimflamatory changes (moderate), in 13 subjects; grade 3 (severe) necroimflamatory changes, in 7 subjects.

- Stage 0 Zone 3 perisinusoidal/pericellular fibrosis, in 5 subjects; stage 1, in 7 subjects; stage 2, in 12 subjects;

stage 3, in 8 subjects.

-None of the subjects with NASH had stage 4 fibrosis.

Serum basal/salt-stimulated paraoxonase and arylesterase activities in patients with NAFLD were found to be significantly lower than controls (for basal paraoxonase, $p<0.05$; for salt-stimulate paraoxonase $p<0.05$; for arylesterase $p<0.05$; respectively), while serum LOOH levels were significantly higher ($p<0.05$) (Table 2).

Serum basal/salt-stimulated paraoxonase and arylesterase activities, LOOH levels was not correlated with neither fibrosis score nor necroinflammatory grades in patients with NAFLD (all $p > 0.05$). Furthermore, neither fibrosis score nor necroinflammatory grades was correlated with serum AST and ALT levels in patients with NAFLD ($p >0.05$ for all). Lipid parameters was also no correlated with basal/salt-stimulated paraoxonase and arylesterase activities, LOOH levels ($p>0.05$ for all). There was also no significant difference between PON1 192 Q and R polymorphism distribution in study groups ($p > 0.05$).

Discussion

In the present study, we hypothesized that PON1 activity may influence carotid intima-media thickness by protecting against atherosclerosis in patients with NAFLD. Although an association between NAFLD and increased carotid intima-media thickness has been described in medical literature, there is no data concerning serum PON1 activity and its relationship with carotid intima-media thickness as a reliable marker of early atherosclerosis in patients with NAFLD. Therefore, in this study, in an attempt to search for a suitable marker for the prediction of carotid intima-media thickness in patients with NAFLD, we measured the serum PON1 activity in these patients. We observed that basal/salt-stimulated paraoxonase and arylesterase activities were significantly lower in patients with NAFLD than in healthy controls, while LOOH levels were significantly higher. Decreased PON1 enzyme activity may have a role in the pathophysiology of increased LDL-cholesterol oxidation and the increased susceptibility to oxidative stress observed in NAFLD. Thus, low serum PON1 activity could contribute to, in part, pathogenesis of carotid arterial atherosclerosis in patients with NAFLD.

Oxidative stress is well documented in NAFLD, one of the second hits. Several studies have suggested that oxidative stress and lipid peroxidation may play an important role in the pathogenesis of NAFLD as their end products can induce hepatocellular injury and fibrogenesis (10, 30). The nature of NAFLD seems to be multifactorial, including derangement in metabolic parameters, endothelial dysfunction, oxidative stress, inflammation, inflammatory cytokines, and abnormal lipid and glucose metabolism (31-33). However, these mechanisms are also closely related to other risk factors for atherosclerosis. Therefore, it is unclear whether NAFLD contributes to the development of atherosclerosis directly. Carotid ultrasound imaging has been widely used for evaluation carotid atherosclerosis (23). Increased carotid intima-media thickness in patients with NAFLD was shown in previous studies (4, 7, 8) and more recently, Bonora et al. shown that carotid intima-media thickness has been related to the severity of liver damage (34).

It has been suggested that the association of NAFLD with atherosclerosis may be linked to visceral fat accumulation, a substantial risk factor for metabolic syndrome (35). However, it has also been speculated that NAFLD itself may be atherogenic through an associated increase in hepatic oxidative stress and inflammation. Also, Targher et al. demostrated that serum inflammatory biomarkers have been found increased in patients with NAFLD which can predict a more atherogenic risk profile (36). Further, atherosclerosis involving the hepatic arteries has been scarcely investigated, but some observations have suggested that these vessels are relatively atherosclerosis-resistant and that pathological mural lesions are most likely to occur as a result of aging alone (37).

PON1 protects plasma lipoproteins from oxidative modification by ROS. The mechanism of the observed decrease in serum PON1 activity in patients with NAFLD remains unclear. This decrease may be related to enhanced lipid peroxidation, since oxidized lipids are reported to inhibit PON1 activity. Thus, increased inactivation of PON1 according to increased generation of ROS in patients with NAFLD can explain the development of carotid artery atherosclerosis directly (13).

Oxidative stress and its consequent oxidation of LDL in arterial walls is a crucial step in the pathogenesis of atherosclerosis (12). PON1 is an antioxidant that inhibits the oxidative modification of LDL and contributes much of the antioxidative activity that has been attributed to HDL. Besides, PON1 can destroy active lipids in mildly oxidized LDL and thus, prevents the induction of inflamatory responses in arterial wall cells (14). On the other hand,

increased low density lipoprotein particle, which are thought to be more atherogenic, in NAFLD patients has been observed (11). In addition, previous studies have also suggested that PON1 activity was decreased in some diseases due to ROS pathogenesis under oxidative stress and atherosclerotic process (38, 39). Alterations in circulating PON1 levels have been reported in a variety of diseases including oxidative stress. Indeed, PON1 activity has been shown to decrease in subjects prone to development of atherosclerosis, as in subjects with coronary artery disease (18) hypercholesterolemia (19), type 2 diabetes (20) and renal failure (21). It is well known that low PON1 activity has also been reported to be an independent risk factor for cardiovascular events (16).

The physiologic role played by PON1 in the liver is unknown, although preliminary observations suggest that the putative function of PON1 is to provide hepatic protection against oxidative stress (40). In only few studies, PON1 activity has been suggested to be increased in patients with NAFLD (25, 26). Baskol et al. (25) investigated serum PON1 activity has been suggested to be decreased in patients with NAFLD. All the patients with low PON1 activity had NAFLD in the study of Baskol et al. (25). Furthermore, they suggested that increased serum oxidative stress in patients with NAFLD may result in a pro-oxidation environment, which in turn could result in decreased antioxidant enzyme activities. Furthermore, Baskol et al. (26) investigated relationship between hepatic antioxidant PON1 activity, lipid peroxidation and liver injury in patients with NAFLD and they observed that increased lipid peroxidation may be either a cause or a result of liver injury in patients with NAFLD. However, in this study, serum PON1 activity and its relationship with carotid intima-media thickness as a reliable marker of early atherosclerosis is not investigated.

Our results suggest that NAFLD is associated with increased oxidative stress and decreased PON1 activity. Decreased PON1 activity could be associated with enhanced lipid peroxidation, since oxidized lipids are reported to inhibit PON1 activity. Thus, low serum PON1 activity could contribute to, in part, pathogenesis of carotid arterial atherosclerosis in patients with NAFLD. Therefore measurement of PON1 activity may be used for assessment of carotid arterial remodeling. Furher prospective studies are needed to investigate the association of low PON1 activity and the development of carotid arterial atherogenesis in NAFLD patients.

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Table 1: Demographic and clinical parameters in patients with NAFLD and controls

	Controls (n=28)	NAFLD (n=32)	*p*
Age (years)	36±9	38±2	NS
Gender (female/male)	18/10	13/19	NS
Glucose (mg/dl)	92.12±5.14	98.10±8.25	< 0.05
BMI (kg/m^2)	27.7±2.4	28.6 ± 2.3	NS
Systolic blood pressure (mm/Hg)	132± 8	125± 7	NS
Diastolic blood pressure (mm/Hg)	81± 5	87 ± 4	NS

Values are expressed as mean ± standard deviation, NS = non significant, BMI: Body mass index, NAFLD: Nonalcoholic fatty liver disease

Table 2: Lipid hydroperoxide**,** basal/salt-stimulated paraoxonase and arylesterase activities in patients with NAFLD and controls

	Controls (n=28)	NAFLD (n=32)	*p*
Paraoxonase (U / L)	110.3±35.3	70.7±38.3	< 0.05
Salt-stimulated paraoxonase (U / L)	116.45±18.5	84.48±8.63	< 0.05
Arylesterase (U / L)	61.5±20.7	52.9±4.4	< 0.05
LOOH (μmol / L)	5.9±1.4	7.6±2.7	< 0.05
AST (U/L)	22.1±5.6	40.5±19.2	< 0.05
ALT (U/L)	23.2±6.9	80.2±39.3	< 0.05
Triglyceride (mg/dl)	92.2±46.3	215.5±100.4	< 0.05
Total Cholesterol (mg/dl)	145.1±31.9	207.4±43.8	< 0.05
HDL Cholesterol (mg/dl)	45.7±10.3	39.4±8.4	< 0.05
LDL Cholesterol (mg/dl)	80.9±27.8	124.8±39.4	< 0.05

Values are expressed as mean ± standard deviation, AST: Aspartate aminotransferase, ALT:

Alanine aminotransferase, LOOH: Lipid hydroperoxide, NAFLD: Nonalcoholic fatty liver disease

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