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Effect of angiotensin II on rhythmic *per2* expression in the suprachiasmatic nucleus and heart and daily rhythm of activity in Wistar rats

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ABSTRACT

Endogenous daily rhythms are generated by the hierarchically organized circadian system predominantly synchronized by the external light (L): dark (D) cycle. During recent years several humoral signals have been found to influence the generation and manifestation of daily rhythm. Since most studies have been performed under *in vitro* conditions, the mechanisms employed under *in vivo* conditions need to be investigated. Our study focused on angiotensin II (angII)-mediated regulation of *Per2* expression in the suprachiasmatic nuclei (SCN) and heart and spontaneous locomotor activity in Wistar rats under synchronized conditions. Angiotensin II was infused (100 ng/kg/min) *via* subcutaneously implanted osmotic minipumps for 7 or 28 days. Samples were taken in 4-h intervals during a 24 h cycle and after a light pulse applied in the first and second part of the dark phase. Gene expression was measured using real time PCR. Locomotor activity was monitored using an infrared camera with a remote control installed in the animal facility. Seven days of angII infusion caused an increase in blood pressure and heart/body weight index and 28 days of angII infusion also increased water intake in comparison with controls. We observed a distinct daily rhythm in *Per2* expression in the SCN and heart of control rats and infused rats. Seven days of angII infusion did not influence *Per2* expression in the heart. 28 days of angII treatment caused significant phase advance and a decrease in nighttime expression of *Per2* and influenced expression of clock controlled genes *Rev-erb alpha* and *Dbp* in the heart compared to the control. Four weeks of angII infusion decreased the responsiveness of *Per2* expression in the SCN to a light pulse at the end of the dark phase of the 24 h cycle. Expression of mRNA coding angiotensin-converting enzyme (ACE) and angiotensin-converting enzyme 2 (ACE2) showed a daily rhythm in the heart of control rats. Four weeks of angII infusion caused a decrease in amplitude of rhythmic expression of *Ace*, the disappearance of rhythm and an increase in *Ace2* expression. The *Ace*/*Ace2* ratio showed a rhythmic pattern in the heart of control rats with peak levels during the dark phase. Angiotensin II infusion decreased the mean *Ace*/*Ace2* mRNA ratio in the heart. We observed a significant daily rhythm in expression of brain natriuretic peptide (BNP) in the heart of control rats. In hypertensive rats mean value of *Bnp* expression increased. Locomotor activity showed a distinct daily rhythm in both groups. Angiotensin II time dependently decreased ratio of locomotor activity in active *versus* passive phase of 24 h cycle. To conclude, 28 days of subcutaneous infusion of angII modulates the functioning of the central and peripheral circadian system measured at the level of *Per2* expression and locomotor activity.

1. Introduction

The circadian rhythms in physiological processes and behavior with 24 h period are generated by the circadian system. The circadian system consists of a central part located in the suprachiasmatic nuclei of the hypothalamus (SCN) and peripheral oscillators. Peripheral oscillators are localized in most tissues and organs of the body (except the SCN). Both central and peripheral oscillators are self-sustained. Under physiological conditions peripheral oscillators are synchronized by the central oscillator, predominantly by neural and humoral pathways [1–4].

The generation of circadian rhythm is based on a feedback loop created by clock genes and their protein products. The basic scheme of this model involves two bHLH-PAS transcriptional factors CLOCK and BMAL1 that heterodimerize and initiate transcription of clock genes *Period* (isoforms *per1*, *per2* and *per3*) and *Cryptochrome* (isoforms *cry1* and *cry2*) *via* a regulatory sequence E-box. As a result of increased

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transcription the PER and CRY proteins accumulate in the cytoplasm. They then heterodimerize and after some delay are translocated into the nucleus where they repress the transcription of their own genes by interacting with the CLOCK/BMAL1 complex. The molecular mechanism generating the circadian rhythms is similar in the central and peripheral oscillators [5]. The functioning of the core feedback loop described above is modulated by several additional loops and many postranscriptional and postranslational modifications, which have been described in detail elsewhere [6].

The essential role of *per2* in the functioning of molecular circadian oscillators was demonstrated by the generation of homozygous *per2*−/− mice, which showed arrhythmicity in locomotor activity in constant darkness [7]. By using echocardiography in *per2* transgenic mice with an altered sequence of amino acids in PER2 it was shown that the circadian system influences heart performance at the level of ejection and the shortening fraction of the heart [8]. In mice carrying a mutation in the *per2* gene, attenuated dipping of blood pressure and heart rate during both light–dark cycle and constant darkness, and a shortening of circadian period during constant darkness, were observed [9]. Recent research has also demonstrated the beneficial role of PER2 in heart protection during ischemia in mammals [10].

While the central oscillator is synchronized predominantly by the external light:dark cycle *via* the retino-hypothalamic tract [11], the functioning of the peripheral oscillators is influenced by several humoral factors. Humoral signals that can synchronize peripheral oscillators are tissue specific and can be mediated by SCN related signals, as shown in a study using parabiotic connection of intact and SCN-lesioned animals in which clock gene expression in the kidney and liver (but not in the heart) was restored [2]. Humoral signals that synchronize peripheral oscillators may also be SCN-independent *e.g.* food provided exclusively during the passive phase of the 24 h cycle can disconnect peripheral oscillators from the SCN [12].

To ascertain which humoral factors are responsible for synchronization of the peripheral oscillators under *in vitro* conditions, several hormones, metabolites and substances were investigated and found to influence clock gene expression [13–15]. Of these, angiotensin II (angII) is able to induce and synchronize the expression of *per2* and *bmal1* in cultured smooth muscle cells isolated from the thoracic aorta of mice. The effect of angII on clock genes was blocked by the specific angII type 1 receptor (AT1) antagonist CV11947 [16]. Similarly angII is able to induce the expression of clock genes *per2* and *bmal1* in cultured cardiomyocytes of neonatal rats. The effect of angII on the expression of clock genes was blocked by the angiotensin II AT1 receptor antagonist telmisartan [17].

The octapeptide angiotensin II is the primary effector molecule of the renin-angiotensin system (RAS), known mainly because of its effects on the cardiovascular system. AngII produced by systemic endocrine RAS is formed from angiotensin I by angiotensin-converting enzyme (ACE), which is primarily present in pulmonary endothelial cells. Angiotensin I is formed *via* the enzymatic cleavage of angiotensinogen originating from the liver by renin that is secreted from the juxtaglomerular apparatus of the kidney [18]. In addition to systemic RAS, angiotensin II can be synthesized by many tissues, including the heart [19,20]. The effects of angiotensin II on the heart are predominantly mediated by angiotensin type 1 (AT1) receptors, and include chronotropic and inotropic effects and myocyte hypertrophy, non-myocyte proliferation and interstitial fibrosis [21,22].

Angiotensin II is cleaved from the circulation within a few minutes [23,24]. Intaperitoneal or subcutaneous low dose infusion of angII (up to 200 ng/kg/min) does not elevate plasma angII or cause an increase in range of physiological values [25–27]. The rapid degradation of angII is assured by several peptidases [28]. Of these, carboxypeptidase angiotensin-converting enzyme 2 (ACE2), which cleaves one amino acid from either angiotensin I or angII, was found to decrease angII levels and increase levels of vasodilatator angiotensin 1–7 [29].

The aim of our study was to demonstrate the effects of angII on *Per2* expression under *in vivo* conditions. We tested the influence of angII infusion on *Per2* expression in the suprachiasmatic nuclei and the heart in rats. Expression of *Bmal1* and clock controlled genes *Dbp* and *Rev-erb alpha* was analyzed to consider possible effect of angII on the whole circadian feedback loop. Locomotor activity was monitored as an output of the central oscillator. In parallel, we measured the endogenous capacity of heart tissue to synthesize angiotensin II at the level of *Ace* and *Ace2* expression and expression of *At1* receptors in the heart. To monitor hypertension *Bnp* (brain natriuretic peptide) expression in heart [30] was analyzed during the 24 h cycle.

2. Material and methods

2.1. Animals

The experimental protocol was approved by the Ethical Committee for the Care and Use of Laboratory Animals at Comenius University, Bratislava. Male Wistar rats (from the Institute of Experimental Pharmacology and Toxicology of the Slovak Academy of Sciences, SR) were obtained at the age of 10–11 weeks with an initial weight of 149 ± 0.5 g. These animals were housed in temperature-controlled rooms (21 ± 2 °C) with a 12:12 light (L):dark (D) cycle. Time is expressed in relative units — Zeitgeber time (ZT), where ZT0 is defined as the start of the light phase and ZT12 corresponds to the start of the dark phase of the 24 h cycle. Food and water were available *ad libitum*. Body weight and water consumption were monitored during the experiment.

2.2. Blood pressure and locomotor activity measurement

Heart rate and blood pressure were measured using the tail-cuff method (AD Instruments, Germany) before and during angiotensin infusion. The locomotor activity of rats was monitored continuously throughout the experiment in home cages in the animal facility. Two cages from the same group containing two rats were in sight of the camera at the same time. The cages were rotated every 2–3 days in such a way that paired comparison of the effect of angII on behavior would be possible. One averaged 24 h record of activity was calculated from each 2–3-day long recording of each pair of cages. In this way we obtained three recordings of activity from the control group (12 rats), three recordings of animals intended for angII infusion before infusion (12 rats) and five recordings of angII-infused rats (20 rats). Any time any animal moved the software switched on the camera and initiated recording. We used an infrared camera with a remote control (StarCam370i, MSI, Taiwan). Activity was detected and recorded using MyGuardLive software (MSI, Taiwan). The length and frequency of locomotor activity was calculated at the end of the experiment using standard statistical programs.

2.3. Implantation of Alzet minipumps containing angiotensin II

Osmotic minipumps (Alzet 2001 for 7-day infusion, Alzet 2004 for 28-day infusion, USA) containing angiotensin II (Calbiochem, USA) were implanted subcutaneously into the intrascapular area under aseptic conditions and total anesthesia (xylazine-ketamine combination applied intramuscularly). Angiotensin II was continually released at a dose of 100 ng/kg/min for 7 or 28 days. Control rats were sham operated.

2.4. Heart and brain tissue sampling for real time PCR

Sampling was performed after 7 or 28 days of angiotensin II infusion with a separate control group for each experiment in 4-h lasting intervals during the 24 h cycle (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22). To