Life Sciences xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Tissue-specific and time-dependent regulation of the endothelin axis by the circadian clock protein Per1

Jacob Richards [a,b], Amanda K. Welch [a,c], Sarah J. Barilovits [a,b], Sean All [a], Kit-Yan Cheng [a], Charles S. Wingo [a,c], Brian D. Cain [b], Michelle L. Gumz [a,b,*]

[a] Department of Medicine, University of Florida, USA
[b] Department of Biochemistry and Molecular Biology, University of Florida, USA
[c] North Florida/South Georgia Veterans Health System, Gainesville, FL, USA

ARTICLE INFO

Article history:
Received 1 November 2013
Accepted 26 March 2014
Available online xxxx

Keywords:
Endothelin-A receptor
Endothelin-B receptor
ETA
ETB
Gene regulation

ABSTRACT

Aims: The present study is designed to consider a role for the circadian clock protein Per1 in the regulation of the endothelin axis in mouse kidney, lung, liver and heart. Renal endothelin-1 (ET-1) is a regulator of the epithelial sodium channel (ENaC) and blood pressure (BP), via activation of both endothelin receptors, ET_A and ET_B. However, ET-1 mediates many complex events in other tissues.
Main methods: Tissues were collected in the middle of murine rest and active phases, at noon and midnight, respectively. ET-1, ET_A and ET_B mRNA expressions were measured in the lung, heart, liver, renal inner medulla and renal cortex of wild type and Per1 heterozygous mice using real-time quantitative RT-PCR.
Key findings: The effect of reduced Per1 expression on levels of mRNAs and the time-dependent regulation of expression of the endothelin axis genes appeared to be tissue-specific. In the renal inner medulla and the liver, ET_A and ET_B exhibited peaks of expression in opposite circadian phases. In contrast, expressions of ET-1, ET_A and ET_B in the lung did not appear to vary with time, but ET-1 expression was dramatically decreased in this tissue in Per1 heterozygous mice. Interestingly, ET-1 and ET_A, but not ET_B, were expressed in a time-dependent manner in the heart.
Significance: Per1 appears to regulate expression of the endothelin axis genes in a tissue-specific and time-dependent manner. These observations have important implications for our understanding of the best time of day to deliver endothelin receptor antagonists.

Introduction

The circadian clock regulates a variety of physiological processes such as metabolism, immune response, sleep–wake cycles, renal function, and blood pressure (BP) (reviewed in Richards and Gumz, 2012, 2013; Stow and Gumz, 2011). On the molecular level, the circadian clock consists of multiple proteins. Four are considered the core proteins that interact with one another to affect transcription of circadian target genes (Dibner et al., 2010). These proteins are Period (Per: homologs 1–3), Cryptochrome (Cry: homologs 1–2), BMAL1, and CLOCK. CLOCK and BMAL1 form a heterodimer, and then bind E-box DNA response elements to transcriptionally regulate CLOCK-controlled genes, including the genes Per and Cry. In the canonical model, Per and Cry presumably interact to repress the transcriptional activity of CLOCK and BMAL1 (Albrecht and Eichele, 2003).

Endothelin-1 (ET-1) is a peptide hormone expressed in multiple tissues and mediates its actions through two receptors: endothelin-A (ET_A) and endothelin-B (ET_B) receptors. ET-1 was first characterized as a potent vasoconstrictor; however, it is now known that ET-1 action is much more complex (reviewed in Kohan et al., 2011). ET-1 in the renal collecting duct is a potent inhibitor of epithelial Na channel (ENaC) activity through both ET_A and ET_B receptors (Bugaj et al., 2012; Ge et al., 2006, 2008; Lynch et al., 2013). This inhibition appears to occur via a nitric oxide-dependent mechanism (Bugaj et al., 2008; Stricklett et al., 2006) (reviewed in Kohan, 2013). The ET-1 gene (*Edn1*) is regulated by epigenetic factors (Welch et al., 2013) and transcription is controlled by mineralocorticoid action (Stow et al., 2009), calcium via the nuclear factor of activated T-cells (NFAT) (Strait et al., 2010), and a variety of other mechanisms (reviewed in Stow et al., 2011). Emerging evidence has demonstrated that *Edn1* is also regulated post-transcriptionally (reviewed in Jacobs et al., 2013; Welch et al., 2013). Our laboratory has demonstrated that ET-1 peptide expression varies in a time-dependent manner in the renal cortex and medulla (Stow et al., 2012). The mechanism of this effect appears to be

* Corresponding author at: 1600 SW Archer Rd Box 100224, Gainesville, FL 32610, USA. Tel.: +1 352 273 6887; fax: +1 352 392 5465.
E-mail address: Michelle.Gumz@medicine.ufl.edu (M.L. Gumz).

http://dx.doi.org/10.1016/j.lfs.2014.03.028
0024-3205/

transcriptional. Indeed, we have previously shown that Per1 interacts with a non-canonical E-box from the *Edn1* promoter (Stow et al., 2012). Per1 is a repressor of renal ET-1 mRNA and peptide levels, and Per1 knockout (KO) animals have elevated levels of ET-1 peptide in the kidney cortex and medulla (Richards et al., 2013; Stow et al., 2012).

Although ET-1 plays an integral role in a variety of physiological processes, circadian regulation of ET-1 and the receptors by the circadian clock and Per1 has not been studied outside of the kidney. Therefore, the goal of this study was to characterize the time-dependence of ET-1, ET_A and ET_B (the "endothelin axis") mRNA expressions and to test the hypothesis that Per1 plays a role in the regulation of the endothelin axis mRNA in the liver, heart, kidney, and lung. It is well established that the circadian clock plays an integral role in the regulation of liver, heart, kidney and lung functions (reviewed in Richards and Gumz, 2013) but the role of Per1 in the regulation of the endothelin axis in these tissues has not been investigated. For the first time, we demonstrate that the endothelin axis is regulated by time and by Per1 in a manner that is unique to each of the tissues tested.

Materials and methods

Animals

All animal-use protocols were approved by the University of Florida and North Florida/South Georgia Veterans Administration Institutional Animal Care and Use Committee in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals. Per1 KO and wild type (WT) mice (129/sv) were originally provided by Dr. David Weaver (University of Massachusetts (Bae et al., 2001)). WT and Per1 heterozygote (het) mice were bred in house by UF Animal Care Services Staff. Animals were maintained on a normal 12 h light: dark cycle. Mice were fed normal lab chow and given free access to water. At noon and midnight, mice were anesthetized and tissues were collected and snap frozen in liquid nitrogen. Kidneys were later dissected and cortex removed for protein or RNA isolation.

Isolation of IMCD

Isolations of inner medullary collecting ducts (IMCDs) were prepared as described below. WT and Per1 het mice were euthanized at midnight and inner medulla was dissected from both kidneys. The inner medulla was minced longitudinally and then digested at 37 °C in a buffer containing 250 mM sucrose, 10 mM Triethanolamine, 3 mg/mL of Collagenase type I, and 2 mg/mL of Hyaluronidase type IV for 30 min with gentle inversion. DNAse I was then added at a concentration of 0.1 mg/mL and incubated for an additional 10 min. The mixture was then filtered over a 100 μm filter and the resulting supernatant spun at 600 ×*g* for 3 min. The pellet was then suspended in a buffer containing 250 mM sucrose and 10 mM Triethanolamine and spun again. The pellet was then suspended in Hanks Buffered Salt Solution (HBSS—10 mM HEPES pH 7.4) and spun at 600 ×*g* for 5 min.

Endothelin-1 ELISA

ET-1 peptide levels were determined as previously described (Stow et al., 2012). Cytosolic extracts were isolated using NE-PER kit (Pierce). Immunoreactive ET-1 peptide was detected by chemiluminescent ELISA (R&D Systems) and normalized to total protein content as determined by BCA assay (Pierce).

RNA isolation and qPCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA (10 μg) was treated with DNA-free DNaseI (Ambion). DNaseI-treated RNA (2 μg) samples were used as template for reverse transcription with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNAs (20 ng) were then used as template in quantitative real-time PCR (qPCR) reactions (Applied Biosystems) to evaluate changes in ET-1, ET_A, and ET_B mRNA levels. Cycle threshold (Ct) values were normalized against β-actin and relative quantification was performed using the ΔΔCt method (Livak and Schmittgen, 2001). Fold change values were calculated as the change in mRNA expression levels relative to the control. TaqMan primer/probe sets were purchased from Applied Biosystems.

Statistical analysis

All data are represented as mean ± standard error of the mean (SEM). Statistics were performed with Graphpad Prism v6. All graphs and plots were made with Graphpad Prism v6. The effects of time and genotype were analyzed by two-way ANOVA with post-hoc Student–Newman–Keuls test. All p values less than 0.05 were considered significant.

Results

Per1 regulates ET-1 expression but not ET_A and ET_B mRNA expression in the kidney

We have previously shown that ET-1 peptide expression varies with time in murine renal cortex and inner medulla, with peak expression during the inactive period. In mice completely lacking the Per1 protein (Per1 knockout), renal ET-1 levels were increased (Stow et al., 2012). We have previously shown that Per1 heterozygous (het) mice have an approximate 50% reduction in Per1 protein expression in the kidney and liver (Richards et al., 2013) and that these mice exhibit a renal sodium wasting phenotype and reduced plasma aldosterone levels (Richards et al., 2013). To determine the effect of reduced Per1 expression on ET-1 expression, renal ET-1 peptide levels were measured in Per1 het mice and compared to wild type (WT). ELISA was used to measure ET-1 peptide levels ex vivo in inner medullary collecting ducts (IMCD) isolated from WT and Per1 het mice at midnight. ET-1 peptide levels were significantly higher in IMCD from Per1 het mice than WT mice (Fig. 1).

To determine the effects of time and reduced Per1 protein levels on expression of the endothelin receptors, mRNA levels of ET_A and ET_B were assessed by qPCR in WT and Per1 het mice at noon and midnight, the middle of murine rest and active phases, respectively. In wild-type mice, ET_A mRNA levels were significantly lower at midnight compared to noon in both the renal cortex and inner medulla (Fig. 2A), similar to the timed regulation of ET-1 peptide levels that we have previously observed (Stow et al., 2012). ET_B mRNA levels also changed with time; however, the pattern was remarkably different between renal inner medulla and renal cortex (Fig. 2B). ET_B mRNA levels were significantly

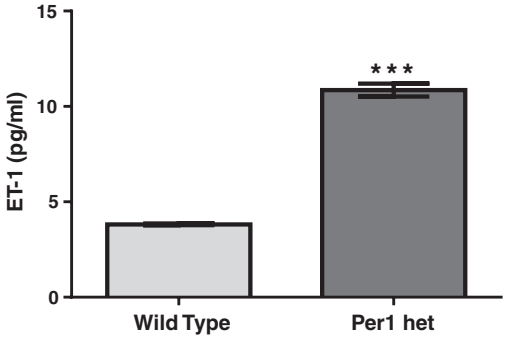

Fig. 1. ET-1 peptide levels are elevated in IMCDs of mice with reduced levels of Per1. Wild type (WT) (light bars) and Per1 het (dark bars) mice were euthanized at midnight. IMCDs were isolated as described in the Materials and methods. ELISA was used to measure ET-1 peptide levels in WT and Per1 het mice. Data are presented relative to WT, ±SEM. ***P < 0.05 vs. genotype. N = 3.

Please cite this article as: Richards J, et al, Tissue-specific and time-dependent regulation of the endothelin axis by the circadian clock protein Per1, Life Sci (2014), http://dx.doi.org/10.1016/j.lfs.2014.03.028