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Research Article
Diurnal Temporal Blood H_2S Variations Correlate with the Circadian Rhythm of Vascular Contraction and Blood Pressure

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Abstract

Background: It is well known that blood pressure has a circadian rhythm in rat. However, the underlying mechanisms that modulate circadian rhythm of blood pressure have not been fully clarified. The aim of this study was to investigate the probable mechanisms that regulate time dependent variation of blood pressure. In present study, the correlations among the followings: α-1 adrenoceptor stimulated aortic contractions, thoracic aortic expression of Rho-kinase II and myosin phosphatase target subunit-1 and blood biomarkers (nitric oxide, hydrogen sulfide [H_2S] and total antioxidant capacity) that regulate blood pressure at six different times of the day and night were examined. **Materials and Methods:** Systolic blood pressure was measured every 4 h during a 24 h period in male albino Wistar rats by tail-cuff plethysmography. At each time point, contraction and relaxation responses of isolated thoracic aortas were recorded. The expression of protein from aortas was determined by western blot method. Nitric oxide, total antioxidant capacity and H_2S levels were measured spectrophotometrically in plasma samples. One-way analysis of variance and student t-test was used to determine statistical differences. **Results:** Rat systolic blood pressure displayed a circadian rhythm, which reached the maximum at 05:00 am and minimum at 09:00 am. Diurnal variation of phenylephrine-induced contractions in the isolated thoracic aorta was also observed. Although, the Rho-kinase inhibitor Y-27632 reduced phenylephrine-induced contractions, the circadian pattern of the contractions did not change. Interestingly, Rho-kinase II and myosin phosphatase target subunit-1 protein expression in the thoracic aorta did not show significant changes throughout the day. Further, plasma levels of nitric oxide and total antioxidant capacity did not vary during the day. However, H_2S levels in the systemic circulation showed circadian variation, which was the maximum at 01:00 am and minimum at 05:00 am. **Conclusions:** These results suggest that, in addition to α-1 adrenoceptor sensitivity of vessels, the circadian rhythm of plasma H_2S could contribute to diurnal blood pressure variations. This highlights a potential novel experimental and therapeutic approach to blood pressure regulation.

Key words: Blood pressure, diurnal rhythm, rho-kinase, nitric oxide, total antioxidant capacity, H_2S

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In mammals, the cardiovascular system shows strong circadian rhythmicity. Physiological cardiovascular and hemodynamic factors, such as blood pressure, heart rate and endothelial function have diurnal rhythm. Types of acute cardiac pathologies such as myocardial ischemia, acute myocardial infarction, ventricular tachycardia and sudden death due to heart failure also exhibit circadian or at least diurnal rhythms[1,2].

Blood pressure has a daily variation in humans such that it is the highest during early to mid-morning and then decreases progressively throughout the day[3]. Circadian rhythm of blood pressure and its neuronal regulation have also been established in rodents. Contrary to humans, blood pressure of rat is higher at night than during day[4,5]. Although, several studies have been worked on regulation of diurnal blood pressure variation, the certain mechanism(s) have not been obtained yet[6,7]. Auto-regulatory mechanisms of cardiovascular system affect circadian clock through many factors, including vasoactive metabolites, endothelial substances and autacoids by changing vascular smooth muscle tone[8]. Recent studies in rodents have shown that administration of vasoactive substances cause intense diurnal variation in sensitivity of aorta[9,10]. Circadian variation in vascular tone may result from daily changes in calcium sensitivity of contractile system. The RhoA/Rho-kinases play an important role in controlling calcium sensitivity in myofilaments[11] and have a crucial effect on regulation of arterial blood pressure[12]. When cytosolic Ca^{2+} levels increase, 20 kDa Myosin Light Chain (MLC) is phosphorylated by Ca^{2+}-calmodulin activated MLC kinase, causing vascular smooth muscle to contract. Relaxation occurs by Ca^{2+}-independent MLC phosphatase (MLCP), which dephosphorylates MLC[13]. Rho-kinase (ROCK) activation causes inhibition of MLCP by phosphorylating MYPT-1, the regulatory subunit of MLCP[14]. The expression levels of ROCK II exhibited a diurnal rhythm in mouse aorta and mesenteric arteries and in porcine coronary artery smooth muscle cells[15,16,17]. However, further studies are needed to elucidate the mechanism(s) of circadian variation of ROCK expression and activation in rats.

In addition, Nitric Oxide (NO) and H_2S both have important roles in maintaining vascular responsiveness and blood pressure regulation. These two gaseous mediators affect production of each other[18] and their bio availability is altered by oxidative stress[19]. Although, vascular responsiveness plays a key role in circadian blood pressure regulation, the correlation between circadian vessel contraction and blood biomarkers including NO, Total Antioxidant Capacity (TAC) and H_2S are not currently described. In this study, diurnal blood pressure variations by aortic contraction and ROCK II and myosin phosphatase target subunit-1 (pMYPT-1) expression were examined and compared that to the levels of NO, TAC and H_2S production in rats.

MATERIALS AND METHODS

Animal care: All animal experiments were approved by the Local Ethical Committee of Animal Care and Use of Gazi University (G.Ü.ET-08.023; 45-5909). Male albino Wistar rats (250–350 g) were obtained from the Laboratory Animal and Experimental Research Center of Gazi University. The rats were housed under a synchronized 12 h light/dark cycle at a constant temperature (22±1°C) with free access to food and water. The lighting regimen was 12 h of light attenuating with 12 h of darkness (lights on 08:00 am-08.00 pm) with a light intensity of approximately 100 lux. Lighting was provided by cool fluorescent bulbs controlled by an automatic timer. Rats were housed in standard cages.

Blood pressure measurements: Systolic blood pressure was measured in prewarmed, restrained rats by tail-cuff plethysmography (Non-invasive indirect blood pressure system for rats, NIBP200A, COMMAT, BIOPAC®, Turkey). The measurements were performed every 4 h during a 24 h period for a total of six measurements. The specific times for each measurement were 09:00 am, 01:00, 05:00, 09:00 pm, 01:00 and 05:00 am.

***In vitro* isometric tension measurements:** Following the blood pressure measurement at each time point, the rats were anesthetized with thiopental sodium (40 mg kg^{-1}, i.p.) and the aorta was rapidly removed. Following removal of fatty and connective tissues, aorta ring segments (3-4 mm) were mounted in tissue baths. The baths were filled with Krebs' buffer solution (119 mM NaCl, 25.0 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 11 mM glucose, pH = 7.4) that was continuously bubbled with 95% O_2-5% CO_2 at 37°C. Rings were then stretched to an optimal resting tension of 1 g. The changes in isometric tension were recorded using an isometric force-displacement transducer (The Integrated Tissue Bath and Heater System [ITBS05], COMMAT, Turkey). Following a 40 min equilibration period, tissues were contracted two times by addition of 75 mM KCl. Tissues were again stabilized for 40 min and then contracted by addition of 10^{-6} M phenylephrine (Phe) and relaxed with 10^{-5} M acetylcholine (ACh). Rings that had ≥80% relaxation

response to ACh were used for further experiments as intact endothelial vessels[20]. After this procedure, the rings were used for three different experiments:

Group 1: Cumulative concentration-contraction response curves to Phe (10^{-9}-3×10^{-5} M) were recorded

Group 2: Cumulative concentration-contraction response curves to Phe (10^{-9}-3×10^{-5} M) were recorded after preincubation with Y-27632 (10^{-6} M) for 30 min. The contractions were expressed as a percentage of the KCl-induce contractions

Analysis of ROCK II and pMYPT-1 protein expression: Following aorta isolation at each time point, a portion of the tissue was flash frozen in liquid nitrogen. Flash frozen tissue was homogenized in lysis buffer containing 50 mM tris-HCl (pH 7.5), 400 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM NaF, protease inhibitor cocktail (0.5 tablet/100 mL, Roche) using an ultrasonic homogenizer (Vibra-Cell; Sonics Materials Inc.). Homogenates were centrifuged at 10,000×g for 10 min and supernatant protein concentration was determined using bicinchoninic acid (BCA) method (Sigma Chemical Co.). Equal amounts of protein were separated by electrophoresis using an 8% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with TBS-T buffer containing 5% non-fat milk powder (for ROCK II and actin) or 3% BSA (for pMYPT-1) for 1 h followed by incubation with ROCK-II (1:500, Mouse IgG1, BD Biosciences), pMYPT-1 (1:500, rabbit polyclonal IgG, Millipore) or pan-actin (1:10,000) primary antibodies for 2.5 h. After washing, membranes were incubated with corresponding secondary peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (1:1000 for ROCK II and pMYPT-1, 1:2000 for pan-actin, Pierce) for 1.5 h. Protein-antibody complexes were detected using the ECL system (Amersham). All film was scanned and densitometry was performed to quantitate expression using Image J software.

NO, H_2S and TAC quantification: Plasma NO levels were measured spectrophotometrically using the method described by Navarro-Gonzalvez[21] as previously described. This method is based on the Griess reaction and involves a shortened incubation period for the reduction of nitrate by cadmium[21]. Modifications to the protocol were made to accommodate a 96-well-plate format.

The H_2S levels of plasma were measured spectrophotometrically as explained in methods which are subject to measuring absorbance of methylene blue at 670 nm, the resultant product of the chemical reaction between $FeCl_3$ and N, N-dimethyl-p-phenylenediamine[22].

The TAC levels were restrained in plasma as described before[23]. Briefly, Cu^{2+} was reduced to Cu^{1+} by antioxidants in the plasma. Neocuproine (Nc) was added to form a colored complex (Nc-Cu^{1+}) that was detected spectrophotometrically at 455 nm.

Chemicals: Acetylcholine, phenylephrine, Y-27632 and all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Statistical analysis: All values were expressed as Mean±SEM. Comparing the difference between groups by the means of significant difference, one-way analysis of variance (ANOVA) (*post-hoc* Bonferroni) and "unpaired" student t-test was used to determine statistical differences between different treatment groups. For all comparisons, $p<0.05$ was considered statistically significant.

RESULTS

It was observed that the mean systolic blood pressure of rats was higher during the dark cycle than the light cycle. Systolic blood pressure was the highest at 05:00 am, which was significantly different from the lowest value obtained at 09:00 am (Fig. 1).

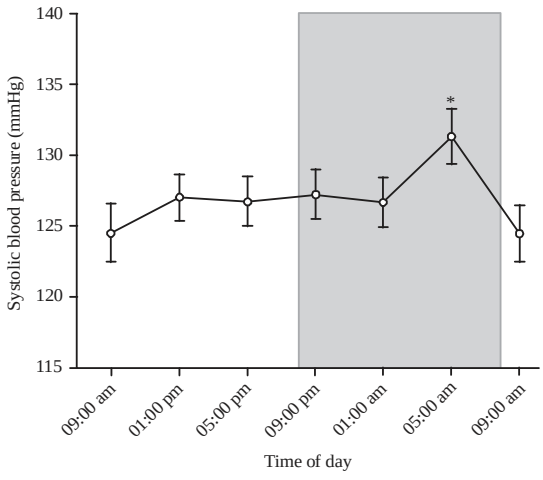

Fig. 1: Circadian variation of mean systolic blood pressures in rats. Mean systolic blood pressure was higher during the dark cycle and displays diurnal variation. *$p<0.05$ as compared to the value at 09:00 am. The dark cycle is shown as the shaded area (n = 6)

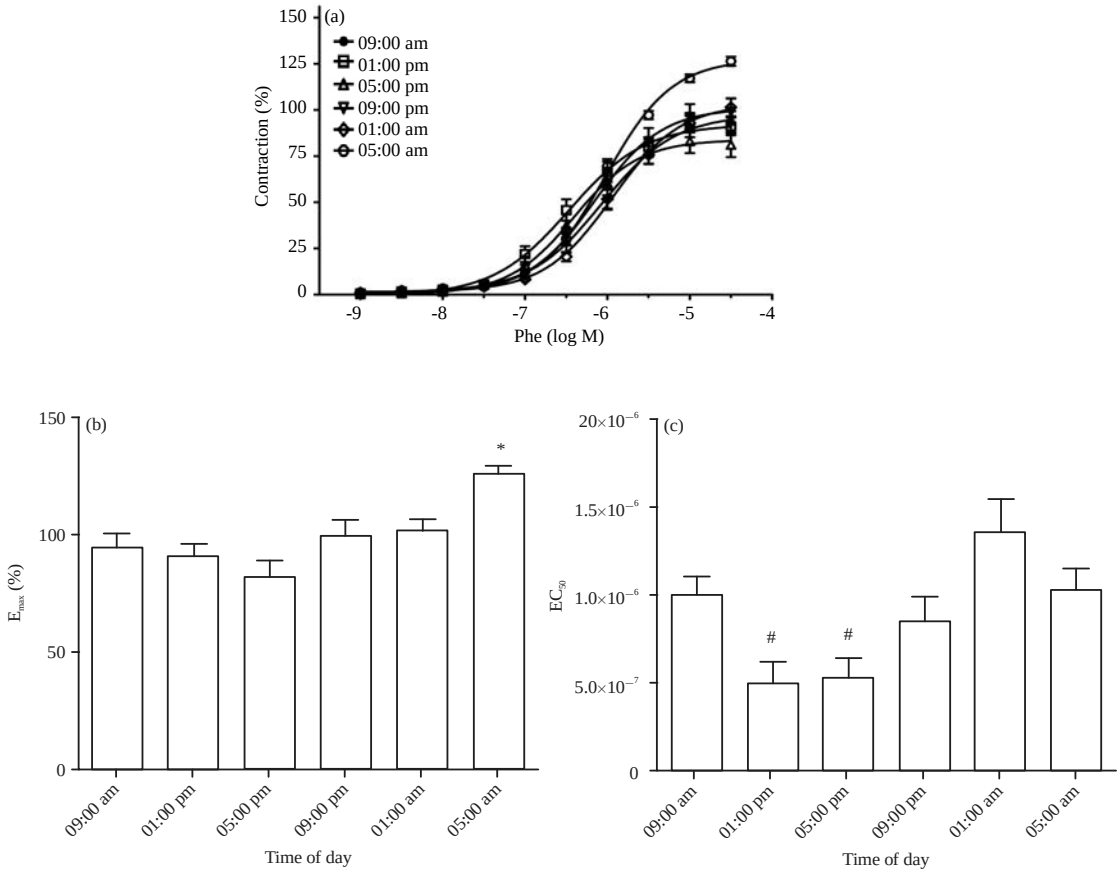

Fig. 2(a-c): Circadian variation of phenylephrine contractions in endothelium-intact isolated rat thoracic aorta, (a) Contraction responses to phenylephrine represent diurnal variation, (b) E_{max} value of phenylephrine was the highest at 05:00 am and the lowest at 05:00 pm and (c) EC_{50} values with phenylephrine were lower at 01:00 pm and 05:00 pm than at 01:00 am. *p<0.05 difference among values at 09:00 am, 01:00, 05:00, 09:00 pm and 01:00 am, #p<0.05 compared to 01:00 am and Phe: Phenylephrine (n = 6)

In the endothelium-intact thoracic aorta rings, the concentration response curves for phenylephrine were generated at six different periods of day. Phenylephrine-induced contractions are augmented during the dark period of day (Fig. 2a). Moreover, the highest and lowest E_{max} values for the phenylephrine response occurred at 05:00 am and 05:00 pm, respectively (Fig. 2b) and the EC_{50} values of phenylephrine at 01:00 pm and 05:00 pm were lower than the value recorded at 01:00 am (Fig. 2c).

As expected, pretreatment of the ROCK inhibitor Y-27632 inhibited phenylephrine-induced contractions. However, the circadian pattern of contractions did not change (Fig. 3a). The E_{max} values of phenylephrine were the highest at 05:00 am and the lowest at 05:00 pm in the presence of Y-27632 (10^{-6} M) (Fig. 3b), similar to data obtained in the absence of inhibitor (Fig. 2b). Moreover, EC_{50} values for phenylephrine after Y-27632 preincubation was lower at 05:00 pm than at 01:00 am and 05:00 am (Fig. 3c). No alteration was noted in the E_{max} for phenylephrine diurnal rhythm profiles in the presence of Y-27632 (Fig. 4).

Although, ROCK II protein expression in thoracic aorta appeared to change in accordance with the daily circadian rhythm, no statistical significance was found (Fig. 5a and b). Similarly, pMYPT-1 expression, which influences ROCK activation, also remained unchanged throughout the day (Fig. 5c and d).

The plasma NO and TAC concentrations were similar with variations occurring throughout the time course and reaching the maximum concentrations at 05:00 am and 01:00 am, respectively (Fig. 6). The plasma level of H_2S also varied significantly at different times of the day with the maximum and minimum concentrations observed at 01:00 and 05:00 am, respectively (Fig. 6).

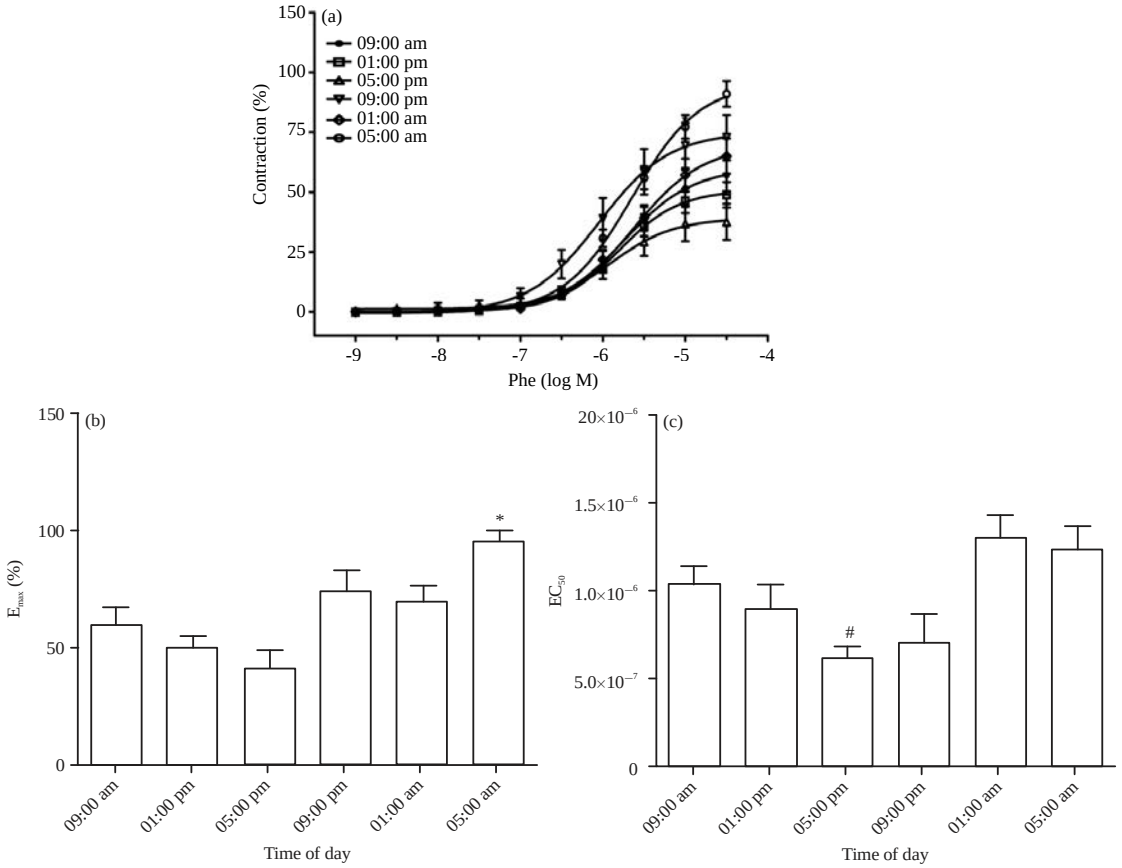

Fig. 3(a-c): Circadian variations of phenylephrine contractions after Y-27632 incubation in endothelium-intact isolated rat thoracic aorta, (a) Contractions with phenylephrine in the presence of Y-27632 have diurnal variation, (b) E_{max} value of phenylephrine in presence of Y-27632 (10-6 M) was the highest at 05:00 am and the lowest at 05:00 pm and (c) EC_{50} values of phenylephrine was lower at 05:00 pm than at 01:00 am and 05:00 am after Y-27632 incubation. *p<0.05 comparison of values at 05:00 pm, 01:00 pm and 09:00 am, #p<0.05 comparison of values at 01:00 am and 05:00 am and Phe: Phenylephrine (n = 6)

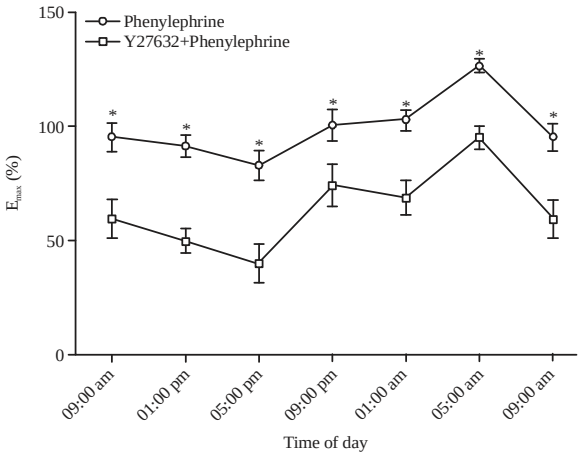

Fig. 4: Effect of Y-27632 on diurnal rhythm with phenylephrine E_{max} values in endothelium-intact isolated rat aorta. The maximal contraction of phenylephrine was decreased by Y-27632, (10-6 M) but the circadian profile of the E_{max} values was not affected. *p<0.05 comparison between Phe E_{max} values and after Y-27362 incubation at the same time point (n = 6)

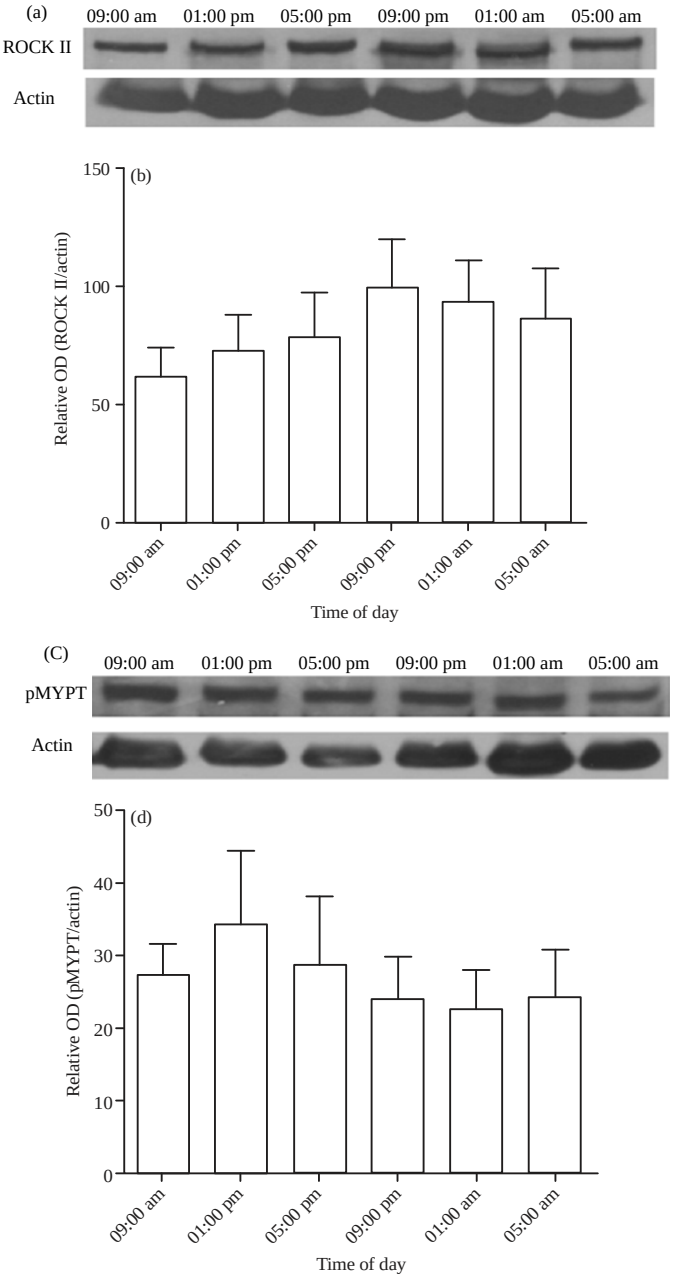

Fig. 5(a-d): Changes of ROCK II and pMYPT-1 expression throughout the day in endothelium-intact rat thoracic aorta, (a) Representative gels for ROCK II protein expression, (b) Relative expression value calculated by densitometry (ROCK II/actin). The ROCK II expression did not change during the light and dark cycles, (c) Representative gels for pMYPT-1 protein expression and (d) Relative expression value calculated by densitometry (pMYPT-1/actin). The pMYPT-1 expression, an indicator of ROCK activation, did not change during the light and dark cycles (n = 6)

DISCUSSION

Blood pressure circadian rhythms can be affected by several external (temperature, physical activities, mood, alcohol intake, caffeine intake, diet, sleep etc.) and internal (ethnicity, gender, autonomic nervous system tonus, vasoactive hormones, hematological and renal varieties etc.) factors[24,25]. However, the current studies are insufficient to clarify this subject. Therefore, investigation of cellular mechanisms involved in blood pressure regulation and

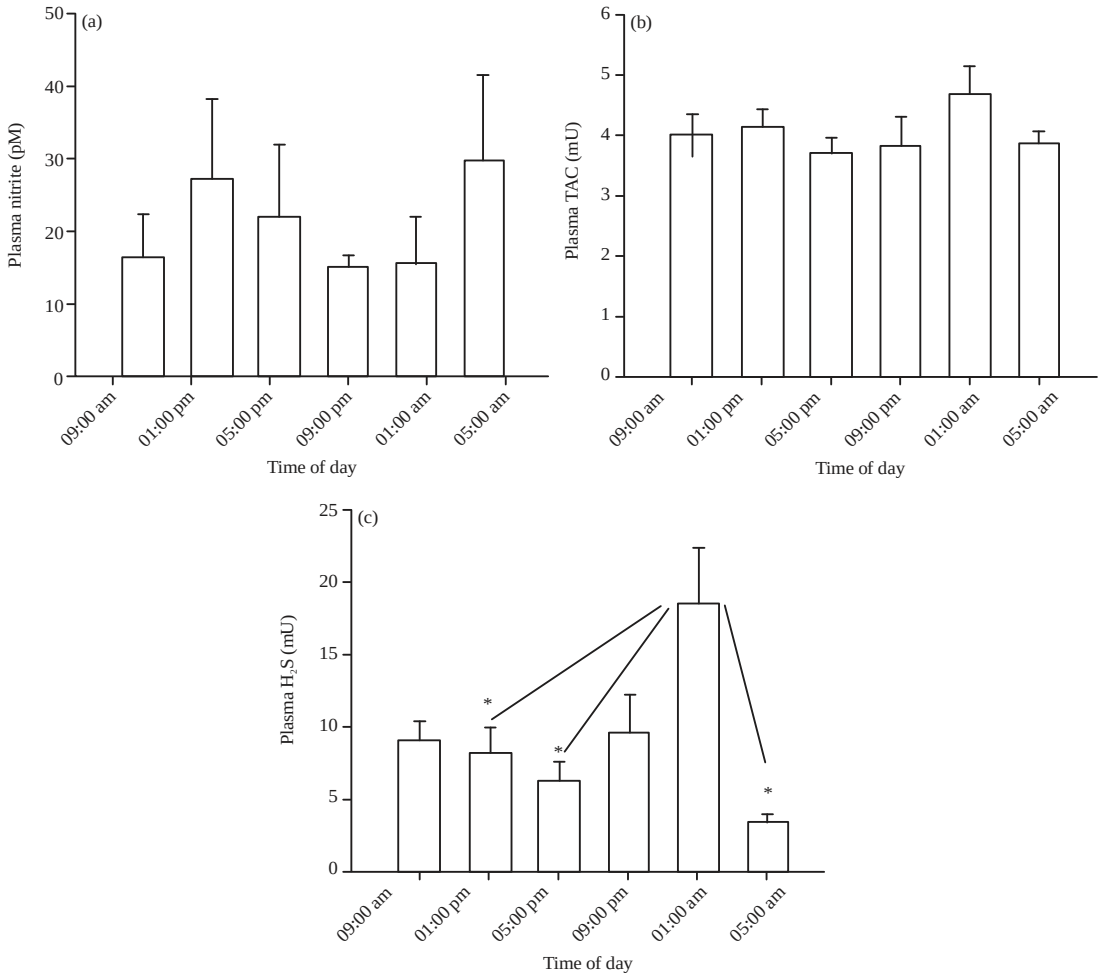

Fig. 6(a-c): Circadian changes in plasma levels of NO, TAC and H_2S at throughout the day. Plasma levels of nitric oxide and TAC did not change during the day but H_2S levels show diurnal variation. *p<0.05, different from the value at 01:00 am (n = 4-6)

circadian rhythm is important for new therapeutic approaches. Circadian rhythm of blood pressure is well established in humans and animals. Contrary to humans, rodents are nocturnal and are the most active during the dark hours. In humans, the average nightly blood pressure is lower than the daytime average but it is exactly the opposite of that in rats[4,5,26]. Data of the present study shows a significant time of day variation in the systolic blood pressure in rats, which was high during the dark-period. Systolic blood pressure reached the maximum at 05:00 am and was significantly different from the minimum value at 09:00 am. Previous data also have shown a similar pattern of the circadian variation in the systolic blood pressure in rodents[5,27,28].

It is important that chemical mediators and biological active compounds (hormones, peptides and neurotransmitters) work together to regulate and provide feedback control systems for phasic oscillation of diurnal cardiovascular function. In addition, it was known that there are important temporal oscillations of tissue sensitivity to these mediators. Time-dependent variations in rat thoracic aorta sensitivity to phenylephrine have been reported[29,30]. Consistent with the previous studies, this study demonstrate that a circadian variation exists for the contractile response of thoracic aortas in response to the vasoconstrictor phenylephrine. The E_{max} values calculated from concentration-response curves with phenylephrine were highest at 05:00 am, which correlated with high blood pressure at night. Aortic smooth muscle isolated during the light cycle exhibited higher sensitivity to phenylephrine than to tissue isolated during the dark cycle[10]. These results support the idea that variation of tissue sensitivity to phenylephrine plays a role in circadian blood pressure rhythm.

Phosphorylation of myosin light chain plays a central role in the regulation of smooth muscle contraction[31]. The ROCK

pathway is an essential regulator of vascular function and has an important role in the regulation of arterial blood pressure[12]. This pathway is implicated in the regulation of vascular tone[32] through Ca^{2+} sensitization of myofibrils[14]. The ROCK modulates myosin light chain phosphorylation by inhibiting MLC phosphatase activity via phosphorylation of MYPT1. There are 2 isoforms of ROCK which share 65% overall homology at the amino acid level[33]. The ROCK II plays a predominant role in the regulation of vascular smooth muscle contraction[34]. In this study, the ROCK inhibitor Y-27632 depressed phenylephrine-stimulated contraction of the aorta. However, the circadian pattern of contractile response with phenylephrine did not change. This suggests that ROCK activity did not affect the daily variation of tissue sensitivity to α-1 adrenoceptor stimulation. On the other hand, previous studies demonstrated that ROCK II mRNA or protein expression exhibited a circadian rhythm in the normal mouse aorta[15], in porcine coronary artery smooth muscle cells[16] and in mouse mesenteric arteries[17]. The circadian changes in the level of MYPT-1 phosphorylation have also been reported in porcine coronary artery smooth muscle cells[16]. In this study, however, ROCK II and pMYPT-1 protein expression remained unchanged in rat thoracic aorta tissue. These differences may have been due to different animal species and tissues.

It is well known that nitric oxide plays important roles in the regulation of blood pressure. Decreased or increased bioavailability of NO could change vascular responsiveness and blood pressure. Differing results have also been reported for the presence of a NO circadian rhythm in plasma. It has been observed that NO levels exhibit diurnal rhythm in mice[35], rats[36,37] and human[38]. Furthermore, strain and age-dependent variation of blood nitrite/nitrate level in rat have been also reported[39, 40]. In this study, NO plasma levels unchanged at six different times of day in rats. These results are similar to a previously study[26]. In several literatures, the plasma level of NO may be different due to changes in experimental protocols such as animal housing, blood collection and test procedure. Because of multi factorial variation of plasma NO level in organism, it is hard to compare all the data and understand the nature of the rhythm of NO.

Oxidative stress due to the elevation of reactive oxygen species has been shown to change vascular reactivity[41,42]. Additionally, diurnal variation in the redox state of plasma has been reported[43,44]. However, variations of plasma TAC levels were not significantly different in this study. The reason for the discrepancies between results is currently unknown and requires further investigation.

The H_2S is a recently described gaseous mediator that is vital for various physiological functions[45,46]. However, the H_2S level in plasma has not been determined at different times of the day. The present study is the first, to our knowledge, to show that the plasma H_2S level has diurnal variation. The minimum plasma level of H_2S occurs at 05:00 am and correlates with peak in blood pressure and phenylephrine-induced contraction. This correlation would be reason or result of vascular responsiveness. The exact source of blood H_2S was not recognized very well. Further studies are needed to identify the mechanism of this inverse correlation and the source of circadian H_2S.

CONCLUSION

As a conclusion, the contribution of various substances on the circadian regulation of blood pressure has been described. The current study demonstrated that plasma levels of H_2S were accompanied by circadian variation in systolic blood pressure and α-1 adrenoceptor-stimulated contraction of vessels. This finding implicates that H_2S production might be involve in regulation of the diurnal blood pressure variation.

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