Dysregulation of CLOCK gene expression in hyperoxia-induced lung injury

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Submitted 28 February 2013; accepted in final form 27 March 2014

Dysregulation of CLOCK gene expression in hyperoxia-induced lung injury. Am J Physiol Cell Physiol 306: C999–C1007, 2014. First published April 2, 2014; doi:10.1152/ajpcell.00064.2013.—Hyperoxic acute lung injury (HALI) is characterized by inflammation and epithelial cell death. CLOCK genes are master regulators of circadian rhythm also implicated in inflammation and lung diseases. However, the relationship of CLOCK genes in hyperoxia-induced lung injury has not been studied. This study will determine if HALI alters CLOCK gene expression. To test this, wild-type and NALP3−/− mice were exposed to room air or hyperoxia for 24, 48, or 72 h. In addition, mice were exposed to different concentrations of hyperoxia (50, 75, or 100% O2) or room air for 72 h. The mRNA and protein levels of lung CLOCK genes, based on quantitative PCR and Western blot analysis, respectively, and their target genes are significantly elevated in mice exposed to hyperoxia compared with controls. Alterations in CLOCK genes are associated with increased inflammatory markers in bronchoalveolar lavage fluid of hyperoxic mice compared with controls. Histological examination of mice lungs exposed to hyperoxia show increased inflammation and alveolar congestion compared with controls. Our results indicate sequential increase in CLOCK gene expression in lungs of mice exposed to hyperoxia compared with controls. Additionally, data suggest a dose-dependent increase in CLOCK gene expression with increased oxygen concentrations. To validate if the expression changes related to CLOCK genes are indeed associated with inflammation, NALP3−/− was introduced to analyze loss of function in inflammation. Western blot analysis showed significant CLOCK gene downregulation in NALP3−/− mice compared with wild-type controls. Together, our results demonstrate that hyperoxia-mediated lung inflammation is associated with alterations in CLOCK gene expression.

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ACUTE LUNG INJURY (ALI) AFFECTS a large number of patients worldwide, with mortality rates reported up to 40% (16). Many patients with ALI require oxygen supplementation to maintain adequate tissue oxygenation; unfortunately, it can exacerbate the condition as it may lead to hyperoxia-induced acute lung injury (HALI) (4). Exposure to hyperoxia can have pathological effects such as lung inflammation and edema accompanied by epithelial and endothelial cell death, suggesting that oxygen supplementation, although necessary, may potentially perpetuate or exacerbate ALI (2, 3). Inflammatory cells flood the lung tissue and proinflammatory cytokines like IL-1β are produced as a result of hyperoxia-induced ALI (11). Recent reports from our laboratory suggest the IL-1β processing machinery known as the “inflammasome” plays an important role in hyperoxic lung injury (14). Inflammasomes are master switches that are involved in caspase-mediated processing of proinflammatory cytokines (14).

Circadian rhythms play important roles in physiology and behavior; disruption of these rhythms can become a major cause in disease development (23). Recent evidence has implicated an important role for CLOCK genes in inflammation in relation to chronic diseases like diabetes and hypertension (17, 21). However, their role in ALI and how CLOCK gene expression is altered due to inflammation in ALI and other pulmonary associated diseases has been unexplored.

The mammalian circadian CLOCK is composed of at least 10 core circadian CLOCK proteins. Circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein (Bmal1) transcription factors form a heterodimeric complex. This complex binds to E-boxes in the promoters of various target genes, including those encoding for negative [e.g., period homolog 1 (Per1), Per2, cryochrome 1 (Cry1), and Cry2] or positive (e.g., Bmal1) loop components, as well as target genes, including D site of albumin promoter-binding protein (DBP), nuclear receptor subfamily 1, group D, member 1 (Rev-erb-α), and peroxisome proliferator-activated receptor-γ (PPARγ). It is well known that CLOCK genes are expressed rhythmically in the suprachiasmatic nucleus of the hypothalamus, the master circadian pacemaker in mammals. Recently, it has become clear that CLOCK genes also express and function in various peripheral tissues (24). In particular, CLOCK genes exhibit circadian expression patterns in organs that play critical roles in blood pressure homeostasis, including the vasculature, more specifically, the mouse aorta (18), heart (19, 25, 27), and kidney (19, 25). However, it is unknown whether CLOCK gene expression levels are altered in mice with HALI. In the present study, we hypothesize that chronic hyperoxia can induce inflammation in mouse lungs which may alter CLOCK gene expression and lead to the development of HALI.

MATERIALS AND METHODS

Mice. C57BL/6J (wild-type) male mice (n = 6), 9 wk of age, were purchased from Harlan Laboratories (Indianapolis, IN). NALP3 knockout mice (NALP3−/−) derived from C57BL/6J strain were a gift from
from Dr. Vishva Dixit, Genentech (San Francisco, CA). The Animal Care and Use Committee of University of South Florida approved all procedures. Six mice were placed in an air tight chamber (75 × 50 × 50 cm) and exposed to 100% hyperoxia for 24, 48, or 72 h (6 mice per hour group). Six mice were also placed in an air tight chamber (75 × 50 × 50 cm) and exposed to 50, 75, or 100% oxygen (hyperoxia) for 72 h (6 mice per O2 concentration group). The oxygen concentration in the chamber was monitored and regulated with proOx P100 (BioSpherix). The control mice group (n = 6) were maintained at room air under standard conditions. Mice exposed to hyperoxia at different time points (24, 48, or 72 h), different concentrations of oxygen (50, 75, or 100% O2), or room air (normoxia) were killed at 72 h to analyze inflammation in bronchoalveolar lavage fluid at these time points and oxygen concentrations.

**Lung perfusion and tissue collection.** Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine mixture. After cervical dislocation, the abdominal cavity was opened and lungs were perfused through the right ventricle using sterile saline. The left lower lobe of the lung was removed and fixed in 10% buffered formalin for standard histological processing and paraffin embedding (FFPE). The remaining portion of the lungs was dissected out carefully, frozen in liquid nitrogen, and stored at −80°C until analysis.

**Bronchoalveolar lavage fluid collection.** Blood was removed from the inferior vena cava (IVC), as described previously, followed by a small transverse incision in the skin of the ventral neck. The trachea was exposed, a catheter was inserted, and a whole lung lavage was performed by using sterile PBS as previously described (6).

**Hyperoxia exposure at different doses of oxygen.** The Animal Care and Use Committee of University of South Florida approved all procedures. Mice (n = 6 per group) were placed in an air tight chamber (75 × 50 × 50 cm) and were exposed to varied oxygen concentrations at 50, 75, or 100% for about 72 h. The oxygen concentration in the chamber was monitored and regulated with proOx P100 (BioSpherix). Control mice (n = 6) were maintained at room air under standard conditions. Mice were killed at 72 h after hyperoxia (50, 75, or 100% O2) or room air exposure (normoxia).

**Quantitative RT-PCR.** Total RNA was extracted from mouse lung tissues using the Trizol reagent (Qiagen, Valencia, CA). RNA was eluted in ribonuclease-free water. Total RNA, 1.5 μg, was used for cDNA synthesis by iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s recommendations. Quantitative PCR (qPCR) was performed in a Bio-Rad iCycler, using SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) and 50 ng of cDNA. Amplification was performed at 95°C for 3 min; followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. The mRNA expression of mouse CLOCK genes, target genes, and inflammatory genes were measured using specific primers (Table 1). All reactions were performed in duplicate, and each target gene expression was normalized to 18S rRNA expression. The relative amount of target gene in each sample was estimated using the ΔΔCt method.

**Western blot.** The lung tissue lysate was prepared by homogenizing ~250 mg of tissue in 1 ml RIPA lysis buffer [20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (Na2EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycercophosphate, 1 mM sodium orthovanadate (Na3VO4), 1 μg/ml leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF)] followed by centrifugation at 12,000 g for 15 min at 4°C. The protein content of the lysate was determined using BCA assay (Bio-Rad laboratories). The lysate was stored at −80°C until analysis.

Lysates were subjected to Western blot analysis as previously described (13) using rabbit anti-CLOCK1, anti-Bmal1, anti-Per1, and anti-Cry1 primary antibodies (1:250 dilution; Santa Cruz Biotechnol-
logy, Santa Cruz, CA). Anti-Cry2 and anti-Per2 primary antibodies (1:250 dilution) were obtained from Abcam and Santa Cruz, respectively. Mouse β-actin was used as a protein loading control. Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used, and protein expression was measured using Pierce ECL Western blotting reagents (Thermo Scientific, Rockford, IL) and detected with the Bio-Rad ChemiDoc System. Protein concentrations were quantified using ImageJ software.

ELISA. Levels of IL-1β (eBioscience, San Diego, CA), TNF-α (RayBiotech, Norcross, GA), MPO (eBioscience, San Diego, CA), and NF-κB (Abcam, Cambridge, MA) in bronchoalveolar lavage fluid (BALF) were measured using commercial ELISA kits as per manufacturer’s instructions.

Evaluation of lung injury. To quantitatively examine lung edema in hyperoxia-induced lung injury, we recorded wet-to-dry weight ratios by removing six lungs per group from the hilum as previously described (22). The lungs were dry blotted and weighed to determine the wet weight. Then, the lungs were desiccated overnight by 130°C incubation in a vacuum oven and reweighted to obtain the dry weight. We then calculated the wet-to-dry ratio (22).

Statistical analysis. Data were statistically analyzed by GraphPad Prism version 10.00 for Windows (GraphPad Software, San Diego, CA). Comparison of continuous variables between two groups was performed using Student’s t-test. All tests were two-tailed, and results were considered significant at $P < 0.05$.

RESULTS

Altered mRNA and protein expression of CLOCK and its targets in hyperoxic lung tissues. To investigate whether CLOCK gene expression is affected in hyperoxic compared with normoxic lung tissue, we measured and compared the mRNA levels of multiple CLOCK and target genes in six hyperoxic and normoxic mouse lungs. The mRNA levels of CLOCK and Bmal1 were significantly increased in hyperoxic mouse lungs compared with control mouse lungs ($P < 0.01$; Fig. 1). Similarly, the expression levels of oscillator genes Per1 and Per2 and the expression levels of Cry1 and Cry2 were significantly elevated in hyperoxic lungs compared with the control mouse lungs (normoxia) ($P < 0.01$; Fig. 1). CLOCK, Bmal1, Per1, Cry1, and Cry2 protein expression was also significantly increased in hyperoxic lungs compared with control lungs; surprisingly, Per2 was decreased in mice exposed to hyperoxia compared with controls (Fig. 2).

To further investigate the function of these core CLOCK genes in hyperoxic lungs, we analyzed the mRNA expression of several CLOCK target genes, Rev-erb-α, DBP, and PPARγ. Rev-erb-α and PPARγ expression levels were dramatically elevated in the hyperoxic mouse lungs compared with the

![Fig. 2. Hyperoxia-mediated increase in CLOCK proteins. Male C57Bl/6 mice were exposed to room air (normoxia) or 100% O2 (hyperoxia) for 72 h as described in MATERIALS AND METHODS. CLOCK protein expression levels were measured in lung homogenates by Western blotting and calculated relative to β-actin in each sample. Levels of CLOCK genes were analyzed densitometrically using ImageJ. A: CLOCK. B: Bmal1. C: Per1. D: Cry1. E: Per2. F: Cry2. A representative blot is depicted. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, when compared between hyperoxic and control mice by Student’s t-test.](http://ajpcell.physiology.org/)
control mouse lungs ($P < 0.01$; Fig. 3, A and B). For DBP, expression levels in hyperoxic lungs were significantly suppressed compared with control ($P < 0.05$; Fig. 3C). Collectively, the expression levels of CLOCK and target genes exhibited gene specific alterations in the hyperoxic mouse lungs.

**Increased inflammation in hyperoxic lung tissues.** To investigate whether extensive alterations in CLOCK and target gene mRNA are associated with changes in inflammation in mice exposed to hyperoxia, we measured mRNA expression of some key genes involved in inflammation. In lungs isolated from hyperoxic mice, NF-κB mRNA levels (subunit 50: $P < 0.001$ and subunit 65: $P < 0.01$) were significantly elevated compared with control, suggesting increased inflammation (Fig. 4A). IL-1β and TNF-α expression levels were also significantly increased in hyperoxic lungs compared with control lungs ($P < 0.01$; Fig. 4, B and C). Interestingly, the mRNA level of the NALP3 inflammasome was also elevated in hyperoxic lungs ($P < 0.01$; Fig. 4D). Since the mRNA levels of inflammatory markers were significantly upregulated in lungs of mice exposed to hyperoxia, we next evaluated if there was a change in inflammatory cytokine release and immune cell infiltration in BALF and lung morphology of these mice. Our results showed a significant increase in inflammatory cytokine release such as IL-1β, TNF-α, and immune cell infiltration with MPO and neutrophils (Fig. 5, A–D). Histological examination of lung tissue sections showed normal appearance of lungs in mice exposed to 100% O2 for 24 h similar to controls (normoxia), but mice exposed to hyperoxia for 48 h showed severe ALI featuring interstitial thickening, inflammatory cell infiltration,
and alveolar congestion (Fig. 5E). Additionally, mice showed mild alveolar hemorrhage due to alveolar edema along with the features mentioned above when exposed to 72 h hyperoxia compared with controls (Fig. 5E). A quantitative representation of the alveolar edema was calculated by obtaining the wet-to-dry ratio (Fig. 5F). These results strongly suggest a positive association between exposure to hyperoxia and inflammation.

**Increased exposure of mice to hyperoxia elevates CLOCK gene expression.** Next, we sought to identify the profiling of CLOCK gene expression with inflammation mediated by hyperoxic exposure over time. To confirm whether induced inflammation at 24, 48, and 72 h exposure to hyperoxia alters CLOCK gene expression in a temporal fashion, we exposed mice to hyperoxia (100% O2) for 24, 48, and 72 h, measured CLOCK gene levels in lung homogenates by Western blot analysis, and densitometrically analyzed the blot using ImageJ (Fig. 6, A–G). Our results showed a sequential increase in the expression levels of CLOCK genes in lungs of mice exposed to different time points at 24, 48, and 72 h compared with room air controls (normoxia). With the exception of Bmal1 at 24 and 72 h and Cry2, CLOCK gene expression levels increased temporally with higher exposure times (24, 48, or 72 h) compared with normoxic controls. This indicates that inflammation induction by hyperoxia alters CLOCK gene expression and these alterations are in part proportional to the degree of hyperoxia-induced lung injury.

**Altered CLOCK gene expression and inflammation in mice exposed to different doses of O2.** To determine if there is a gradual increase in inflammation-mediated lung injury and altered CLOCK gene expression with increased doses of oxygen, we exposed mice to 50, 75, and 100% oxygen concentrations for 72 h. Lung histochemistry demonstrated normal appearance of lungs in mice exposed to room air (normoxia). However, at 50% O2 exposure, lungs showed mild interstitial thickening and inflammatory cell infiltration. At 75% O2 exposure, lungs showed moderate interstitial thickening, inflam-
matory cell infiltration, and mild alveolar hemorrhage due to edema. At 100% O₂, the lungs showed similar findings to that of lungs exposed to 75% hyperoxia but also showed mild increase in alveolar hemorrhage from edema (Fig. 7A). The wet-to-dry ratio of alveolar edema was calculated to assess the extent of lung injury (Fig. 7B). These findings suggest that increased oxygen concentrations amplified inflammation and induced hyperoxia-mediated lung injury. Alterations in lung morphology further prompted us to analyze the CLOCK gene expression in mice exposed to different doses of oxygen under hyperoxia. Lung homogenates of mice exposed to different concentrations of oxygen (50, 75, and 100%) were used to determine the alterations in CLOCK gene expression by Western blot analysis and were densitometrically analyzed using ImageJ (Fig. 7, C–I). With the exception of Per1 and Per2, CLOCK gene expression significantly increased with increase in percent O₂ exposure. CLOCK gene expression increased sequentially with significant increase at 100% O₂ exposure compared with the control (normoxia). This illustrates a strong association between the increase in concentration of hyperoxia and increased CLOCK gene expression.

Deletion of the inflammation component NALP3 decreases CLOCK gene expression in mice exposed to hyperoxia. Since our results strongly revealed a link between hyperoxia-induced lung inflammation and CLOCK gene expression (Figs. 5–7), we next validated if the expression changes related to CLOCK genes are indeed associated with inflammation. We hypothesized that if a link exists between inflammatory marker regulation and CLOCK gene expression, then the deletion of NALP3, an inflammasome component, should alter CLOCK gene expression significantly. To test this hypothesis, we utilized NALP3 KO mice, where inflammation was abolished as demonstrated recently in our laboratory (6). Wild-type (C57Bl/6) (n = 6) mice and NALP3 knockout mice (NALP3⁻/⁻) were exposed to hyperoxia (100% O₂) for 72 h to compare the degree in which NALP3 influenced CLOCK gene expression. Western blot was
performed from lung homogenates of both C57Bl/6 mice and NALP3 knockout mice to measure CLOCK genes (CLOCK, Bmal1, Cry1, Cry2, Per1, and Per2). The evidence from Western blot analysis showed downregulation of CLOCK genes in NALP3−/− mice compared with wild-type controls (Fig. 8A). Densitometric analysis was performed to measure the downregulation of CLOCK genes in NALP3−/− mice compared with wild-type controls (Fig. 8, B–G). These results suggest and further support our hypothesis that, with the deletion of NALP3, there is a decrease in CLOCK gene expression in mice exposed to hyperoxia.

DISCUSSION

The major finding of this current study is that hyperoxia may alter CLOCK gene expression in lungs. Furthermore, these alterations may be involved due to hyperoxia-mediated inflammation. This finding is substantial as CLOCK genes in peripheral organs regulate important mammalian circadian rhythms (10). Dysfunction of CLOCK genes can contribute to sleep disorders, psychiatric diseases, tumorigenesis, dyslipidemia, and cardiovascular disease; therefore, proper regulation of CLOCK gene expression is critical for cellular functions such as cell cycle control (15), tumor suppression (5), and xenobiotic metabolism (7, 8).

It is well recognized that gene products of CLOCK and Bmal1 form a heterodimer that activates the transcription of Per and Cry genes. In agreement with the literature, our study showed increased activation of CLOCK, Bmal1, oscillatory gene expression (Per1, Per2, Cry1, and Cry2) in mice exposed to hyperoxia for 72 h. We also observed that CLOCK, Bmal1, Per1, and Cry1 protein expression was significantly increased in hyperoxic mouse lungs compared with control mouse lungs. This increase is associated with inflammation induced in mice exposed to hyperoxia for 72 h. Additionally, our results showed a sequential increase in the expression levels of CLOCK genes in lungs of mice exposed to increasing time.
points at 24, 48, and 72 h compared with room air controls (normoxia). We examined if different concentrations of O₂ exposure induces inflammation and alters CLOCK gene expression. Our results indicated a progressive increase in inflammation with increase in percentage of oxygen used in hyperoxia. These results suggest that oxygen toxicity alters CLOCK gene expression, which is associated with progressive increase in lung inflammation as indicated by enhanced injury markers like IL-1β and NALP3. Moreover, the expression of CLOCK genes was also modulated by target genes Rev-erb-α, DBP, and PPARγ, which control downstream events by regulating the expression of CLOCK-controlled output genes. Although the mRNA expression of DBP decreased, the mRNA expression of Rev-erb-α and PPARγ was elevated in lungs of mice exposed to hyperoxia. These data suggest that hyperoxia-induced modification in core CLOCK genes increases the expression of CLOCK target genes, which may influence actual physiological functions. It is well known that environmental factors like light and temperature influence the circadian CLOCK and may disrupt the physiological behaviors. To our knowledge, this is the first report showing that hyperoxia may modulate CLOCK gene/protein expression and further influences CLOCK-controlled target genes.

Previous studies suggest that inflammation will modulate the abnormal expression of CLOCK genes in cells (26). Hyperoxia-mediated lung inflammation plays a major role in the development of ALI and several other pulmonary diseases (12, 20). In the present study, hyperoxia-induced key inflammatory markers like NF-kB (subunits 50 and 65), IL-1β, and TNF-α were significantly increased. This was also associated with an increase in expression of a key component of the NALP3 inflammasome that processes the secretion of IL-1β. Inflammatory markers in BALF like IL-1β, TNF-α, MPO, and neutrophils also showed significant elevations in hyperoxia-induced mice with increasing exposure. Based on wet-to-dry ratio of edema from lung histology, lung damage is present in mice exposed to 24 h hyperoxia, but there was a greater extent of lung injury in mice exposed to hyperoxia for 48 and 72 h. These observations suggest that in the lung, hyperoxia modulates the inflammatory pathways and induces abnormal CLOCK gene expression. In contrast to previous studies where suppressed CLOCK genes modulated inflammation (19), we report that inflammation plays a role in hyperoxia-mediated elevated CLOCK gene expression. To further analyze the effects of hyperoxia-mediated inflammation on CLOCK gene expression, we exposed NALP3 knockout mice (mice with...
suppressed inflammatory genes) to hyperoxia for 72 h and analyzed the expression levels of CLOCK genes. Our data revealed alterations in CLOCK gene expression and in Cry1, Cry2, Per1, and Per2 protein expression in NALP3 knockout mice compared with the wild type. These results reveal that protein was suppressed in NALP3 mice showing that deletion of inflammation decreases CLOCK gene expression in mice exposed to hyperoxia. CLOCK genes were suppressed in NALP3 knockout mice since the inflammation gene was blocked. It is evident that inflammation in hyperoxic lung injury occurs through NALP3 (6), and suggest that CLOCK gene expression changes are indeed associated with hyperoxia-induced lung inflammation.

Taken together, we demonstrate for the first time that mice exposed to hyperoxia had alterations in CLOCK genes and target gene mRNA. Sequential increase in exposure time and dosage of hyperoxia are associated with variations in CLOCK genes and are proportional to the degree of hyperoxia-induced inflammation as demonstrated by analysis of IL-1β, TNF-α, NF-κB, neutrophil accumulation, and lung edema. Hyperoxia-induced expression and its alteration on CLOCK genes expression are further supported by the deletion of NALP3, a master switch of inflammation, as it decreased CLOCK gene expression in mice exposed to hyperoxia. These findings implicate a potential role of CLOCK genes in the pathogenesis of ALI and may be useful in the development of new therapeutic strategies.

ACKNOWLEDGMENTS

We thank Dr. Brenda Flam for critical reading of this manuscript.

GRANTS

N. Kolliputi was funded by the American Heart Association National Scientist Development Grant 09SDG2260957 and National Heart, Lung, and Blood Institute Grant R01-HL105932 and the Joy McCann Culverhouse Endowment to the Division of Allergy and Immunology.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.L., O.P., J.F., I.F., H.B, and L.G. performed experiments; V.L., P.T.P., and R.C. prepared figures; V.L. and P.T.P. drafted manuscript; Y.C. edited and revised manuscript; R.F.L. and N.K. approved experiments; V.L., P.T.P., and R.C. drafted final version of manuscript; N.K. conception and design of research; N.K. interpretation of results of experiments.

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