Oxidative Stress and Enzymatic Antioxidant Status in Patients with Nonalcoholic Steatohepatitis

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Abstract. Oxidative stress is an important pathophysiological mechanism in nonalcoholic steatohepatitis (NASH). To assess whether there are relationships between oxidative stress and antioxidant enzymes in the development of NASH, we investigated oxidative stress by measuring serum malondialdehyde (MDA) and nitric oxide (NO) and antioxidant status by measuring serum glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and superoxide dismutase (SOD). The study included 18 patients (13 men, 5 women; mean age 42 yr) with biopsy proven NASH and 16 healthy volunteers (10 men, 6 women; mean age 38 yr). Serum levels of MDA, NO, GSH, GSH-Px, GR and SOD were determined by spectrophotometric methods. Serum levels (mean ± SD) of MDA (6.7±1.6 vs 2.8±1.7 nmol/ml, p 0.0001), NO (135±28 vs 113±35 mmol/L, p 0.04), GSH (919±137 vs 770±128 mmol/L, p 0.003) were increased in patients with NASH vs controls. Serum levels of GSH-Px (1063±152 vs 1000±94 U/L) and GR (47±22 vs 40±21 U/L) were not significantly different in the patients vs controls. However, the serum level of SOD (1.24±0.32 vs 1.51±0.37 U/ml, p: 0.04) was significantly decreased. Impaired antioxidant defense mechanisms may be an important factor in the pathogenesis of NASH. Treatment approaches that affect the antioxidant enzymes may be beneficial in patients with NASH. (received 18 March 2003; accepted 23 July 2003)

Keywords: nonalcoholic steatohepatitis, malondialdehyde, nitric oxide, antioxidant enzymes

Introduction

Nonalcoholic steatohepatitis (NASH), an advanced form of nonalcoholic fatty liver disease (NAFLD), is a cause of hepatitis that may progress to cirrhosis. The pathogenesis of NASH involves two major stages; accumulation of triglyceride within hepatocytes as the first step, and oxidative stress that leads to inflammation, cellular injury, and progressive fibrosis as the second step. Cellular damage induced by mitochondrial oxidative stress is a widely accepted explanation for steatosis to the progression of steatosis to necroinflammation and fibrosis [1,2]. Increased intrahepatic levels of fatty acids provide a source of oxidative stress. It has been proposed that the vulnerable fatty liver is injured by reactive oxygen species (ROS) generated from microsomal, mitochondrial, and/or other hepatocellular pro-oxidant pathways when the antioxidant defenses are critically lowered [3]. ROS-mediated liver injury may be triggered by 3 main mechanisms: lipid peroxidation, cytokine induction, and Fas ligand induction. Cytotoxic products of lipid peroxidation (eg, malondialdehyde, MDA, and 4-hydroxynonenal, 4-HNE) may impair cellular functions including nucleotide and protein synthesis and may play a role in hepatic fibrogenesis [4-6]. The generation of excess reactive oxygen species, in addition to triggering lipid peroxidation of cellular membranes, leads to the release of tumor necrosis factor-alpha (TNFα) via hepatocytes, Kupffer cells, and adipose tissue [7]. The release of TNFα activates specific redox sensitive kinases that can upregulate proinflammatory pathways and enhance insulin resistance [8].

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Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase, is a relaxing factor for the vascular endothelium that may mediate hepatic injury from reactive oxygen species and lipid peroxidation products. Liver is the only organ that is normally exposed to bacterial endotoxin, an inducer of NO synthase; high levels of circulating endotoxin are frequently found in patients with cirrhosis and NASH [9-11]. NO and superoxide radical interact to form peroxynitrite, which is an important mediator of free radical toxicity.

The body protects itself from oxygen free radical toxicity by enzymatic antioxidant mechanisms (eg, glutathione peroxidase, GSH-Px; glutathione reductase, GR; superoxide dismutase, SOD; and catalase) and by non-enzymatic antioxidants (eg, vitamins, uric acid, albumin, bilirubin, and many others). Antioxidant enzymes reduce the levels of lipid peroxides as well as hydrogen peroxide and are important in preventing lipid peroxidation and maintaining the structure and function of biologic membranes. SOD catalyses the dismutation of peroxide to hydrogen peroxide and GSH-Px catalyses the oxidation of glutathione. It has been reported that certain antioxidants (eg, vitamin E, betaine, and acetylcysteine) are effective in decreasing serum transaminases and improving hepatic histopathology in patients with NAFLD [12-14]. However, changes in the activities of the serum antioxidant enzymes and their relationships to indices of oxidative stress have inadequately been studied in patients with NASH. To see if relationships exist between oxidative stress and serum antioxidant enzymes in the development of NASH, we measured serum levels of MDA, NO, glutathione (GSH), and serum activities of antioxidant enzymes (SOD,GSH-Px, GR) in patients with NASH.

Materials and Methods

Subjects. The study included 18 patients with NASH and 16 healthy volunteers. The NASH diagnosis was based on the following criteria: (1) abnormal liver function tests at least for 3 mo and no history of prior liver disease, (2) sonographic diagnosis of liver steatosis, (3) histologic diagnosis of fatty infiltration, lobular or portal inflammation, and/or Mallory bodies, fibrosis, or cirrhosis. Informed consent was obtained from each subject. The experimental protocol was approved by the Ethics Committee of Ataturk University Medical School.

Absence of alcohol consumption was required in all patients and was confirmed by family members. The patients had not consumed drugs that might cause liver steatosis (eg, corticosteroids, estrogen, methotrexate, tetracyclin, calcium channel blockers, amiodarone) and they had not undergone surgical operations. The body mass index (BMI) was calculated using the formula: BMI = [body weight (kg)/(height (m))2. Individuals with BMI >30 were defined as obese [15]. The following blood tests were performed in all patients: serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), albumin, total protein, total cholesterol, triglyceride, plasma protrombin time, hepatitis B serology (HbsAg, anti-HBs, anti-HBc), anti-HCV, HCV RNA, and autoantibodies (ie, antinuclear, anti-smooth muscle, anti-mitochondrial antibodies).

The 16 healthy volunteers in the control group were workers who gave no history of liver disease or alcohol consumption. Liver function tests and sonographic liver examinations were normal in controls.

Assay procedures. MDA was determined by the thiobarbituric acid method [16]. Aliquots of 0.2 ml of serum were mixed thoroughly with 0.8 ml of phosphate-buffered saline (pH 7.4) and 25 µl of butylated hydroxytoluene solution. After addition of 0.5 ml of 30% trichloroacetic acid, the samples were placed on ice for 2 hr and then centrifuged at 2000 x g at 25°C for 15 min. One ml of supernatant was mixed with 0.075 ml of 0.1 mol/L EDTA and 0.25 ml of 1% thiobarbituric acid in 0.05 N sodium hydroxide. The samples were kept in boiling water for 15 min, cooled to room temperature, and the absorbance at 532 nm was measured. Total thiobarbituric acid-reactive substances (TBARS) were expressed as MDA, using a molar extinction coefficient for MDA of 1.56 x 105 cm-1 M-1; results were expressed as nmol/ml.

Serum NO levels were measured with Griess’s reagent as previously described [17]. The first step
was conversion of nitrate to nitrite using nitrate reductase. The second step was addition of Griess’s reagent, which converts nitrite to a purple azocompound. Protein interference is avoided by treatment of the reacted samples with zinc sulphate and centrifugation for 5 min at 10000 x g. Spectrophotometry of the azochromophore was performed at 540 nm; sodium nitrate was used as the standard and results were expressed as mmol/L.

Serum SOD activity was assayed according to Sun et al [18]. The SOD activity was measured at 560 nm by detecting the inhibition of the NBTH₂ reduction rate. One SOD unit was defined as the enzyme activity that caused 50% inhibition of the NBTH₂ reduction rate. SOD activity was expressed as U/ml.

GSH-Px activity was measured according to Paglia and Valentine [19]. When oxidized glutathione was converted to the reduced form in the presence of glutathione reductase (GR), NADPH was oxidized to NADP. The diminished absorbance of NADPH was measured at 340 nm. By measuring the ΔA/min and using the molar extinction coefficient of NADPH, GSH-Px activity was calculated and expressed as IU/L.

GR activity was assayed using oxidized glutathione as substrate [20]. The GR assay depended on the absorbance change at 340 nm owing to oxidation/reduction of the NADPH/NADP system. Absorbance of the reduced chromogen, measured at 412 nm, was directly proportional to the GSH concentration [21].

Liver histopathology. Liver biopsy was performed on all patients; the biopsy specimens were stained with H&E and Sudan-black. All of the liver biopsies were interpreted by pathologists with coded patient identifications and without access to the clinical and biochemical data. Histologic grading and staging was performed by a modified scoring system based on the classification of Brunt et al [22].

Statistical analyses. Means were compared by unpaired Student’s t test for parametric data and by Mann-Whitney U test for non-parametric data. Correlations were analyzed by Pearson’s correlation coefficient (r). The chi-square test was used to compare ratios. The computer software for the statistical analyses was SPSS for Windows 9.0 (SPSS Inc. Chicago, IL, USA); p values <0.05 were deemed statistically significant.

Results

Clinical and laboratory characteristics of the patients with NASH and the controls are summarized in Table 1. Most of the patients (72%) were men. The median BMI in patients with NASH was 27.6. Nine patients were obese; 4 patients had diabetes mellitus; and 12 patients had hyperlipidemia, of which hypertriglyceridemia was present in 10 (83%).

Histologic data for the patients with NASH are listed in Table 2. Mild or moderate liver fatty infiltration was evident in 16 patients; only 2 patients had severe fatty infiltration. Minimal, mild, or moderate hepatic inflammation was noted in all patients with NASH (39; 44; and 17%, respectively), but none had severe hepatic inflammation. Ballooning degeneration in seen in 11 patients. Ten patients had no fibrosis, whereas 8 had pericellular, perisinusoidal fibrosis, or periportal fibrosis. None had bridging fibrosis, cirrhosis, or Mallory bodies.

Lipid peroxidation, measured by serum MDA level, was significantly higher in patients with NASH than in controls (Table 3). Likewise, the serum NO

<table>
<thead>
<tr>
<th>Patients</th>
<th>NASH patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>41±5.2</td>
<td>38±8.5</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>13/5</td>
<td>10/6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.6±3.3</td>
<td>26.4±4.5</td>
</tr>
<tr>
<td>Obesity</td>
<td>9 (50%)</td>
<td>7 (44%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4 (22 %)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>12 (66 %)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>5 (28 %)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>10 (55 %)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Serum AST (U/L)</td>
<td>42±6±17.9</td>
<td>26.4±16.4</td>
</tr>
<tr>
<td>Serum ALT (U/L)</td>
<td>92.6±58.6</td>
<td>28.8±18.6</td>
</tr>
<tr>
<td>AST/ALT &gt;1</td>
<td>4 (22%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Serum ALP (U/L)</td>
<td>172±64</td>
<td>159±63</td>
</tr>
<tr>
<td>Serum total bilirubin (mg/dl)</td>
<td>0.98±0.86</td>
<td>0.76±0.22</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>4.9±0.1</td>
<td>4.5±0.1</td>
</tr>
<tr>
<td>Plasma prothrombin time (sec)</td>
<td>12.7±0.9</td>
<td>12.2±1.1</td>
</tr>
</tbody>
</table>
level in patients with NASH was higher than in the controls. The serum activity of SOD was lower and the serum level of GSH was higher in patients with NASH than in the controls. Slightly increased serum levels of GSH-Px and GR in NASH patients were not significantly different from the controls. Mean serum levels of MDA, NO, and serum activities of antioxidant enzymes (GSH, GSH-Px, GR, SOD) in 5 female patients with NASH did not differ significantly from the 13 male patients with NASH.

When the relationships of serum MDA and NO levels vs serum antioxidant enzyme activities were examined in the patients with NASH, no significant correlations were found. Moreover, there were no significant correlations between these parameters and the degrees of hepatic inflammatory activity or liver fibrosis in patients with NASH.

**Discussion**

Increased intrahepatic levels of fatty acids are a source of oxidative stress, which may be responsible for the progression from hepatic steatosis to steatohepatitis and to cirrhosis. Gut-derived endotoxins may play an important role in activating Kupffer cells, causing collateral damage to hepatocytes, and promoting an inflammatory response in the liver acinus [23]. Lipid peroxidation products alter mitochondrial DNA and also react with mitochondrial proteins to inhibit electron transfer along the respiratory chain, further increasing ROS production, and generating a self-propagating cycle of oxidative stress and lipid peroxidation [24].

Evidence of lipid peroxidation in the form of increased MDA production, a surrogate marker of oxidative stress, has been noted in previous studies, and serum levels of MDA have been correlated with the severity of chronic hepatitis [25,26]. In the present study, serum MDA levels were significantly increased in patients with NASH, indicating increased oxidative stress, but no correlations were found between serum MDA levels and the histopathologic findings in NASH patients. This may reflect the fact that most of NASH patients had minimal or mild hepatic inflammation and absent or minimal evidence of liver fibrosis; none had severe of hepatic inflammation and fibrosis.

Reports concerning the role of NO in liver damage during inflammatory conditions are contradictory. Zhu and Fung [27] found that NO protects against liver injury by scavenging lipid radicals and inhibiting the lipid peroxidation chain reaction. On the other hand, Sass et al [28] reported that iNOS-derived NO regulates proinflammatory genes in vivo, contributing to inflammatory liver injury. Other investigators have reported that, in the pathogenesis of NASH, NO may potentiate cytotoxicity by reaction with superoxide anion to form peroxynitrite, a strong oxidant that promotes nitration of tyrosine to form nitrotyrosine [29,30]. The finding that intrahepatic accumulation of nitrotyrosine is associated with the histological...
severity of NASH strongly suggests that oxide-related oxidative injury may play a significant role in the pathogenesis of NASH [30]. Although our NASH patients had increased serum levels of NO, we did not find correlations between the histologic severity of the disease and serum NO concentration. This may reflect the early stage of the disease in most of our NASH patients. In addition, NO may have a protective role during an early stage of NASH. At the beginning of hepatic injury, when only a small amount of NO is being produced, NO may protect the liver through vasodilatatory, antioxidative, and antiapoptotic effects. However, in the presence of massive injury (eg, high level of inducers and elevated oxidative stress), greatly increased NO production might induce the hepatocytes to progress to irreversible channel necrosis and cell death [31].

The defenses against free radical-mediated-injury include enzymatic deactivation and direct reaction with free radicals [32]. Cells have various antioxidant systems (eg, SOD, GSH-Px, GR, and catalase) and non-enzymatic scavengers (eg, vitamins E, A, and C; carotenoids, flavonoids, and thiols) [33]. SOD, the first line of defense against oxygen-derived free radicals, converts superoxide anion into H₂O₂, forming as neutral products O₂ and H₂O. GSH-Px catalyses reductive destruction of hydrogen and lipid hydroperoxides, using glutathione as an electron donor [34].

It is now generally accepted that oxidative stress due to increased ROS production has a role in the pathogenesis of NASH. Hepatocytes are continuously exposed to ROS and are protected from oxidative injury by a range of antioxidant pathways [35]. The state of oxidative stress exists when there is imbalance between pro-oxidant and antioxidant chemical species. There is insufficient knowledge about antioxidant defense mechanisms, particularly the enzymatic components, in the pathogenesis of NASH. In our study, we observed decreased serum SOD activity, increased serum GSH, and slight but statistically insignificant increases of serum GSH-Px and GR levels in NASH patients. This finding may indicate that the hepatic antioxidant enzymatic defense system in NASH is impaired.

Previous studies suggested that mitochondrial ROS can deplete hepatic antioxidants, allowing the accumulation of more ROS [36,37]. Robertson et al [38] reported that when antioxidant reserves are depleted, hepatic CYP 2E1 and 4A, which are microsomal oxidases involved in fatty acid oxidation, could induce the cellular injury from oxidative stress in NASH. Moreover, down-regulation of the cytosolic isoform of SOD sensitizes tissues and organs, including the kidney and liver, to superoxide mediated tissue injury [39].

The balance between oxidative stress and antioxidant defense mechanisms may be impaired by depletion of enzymatic antioxidants and increased serum levels of MDA and NO in patients with NASH. We perceive that failure of antioxidant defense mechanisms against oxidative stress may be an important factor in the pathogenesis of NASH. Treatment approaches that address the antioxidant enzymes and the antioxidant vitamins may be helpful in the therapy of patients with NASH.

References


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