NADPH oxidase, uncoupled endothelial nitric oxide synthase, and NF-KappaB are key mediators of the pathogenic impact of obstructive sleep apnea – Therapeutic implications

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Abstract
Obstructive sleep apnea (OSA) markedly increases risk for atherosclerosis, systemic and pulmonary hypertension, ventricular hypertrophy, myocardial infarction, stroke, and sudden-death arrhythmias; it also has adverse psychological effects, including daytime somnolence. Clinical and rodent studies indicate that oxidative stress and inflammation underlie the pathogenesis of these complications. NADPH oxidase complexes have emerged as a key source of this oxidative stress, which is amplified in vascular endothelium by uncoupled nitric oxide synthase (eNOS). Proteolytic conversion of xanthine dehydrogenase to xanthine oxidase triggered by hypoxia appears to be upstream from NADPH oxidase activation during intermittent hypoxia. Activation of NF-kappaB downstream from this oxidative stress promotes the inflammation associated with OSA. These considerations suggest therapeutic avenues that, when OSA cannot be fully controlled with CPAP, may lessen the pathogenic impact of this syndrome. The elevated NADPH oxidase activity in OSA might be addressed with spirulina, whose chromophore phycocyanobilin can potently inhibit NADPH oxidases in a manner analogous to bilirubin. Xanthine oxidase inhibitor allopurinol may suppress NADPH oxidase activity and may lessen the pathogenic impact of this syndrome. 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) imaging was found to be increased in a range of organs, including the brain [15,25-28]. Clinically, studies comparing OSA patients with matched controls, or comparing patients whose OSA was of greater or lesser severity, have documented increased markers of oxidative stress and/or inflammation associated with significant OSA. The markers found to be higher in OSA included TBARs in LDL particles, lag phase of LDL oxidation ex vivo, paraoxonase level, superoxide production by neutrophils stimulated ex vivo, urinary excretion of 8-hydroxydeoxyguanosine and 8-isoprostane, exhalation of 8-isoprostane, plasma levels of C-reactive protein and adhesion molecules (ICAM-1, VCAM-1, selectin, CD15, CD11c), and diacron reactive oxygen metabolites [29-38]. Conversely, the antioxidant capacity of whole blood (trolue equivalent antioxidant capacity) was found to be decreased relative to controls in OSA patients [39]. In most of these studies, markers of oxidative stress and inflammation were reported to decline significantly following a course of CPAP therapy.

Considerable evidence points to NADPH oxidase complexes as a key source of the oxidative stress underlying OSA, and to increased activation of NF-kappaB as a mediator of the associated inflammation. Additionally, in vascular endothelium, uncoupling of the endothelial nitric oxide synthase (eNOS) contributes both to oxidative stress and a loss of protective NO bioactivity.18, 41

Adverse health consequences of obstructive sleep apnea
Epidemiological studies reveal that, independent of associated risk factors, obstructive sleep apnea (OSA) increases risk for heart attack and stroke, systemic hypertension associated with sympathetic activation, pulmonary hypertension, ventricular hypertrophy and heart failure, arrhythmias, erectile dysfunction, and sudden death [1-9]. Patients with OSA have an impairment of flow-mediated vasodilation, and increased circulating markers of endothelial activation, that improve with CPAP (chronic positive airway pressure) therapy [10-13]. When rodents are subjected to chronic intermittent hypoxia, a manner intended to mimic the episodic hypoxia of OSA, systemic and pulmonary hypertension, ventricular hypertrophy, endothelial dysfunction, and an exacerbation of diet-induced atherosclerosis can be observed [14-18]. Cardiac ischemia-reperfusion produces greater infarct sizes in rats previously subjected to chronic intermittent hypoxia [19]. OSA also has adverse psychological effects; OSA patients tend to experience daytime hypersomnolence and are prone to depression, and rodents subjected to intermittent hypoxia experience a deficit in hippocampus-dependent spatial learning and increases in anxiety responses [20-25].

There is general agreement, based on clinical studies of OSA patients, as well as rodent studies of chronic intermittent hypoxia, that OSA is attended by an increase in oxidative stress and inflammation in a range of organs, including the brain [15,25-28]. Clinically, studies comparing OSA patients with matched controls, or comparing patients whose OSA was of greater or lesser severity, have documented
NADPH oxidase and uncoupled eNOS are key mediators of the pathogenic impact of OSA

The central role of NADPH oxidase as a mediator of many pathogenic effects of chronic intermittent hypoxia (CIH) clearly emerges in rodent studies. Mice that are genetically deficient in gp91phox (a.k.a. Nox2) are protected from the pulmonary hypertension, systemic hypertension, ventricular remodeling, hypersomnolence, cognitive deficits, and anxiety produced by CIH in wild-type mice [17,24,42-45]. Analogously, treatment with apocynin, an inhibitor of NADPH oxidase complexes, protects rodents from the systemic hypertension, erectile dysfunction, hypersomnolence, and brain oxidative damage provoked by CIH [16,42,45,46]. Increased expression of various subunits of NADPH oxidase has been observed in the tissues of rodents exposed to CIH, and increased levels of p22phox mRNA in macrophages and neutrophils derived from morning sputum – as contrasted to sputum collected before sleep – were observed in patients with untreated OSA [10,17,19,47,48].

How NADPH oxidase is activated by intermittent hypoxia is not yet entirely clear. The mechanism whereby Nox2-dependent NADPH oxidase activity is increased by intermittent hypoxia in the carotid body – an effect which is key to sympathetic activation and systemic hypertension in CIH – has been studied in PC12 cells [49]. Proteolytic conversion of xanthine dehydrogenase to xanthine oxidase appears to be upstream from NADPH oxidase activation [50]. This is mediated by proteolytic activity, evoked by hypoxia, that is suppressed by a trypsin inhibitor [51]. The resultant initial production of superoxide by xanthine oxidase, provided with purine catabolites owing to hypoxic dephosphorylation of adenine nucleotides, increases intracellular free calcium, and this in turn promotes activation of protein kinase C activities, leading to membrane assemblage of intact and active NADPH oxidase complexes [50]. The greater level of superoxide production associated with NADPH oxidase activation boosts expression of hypoxia-inducible factor-1alpha (HIF-1alpha) by inhibiting prolyl hydroxylases that induce its proteasomal degradation [52]. One of the effects of increased HIF-1 activity is increased transcription of the Nox2 gene [49] hence, a vicious cycle is established in which HIF-1 activity increases expression of Nox2, leading to greater superoxide production that further promotes HIF-1alpha expression. Notably, in mice that are heterozygous for knockout of HIF-1alpha, the systemic hypertension and brain oxidative stress evoked by CIH are not observed [49]. The extent to which these mechanisms can account for CIH-mediated activation of NADPH in other tissues, such as the vasculature, requires further study.

Other recent research points to uncoupled eNOS as a source of oxidative stress, and a cause of impaired endothelium-dependent vasodilation, in OSA patients and in rats subjected to CIH [40,41]. This likely is a downstream consequence of the oxidative stress generated by NADPH oxidase, and would be expected to further amplify this oxidative stress [53]. Arginase inhibition (in rats) and provision of the eNOS cofactor tetrahydrobiopterin (in humans) have been shown to rescue this enzyme [40,41]. These findings suggest that both an imbalance of arginine and asymmetric dimethylarginine (ADMA), as well as of tetra- and dihydrobiopterin, contribute to the uncoupling associated with OSA. With respect to ADMA, clinical studies have reported elevated ADMA levels in OSA patients, pointing to ADMA as a mediator of eNOS uncoupling in OSA [54,55].

The impairment of endothelium-dependent vasodilation which characterizes OSA appears to be attributable to loss of NO bioactivity, [56-58] and may therefore reflect both the uncoupling of eNOS, and the fact that superoxide generated by either NADPH oxidase or uncoupled eNOS can react spontaneously with NO to form peroxynitrite. The ability of intravenous vitamin C to acutely improve flow-mediated vasodilation in OSA patients likely is attributable to scavenging of superoxide by elevated levels of ascorbate. [59]

**NF-KappaB activation drives inflammation in OSA**

In untreated patients with OSA, increased NF-kappaB activity has been observed in monocytes, neutrophils, and freshly harvested venous endothelial cells; CPAP therapy reversed this effect [13, 60-62]. In rodents subjected to CIH, activated NF-kappaB has been reported in arterial tissues, cardiomyocytes, vascular endothelium, lung, and liver [14,61,63-66]. Activation of NF-kappaB has also been reported in cultured cells exposed to intermittent hypoxia [67-69]. In some cases, this activation was not accompanied by activation of HIF-1, but was dependent on activation of p38 MAP kinase and IkappaB kinase-beta; hence activation occurred via the canonical pathway [68,69]. The activation of NF-kappaB in cardiomyocytes of mice exposed to CIH was not seen in gp91phox knockout mice similarly treated, suggesting a role for NADPH oxidase as an upstream mediator of NF-kappaB activation [14]. Whereas wild type mice fed a high cholesterol diet developed atherosclerosis when exposed to CIH, no such effect was observed in mice genetically deficient in the p50 subunit of NF-kappaB [18].

These considerations suggest that measures capable of safely down-regulating the activation of NADPH oxidase, recoupling eNOS, and/or diminishing NF-kappaB activation may have potential for alleviating the pathological consequences of OSA. While CPAP therapy, applied regularly and properly, may provide all of the protection that is needed in this syndrome, compliance with CPAP therapy, which can be unwieldy and uncomfortable for some patients, is less than ideal – and CPAP equipment sometimes malfunctions [70,71]. Hence, it would seem prudent to use safe and convenient adjunctive measures in conjunction with CPAP, if these were indeed effective for mitigating the risks associated with OSA.

**Spirulina as an antagonist of NADPH oxidase activity**

There is recent evidence that unconjugated bilirubin functions within cells as an inhibitor of NADPH oxidase complexes; this likely explains much of the profound antioxidant activity of the heme oxygenase, which, in response to perceived oxidative stress, cleaves heme to generate carbon monoxide, free iron, and (via biliverdin) bilirubin [72-75]. Moreover, the spirulina chromophore phycocyanobilin (PhyCB), a structural analog and derivative of biliverdin that constitutes about 0.6% of the dry weight of spirulina, has been shown to mimic the ability of biliverdin/bilirubin to inhibit NADPH oxidase complexes – even when administered orally in rodents. [76,77]. This may account for the manifold anti-inflammatory and antioxidant effects of oral spirulina (or of oral phycocyanin, the spirulina protein which incorporates PhyCB as a chromophore) reported in rodent studies [76-80]. If the metabolism of PhyCB in humans is reasonably analogous to its metabolism in rodents, it should follow that a sufficiently high intake of spirulina, phycocyanin, or free PhyCB will be a clinically effective inhibitor of NADPH oxidase activity. Hence, PhyCB/spirulina may have important potential in the management of OSA. Extrapolations from rodent studies suggest that a spirulina intake of 15-30 g daily might be needed for an optimal antioxidant effect [76].
Inhibiting xanthine oxidase with allopurinol

In light of evidence that conversion of xanthine dehydrogenase to xanthine oxidase is upstream from NADPH oxidase activation in CIH, it is reasonable to suspect that allopurinol treatment might have a favorable clinical impact on OSA. Indeed, in a crossover placebo-controlled clinical study, 2 weeks of allopurinol (300 mg daily) improved flow-mediated vasodilation and lessened plasma markers of oxidative stress in OSA patients [81]. Analogously, in rats exposed to CIH, allopurinol administration boosted acetylcholine-induced vasodilation, and favorably influenced cardiac function and cardiac markers of oxidative stress and apoptosis [82,83].

Supporting glutathione synthesis with N-Acetylcysteine

Some of the downstream effects of excessive superoxide production – whether stemming from NADPH oxidase activation, uncoupling of eNOS, or other sources – can be antagonized by boosting the reduced glutathione content of tissues. Superoxide gives rise to hydrogen peroxide, which oxidizes mildly acidic cysteine groups in proteins, altering their structure and function; reduced glutathione often acts to reverse these effects, while also aiding catabolism of hydrogen peroxide [84-87]. Cysteine supplementation – best achieved with N-acetylcysteine (NAC) – can boost tissue glutathione levels, and there is evidence that this may be particularly beneficial in the elderly, who have a diminished capacity for glutathione synthesis [88-92]. Most clinical trials with NAC have employed daily doses of 1200-1800 mg.

In rodent studies, NAC administration has been reported to alleviate the adverse impact of CIH on erectile dysfunction, and prevent the pro-inflammatory, pro-oxidant effects of CIH on the liver [93,94]. But of particular interest is a clinical study in which 20 OSA patients were randomized to receive NAC or placebo for 30 days; sleep function was assessed by polysomnography before and after the supplementation [95]. A number of the parameters measured – including sleep efficiency, oxygen desaturation events per hour, and Epworth sleepiness score – improved markedly and significantly in the NAC group, but not the placebo group. If the findings of this small study prove to be replicable, they suggest that oxidative stress in the central nervous syndrome tends to promote apneic episodes. The authors propose that “long-term treatment with NAC in patients with OSA may reduce their dependency on CPAP.” An attempt to replicate this intriguing clinical study would be warranted.

Citrulline and high-dose folate for recoupling eNOS

eNOS is only fully coupled when it binds its substrate arginine and its cofactor tetrahydrobiopterin (BH4) [53]. The oxidation product of BH4, dihydrobiopterin (BH2), competes with BH4 for binding to eNOS; an elevation of the ratio of BH2 to BH4 therefore is associated with eNOS uncoupling [96-98]. Analogously, ADMA competes with arginine for binding to eNOS, and a relatively high ratio of ADMA with eNOS uncoupling [99]. Endothelial oxidative stress, whether originating from NADPH oxidase activity or other sources, tends to boost levels of both BH2 and ADMA [53]. BH2 increases under oxidative stress owing to direct oxidation of BH4 by oxidants such as peroxynitrite; in addition, oxidative stress may somehow down-regulate endothelial expression of dihydrofolate reductase, an enzyme capable of recombining BH2 to BH4 [97,98,100]. Oxidative stress also tends to boost endothelial ADMA levels by inhibiting the enzyme responsible for its catabolism, dimethylarginine dimethylaminohydrolase (DDAH) [101-103]. And eNOS can be uncoupled by glutathionylation, which is more likely when oxidants boost cellular levels of glutathione [106-106]. Hence, uncoupling of eNOS may be a consequence of NADPH oxidase activation in OSA – leading to a further increase in oxidant production that would tend to maintain the uncoupling of eNOS in a vicious cycle.

Fortunately, there are practical nutraceutical strategies that can promote the recoupling of eNOS. The adverse impacts of elevated ADMA and of increased arginase activity can be offset by increasing endothelial levels of arginine. This is best achieved by supplementing with the amino acid citrulline, rather than arginine per se [107-109]. The utility of supplemental arginine is compromised by inductive arginase activities in the gastrointestinal tract and liver, such that only a small proportion of supplemental arginine reaches peripheral tissues intact. Citrulline, however, is well absorbed and well distributed to the body’s tissues; within cells, it is efficiently converted to arginine. Hence, supplemental citrulline functions as an efficient delivery form for intracellular arginine. The utility of supplemental citrulline – usually administered in a range of 3-6 g daily, in divided doses – has been documented in clinical circumstances associated with eNOS uncoupling [107,110-114].

With respect to BH2/BH4 imbalance, high-dose folate has the potential to correct this imbalance. This appears to reflect two phenomena. When taken up by cells, folate is rapidly converted to reduced forms, such as a 5-methyltetrahydrofolate, which can serve as highly effective scavengers for peroxynitrite and possibly other oxidants which tend to oxidize BH4; this effect is most meaningful when supraphysiological concentrations of folate are employed [115-117]. In addition, there is recent evidence that high-dose folate has an inductive effect on dihydrofolate reductase in endothelial cells, reflecting increased transcription of its gene [118-120]. Since this enzyme plays a key role not only in folate metabolism, but also in mediating the reconversion of BH2 to BH4 [97,98] folate-mediated induction of dihydrofolate reductase may also help to recouple eNOS. Recoupling of eNOS with high-dose folate has been documented in clinical and rodent studies [121-125]. Folate doses in the range of 10-80 mg daily have been employed in the management of vascular disorders, without evident toxicity or side effects; several decades ago, cardiologist Kurt Oster claimed anecdotally that such doses were useful in the management of angina and intermittent claudication [126-128]. Concurrent supplementation with high-dose vitamin B12 – around 1 mg daily – could be used to correct any coexisting B12 deficiency, and hence eliminate the risk that high-dose folate could mask the early symptoms of pernicious anemia [129] (This risk is the FDA’s justification for banning high-dose folate products [130]).

Docosahexaenoic acid functions as a sensor of oxidative stress

The long-chain omega-3 fatty acid docosahexaenoic acid (DHA), when incorporated in cellular membranes, is readily oxidized in tissues under oxidative stress. Subsequent metabolism of oxidized DHA leads to generation of the compound 4-hydroxyhexenal (4-HHE) [131] (In contrast, oxidation of linoleic or eicosapentaenoic acids yields 4-hydroxynonenal.) Recent studies show that 4-HHE is an exceptionally potent activator of the nrf2 transcription factor which mediates the transcription of genes coding for a range of phase 2 antioxidant enzymes – including heme oxygenase-1 – as well as for glutathionylcysteine synthetase, rate-limiting for glutathione synthesis [131-133]. Hence, DHA can be viewed as a sensor of oxidative stress that promotes feedback induction of natural cellular antioxidant mechanisms.
This recent discovery may rationalize two rodent studies in which DHA supplementation was shown to provide protection from CIH. In atheroma-prone apolipoprotein-E-deficient mice, CIH exacerbates atherosclerosis. Concurrent supplementation with DHA was found to oppose this up-regulatory impact of CIH on atherogenesis, whereas DHA did not influence atherogenesis when these mice were not subjected to CIH [134]. In rats, CIH has been used to promote pulmonary hypertension; concurrent DHA administration was found to have a favorable impact on elevated pulmonary arterial blood pressure, thickening of the pulmonary artery wall, and ventricular hypertrophy [135]. It is reasonable to suspect that DHA’s antioxidant function contributed to these benefits. However, the doses of DHA employed in these rodent studies were high relative to doses that would be feasible for long-term human use; it remains to be seen whether practical clinical doses of DHA could confer benefit in OSA.

A study evaluating OSA patients has observed that DHA levels in erythrocyte membranes tend to be significantly lower in patients with severe OSA, as opposed to less severe OSA [136]. Could DHA be mitigating the severity of OSA – or does severe OSA deplete membrane OSA by oxidative stress or some other mechanism? In any case, clinical evaluation of supplementation with DHA-rich fish oil concentrates in OSA appears to be warranted – as has previously been suggested [136,137].

Addressing NF-KappaB activation with salsalate

With respect to NF-kappaB, clinically tolerable concentrations of salicylic acid can inhibit its activation via the canonical pathway by directly inhibiting the kinase activity of IkappaB kinase-beta [138-140]. Indeed, this effect is currently being employed in the treatment of type 2 diabetes, and is likely responsible for anti-inflammatory impact of salicylate therapy in rheumatoid arthritis (as salicylate per se – as opposed to its derivative aspirin – is only a very weak reversible inhibitor of cyclooxygenase activity) [141-144]. Since salicylate does not produce a physiologically significant inhibition of cyclooxygenase activity, it does not induce the GI bleeds or renal damage that can complicate NSAID therapy [145]. The dose-limiting adverse effect of high-dose salicylate is fully reversible otoxicity (tinnitus, mild hearing loss); this is seen only in a small minority of patients when a dose of 1.5 g twice daily is employed, a dose sufficient to achieve clinically useful anti-inflammatory effects [142,145,146]. Salicylic acid is best administered as the synthetic dimer salasaril (disalacid), which is cleaved in the alkaline intestinal tract to release free salicylate; salutaril is less prone than salicylate to provoke mild gastric irritation [147-152]. Salutaril, in a dose of 1.5 g twice daily, is usually well tolerated and may have worthwhile potential in the management of OSA.

However, several recent clinical studies have failed to show a favorable effect of salasaril on endothelium-dependent vasodilation – and in some instances a negative effect [153,154]. This suggests that salutaril may have some additional unknown target that compromises its utility for cardiovascular health. A study examining the impact of salutaril on endothelial function in patients with OSA would be of interest.

Figure 1 provides a summary of the molecular mechanisms driving oxidative stress and inflammation in OSA, and of the agents thought to have potential for opposing these mechanisms.

Practical considerations

Daily ingestion of spirulina (or of spirulina extracts enriched in

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