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Nocturnal Hypoxia Induced Oxidative Stress Promotes Progression of Pediatric Non-Alcoholic Fatty Liver Disease

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Abstract

Background—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.

Aim—To determine if OSA/nocturnal hypoxia induced oxidative stress promotes the progression of pediatric NAFLD.

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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 $\text{SaO}_2 < 90\%$ ($r=0.56$, $p=0.0008$) 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.

Graphical Abstract

A graphical summary of this work is presented in the manuscript as Figure 5.

Keywords

Hypoxia; NASH; Sleep Apnea; Reactive Oxygen Species; F (2)-isoprostanes; anti-oxidants

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 reactive oxygen species (ROS), compounded by insufficient scavenging by depressed anti-oxidant 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.

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, thiazolidinediones 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 < 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 ≥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 ≥5) versus borderline or not NASH (NAS <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 ×20 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% nonfat 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 O₂ saturation (SaO₂) > 90% and oxygen desaturation index (the number of SaO₂ drops below 95% by pulse oximeter). The presence of OSA was defined as an apnea/hypopnea index (AHI) > 2.0, indicating total apneas and hypopneas per hour of total sleep time [22, 23]. Apnea was defined as cessation of airflow over ≥ 2 attempted respiratory cycles and hypopnea was defined as a decrease in nasal pressure of ≥ 50%, with a corresponding decrease in SaO₂ of ≥ 3% and/or arousal. Hypoxia was defined as SaO₂ < 90% for ≥ 1% of total sleep time [24]. At the time of the polysomnogram, each subject's height, weight and waist circumference were measured and BMI calculated [14].

The morning following the sleep study, fasting blood specimens were obtained for serum ALT, AST, GGT, ultra-sensitive CRP, total cholesterol, triglyceride, HDL, glucose and insulin (used to calculate the homeostasis model assessment of insulin resistance (HOMA-IR)) [25]. A first morning urine sample was obtained by clean catch and stored at -70 °C for analysis of urine F(2)-isoprostanes by liquid chromatography/tandem mass spectrometry, and normalized to urine creatinine as a measure of oxidative injury [26, 27]. F(2)-isoprostanes, prostaglandin like substances formed by non-enzymatic free radical induced peroxidation of arachidonic acid, have emerged as reliable markers of *in vivo* oxidative stress [28]. Serum anti-oxidants, alpha tocopherol and alpha and beta carotene, were measured on fasting blood specimens by ultra-high performance liquid chromatography and normalized to total serum lipid concentrations measured by a spectrophotometric assay in the Children's Hospital Colorado Clinical and Translational Research Center (CTRC) Core Laboratory [29].

Lean control subjects were evaluated in the outpatient CTRC facility during an early morning appointment. Demographic data, medical history, physical exam and clinical symptoms of sleep apnea (snoring, witnessed apnea, non-restorative sleep, and daytime sleepiness) were recorded. Height, weight and waist circumference were measured and BMI calculated [14]. Similar to NAFLD subjects, lean control subjects had fasting blood specimens obtained for serum ALT, AST, GGT, ultra-sensitive CRP, total cholesterol, triglyceride, HDL, glucose, insulin, total lipids and serum alpha tocopherol and alpha and beta carotene. They also provided a urine sample for F(2)-isoprostane analysis. Lean control subjects did not undergo liver biopsy or polysomnogram.

Statistical analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC, USA). Descriptive statistics are presented as either mean ± SD for continuous measures or

percentages for categorical responses. Two sample t-test, chi square or Fisher's exact test were used, as appropriate, to assess differences between subjects with and without OSA/hypoxia. In addition, to account for the potential confounding effect of BMI changes over time in subjects with and without OSA/hypoxia, a linear regression analysis was performed when appropriate. Pearson or Spearman correlation coefficient, as appropriate, was used to quantify the relationships between ALT, AST and histologic parameters with polysomnographic parameters and oxidative stress measurements. Ln transformation was applied to Urine F(2)- isoprostanes and arcsine-root transformation was applied to proportional type polysomnographic data, such as percent time with $\text{SaO}_2 < 90\%$, however, results are presented in the original scale for ease of interpretation. A p value < 0.05 was considered statistically significant.

Results

Thirty-six obese subjects with liver biopsy confirmed NAFLD were studied (Supplementary Figure 1); 67% were male, 89% Hispanic, with a mean age of 12.9 ± 1.9 years, and mean BMI of 32.3 ± 5.5 (Table 1). Fourteen lean controls were studied; 50% were male, 36 % Hispanic, with a mean age of 13.1 ± 1.8 years, and mean BMI of 18.9 ± 2.3 . As expected, NAFLD subjects had significantly ($p < 0.05$) elevated aminotransferases, inflammatory markers and evidence of the metabolic syndrome, as compared to lean controls (Table 1).

Relationship of OSA/hypoxia to NAFLD

Twenty five NAFLD subjects (69 %) met the study criteria for OSA and/or nocturnal hypoxia: 1 had isolated hypoxia; 15 isolated OSA and 9 subjects had both OSA and hypoxia. NAFLD subjects with and without OSA/hypoxia reported similar symptoms of sleep disordered breathing (Table 2). There were no significant differences in elapsed time between performance of liver biopsy and polysomnogram between subjects with (111 ± 94 days) and without (108 ± 71 days) OSA/hypoxia, $p = 0.9$. There were also no significant changes in BMI in subjects with and without OSA/hypoxia from 3–6 months prior to liver biopsy, to the time of biopsy and at polysomnogram (Table 2). In addition, linear regression analyses adjusting for BMI over time showed no difference from unadjusted models. In all NAFLD subjects, polysomnograms were of adequate length and $> 12\%$ of total sleep time was spent in REM sleep, allowing all studies to be considered valid (Table 2). NAFLD subjects with OSA/hypoxia had a significantly higher ($p < 0.001$) mean AHI score (8.5 ± 7.6) versus those without OSA/hypoxia (1.0 ± 0.6 than), indicating moderate to severe sleep disordered breathing. While oxygen nadirs were similar in those with OSA/hypoxia compared to those without OSA/hypoxia ($83.3 \pm 6.1\%$ vs. $87.2 \pm 23.4\%$, $p = 0.06$), the percent of sleep time spent with SaO_2 less than 90% was significantly longer (2.1 ± 3.6 vs. 0.1 ± 0.2 , $p = 0.01$) and oxygen desaturation index higher (13.6 ± 24.2 versus 2.3 ± 4.0 , $p = 0.03$) in those with OSA/hypoxia. These differences were not explained by differences in age, gender, ethnicity, BMI, waist circumference, clinical symptoms or laboratory testing (Table 2). Subjects with OSA/hypoxia had higher blood hematocrit ($43.5 \pm 2.6\%$) compared to subjects without OSA ($41.3 \pm 1.9\%$), $p = 0.03$. Moreover, there were no significant correlations between hematocrit and AHI, oxygen nadir, percent of sleep time spent with SaO_2 less than 90% or oxygen desaturation index higher (data not shown).

NAFLD subjects with definite NASH (NAS histologic score ≥ 5 , $n=23$) had more severe sleep disordered breathing compared to those without definite NASH (NAS score < 5 , $n=13$). Subjects with definite NASH had significantly higher ($p=0.04$) mean AHI scores (7.7 ± 8.5) than those without definite NASH (3.5 ± 2.7). Subjects with definite NASH also spent significantly more time ($p=0.01$) with oxygen SaO_2 less than 90% (2.2 ± 3.7) than those without definite NASH (0.17 ± 0.33). There were no differences in oxygen nadir or oxygen desaturation index between those with and without definite NASH (data not shown).

NAFLD subjects with and without OSA/hypoxia had similarly elevated aminotransferases, HDL, triglycerides, total cholesterol, inflammatory markers and evidence of insulin resistance (Table 2). Liver histology scores for steatosis, inflammation, ballooning degeneration, NAS summary score (Mean NAS: 4.84 ± 1.28 vs. 4.82 ± 1.40) and histologic grade were also similar in those with and without OSA/hypoxia (Supplementary Table 1). However, NAFLD subjects with OSA/hypoxia had more severe fibrosis (60% Stage 0–2; 40 % Stage 3) than those without OSA/hypoxia (100% Stage 0–2), $p=0.03$. There were no differences in the distribution of histologic Type 1 versus Type 2 NAFLD in subjects with and without OSA/hypoxia.

NAFLD subjects with and without OSA/hypoxia had similar numbers of CD163⁺ cells in both portal and lobular areas (Table 2). A significant relationship was found between lobular but not portal CD163⁺ cells and ALT ($r=-0.40$, $p=0.04$) and portal but not lobular CD163⁺ cells and fibrosis stage ($r=0.40$, $p=0.02$). There were no significant correlations between portal or lobular CD163⁺ cells and AHI, oxygen nadir, histologic grade, degree of steatosis, inflammation, ballooning or NAS summary score (data not shown).

We next examined relationships (r values) between key polysomnographic measurements and markers of NAFLD disease severity (aminotransferases and histologic parameters) across all NAFLD subjects. A significant relationship was found between aminotransferase values and % time with $\text{SaO}_2 < 90\%$ (Figure 1A and 1B). Moreover, an increasing percentage of time with $\text{SaO}_2 < 90\%$ was associated with increasing hepatic steatosis ($r=0.4$, $p=0.01$), inflammation grade ($r=0.31$, $p=0.06$) and NAS summary score ($r=0.32$, $p=0.05$) (Figure 1C, 1D and 1E). In addition, a significant relationship was found between hematocrit and NAFLD fibrosis stage ($r=0.36$, $p=0.03$), but not other histologic parameters. There were no significant correlations between the AHI, oxygen nadir or oxygen desaturation index and histologic grade, fibrosis stage, degree of steatosis, inflammation, ballooning or NAS summary score (data not shown).

Relationship of OSA/hypoxia and Oxidative Stress

Urine F(2)-isoprostanes normalized to urine creatinine were utilized as a biomarker of oxidative stress. Urine F(2)-isoprostanes were significantly higher ($p=0.04$) in NAFLD with (725 ± 53 pg/mg creatinine) and without OSA/hypoxia (573 ± 85 pg/mg creatinine) compared to lean controls (310 ± 77 pg/mg creatinine). Higher levels of F(2)-isoprostanes in NAFLD subjects correlated with more severe sleep apnea, denoted by the AHI ($r=0.39$, $p=0.03$), and more severe hypoxia, denoted by percent time SaO_2 was less than 90% ($r=0.56$, $p=0.0008$), oxygen nadir ($r=-0.46$, $p=0.008$) and oxygen desaturation index ($r=0.45$, $p=0.01$) (Figure 2A, 2B, 2C and 2D). A strong correlation was observed between

urine F(2)-isoprostanes and 4HNE liver immunohistochemistry, a marker of *in situ* hepatic lipid peroxidation ($r=0.56$, $p=0.002$). Representative images of mild 4HNE staining (score of 1) versus severe staining (score of 3) are shown in Figure 3A and 3B. Sleep disordered breathing was most severe in subjects with the greatest 4HNE staining. NAFLD subjects with 4HNE scores of 1 had a mean AHI of 2.83 ± 1.26 and spent 0.10 ± 0.14 % of total sleep time with $\text{SaO}_2 < 90\%$ compared to those with elevated 4HNE scores of 2/3 who had a mean AHI of 6.23 ± 7.24 and spent 1.46 ± 3.06 % of total sleep time with $\text{SaO}_2 < 90\%$ ($p=0.03$), Figures 3C and 3D. Thus, evidence of hepatic oxidative stress in NAFLD was associated with more severe sleep apnea and nocturnal hypoxia.

Relationship of Oxidative Stress and Liver Histology

Next we examined the relationship between oxidative stress and liver histology. Increasing F(2)-isoprostanes ($r=0.32$, $p=0.04$, Figure 2E) and hepatic lipid peroxidation by 4HNE staining ($r=0.47$, $p=0.007$) were associated with worsening steatosis, but not with inflammation, ballooning degeneration, or fibrosis. However, greater lipid peroxidation occurred in subjects with definite NASH (NAS score ≥ 5 , $n=23$), with F(2)-isoprostanes values of 753 ± 308 pg/mg creatinine, compared to those without definite NASH (NAS score < 5 , $n=13$), with values of 548 ± 204 pg/mg urine creatinine, $p=0.06$ (Figure 2F). Similarly, 4HNE staining (hepatic lipid peroxidation) was significantly ($p=0.03$) more marked in subjects with definite NASH (2.53 ± 0.6) compared to those without definite NASH (2.0 ± 0.7) (Figure 3E). Moreover, the severity of 4HNE staining correlated with increasing ALT ($r=0.40$, $p=0.02$) (Figure 3F). Thus, hepatic oxidative stress was associated with definite NASH and evidence of hepatocellular injury. There were no significant correlations between the numbers of portal or lobular CD163⁺ cells and urine F(2)-isoprostanes or hepatic 4HNE.

Relationship of Anti-oxidants to NAFLD and Lipid Peroxidation

Selected circulating serum anti-oxidants were measured and normalized to serum total lipids in NAFLD and lean control subjects. NAFLD subjects had lower serum alpha tocopherol and beta carotene levels compared to lean control subjects (Table 1). These difference in anti-oxidant levels were most striking between NAFLD subjects with OSA/hypoxia and lean subjects ($p < 0.01$) (data not shown). There were no differences in antioxidants between NAFLD subjects with and without OSA/hypoxia. However, alpha tocopherol/total lipid and beta carotene /total lipid ratios were inversely related to elevations of AST and ALT (Figure 4A, 4B, 4C and 4D), but not to liver histology. No significant correlations were found between alpha carotene/total lipid ratio and AST, ALT or liver histology. In addition, no differences in measured antioxidants were found between NAFLD subjects with and without definite NASH. Furthermore, measured anti-oxidant levels did not correlate with AHI, oxygen nadir or percent time SaO_2 was less than 90%. Thus, antioxidant status was not related to the presence of OSA/hypoxia although it did correlate with biochemical evidence of liver injury.

In contrast, alpha-tocopherol/total lipids ($r = -0.27$, $p=0.07$) and beta carotene/total lipids ($r = -0.48$, $p=0.001$) were inversely correlated with urinary F(2)-isoprostanes (Figure 4E and 4F). In addition, lower beta-carotene/total lipid ratios ($p < 0.01$) were found in NAFLD subjects with 4HNE staining scores of 2 (mean: 0.01 ± 0.005 mg/gm) or 3 (mean: 0.01 ± 0.008

mg/gm) compared to those with minimal 4HNE staining scores of 1 (0.04 ± 0.06 mg/gm). Thus, antioxidant levels were inversely related to the measures of oxidative stress and hepatic lipid peroxidation.

Discussion

The results of this study provide evidence implicating OSA/nocturnal hypoxia as a trigger of oxidative stress and injury that promotes the progression of pediatric NASH. In addition, this study confirms that OSA/hypoxia is common in pediatric NAFLD and that more severe OSA/hypoxia is associated with elevated aminotransferases, hepatic steatosis, inflammation, NAS and fibrosis. Higher blood hematocrit levels, a potential biomarker of chronic hypoxia, were also associated with more severe hepatic fibrosis. Moreover, patients with definite NASH experience more severe obstructive sleep apnea and hypoxia than those without definite NASH. The results of this study support the role of nocturnal hypoxia in obese pediatric patients in inducing localized hepatic oxidative stress, a factor associated with the progression of NASH and hepatic fibrosis [5]. These data also implicate low antioxidant status as an additional independent risk factor for hepatic oxidative stress in NAFLD.

In this study, we used two validated markers of oxidative stress-induced lipid peroxidation, urinary excretion of F(2)-isoprostanes and hepatic staining for 4HNE adducts. Studies in animal models of NAFLD demonstrate increased free radical generation in the liver, indicated by increased mitochondrial superoxide radical generation and induction of CYP2E1 isoforms [30] [31]. Adults with NASH have elevated serum markers of oxidative stress (thiobarbituric acid reactive substances [TBARS], oxidized LDL, oxidized glutathione and malonyldialdehyde) compared to those with simple steatosis and/or controls [4, 32, 33]. Limited pediatric data utilizing serum TBARS or malondialdehyde (MDA) suggest increased oxidative stress in children with NAFLD [34] [35]. Although widely used, both TBARSs and MDA can be formed by multiple oxidants and are suboptimal for analysis of active lipid oxidation species *in vivo* [28] [36]. Isoprostanes, a series of stable prostaglandin-like compounds formed by non-enzymatic, free radical-catalyzed peroxidation of arachadonic acid, have emerged as validated biomarkers of lipid peroxidation that are well suited for non-invasive measurement of systemic oxidative stress [28]. Using this biomarker, we show that oxidative stress was significantly higher in children with NAFLD, both with and without OSA/hypoxia, compared to healthy, lean children. Importantly, increasing F(2)-Isoprostanes were associated with worsening histologic steatosis and definite NASH (NAS score 5). In this study, we demonstrated a strong relationship between urine F(2)-isoprostanes and hepatic 4HNE immunostaining, suggesting that urinary excretion of F(2)-isoprostanes reflected ongoing hepatic oxidative injury in our patients.

As a major aldehyde metabolite of lipid peroxidation, 4HNE is a reliable marker of *in situ* lipid peroxidation. Increasing formation of stable 4HNE protein adducts have been described in the oxidative injury that occurs in several chronic liver diseases, including viral hepatitis, hereditary hemochromatosis, alcoholic liver disease and adult NAFLD [21] [37] [38] [39]. Using similar 4-HNE immunohistochemical methodology, we have demonstrated evidence of increased oxidative injury in the liver of pediatric NAFLD patients that was most pronounced in those with severe sleep disordered breathing, supporting our hypothesis that

OSA and nocturnal hypoxia are significant triggers of hepatic oxidative stress and NAFLD progression. Moreover, 4HNE is a strong neutrophil chemo-attractant and may also attract hepatic stellate cells [37] [21], providing a plausible physiologic explanation for its role in disease and fibrosis progression.

In obstructive sleep apnea, liver tissue is subjected to repeated episodes of nocturnal hypoxia and normoxia, resembling the pathophysiology of ischemia/reperfusion injury. During hypoxia, cells adapt to a lower than normal oxygen environment, but with normoxia due to subsequent re-oxygenation, there is a sudden increase in oxygen consumption with enhanced mitochondrial generation of ROS and oxidative stress [40, 41]. This relationship has been established experimentally wherein animals subjected to intermittent cycles of hypoxia demonstrate elevated markers of oxidative stress [42] [43] [44] [45]. This effect may be mediated in part by alterations in oxygen responsive hypoxia inducible factor (HIF-1 α) and nuclear factor kappa B (NF- κ B), thereby triggering the production of cytotoxic, pro-inflammatory (e.g., tumor necrosis factor alpha (TNF- α)) and fibrogenic mediators by Kupffer cells and hepatic stellate cells [45] [46], as summarized in Figure 5. This is supported by the significant correlation between CD163⁺ cells, suggestive of Kupffer cell activation, and liver fibrosis noted in this as well as previous studies [12] [18]. Alterations in gut permeability with resultant endotoxemia in patients with NAFLD and OSA/hypoxia suggest an additional mechanistic link between nocturnal hypoxia, inflammatory activation and NASH [47].

Increased oxidative stress has been reported in adults with OSA compared to healthy controls, which improves following Continuous Positive Airway Pressure (CPAP) treatment [48–50]. Other studies, however, suggested that oxidative stress in OSA patients was related to obesity rather than intermittent hypoxia [51] [52]. Limited small pediatric studies also have yielded conflicting results. Elevated plasma oxidized LDL and exhaled breath concentrate levels of hydrogen peroxide and 8-Isoprostane levels were reported in children with OSA [53] [54]. However, in other reports, urinary F(2) - Isoprostanes were not correlated with sleep disordered breathing [55, 56]. In contrast, our study demonstrates a clear relationship between oxidative stress and the severity of sleep apnea and hypoxia in obese children with NAFLD, both systemically and specifically in the liver. We speculate that nocturnal CPAP therapy may be a potential treatment modality for pediatric NAFLD patients with OSA/hypoxia, exerting its effect by reducing intermittent nocturnal hypoxia-induced oxidative stress.

In addition to increased ROS generation, insufficient anti-oxidant defenses may compound oxidative injury in NAFLD. In adults, reduced levels of the hepatic anti-oxidants coenzyme Q10 and glutathione correlated with the severity of NAFLD, as did serum and hepatic retinol stores [4] [57]. In children, reduced serum beta-carotene and alpha tocopherol were associated with NAFLD [58, 59]. These relationships formed the basis for clinical trials of alpha tocopherol supplementation in adult and pediatric NAFLD patients [60, 61]. In contrast, no clear relationship of anti-oxidant levels with OSA has been reported. On the one hand, decreased total anti-oxidant status was described in adults with OSA compared to healthy controls and an inverse linear relationship between anti-oxidant capacity and AHI in adults was reported [48, 62]. However, others have observed lower total anti-oxidant levels

in adults with mild to moderate but not severe OSA[63]. In the present study, lower serum alpha tocopherol and beta carotene normalized to total serum lipids were observed in NAFLD patients with OSA/hypoxia compared to lean controls. NAFLD subjects with the highest aminotransferases were found to have lower alpha tocopherol and beta carotene. Consistent with the adult experience, anti-oxidant levels did not relate to severity of sleep disordered breathing in our NAFLD subjects. However, NAFLD subjects exhibiting the highest levels of F(2)-isoprostanes and hepatic 4HNE immunohistochemical staining manifested the lowest anti-oxidant levels. Thus, lower anti-oxidant levels in NAFLD patients may be an additional independent risk factor promoting oxidative stress, unrelated to the presence of OSA/hypoxia.

The limitations of this study include the modest sample size, raising the possibility of a type 2 error in detecting relationships between OSA/hypoxia, oxidative stress and severity of NAFLD. While this largely Hispanic male cohort of subjects reflects the demographics of pediatric NAFLD in the United States, these findings may not be generalizable to other populations. Furthermore, the lean control group in this study was disproportionately non-Hispanic white, reflecting racial disparities in BMI among children in the United States [64]. In addition, our sample may be biased towards more severe liver disease as all enrolled NAFLD subjects underwent liver biopsy for clinical indications that included chronic elevation of aminotransferases. Finally, although we recognize that visceral adiposity and its associated adipocytokines may be important in both NAFLD and OSA, this study did not include an obese control group without these diseases [65, 66]. However, similarities noted in waist circumference, a surrogate for visceral adiposity [67] [68], serum leptin and adiponectin between those with and without OSA/hypoxia suggest that these factors were not major confounders in this study.

In conclusion, this study demonstrates a strong association between OSA/hypoxia in obese adolescents, evidence of increased systemic and hepatic oxidative stress and reduced circulating antioxidants with more advanced NAFLD histology and aminotransferase elevation. These data support sleep disordered breathing as an important trigger of oxidative stress that promotes progression of pediatric NAFLD to NASH. Further proof of this hypothesis will require additional investigations to demonstrate prevention or reversal of NASH following effective therapy of OSA and nocturnal hypoxia in obese patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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
SaO₂	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

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Lay Summary

Obstructive sleep apnea (OSA) 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.

Fig.1.

Nocturnal hypoxia is associated with the degree of aminotransferase elevation and histologic disease severity in pediatric NAFLD. A significant relationship was observed between % time with SaO₂ < 90% and elevations of AST (A) and ALT (B), as well as hepatic steatosis (C), inflammation grade (D) and NAFLD Activity Score (E).

Fig. 2.

Severity of OSA and nocturnal hypoxia are associated with increasing oxidative stress (urinary F (2)-Isoprostanes) in pediatric NAFLD: AHI (A), % time with $\text{SaO}_2 < 90\%$ (B), oxygen nadir (C) and oxygen desaturation index (D). In addition, oxidative stress is associated with worsening histologic steatosis (E) and NAFLD disease severity (F).

Fig. 3.

Representative liver histology demonstrates examples of mild 4HNE staining (4HNE score of 1) with the presence of minimal cytoplasmic staining and indistinct granules (A) and severe 4HNE staining (4HNE score of 3) with distinct staining of fat globules and cytoplasmic staining (B). NAFLD subjects with low 4HNE scores had lower AHI scores (C) and spent less of their total sleep time with $\text{SaO}_2 < 90\%$ (D) compared to those with higher 4HNE scores. Subjects with definite NASH had more marked 4HNE staining than those

without definite NASH (E). The severity of 4HNE staining was associated with increasing ALT (F).

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Fig. 4.

Serum alpha tocopherol/total lipid ratios were inversely related to AST (A) and ALT (B). Serum beta carotene/total lipid ratios were inversely correlated with AST (C) and ALT (D). Urinary F(2)-isoprostanes were inversely correlated with alpha-tocopherol/total lipids (E) and beta carotene/total lipids (F).

Fig. 5.

Several underlying mechanisms may explain the progression of NAFLD by oxidative stress. Obese individuals are prone to insulin resistance and hyperlipidemia, which may lead to both hyperglycemia and increased free fatty acids with resultant hepatic steatosis and lipotoxicity. Multiple pathways including sleep apnea and chronic intermittent hypoxia may lead to ROS generation. Oxidative hepatic injury may result from direct attack of ROS on essential biomolecules, including lipids, proteins and DNA, with subsequent activation of cell death pathways and loss of biologic function and hepatocyte viability. In addition to direct attack, ROS may indirectly activate redox sensitive transcription factors, including NFKB and HIF-1, thereby triggering the production of cytotoxic, pro-inflammatory and fibrogenic mediators by Kupffer cells and hepatic stellate cells, promoting NAFLD disease progression [69] [70] [71].

Table 1

Demographic and laboratory findings in NAFLD and lean control subjects.

Demographic/Laboratory Testing	All NAFLD (n=36)	Lean (n=14)	p value	Normal Values for Laboratory tests
Mean Age	12.9 ± 1.9	13.1 ± 1.8	0.8	--
Male Gender	67%	50%	0.3	--
Hispanic Ethnicity	89%	36%	0.0009	--
BMI (±SD)	32.3 ± 5.5	18.9 ± 2.3	<0.0001	--
ALT(IU/L)	115 ± 93	30 ± 5	<0.0001	10–45 IU/L
AST(IU/L)	68 ± 46	36 ± 8	0.0002	15–40 IU/L
CRP (mg/dL)	2.5 ± 2.8	0.6 ± 0.8	0.0005	<0.5 mg/dL
Uric Acid (mg/dL)	6.4 ± 1.5	4.7 ± 1.1	0.0006	2.3–5.4 mg/dL
Cholesterol (mg/dL)	151 ± 42	134 ± 21	0.07	170 mg/dL
Triglyceride mg/dL)	158 ± 83	84 ± 23	<0.0001	150 mg/dL
HDL (mg/dL)	38 ± 8	46 ± 7	0.002	< 35 mg/dL
Glucose (mg/dL)	91 ± 9	84 ± 9	0.02	60–105 mg/dL
Insulin (mIU/mL)	39.6 ± 27.1	14.0 ± 10.5	<0.0001	0.0–29.1 mIU/mL
HOMA-IR	9.2 ± 6.9	3.0 ± 2.6	<0.0001	<2.60
Adiponectin (ug/mL)	7.0 ± 3.3	12.0 ± 3.8	<0.0001	3.5–14.4 ug/mL
Leptin (ng/mL)	30.8 ± 13.9	7.9 ± 7.9	<0.0001	3.7–11.4 ng/mL
Alpha-Tocopherol/Total Lipids (mg/gm)	1.42 ± 0.33	1.67 ± 0.25	0.02	>0.8 mg/gm
Beta-Carotene/Total Lipids (mg/gm)	0.02 ± 0.02	0.04 ± 0.03	0.05	--
F(2)- isoprostanes/urine creatinine (pg/mg creatinine)	682 ± 290	310 ± 130	<0.0001	

BMI: Body Mass Index

All values are mean ± SD unless otherwise indicated.

Table 2

Demographics, laboratory data, symptoms of sleep disordered breathing and polysomnogram results in NAFLD subjects with and without OSA/hypoxia.

Demographic/Laboratory Test	NAFLD with OSA/hypoxia (n=25)	NAFLD without OSA/hypoxia (n=11)	p value	Normal Values for Laboratory tests
Sleep parameter				
Sleep Disordered Breathing Symptoms (% of subjects)				
Snoring	72%	55%	0.3	--
Apnea/Gasping for Air	36%	40%	0.8	--
Restorative Sleep	64%	60%	0.8	--
Daytime Sleepiness	48%	40%	0.7	--
Total Sleep Time (minutes)	390 ± 85	406 ± 53	0.5	--
% REM Sleep	19 ± 8	19 ± 4	0.9	--
Apnea Hypopnea Index (AHI)	8.5 ± 7.6	1.0 ± 0.6	<0.001	--
Oxygen Nadir	83.3 ± 6.1	87.2 ± 3.4	0.06	--
% Time SaO ₂ <90%	2.1 ± 3.6	0.1 ± 0.2	0.01	--
Oxygen Desaturation Index	13.6 ± 24.2	2.3 ± 4.0	0.03	--
Mean Age	12.9 ± 1.8	13.0 ± 2.2	0.8	--
Male Gender	72.0%	54.5%	0.4	--
Hispanic Ethnicity	87.5%	90.9%	1.0	--
BMI *				
BMI pre-liver biopsy (± SD)	33.1 ± 5.8	30.3 ± 4.3	0.2	--
BMI at liver biopsy (± SD)	33.0 ± 6.0	30.7 ± 5.4	0.3	--
BMI at polysomnogram(± SD)	33.0 ± 5.7	30.5 ± 5.0	0.2	--
Waist Circumference (cm)	106.3 ± 14.2	101.0 ± 14.6	0.4	--
ALT(IU/L)	128 ± 102	89 ± 61	0.2	10–45 IU/L
AST(IU/L)	74 ± 52	56 ± 26	0.2	15–40 IU/L
CRP (mg/dL)	2.8 ± 3.2	1.7 ± 1.2	0.1	<0.5 mg/dL
Uric Acid (mg/dL)	6.5 ± 1.5	6.0 ± 1.4	0.4	2.3–5.4 mg/dL
Cholesterol (mg/dL)	161 ± 38	127 ± 44	0.03	170 mg/dL
Triglyceride mg/dL)	153 ± 71	170 ± 112	0.6	150 mg/dL

Demographic/Laboratory Test	NAFLD with OSA/hypoxia (n=25)	NAFLD without OSA/hypoxia (n=11)	p value	Normal Values for Laboratory tests
HDL (mg/dL)	39 ± 8	36 ± 7	0.3	< 35 mg/dL
Glucose (mg/dL)	91 ± 9	92 ± 11	0.9	60–105 mg/dL
Insulin (mIU/mL)	42.7 ± 25.2	32.9 ± 31.2	0.3	0.0–29.1 mIU/mL
HOMA-IR	9.8 ± 6.1	7.8 ± 8.6	0.5	<2.60
Adiponectin (ug/mL)	7.1 ± 3.5	6.6 ± 2.9	0.7	3.5–14.4 ug/mL
Leptin (ng/mL)	31.5 ± 15.1	29.1 ± 10.9	0.6	3.7–11.4 ng/mL
Hematocrit (%)	43.5 ± 2.6	41.3 ± 1.9	0.03	30.5–39.7 %
CD163 ⁺ cells, n ^{**}	--	--		
Portal	24.7 ± 5.6	22.0 ± 7.3	0.3	--
Lobular	104.0 ± 21.6	111.2 ± 23.6	0.4	--
Alpha-Tocopherol/Total Lipids (mg/gm)	1.40 ± 0.3	1.46 ± 0.4	0.6	> 0.8 mg/gm
Beta-Carotene/Total Lipids (mg/gm)	0.01 ± 0.01	0.03 ± 0.03	0.2	--

REM: Rapid Eye Movements; OSA: Obstructive Sleep Apnea. All values are mean ± SD unless otherwise indicated.

* There were no differences in BMI prior to liver biopsy, at the time of liver biopsy and at the time of polysomnogram based on linear regression analysis.

** The density of CD163⁺ 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 ×20 under light microscopy.