The effect of chronic intermittent hypoxia in the evolution of NASH

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Received 10 May 2012; accepted 20 May 2012
Available online 7 March 2013

KEYWORDS
Obstructive sleep apnea; Non alcoholic steatohepatitis

Abstract Background: Obstructive sleep apnea (OSA) causes chronic intermittent hypoxia (CIH) during sleep. OSA is associated with nonalcoholic steatohepatitis (NASH) in obese individuals and may contribute to progression of nonalcoholic fatty liver disease (NAFLD) from steatosis to steatohepatitis.

Objective: To assess the potential role of hypoxia in the development of NASH in obstructive sleep apnea patients.

Methods: Nocturnal polysomnography was performed in 60 consecutive patients for clinical suspicion of OSA. We investigated fasting blood glucose, serum insulin, TNF-α, ABG and liver enzymes for 30 patients with nocturnal polysomnographic recording of OSA and for 15 patients without recording OSA used as controls. Liver biopsy was offered to 15 of 30 patients with elevated liver enzymes.

Results: Patients with OSA had significantly higher levels of insulin and were more insulin-resistant according to HOMA-IR than in controls. We found that the parameters which significantly correlated with AHI were elevated liver enzyme, BMI, ultrasound grading, TNF-α and HOMA-IR in patients group but did not find a similar correlation in controls. Liver biopsy showed steatosis with lobular necrosis or hepatocyte ballooning in the 15 patients, associated with fibrosis in 5 patients.

Conclusion: Hypoxic stress of obstructive sleep apnea may be implicated in the evolution of insulin resistance and steatohepatitis in obese individuals.

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Introduction

Obstructive sleep apnea (OSA) is a condition that affects 1–4% of the general population and 25–35% of obese individuals [1]. According to the criteria defined by the American Academy of Sleep Medicine (AASM), OSA is a medical condition presenting the various symptoms caused by excessive day time sleepiness (EDS) and obstructive sleep apnea (OSA) with an
apnea–hypopnea index (AHI) of 5 or more [2]. Obstructive sleep apnea (OSA) is a complex disorder that consists of upper airway obstruction, chronic intermittent hypoxia and sleep fragmentation. OSA is well known to be associated with hypoxia, insulin resistance and glucose intolerance, and these factors can occur in the presence or absence of obesity and metabolic syndrome [1,3]. Obstructive sleep apnea (OSA) causes chronic intermittent hypoxia (CIH) during sleep. Importantly, and potentially relevant to OSA, hypoxia is now considered as one of the aggravating factors for development of NAFLD [4], and interestingly, OSA is also regarded as one of the factors that accelerate the progression of NAFLD from steatosis to nonalcoholic steatohepatitis (NASH) [5].

NAFLD is emerging as an important public health problem across the globe [6]. NAFLD refers to a wide spectrum of liver damage, which ranges from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. NAFLD is strongly associated with insulin resistance and is defined by accumulation of liver fat > 5% per liver weight, in the presence of < 10 g daily alcohol consumption [7]. The diagnosis of NAFLD can be established by ultrasound and can be confirmed by liver biopsy. The characteristic histology of NAFLD resembles that of alcohol-induced liver injury, but occurs in people who consume minimal or no alcohol. NAFLD is regarded as the most common cause of elevated liver enzymes and is associated with type 2 diabetes, obesity and hyperlipidemia [8].

In routine clinical practice, most cases of fatty liver disease are attributable to alcohol excess; however, fatty liver disease can also occur in association with a wide range of toxins, drugs, and diseases, such as morbid obesity, cachexia, type 2 diabetes, hyperlipidemia, and after jejunointestinal bypass surgery. As important risk factors for NAFLD such as obesity and type 2 diabetes are increasing in prevalence this could explain the marked increase in numbers of individuals with NAFLD [9].

We aimed from this study to assess the potential effect of chronic intermittent hypoxia on the progression of hepatic steatosis into non alcoholic steatohepatitis (NASH).

Patients and methods

Patients

The patients included in this study were referred to sleep unit in Al Rashd Allergy center, Between December 2009 and August 2010, with clinical suspicion of obstructive sleep apnea (OSA) and without known liver diseases. Subjects with a history of excessive alcohol use, decompensate cardiac or respiratory insufficiency, treatment for OSA, regular use of hepatotoxic drugs and known liver disease (e.g. chronic hepatitis C, B or autoimmune hepatitis etc.) were excluded.

Polysomnography

Level III sleep study applied on all patients which records nasal airflow (nasal cannulae) and oral airflow (thermistor), rib cage and abdominal wall motion and arterial oxygen saturation (SaO2). Sleep analysis was performed according to standard criteria [2]. The following data were recorded at the time of polysomnography: age, sex, body mass index (BMI), history of diabetes, hypertriglyceridemia, or high blood pressure. Overweight was defined as a BMI higher than 25 kg/m². All patients underwent a full-night sleep study to characterize their sleep and breathing patterns. Standard criteria were employed to evaluate the breathing patterns during sleep. Apnea was defined as cessation of oronasal airflow for 10 s or more. Hypopnea was defined as a reduction of oronasal airflow, with a more than 4% fall in SaO2. The number of apnea and hypopnea episodes per hour of sleep, or apnea hypopnea index (AHI), was calculated. OSA was defined as severe when the AHI was above 30/hr, moderate when it was 15–30/hr, and mild when it was from 5 to 10/hr and normal when less than 5/hr [10,16].

Laboratory investigations

Immediately after the termination of the sleep study at 7 A.M., fasting venous blood samples were collected for liver function test, glucose, insulin, lipid profile and also arterial blood gas (ABG) was measured. Insulin resistance was calculated via homeostasis model assessment (HOMA) as follows: fasting serum insulin x fasting serum glucose/22.5, where insulin is expressed in mU/L and glucose in mmol/L [11].

Patients with elevated liver enzymes were proposed for additional laboratory investigations at the time of liver biopsy. The following chronic liver diseases were excluded by using the appropriate tests: hepatitis B, hepatitis C, autoimmune hepatitis.

Ultrasonography

All patients included in the study underwent abdominal ultrasonography for exclusion of liver cirrhosis, ascites, focal lesion, splenomegaly and renal abnormality, and also for determination of bright echo-pattern grading which correlated with the degree of fatty infiltration of liver. Grade I (mild): Echogenicity is slightly increased with normal visualization of the diaphragm and the intrahepatic vessels borders. Grade II (moderate): Echogenicity is moderately increased with slightly impaired visualization of the diaphragm or the intrahepatic vessels borders. Grade III (severe): Echogenicity is markedly increased with poor or no visualization of the diaphragm, the intrahepatic vessels borders and posterior border of the right lobe [33].

Liver biopsy

A liver biopsy was performed for histopathologic analysis after taking a specific written informed consent.

Pathology

Liver biopsy was proposed to patients with elevated liver enzymes. Liver biopsy was scored according to Brunt et al., with modifications [34]. Steatosis was graded as follows: mild (10–30% of hepatocytes affected), moderate (30–60% of hepatocytes affected), and severe (60% of hepatocytes affected). Fibrosis was graded on a scale of 0 to 4 (0, absent; 1, perisinusoidal/pericellular fibrosis; 2, periportal fibrosis; 3, bridging fibrosis; 4, cirrhosis). Intralobular necrosis was graded on a scale of 0 to 3 (0, absent; 1, less than one necrosis injury per lobule; 2, one or more necrosis injury per lobule; 3, more than two necrosis injuries per lobule). Hepatocyte ballooning was graded on a scale of 0 to 2 (0, absent; 1, ballooned hepatocytes in less than 50% of lobules; 2, ballooned hepatocytes in more than 50% of lobules). Nonalcoholic steatohepatitis (NASH) was defined by the presence of steatosis associated with lobular
necrosis (grade 1 or more) or hepatocyte ballooning (grade 1 or more), with or without fibrosis (grade 1 or more) [34].

Statistical analysis

The data was analyzed using the statistical package for social sciences (spss) version 8.0 software. The significance of differences between mean values of the study variables was evaluated by using t-test. The significance of differences between proportions was performed using the Chi-square test. Correlation coefficient (r) between quantitative variables was calculated. The P value less than 0.05 is considered significant.

Results

Nocturnal polysomnography was performed in 60 consecutive patients for clinical suspicion of OSA. Fifteen patients were excluded: 3 because of known hepatitis B and C, 2 because use of hepatotoxic drugs (e.g., rifampicin and nonsteroidal anti-inflammatory drugs), and 10 because of incomplete data. Therefore, 45 patients were included in the study: 30 of 45 patients had OSA with mean AHI of 40.7 and mean Spo2 of 83 and were used as patients group. 15 of 45 patients did not have OSA with mean AHI of 10.07 and mean Spo2 of 95.6 and were used as control group (Table 2).

Of these, 20 (66.6%) patients were females and 10 (33.4%) patients were males in patient group, varying in age from 24 years to 53 years with the mean age of 41 years compared with 9 (60%) females and 6 (40%) males in control group, varying in age from 29 years to 51 years with the mean age of 39.3 years (Table 2). BMI was significantly higher in patients group than in controls (Table 2). We observed a positive correlation between BMI and AHI in patients group but did not find a similar correlation in controls (Table 1). Patients with OSA had significantly higher levels of insulin and were more insulin-resistant according to HOMA-IR than in controls (Table 2). There was relationship observed between AHI and the level of insulin resistance as assessed by HOMA index in patients group (Table 1). HOMA-IR was significantly correlated with BMI in patients group (r = 0.39; p = 0.03).

Patients group exhibited elevated levels of liver enzymes (ALT and AST) with normal serum levels of bilirubin, total protein, and albumin. We found that liver enzymes were significantly higher in patients group than in controls (Table 2). The parameters which significantly correlated with elevated liver enzyme were AHI (Table 1) and TNF-α (ALT, r = 0.4; p = 0.02 and AST, r = 0.38; p = 0.03).

Liver biopsy was performed in 15 of 30 patients with elevated liver enzymes. 11 of 15 patients had severe OSA (AHI) and 4 of 15 patients had moderate OSA. Liver biopsy showed steatosis in the 15 patients: mild in one patient, moderate in nine patients, and severe in five patients. Steatosis was associated with lobular necrosis or hepatocyte ballooning in the 15 patients, defining necro-inflammatory activity (steatohepatitis) according to Brunt grading [34]. Among the 15 patients with steatohepatitis, perisinusoidal fibrosis was present in 5 patients especially in those had severe OSA. Regarding histological findings, The percentage of steatosis, Brunt grades (necro-inflammatory activity) and Brunt stages (fibrosis) were positively associated with AHI (r = 0.715; P = 0.001 and r = 0.65, P = 0.01 and r = 0.46; P = 0.05, respectively). There were no significant differences as regard any of the histopathological findings between patients with severe OSA and patients with moderate OSA.

In this research, we observed that grad III of ultrasonography was the most common finding in patient group and grad I was the most common in control group (Table 3). We observed a positive correlation between ultrasonography grades and AHI in patients group (r = 0.39; P = 0.03) but did not find

Table 1  Correlation between AHI and Spo2 and clinical and biochemical parameters in studied groups.

| Items          | Patients group | Control group |
|----------------|----------------|---------------|
|                | AHI            | Spo2          |                  |
|                | r   | p   | r   | p   | r   | p   | r   | p   |
| Age            | 0.06 | 0.7 | 0.2 | 0.18 | 0.01 | 0.95 | 0.42 | 0.11 |
| BMI            | 0.40 | 0.02 | -0.26 | 0.33 | 0.19 | 0.48 | 0.41 | 0.12 |
| ALT            | 0.44 | 0.01 | -0.11 | 0.55 | 0.25 | 0.36 | 0.46 | 0.08 |
| AST            | 0.40 | 0.02 | -0.18 | 0.32 | 0.13 | 0.62 | 0.01 | 0.95 |
| TNF            | 0.39 | 0.03 | -0.36 | 0.04 | 0.2 | 0.4 | 0.46 | 0.08 |
| Cholesterol    | 0.12 | 0.5 | 0.12 | 0.51 | 0.14 | 0.6 | 0.12 | 0.65 |
| FBS            | 0.04 | 0.82 | 0.04 | 0.82 | 0.45 | 0.09 | 0.01 | 0.95 |
| FSI            | 0.03 | 0.8 | 0.12 | 0.51 | 0.2 | 0.46 | 0.24 | 0.38 |
| HOMA-IR        | 0.46 | 0.01 | 0.13 | 0.47 | 0.23 | 0.41 | 0.21 | 0.43 |
| Abd. U/S grading | 0.39 | 0.03 | 0.09 | 0.61 | 0.19 | 0.49 | 0.07 | 0.78 |
| Spo2           | 0.02 | 0.88 | 1.0 | 0.88 | 0.21 | 0.44 | 0.1 | 0.0 |
| AHI            | 1   | 0   | 0.02 | 0.88 | 1   | 0   | 0.21 | 0.44 |
a similar correlation in controls ($r = 0.19; P = 0.49$). Similarly, we found a positive correlation between ultrasonography grades and HOMA-IR in patients group but not in control group (Table 4). BMI was positively correlated with ultrasonography grades in both groups (Table 4). No correlation was observed between ultrasonography grades and liver enzymes in both groups (as shown in Table 4).

TNF-$\alpha$ was positively correlated with AHI and negatively correlated with Spo2 in patients groups but these correlations were not observed in controls (as shown in Table 1).

### Discussion

Nonalcoholic fatty liver disease (NAFLD) is a wide pathological complex ranging from simple steatosis to steatohepatitis, advanced fibrosis, cirrhosis and hepatocellular carcinoma [12]. Nonalcoholic steatohepatitis (NASH) is considered to be the cornerstone of NAFLD, showing abnormal triglyceride deposition in hepatocytes along with acute and chronic lobular and portal inflammation [13]. Experimental intermittent hypoxia (IH) has been shown to induce NASH [14]. The metabolic disorders that predispose patients to NASH include insulin resistance and obesity but the mechanism by which repeated hypoxic events, such as occur in OSA, can lead to the progression of liver disease is unclear. This study assesses the relationship between the presence of OSA and liver injury in patients with steatosis and we try to clarify the mechanism by which OSA induce NASH.

Insulin resistance is thought to be the “first hit” in nonalcoholic fatty liver disease, leading to excess free fatty acid accumulation in the liver [15]. In our study, we found a significant link between insulin resistance and OSA, dependent of BMI. This could be explained by the fact that OSA patients included in this study have BMI more than 27, and BMI was positive correlated with OSA and IR. This result suggests that obesity is considered as a risk factor for OSA and IR, confirming previous published studies [16,17]. Our data revealed that nocturnal chronic intermittent hypoxia (CIH) in obese individuals with OSA were associated with marked increase in pro-inflammatory cytokine TNF-$\alpha$. TNF-$\alpha$ is known to play a role in the pathogenesis of insulin resistance [18]. Furthermore, abnormal nocturnal sympathetic output has been proposed as the mediating mechanism in the causal link between insulin resistance and OSA [19]. Adipose tissue hypoxia (ATH) is a new concept in understanding the pathogenesis of insulin resistance and inflammation in OSA. The concept suggests that hypoxia stimulate lipolysis, inhibit uptake of free fatty acid (FFA) in adipocytes, and inhibit adipogenesis and triglyceride synthesis, which leads to FFA elevation in the plasma of obese subjects [1]. Recent data indicate that high plasma FFA have a significant role in contributing to insulin resistance [20]. Improvement in insulin responsiveness after continuous positive airway pressure treatment (CPAP) was reported [16]. Therefore, it could be postulated that OSA contributes to increased insulin resistance and steatosis.

In clinical practice, detection of liver injury is based on abnormal liver tests, and liver biopsy is usually proposed only for patients with elevated liver enzymes. In our study, the diagnosis of NASH was based on elevated liver enzymes, exclusion of other causes of chronic liver disease “most notably viral hepatitis and autoimmune hepatitis” and liver biopsy. As expected, liver biopsy revealed steatosis in all patients that associated with hepatocyte ballooning or lobular necrosis, allowing the diagnosis of NASH in 15 cases, associated with fibrosis in 5 cases [12,21]. In our study, detection of liver damage was probably underestimated because diagnosis was based on elevated liver enzymes, whereas cases of NASH without abnormal liver tests have been reported [22]. In this study, OSA was significantly correlated with elevated liver enzymes. This finding has been endorsed by other human studies which have shown that OSA is associated with an increase in liver enzymes, and treatment of OSA with continuous positive airway pressure (CPAP) therapy has been shown to decrease liver enzymes (ALT and AST) [1,23].

Progression of hepatic steatosis into NASH has been linked to oxidative stress through lipid peroxidation, however, accurate understanding of the trigger of inflammation in the so-called “second hit” remain unclear [24]. The relationship between AHI and lobular necrosis, hepatocyte ballooning and liver fibrosis in our biopsied patients suggests that OSA may play a role in the pathogenesis of NASH. A possible mechanism may be CYP2E1 activation which is a major microsomal source of oxidative stress, as recently shown by Chalasani et al., who found a significant correlation between hepatic CYP2E1 activity, nocturnal hypoxia, and serum insulin levels in non-diabetic patients with biopsy-proven NASH [25]. Liver injury in OSA could also be the result of direct hypoxia, as suggested in two previous case reports [16].

The previous experimental studies in the mouse model showed that CIH increases lipid peroxidation and activates a redox-dependent transcription factor, NF-kB, in the liver [26]. NF-kB is the master regulator of inflammatory process and its activation with hypoxia leads to activation of TNF-$\alpha$, IL-1, IL-6, monocyte chemoattractant protein-1, macrophage migration-inhibition factor and inducible nitric oxide synthase [27,28]. Importantly, NF-kB is also increased not only with OSA but also with obesity and metabolic syndrome [27]. It

### Table 3 Grading of ultrasonography in studied groups.

| Groups       | Grade I (mild) | Grade II (moderate) | Grade III (sever) |
|--------------|----------------|---------------------|-------------------|
| N            | %              | N                   | %                 |
| Patients group | 6              | 20.0                | 9                 | 30.0               | 15                | 50.0               |
| Control group | 6              | 60.0                | 5                 | 33.3               | 1                 | 6.7                |
| $k^2$        | Fisher exact   | 7.2                 | 1.0               | 0.04               |
| $P$ value    | 0.007*         | 0.004*              |

### Table 4 Correlation between grading of ultrasonography and some biochemical parameters in studied groups.

| Items        | Patients group | Control group |
|--------------|----------------|---------------|
|              | $r$ $p$        | $r$ $p$       |
| BMI          | 0.799 0.001*   | 0.515 0.05*   |
| ALT          | 0.072 0.704    | 0.056 0.842   |
| AST          | 0.134 0.48     | 0.097 0.73    |
| HOMA-IR      | 0.41 0.02*     | 0.24 0.35     |
| AHI          | 0.39 0.03*     | 0.19 0.49     |
| Spo2         | 0.09 0.61      | 0.07 0.78     |
has also been shown that CIH itself increases liver levels of pro-inflammatory cytokines IL-1β, IL-6, macrophage inflammatory protein-2, and TNF-α [29]. Our present report and previous experimental data suggest that the hypoxic stress of OSA may induce oxidative stress in the livers of patients with severe obesity, leading to inflammation and fibrosis, which culminate in NASH [30].

Increase in plasma leptin level is considered another possible mechanism by which OSA could induce the progression of hepatic steatosis to steatohepatitis. Leptin is a hormone that is secreted by adipose tissue and increases with obesity. The main role of leptin is to reduce appetite [31]. OSA is known to be associated with an increase in plasma leptin levels, and the increase in leptin occurs in proportion to the severity of OSA [31]. Increased leptin is known to be associated with NAFLD [32].

Conclusion

Our findings suggest that the hypoxic stress of OSA could play a role in the pathogenesis of increasing insulin resistance and the progression of hepatic steatosis to steatohepatitis in obese individuals. Histopathological evidence from 15 obese individuals has shown that OSA is associated with NASH. In addition, we have shown that OSA is a risk factor for abnormal liver enzymes and should be investigated in patients without other cause of liver disease. Future studies are needed to elucidate whether treatment of OSA with CPAP can improve liver injury or not.

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