Oxidative Balance in Lymphocytes From Patients With Nonalcoholic Steatohepatitis

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Abstract: Oxidative stress is linked to several human diseases, including nonalcoholic steatohepatitis (NASH). In this study, lymphocytes were used as a model to study this disease. These cells offer several advantages for cellular and molecular studies such as easy accessibility, and they are easily accessible and constitute a "time-persistent" system capable of reflecting the condition of the whole organism. Lymphocytes from patients with NASH display oxidative stress features. Among the possible causes for the overproduction of reactive oxygen species in NASH lymphocytes, there might be alterations of enzymatic pathways, auto-oxidation of glucose and mitochondrial superoxide production, which, in turn, would lead to protein oxidative damage. Increased oxidative stress in lymphocytes from patients with NASH may result in a pro-oxidative environment, which, in turn, could modify the pathway of the enzymatic activities. The data confirm that an imbalance between pro-oxidant and antioxidant defense mechanisms may be an important factor in NASH.

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Nonalcoholic fatty liver disease (NAFLD) is defined as fat accumulation in the liver exceeding 5% to 10% by weight, as microscopically determined from the percentage of fat-laden hepatocytes. The diagnosis of NAFLD implies the exclusion of the other causes of steatosis, such as viruses, alcohol, autoimmunity, toxicity, hypobetalipoproteinemia and Wilson's disease.

Nonalcoholic steatohepatitis (NASH) is the advanced form of NAFLD that progresses from simple steatosis to liver inflammation,[1,2] fibrosis, cirrhosis and, possibly, hepatocarcinoma.[3] NAFLD/NASH are strictly linked with obesity and insulin resistance and are frequently associated with hypertension and dyslipidemias, a constellation of abnormalities known as metabolic syndrome, and in many cases with type 2 diabetes mellitus. Metabolic syndrome is also a risk factor for NASH and for advanced fibrosis in liver tissue.[4] Taken together, NAFLD and NASH affect between 9% and 33% of the general population in the developed countries. However, although NAFLD is considered a common and relatively benign liver condition, NASH is thought to potentially lead to morbidity and increased mortality, thus demanding more attention.

The pathogenesis of NASH is thought to be multifactorial: the inducing factors include oxidative stress, iron deposits, overexpression of cytochrome P450E1, participation of endotoxin and tumor necrosis factor-α and mitochondrial disorders.

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Some authors have proposed the so-called "two-hit" model to describe the underlying mechanism. The "first hit" involves the accumulation of triglycerides within hepatocytes and the "second hit" regard oxidative stress that leads to inflammation, cellular injury and progressive fibrosis.[5] Abnormal mitochondrial and cellular redox homeostasis has been documented in cases of steatohepatitis, which suggests that there are alterations in the signaling cascades, which would ultimately alter the function of the enzymes and proteins that are critical for mitochondrial and cellular function.[6] Mitochondria play a major role in fat oxidation and energy production. They are also the main source of the reactive oxygen species (ROS) that trigger lipid peroxidation, cytokine overproduction and cell death.[7] The products of ROS-induced lipid peroxidation impair the respiratory chain, leading to increased mitochondrial ROS formation, which could deplete the antioxidants and impair ROS inactivation.[8]

In patients with NASH, insulin resistance is the pathogenic factor that favors the accumulation of free fatty acids in the liver. Insulin resistance is also believed to induce oxidative stress by stimulating microsomal lipid peroxidases and to decrease mitochondrial beta-oxidation because of the direct effect of high insulin levels.[3]

To evaluate whether the pathogenesis of NASH is associated with increased levels of ROS, the following had to be ascertained: (1) significant accumulation of ROS derived from used cell metabolism; (2) definite oxygen radical damage; and (3) abnormalities in the antioxidant defenses in pathological patients. The validation of these conditions was necessary to establish the possible relationship with cascade signaling events directly correlated with the pathogenesis of the disease.

Lymphocytes from peripheral blood were chosen for this study, because they can act as biosensors of the whole organism in response to the disease. These cells are not only involved in the inflammatory processes and immune responses but also contribute to homeostasis and adaptation to environmental variations and pathological conditions.[9] Lymphocytes also have a remarkably long lifetime and have the capacity to keep memory of their original phenotype even after they are cultured. Most importantly, there is no evidence that highly reactive chemical species capable of interacting with biological macromolecules and altering cellular functions have any effect on lymphocytes of patients with NASH.[10] For all of these reasons, lymphocytes are a robust, "time-resistant" system capable of monitoring the conditions of the entire body and keeping track of cumulative long-term stressors.[9]

To evaluate the involvement of oxidative stress in the pathogenesis of NASH, experiments were carried out to aim at verifying the occurrence and the level of the oxidative stress in lymphocytes derived from patients with NASH.

MATERIALS AND METHODS

Materials

All media, sera, antibiotics and culture solutions were purchased from Sigma—Aldrich, Corp. (St. Louis, MO). All

sterile culture plastics were provided by Falcon (Plymouth, United Kingdom). All other reagents were analytical grade.

Patients

A total of 15 overweight/obese patients (4 women and 9 men; mean age, 42 ± 14 years, body mass index [BMI] 30 ± 5.4 kg/m^2), with a clinical diagnosis of NAFLD were included in the study. The diagnosis of NAFLD was based on the following criteria: (1) elevated aminotransferases (aspartate aminotransferase [AST] and/or alanine aminotransferase) in at least 2 determinations; (2) clinical and hematochemical exclusion of alcohol-induced or drug-induced liver disease, autoimmune or viral hepatitis and cholestatic or metabolic/genetic liver disease; and (3) sonography documenting liver steatosis.

A total of 12 age-matched normal weight subjects (5 women and 8 men) from the Hospital staff were included as control group.

A complete medical history and clinical examination was performed in all patients and controls. BMI was calculated using the formula: weight (in kilograms)/height (in square meters). Waist circumference was assessed by wrapping the tape around the waist midway between 2 measure points: the top of hip bone and the bottom of the ribs. Blood was drawn in the morning after a 13-hour fast and the following parameters were determined: total cholesterol, triglycerides, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol (Friedewald's formula), glycemia, insulinemia, AST, alkaline phosphatase, γ-glutamyl transferase (GT) and apolipoprotein B.

Liver Sonography

Sonography of the liver was performed by a skilled ultrasonographer, unaware of clinical and laboratory patients details, using real-time l'ATL (Philips Electronics, Koninklijke, The Netherlands) equipped with a 3.5- to 5-MHz probe. Steatosis was defined sonographically by the appearance of hyperechoic liver tissue with fine, tightly packed echoes and posterior beam attenuation.

Bioimpedentiometry

All patients and controls underwent bioimpedentiometry (50 kHz, amplitude 50 mA; Tanita Europe B.V., Amsterdam, The Netherlands), using electrodes applied to the foot-plantar surface. This methodology allows the measurement of fat mass in kilogram and as percentage of body weight, free fat mass and water in kilogram.

Vascular Ultrasound Measurement

Carotid and femoral arteries were examined by high-resolution B-mode ultrasonography.[1] The examination was performed with a commercially available ultrasound device (ESAOTE Technos MP; ESAOTE S.p.A, Genoa, Italy) equipped with a linear multifrequency 7.5- to 12-MHz transducer. Subjects were examined in the supine position, and all measurements were obtained at end-diastole with electrocardiographic triggering. The ultrasound images were stored on a digital support and analyzed using an image processing workstation (AMS System, Gothenburg, Sweden). On a longitudinal 2-dimensional ultrasound scanning, the image of the far wall of common carotid and common femoral artery, at the prebifurcation tract, was displayed as 2 bright white lines separated by a hypoechogenic space. The intima-media thickness (IMT) of the far wall was automatically measured by the computerized system mentioned earlier (IMT common carotid and IMT common femoral).

The intraobserver coefficient of variation was 1.1% (mean ± standard deviation of the difference 0.018 ± 0.031 mm), whereas interobserver values were 1.8% (0.028 ± 0.032 mm).

Flow-Mediated Vasodilation

Flow-mediated vasodilation (FMV) was assessed on the brachial artery by ultrasonography. Any drug known to affect endothelial function, including nitrates, hypolipidemic drugs and aspirin, was withdrawn ≥1 week before the examination. Details of the procedure, which was performed according to the International Brachial Artery Task Force guidelines,[3] have been reported elsewhere.[4] Briefly, the measurements were performed in supine position on the nondominant arm, after 10 to 20 minutes resting in a quiet, dark room with a temperature of 22°C. The brachial artery was scanned longitudinally just above the antecubital crease using a 10-MHz probe (HDI 3500; Advanced Technology Laboratories). Diameter of the brachial artery was measured at the R-wave of the electrocardiogram, on the interface between media and adventitia of the anterior and posterior wall. Gain settings were optimized to identify the lumen and the vessel wall interfaces and were not modified during the examination. Hyperemia was induced by inflation of a pneumatic cuff (12.5 cm wide) at 230 to 250 mm Hg for 4 minutes on the most proximal portion of the upper arm. Arterial diameter measurement was repeated 45 to 60 seconds after sudden deflation of the cuff. Tracings were recorded on videotape and read by 1 investigator, who was unaware of the subject's clinical data and temporal sequence. The average of 3 measurements of basal and posthyperemia diameter was used for the analysis. FMV was expressed as the relative increase in brachial artery diameter during hyperemia, and defined as 100 × ([posthyperaemia diameter − basal diameter]/basal diameter). Blood flow was measured as arterial cross-sectional area ($\pi \times r^2$) times mean Doppler velocity corrected for angle. The intraobserver between-occasion reproducibility of FMV in our laboratory was assessed in 10 subjects examined 2 days apart. The mean ± SD difference between the 2 examinations was 1.0% ± 1.5%.[11–14]

Isolation of Human Lymphocytes

Peripheral venous blood samples from healthy donors and patients with NASH were collected in sodium-heparinized vacutainers. Peripheral blood lymphocytes were separated under sterile conditions on a Ficoll-Paque PLUS (Amersham, Piscataway, NY) gradient using the method by Boyum.[15] Aliquots of heparinized whole blood diluted with an equal volume of Dulbecco's phosphate-buffered saline (1:1) were gently applied to an equal volume of Ficoll-Paque PLUS in centrifuge tubes. Samples were centrifuged at 400*g* for 30 minutes. The resultant interface (buffy coat) was carefully aspirated from the gradient and washed twice in Dulbecco's phosphate-buffered saline by centrifugation at 200*g* for 10 minutes. The subsequent pellet was resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2% L-glutamine and 1% penicillin/streptomycin. Monocytes were removed from the mononuclear fraction by adherence to Petri dishes during overnight incubation at 37°C. Purified lymphocytes were finally resuspended in complete RPMI 1640 medium (1–2 × 10^6 cells/mL). Cell viability was determined by Trypan blue dye exclusion. The purified lymphocytes were used for experimental analyses within 1 day from their isolation.

ROS Generation

Determination of Hydrogen Peroxide Production

Hydrogen peroxide (H_2O_2) generation in lymphocytes from healthy subjects and patients with NASH was assayed using a colorimetric method involving the oxidation