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Metallothionein deletion exacerbates intermittent hypoxia-induced renal injury in mice

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HIGHLIGHTS

- MT deletion aggravated IH-induced albuminuria.
- MT deletion enhanced and accelerated IH-induced renal oxidative stress, inflammation and fibrosis.
- MT deletion resulted in the attenuation of Nrf2 dependent anti-oxidative responses.

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ABSTRACT

As a main clinical feature of obstructive sleep apnea (OSA), intermittent hypoxia (IH) induces oxidative stress, leading to damage to a variety of organs, including kidney. Metallothionein (MT) is a potent antioxidant that protects kidney against oxidative damage. Our previous studies demonstrated that MT prevented IH-induced cardiomyopathy in mice. However, the role of MT in protecting against IH-induced renal injury is unknown. Therefore, MT knockout (MT KO) mice and wild type (WT) control mice (129S) were culled for exposure to intermittent air as control or IH for a time course of 3 days, 1 week, 3 weeks and 8 weeks. MT KO mice developed higher urinary albumin to creatinine ratio (UACR) after exposure to IH for 8 weeks. Compared with either MT KO control or WT IH mice, MT deletion significantly aggravated IH-induced renal oxidative damage and inflammation at all four time points, along with significant acceleration of renal fibrosis after exposure to IH for 3 weeks and 8 weeks. Antioxidants including MT, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), heme oxygenase 1 (HO1) and NAD (P) H dehydrogenase [quinone] 1 (NQO1) were increased in response to short-term IH (3 days, 1 week and 3 weeks) but decreased after long-term IH (8 weeks) in WT mice. Interestingly, Nrf2, HO1 and NQO1 were significantly attenuated under IH conditions in the absence of MT, which were in parallel with the inactivation of protein kinase B (Akt) and extracellular signal-regulated kinase (ERK). These findings demonstrated that MT played a key role in preventing IH-induced renal injury possibly via preserving Nrf2 signaling pathway.

1. Introduction

Obstructive sleep apnea (OSA) is caused by recurrent collapse of the upper airway during sleep (Joosten et al., 2014), which leads to intermittent hypoxia (IH), along with intrathoracic pressure changes and arousal-induced reflex sympathetic activation. Up to 30% of the adult population in Western countries are affected by asymptomatic OSA and around 2–4% by symptomatic OSA (Kohler and Stradling, 2010), which is related to diseases in multiple organs and systems. OSA increases the risk of hypertension (Marin et al.,

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2012; Peppard et al., 2000), atrial fibrillation (Gami et al., 2004), stroke (Redline et al., 2010), atheroma (Tan et al., 2014) and diabetes (Kendzerska et al., 2014). OSA is also known to be prevalent in patients with chronic kidney disease (CKD) (Nicholl et al., 2012; Sakaguchi et al., 2011). In turn, CKD is also highly prevalent in severe OSA patients without hypertension or diabetes (Linz et al., 2014). Furthermore, accumulating clinical evidence has indicated that OSA is an independent risk factor for loss of kidney function (Ahmed et al., 2011; Chou et al., 2011; Nicholl et al., 2012).

As a critical feature of OSA, IH causes oxidative stress (Kohler and Stradling, 2010; Nair et al., 2013; Quintero et al., 2013), leading to injuries to a variety of organs, including kidney. Oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage (Sheng et al., 2014). 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). We and others have already demonstrated MT as a potent antioxidant, which effectively protects kidney against oxidative damage (Kojima et al., 2009; Shimazu et al., 2013; Sun et al., 2014b). More importantly, our previous studies defined MT as a potent antioxidant that prevented IH-induced cardiomyopathy in mice (Yin et al., 2014; Zhou et al., 2014). However, the role of MT in protecting against IH-induced renal injury remains largely unknown.

In our previous study, we observed the increase of MT in kidneys of FVB mice in response to 3 days of IH exposure, which reflects an urgent protection from acute renal injury by IH (Sun et al., 2012). Interestingly, MT was significantly down-regulated after 8 weeks of IH exposure, which was accompanied by severe renal oxidative stress, inflammation and fibrosis, while other antioxidants such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2), heme oxygenase 1 (HO1) and NAD (P) H dehydrogenase [quinone] 1 (NQO1) were not down-regulated. Thus, it seems that low level of MT was account for heavy renal damage after 8 weeks of IH exposure, indicating that MT might be a key antioxidant in preventing IH-induced renal damage.

With the aim of investigating the importance of MT in protecting kidney during IH, MT knockout (MT KO) mice and 129S1 wild type (WT) control mice were culled for exposure to intermittent air as control or IH for a time course of 3 days, 1 week, 3 weeks and 8 weeks.

2. Materials and methods

2.1. Experimental animals and IH exposures

MT KO and WT 129S1 mice (8–10 weeks old) were purchased from Jackson Lab (Bar Harbor, Maine) and housed in the University of Louisville Research Resources Center at 22 °C with a 12 h light/dark cycle with free access to standard rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee, 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, 2012b). Briefly, adult mice were exposed to an IH profile designed to produce similar nadir hemoglobin oxygen saturations (50–60%) and apnea/hypopnea index (21–50 times/h) as observed in moderate to severe OSA patients. The IH paradigm consisted alteration cycles of 20.9% O_2/8% O_2 FIO_2 (30 episodes/h) with 20 s at the nadir FIO_2 during the 12 h light phase. Urine was collected and mice were then euthanized for tissue collection after IH exposures for 3 days, 1 week, 3 weeks, and 8 weeks, respectively.

2.2. Analysis of the kidney function

Urine albumin and urinary creatinine were measured by a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) and a QuantiChrom™ Creatinine Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturers' instructions, respectively. Urinary albumin to creatinine ratio (UACR) was calculated as UACR = urine albumin/urine creatinine (μg/mg).

2.3. Kidney histopathological examination and immunohistochemical staining

Kidneys were collected and immersion-fixed in 10% neutral formalin, embedded in paraffin and sectioned into 5 μm-thick sections onto glass slides. To examine overall morphology, kidney sections were stained with hematoxylin and eosin (H&E). Sirius red staining was used to examine collagen accumulation, as previously described (Song et al., 2005). For immunohistochemical staining, sections were incubated with the following primary antibodies: vascular cell adhesion molecule 1 (VCAM-1, 1:100 dilution, Santa Cruz Biotechnology, Dallas, TX), transforming growth factor beta 1 (TGF-β1, 1:100 dilution, Santa Cruz Biotechnology), Nrf2 1 (1:100 dilution, Santa Cruz Biotechnology) and MT (1:100 dilution, Dako, Carpinteria, CA) overnight at 4 °C. After sections were washed with PBS, they were incubated with horseradish peroxidase-conjugated secondary antibodies (1:300–400 dilutions in PBS) for 2 h at room temperature. For the development of color, sections were treated with peroxidase substrate 3,3′-diaminobenzidine in the developing system (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin.

2.4. Western blot for protein expression

Kidney tissues were homogenized in lysis buffer using homogenizer. Proteins were collected by centrifuging at 12,000 × *g* at 4 °C for 15 min. The protein concentration was measured by Bradford assay. Anti-MT antibody was purchased from Dako. MT expression was detected by a modified Western blot protocol as previously described (Wang et al., 2006). Routine Western blot for other protein expressions was performed as described in previous studies (Cai et al., 2005; Wang et al., 2006).

2.5. Real-time qPCR

Total RNA was extracted from kidney tissues with TRIzol reagent (Invitrogen, Grand Island, NY). RNA concentration and purity were quantified using a Nanodrop ND-1000 spectrophotometer. Complementary DNA (cDNA) was synthesized from total RNA according to the manufacturer's protocol from the RNA PCR kit (PromegA, Madison, WI). Real-time quantitative PCR (qPCR) was carried out in a 20 μl reaction buffer that included 10 μl of TaqMan Universal PCR Master Mix 1 μl of primer, and 9 μl of cDNA and performed in duplicate for each sample in the ABI 7300 Real-Time PCR system. TaqMan primers for HO1, NQO1 and β-actin control primer were purchased from Applied Biosystems (Carlsbad, CA). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative cycle time (CT) method was used to determine fold differences between samples. The comparative CT method determined the amount of target normalized to an endogenous reference (β-actin) and relative to a calibrator ($2 - \Delta\Delta CT$).

2.6. Quantitative analysis of lipid peroxides

The lipid peroxide concentration was determined by a previously described method (Cai et al., 2005) which measures