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Metallothionein as a compensatory component prevents intermittent hypoxia-induced cardiomyopathy in mice

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ABSTRACT

Obstructive sleep apnea (OSA) causes chronic intermittent hypoxia (IH) to induce cardiovascular disease, which may be related to oxidative damage. Metallothionein (MT) has been extensively proved to be an endogenous and highly inducible antioxidant protein expressed in the heart. Therefore, we tested the hypotheses that oxidative stress plays a critical role in OSA induced cardiac damage and MT protects the heart from OSA-induced cardiomyopathy. To mimic hypoxia/reoxygenation events that occur in adult OSA patients, mice were exposed to IH for 3 days to 8 weeks. The IH paradigm consisted of alternating cycles of 20.9% O_2/8% O_2 F_IO_2 (30 episodes per hour) with 20 s at the nadir F_IO_2 for 12 h a day during daylight. IH significantly increased the ratio of heart weight to tibia length at 4 weeks with a decrease in cardiac function from 4 to 8 weeks. Cardiac oxidative damage and fibrosis were observed after 4 and 8 weeks of IH exposures. Endogenous MT expression was up-regulated in response to 3-day IH, but significantly decreased at 4 and 8 weeks of IH. In support of MT as a major compensatory component, mice with cardiac overexpression of MT gene and mice with global MT gene deletion were completely resistant, and highly sensitive, respectively, to chronic IH induced cardiac effects. These findings suggest that chronic IH induces cardiomyopathy characterized by oxidative stress-mediated cardiac damage and the antioxidant MT protects the heart from such pathological and functional changes.

Introduction

Obstructive sleep apnea (OSA) is a highly prevalent respiratory disorder of sleep, with a prevalence of 3–7% (Punjabi, 2008; Young et al., 2002) and has been shown to be an independent cardiovascular risk factor. For instance, OSA increases the risk of heart failure by 140%, stroke by 60%, and coronary heart disease by 30% (Rosamond et al., 2007). OSA is characterized by recurrent episodes of partial or complete collapse of the upper airway during sleep, which leads to repetitive hypopneas or apneas, respectively. Each of these obstructive respiratory events results in an episode of hypoxia and re-oxygenation, constituting a hallmark pattern of nocturnal intermittent hypoxia (IH) (Khayat et al., 2009; Patil et al., 2007; Pinto et al., 1993).

The heart is highly dependent on oxygen metabolism to maintain normal function (Stanley et al., 2005), and, consequently, cardiac tissue is susceptible to lack of oxygen. Therefore, despite innate defense systems can be induced by exposures to repeated and relatively brief episodes of hypoxia (Murry et al., 1986), patients with severe OSA still show cardiovascular damage, leading to myocardial fibrosis, atrial dilatation, and left ventricular (LV) systolic and diastolic dysfunction (Alchanatis et al., 2002; Baquet et al., 2012; Fung et al., 2002; Kraiczi et al., 2001). However, the underlying mechanisms of cardiac dysfunction in OSA patients are still unclear. To mechanistically study the biological consequence of cyclic episodes of hypoxia and re-oxygenation seen in patients with OSA, animal models exposed to IH have been established and extensively used (Chen et al., 2007; Greenberg et al., 2006; Williams et al., 2010). Mouse models are particularly useful because the impact of specific genes can be investigated by transgenic manipulation (Campen et al., 2005).

Oxidative stress has been recognized as one major cause for OSA-induced cardiac damage based on extensive studies in animal models (Williams et al., 2010; Yin et al., 2012), which is defined as an imbalance between the production of reactive oxygen or nitrogen species (ROS or

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RNS) and the antioxidant capacity. Metallothionein (MT) is a family of cysteine-rich, low molecular weight proteins that have the capacity to bind both physiological (such as zinc and copper) and xenobiotic heavy metals through the thiol group of its cysteine residues, which represents nearly 30% of its amino acid content (Cai et al., 1999). Experimental data suggest that MT provides protection not only against metal toxicity, but also against a variety of oxidative stressors (Cai, 2007; Kang, 1999). Previous reports have shown that the diabetic hearts are significantly protected by MT (Cai, 2007; Cai et al., 2005; Zhou et al., 2008). Thus, it seems reasonable to assume that MT expression in the heart may also be responsible to IH exposures and protect the heart from IH-induced damage. However, it should be mentioned that the oxidative stress profiles caused by chronic IH (CIH) are different from the above oxidative models: The CIH mainly induces hydrogen peroxide (Yin et al., 2012) while diabetes or doxorubicin predominantly generates superoxide that interacts with NO to form peroxynitrite (Cai, 2006, 2007; Mukhopadhyay et al., 2009). Therefore, whether MT protects the heart from CIH cannot be simply extrapolated and has to be investigated experimentally.

The present study was designed to determine if 1) CIH induces the similar cardiac pathological and functional changes in mice as occurred in OSA patients? 2) CIH impacts on cardiac MT expression? 3) If so, what the effect is of the change in endogenous MT expression? To address these issues, we exposed FVB mice to IH from 3 days to 4 weeks to detect the cardiac MT expression. Then, we examined whether mice with cardiac overexpression of the MT1/2 gene (MT-TG) or with global deletion of the MT1/2 gene (MT-KO) are resistant or more sensitive to CIH exposures, which cause cardiac pathological and functional alterations.

Materials and methods

Experimental animals and IH exposures. Male MT-TG mice (8–10 weeks old) were produced from FVB (wild-type, WT) mice (Cai et al., 2005; Zhou et al., 2008). MT-KO mice and their WT 129S1 mice were purchased from Jackson Lab. All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care.

The murine model of IH exposures during sleep was used in this study as previous reports (Cai et al., 2011, 2012). Briefly, adult mice were exposed to an IH profile designed to produce similar nadir hemoglobin oxygen saturations (50–60%) and apnea/hypopnea index (AHI: 21–50 times/h) as observed in moderate to severe OSA patients. The IH paradigm consisted alteration cycles of 20.9% O2/8% O2 FIO2 (30 episodes per hour) with 20 s at the nadir FIO2 during the 12-hour light phase. We have conducted three sets of experiments: The first study was designed to investigate cardiac responses to IH from 3 days to 4 weeks using WT mice only, by measuring cardiac function with echocardiograph, cardiac remodeling by morphology and biochemical assay, and cardiac MT expression by Western blotting and immunohistochemistry. The second study was to investigate the protective effect of cardiac MT on CIH-induced cardiac damage by exposure of both MT-TG mice and WT mice for 4 and 8 weeks. The third study was to define the role of endogenous MT in preventing IH-induced cardiac damage in MT-KO mice. After IH exposures, mice were transferred to room air (RA) and detected cardiac function with echocardiography, and then euthanized for tissue collection.

Echocardiography. To assess cardiac function, transthoracic echocardiograms were performed on mice using a Visual Sonics Vevo 770 high-resolution imaging system, as described before (Zhou et al., 2008). Under sedation with Avertin (240 mg/kg IP), mice were placed in a supine position on a heating pad. Two-dimensional and M-mode echocardiography was used to assess wall motion, chamber dimensions and cardiac function.

Assays for lipid oxidation. Thiobarbituric acid (TBA) assay was used to measure relative malondialdehyde (MDA) production as an index of lipid peroxidation as described before (Cai et al., 2005). Briefly, tissue proteins were collected by centrifuging at 12,000 *g* at 4 °C for 15 min. The protein concentration was measured by Bradford assay. Then, 50 μl sample was mixed with 20 μl 8.1% sodium eodecyl sulfate (SDS) and 150 μl 20% acetic acid and 210 μl 0.0571% TBA, which then was incubated at 90 °C for 70 min. Samples were centrifuged at 4000 RPM for 15 min at 4 °C, collected, and transferred to the 96-well plates for reading at 540 nm using Micro-Plate Auto Reader.

Western blotting for protein expression. Heart tissues were homogenized in lysis buffer using homogenizer. Tissue proteins were collected by centrifuging at 12,000 *g* at 4 °C for 15 min. The protein concentration was measured by Bradford assay. MT expression was detected by a modified Western blotting protocol (Wang et al., 2006). For other protein expressions, routine Western blotting was performed as described in our previous studies (Cai et al., 2005; Wang et al., 2006).

Sirius-red staining. Tissue sections of 5 μm were deparaffinized and rehydrated for Sirius red staining of collagen using 0.1% Sirius red F3BA and 0.25% fast green FCF (Cai et al., 2006).

Immunohistochemical staining. Slides were used for immunohistochemical staining of MT with the same antibody as used for Western blotting assay (Wang et al., 2006). Briefly, slides were deparaffinized, rehydrated, boiled in target retrieval solution (Dako) for 15 min, and treated in 3% hydrogen peroxide for 30 min at room temperature, followed by blocking non-specific antigen with 10% animal serum for 1 h. These slides were incubated with primary anti-MT antibody (Dako) in a dilution of 1:100 at 4 °C overnight, and then incubated with secondary antibody at 1:400 dilution in room temperature for 30 min.

Statistical analysis. Data were expressed as mean ± SD for normally distributed variables. The comparisons of treatments (air vs. 8% O2) in mice with different genotypes (WT vs. MT-TG or WT vs. MT-KO) were performed at the same time periods of treatment using two-way analysis of variance (ANOVA). F-tests were performed to test whether there was treatment effect and/or interactions between treatment and time. When F-tests indicated treatment effect and/or interactions between treatment and time, the post-hoc *t*-tests were performed to examine which groups were significantly different (Little et al., 2002). Linear mixed effect models along with the Wald test statistics (Hedeker and Gibbons, 2006) were applied to compare the body weight between different groups. The significance level was considered if $p < 0.05$.

Results

IH induced cardiac remodeling and dysfunction

In the FVB mice exposed to IH, the body weights were significantly lower than those in the controls on day 3 and not recovered until day 28 (Fig. 1A, $p < 0.05$). The tibia lengths did not show statistic differences between control and IH-treated groups at all time-points (data not shown). IH initially resulted in lower heart weight (Fig. 1B) and ratio of heart weight to tibia length (Fig. 1C) after 7 days of hypoxic insults ($p < 0.05$). And then, the regain of heart weights in IH mice occurred till day 28, when the heart weights and ratio of heart weight to tibia length in IH group were even higher than those in control group (Fig. 1B and C, $p < 0.05$). Cardiac function analysis measured by echocardiography revealed significant increases in LV internal dimensions in diastole (LVID,d) and systole (LVID,s) after 28 days of IH exposures(Fig. 1D, $p < 0.05$), but not in interventricle septum thicknesses in diastole (IVS,d) and systole (IVS,s) and LV posterior wall thicknesses in diastole (LVPW,d) and systole (LVPW,s) (Supplemental Table 1). The cardiac systolic function also declined on the 28th day, reflected by decreased