Research Article

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Nocturnal hypoxia-induced oxidative stress promotes progression of pediatric non-alcoholic fatty liver disease

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Background & Aims: Oxidative stress is proposed as a central mediator in NAFLD pathogenesis, but the specific trigger for reactive oxygen species generation has not been clearly delineated. In addition, emerging evidence shows that obesity related obstructive sleep apnea (OSA) and nocturnal hypoxia are associated with NAFLD progression in adults. The aim of this study was to determine if OSA/nocturnal hypoxia-induced oxidative stress promotes the progression of pediatric NAFLD.

Methods: Subjects with biopsy proven NAFLD and lean controls were studied. Subjects underwent polysomnograms, liver histology scoring, laboratory testing, urine F(2)-isoprostanes (measure of lipid peroxidation) and 4-hydroxynonenal liver immunohistochemistry (*in situ* hepatic lipid peroxidation).

Results: We studied 36 adolescents with NAFLD and 14 lean controls. The OSA/hypoxia group (69% of NAFLD subjects) had more severe fibrosis (64% stage 0–2; 36% stage 3) than those without OSA/hypoxia (100% stage 0–2), $p = 0.03$. Higher F(2)-isoprostanes correlated with apnea/hypoxia index ($r = 0.39$, $p = 0.03$), % time SaO_2 <90% ($r = 0.56$, $p = 0.0008$) and inversely with SaO_2 nadir ($r = -0.46$, $p = 0.008$). OSA/hypoxia was most severe in subjects with the greatest 4HNE staining ($p = 0.03$). Increasing F(2)-isoprostanes($r = 0.32$, $p = 0.04$) and 4HNE hepatic staining ($r = 0.47$, $p = 0.007$) were associated with worsening steatosis. Greater oxidative stress occurred in subjects with definite NASH as measured by F(2)-isoprostanes ($p = 0.06$) and hepatic 4HNE ($p = 0.03$) compared to those with borderline/not NASH.

Conclusions: These data support the role of nocturnal hypoxia as a trigger for localized hepatic oxidative stress, an important factor associated with the progression of NASH and hepatic fibrosis in obese pediatric patients.

Lay summary: Obstructive sleep apnea and low nighttime oxygen are associated with NAFLD progression in adults. In this study, we show that adolescents with NAFLD who have OSA and low oxygen have significant scar tissue in their livers. NAFLD subjects affected by OSA and low oxygen have a greater imbalance between the production of free radicals and their body's ability to counteract their harmful effects than subjects without OSA and low oxygen. This study shows that low oxygen levels may be an important trigger in the progression of pediatric NASH.

Keywords: Hypoxia; NASH; Sleep apnea; Reactive oxygen species; F (2)-isoprostanes; Antioxidants.

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Abbreviations: ROS, reactive oxygen species; NAFLD, non-alcoholic fatty liver disease; OSA, obstructive sleep apnea; NASH, non-alcoholic steatohepatitis; NAS, NAFLD activity score; AHI, apnea-hypopnea index; SaO_2, O_2 saturation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; CPAP, continuous positive airway pressure; HPF, high powered field; REM, rapid eye movement; GGT, gamma-glutamyltranspeptidase; CRP, c-reactive protein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; SD, standard deviation; CYP2E1, cytochrome P450, family 2, subfamily E, polypeptide 1; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; HIF-1α, hypoxia inducible factor; NF-κB, nuclear factor kappa B; TNF α, tumor necrosis factor alpha; LDL, low-density lipoprotein; DNA, deoxyribonucleic acid; 4HNE, 4-hydroxynonenal.

Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized by abnormal lipid deposition in hepatocytes in the absence of excess alcohol intake, is a disease of epidemic proportions in both children and adults, paralleling the obesity epidemic [1]. NAFLD affects up to 9.6% of all children and 38% of obese children across a spectrum of disease, including isolated hepatic steatosis, non-alcoholic steatohepatitis (NASH, defined as steatosis, hepatocyte ballooning and inflammation), and cirrhosis [1,2]. Although

isolated hepatic steatosis may have no apparent consequences, NASH progresses to liver fibrosis and cirrhosis in about 20% of cases and is associated with hepatocellular carcinoma in adults [1,3].

Substantial evidence suggests oxidative stress is a central mediator in NAFLD pathogenesis and progression, although the specific trigger for reactive oxygen species (ROS) generation has not been clearly delineated. The initial pathology in NAFLD is excessive accumulation of hepatocyte lipid as free fatty acids and triglycerides, in part due to insulin resistance. This lipid is believed to be the substrate for lipid peroxidation upon exposure to ROS, compounded by insufficient scavenging by depressed antioxidant defenses [4]. Oxidative stress induces cytokine activation, inflammation and fibrogenesis, thus promoting the progression from fatty liver to NASH [5]. However, the trigger and source of oxidative stress in NAFLD has not been elucidated.

Emerging evidence demonstrates that obesity related obstructive sleep apnea (OSA) and intermittent nocturnal hypoxia are associated with NAFLD progression. Patients with OSA experience repeated episodes of nocturnal hypoxia alternating with normoxia (so called chronic intermittent hypoxia), resembling the pathophysiology of ischemia/reperfusion injury [6,7]. In obese mice with hepatic steatosis induced by a high fat, high cholesterol diet, exposure to chronic intermittent hypoxia leads to significant increases in ALT and histologic evidence of hepatic inflammation and fibrosis [8,9]. Moreover, morbidly obese adults with moderate to severe OSA and hypoxia have more severe hepatic inflammation than those without hypoxia [10]. Recent reports demonstrate that pediatric NAFLD patients with OSA/hypoxia have more advanced liver disease and fibrosis [11,12]. While these data support a role for OSA/hypoxia in NASH pathogenesis, the mechanism underlying this relationship has not been elucidated.

To address nocturnal hypoxia as a potential source of oxidative stress in NAFLD, this study was conducted to determine if OSA/nocturnal hypoxia-induced oxidative stress promotes the progression of pediatric NAFLD. We hypothesized that systemic and hepatic evidence of increased ROS generation would be strongly associated with both nocturnal hypoxia and histologic severity of pediatric NAFLD.

Patients and methods

Children cared for at the Children's Hospital Colorado Pediatric Liver Center between June 2009 and January 2014 were eligible for this study if they had suspected NAFLD and were scheduled to undergo a clinically indicated liver biopsy. In our center, a clinical liver biopsy for suspected NAFLD is performed in overweight or obese children (body mass index (BMI) >85% for age and gender) with chronically elevated aminotransferases in whom a diagnosis is unclear based on serologic testing, including testing for Wilson's disease, alpha-1-antitrypsin deficiency, viral hepatitis, and autoimmune hepatitis [13]. Inclusion criteria for the study were males and females, ages 8 through 18 years, and Tanner stage 2–4 with liver biopsy evidence of NAFLD. Liver biopsies were performed for clinical indications and not as part of the research protocol. This Tanner stage range was chosen to minimize variations in insulin sensitivity that may confound the interpretation of potential associations between OSA/hypoxia and NAFLD. Exclusion criteria included the presence of Wilson's disease, alpha-1-antitrypsin deficiency, viral hepatitis, autoimmune hepatitis, other known chronic liver disease or cholelithiasis, or use of anticonvulsants, sedatives, corticosteroids, drugs that promote or reduce insulin resistance (including insulin sensitizers, thiazolidenediones and metformin), or other treatments known to induce hepatic steatosis (amiodarone or parenteral nutrition) in the past 2 weeks. Additional exclusion criteria included regular tobacco or alcohol use, current use of continuous positive airway pressure (CPAP), insulin dependent diabetes, neuromuscular disorders, and genetic or craniofacial abnormalities. Data from a subset of these patients has previously been reported [11].

Lean age-matched control subjects (BMI <85%) with no evidence of hepatomegaly or liver disease (AST and ALT $\leqslant$40 IU/L), Tanner stage 2–4, were also enrolled. Subjects were excluded if they had clinical or biochemical evidence of liver disease, with all other exclusion criteria the same as for suspected NAFLD subjects. This study was approved by the Colorado Multiple Institutional Review Board and informed written consent was obtained from parents/guardians and written assent from all subjects.

At enrollment, demographic data, medical history, physical exam and clinical symptoms of sleep apnea (snoring, witnessed apnea, non-restorative sleep, and daytime sleepiness) were recorded. A retrospective review of patient charts was conducted to collect height, weight and BMI in the 3–6 months prior to study enrollment. Height, weight and BMI at liver biopsy were also recorded [14].

Liver biopsies, obtained only for clinical indications by standard percutaneous technique, were subsequently examined in this study. Blood hematocrit level, obtained prior to the liver biopsy, was recorded. Liver histology (Hematoxylin and Eosin and Masson's trichrome stains) was reviewed and scored by a single pediatric pathologist blinded to subject information. Biopsies with histologically confirmed NAFLD (defined as $\geqslant$5% of hepatocytes containing macrovesicular fat) were assigned a grade of necro-inflammation (0–3) and a stage of fibrosis (0–4) based on the histologic criteria of Brunt *et al.* [15]. Biopsies were also scored for steatosis, inflammation, and ballooning degeneration following the criteria established by the NASH Clinical Research Network (CRN) [16]. A NAFLD Activity Score (NAS) was calculated by summing the scores for steatosis, lobular inflammation and ballooning degeneration [16]. In addition, subjects were classified as definite NASH (NAS $\geqslant$5) *vs.* borderline or not NASH (NAS $\leqslant$4). Hepatic fibrosis was scored as stage 0 [none], stage 1 [mild to moderate perisinusoidal or portal/periportal fibrosis only], stage 2 [zone 3 and periportal fibrosis], stage 3 [bridging fibrosis] or stage 4 [cirrhosis] [16] (20). Subjects were also classified as either type 1 (classic adult pattern), type 2 (portal-based) or an overlap of the two NASH histologic subtypes [17].

Immunohistochemical analysis of CD163, expressed on activated cells of monocyte/macrophage origin including Kupffer cells, was performed on paraffin embedded and formalin fixed liver tissue using a primary monoclonal mouse antibody raised against CD163 (clone MRQ-26, Ventana, Tucson, AZ) [12,18]. Staining was performed with the Benchmark Ventana system. The density of positive cells within the portal tract and liver lobule was determined by counting the number of positive cells in an average of ten random portal tracts and ten lobular areas at a magnification of x20 under light microscopy. CD163 was selected to demonstrate activated immune cells during oxidative stress in the current study because it was a significant cell surface marker in a previous study of OSA/hypoxia and pediatric NAFLD [12].

Immunohistochemical analysis of 4-hydroxynonenal (4HNE), an *in situ* marker of lipid peroxidation, was performed on paraffin embedded and formalin fixed liver tissue using a rabbit polyclonal anti-4-HNE primary antibody (generated and validated by C. Shearn in his laboratory in the Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Anschutz Medical Center, Aurora, CO.) and goat anti-rabbit secondary polyclonal antibody with a Vectastain ABC IHC kit (Vector Laboratories, Burlingame, CA), as previously described [19,20]. The primary antibody was diluted 1:750 in tris- buffered saline plus 5% non-fat dry milk and incubated overnight at 4 °C. The secondary antibody was diluted as per manufacturer's instructions and slides were developed using DAB (3,3′-diaminobenzidine) and horseradish peroxidase [19,20]. The 4HNE immunostains were reviewed by a single pediatric pathologist blinded to subject information and scored as follows: 0, no staining; 1, cytoplasmic staining within hepatocytes with indistinct granules and no staining of fat globules; 2, cytoplasmic staining within hepatocytes with distinct, well-formed granules and no staining of fat globules; and 3, cytoplasmic staining within hepatocytes with large cytoplasmic granules and distinct staining around the circumference of fat globules [21]. Staining was uniform throughout the biopsies and when present, was noted within 100% of the hepatocytes.

Following clinical confirmation of NAFLD on liver biopsy, NAFLD subjects underwent a standard multi-channel sleep study (polysomnogram), which was scored by a research trained technician and interpreted by a single sleep medicine physician, both of whom were blinded to liver biopsy results. The following data were analyzed: total sleep time, percent REM sleep, apnea/hypopnea index (AHI), oxygen nadir, percent of time O_2 saturation (SaO_2) $\leqslant$90% and oxygen desaturation index (the number of SaO_2 drops below 95% by pulse oximeter). The presence of OSA was defined as an AHI >2.0, indicating total apneas and hypopneas per hour of total sleep time [22,23]. Apnea was defined as cessation of airflow over $\geqslant$2 attempted respiratory cycles and hypopnea was defined as a decrease in nasal pressure of $\geqslant$50%, with a corresponding decrease in SaO_2 of $\geqslant$3% and/or arousal.