Mutation of the Circadian Clock Gene Per2 Alters Vascular Endothelial Function
Hema Viswambharan, João M. Carvas, Vladan Antic, Ana Marecic, Corinne Jud, Christian E. Zaugg, Xiu-Fen Ming, Jean-Pierre Montani, Urs Albrecht and Zhihong Yang

Circulation 2007;115;2188-2195; originally published online Apr 2, 2007;
DOI: 10.1161/CIRCULATIONAHA.106.653303
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/cgi/content/full/115/16/2188
Mutation of the Circadian Clock Gene Per2 Alters Vascular Endothelial Function

Hema Viswambharan, PhD*; João M. Carvas, MSc*; Vladan Antic, MD, PhD; Ana Marecic, MSc; Corinne Jud, MSc; Christian E. Zaugg, PhD; Xiu-Fen Ming, MD, PhD; Jean-Pierre Montani, MD; Urs Albrecht, PhD; Zhihong Yang, MD

Background—The circadian clock regulates biological processes including cardiovascular function and metabolism. In the present study, we investigated the role of the circadian clock gene Period2 (Per2) in endothelial function in a mouse model.

Methods and Results—Compared with the wild-type littermates, mice with Per2 mutation exhibited impaired endothelium-dependent relaxations to acetylcholine in aortic rings suspended in organ chambers. During transition from the inactive to active phase, this response was further increased in the wild-type mice but further decreased in the Per2 mutants. The endothelial dysfunction in the Per2 mutants was also observed with iomycin, which was improved by the cyclooxygenase inhibitor indomethacin. No changes in the expression of endothelial acetylcholine-M₁ receptor or endothelial nitric oxide synthase protein but increased cyclooxygenase-1 (not cyclooxygenase-2) protein levels were observed in the aortas of the Per2 mutants. Compared with Per2 mutants, a greater endothelium-dependent relaxation to ATP was observed in the wild-type mice, which was reduced by indomethacin. In quiescent aortic rings, ATP caused greater endothelium-dependent contractions in the Per2 mutants than in the wild-type mice, contractions that were abolished by indomethacin. The endothelial dysfunction in the Per2 mutant mice is not associated with hypertension or dyslipidemia.

Conclusions—Mutation in the Per2 gene in mice is associated with aortic endothelial dysfunction involving decreased production of NO and vasodilatory prostaglandin(s) and increased release of cyclooxygenase-1–derived vasoconstrictor(s). The results suggest an important role of the Per2 gene in maintenance of normal cardiovascular functions.

(Circulation. 2007;115:2188-2195.)

Key Words: acetylcholine ■ circadian rhythm ■ cyclooxygenase 1 ■ endothelium ■ nitric oxide ■ vasodilation

The master circadian clock in mammals, located in the suprachiasmatic nuclei of the hypothalamus, regulates many biochemical, physiological, and behavioral processes and allows an organism to anticipate diurnal changes. In mammals, a set of clock genes constitutes the molecular machinery of the circadian clock. The 2 transcription factors CLOCK and BMAL1 form heterodimers and induce expression of several genes by binding to E-box enhancer elements in the promoters of target genes. Among these genes are the Period genes (Per1/2/3) and Cryptochrome genes (Cry1/2), whose protein products PER and CRY then negatively regulate the activation of their own expression by inhibiting the activity of the CLOCK/BMAL1 complex, thereby constituting a negative feedback loop. Genetic studies revealed important roles of the circadian genes not only in the regulation of behavior but also in metabolism. For example, an autosomal dominant mutation in the human Per2 gene results in familial advanced sleep phase syndrome, and Per2 mutant mice display impaired clock resetting and loss of circadian rhythmicity in constant darkness. Recent studies suggest an important role of CLOCK and BMAL1 in glucose homeostasis. Homozygous Clock mutant mice reveal not only behavioral changes but also phenotypes resembling metabolic syndrome, a cluster of cardiovascular risk factors including obesity, dyslipidemia, hypertension, insulin resistance, and hyperglycemia. These risk factors can cause vascular injury, and in particular they promote endothelial dysfunction leading to cardiovascular diseases.

The endothelium regulates vascular function by releasing vasoactive factors including relaxing and contracting factors. An imbalance between the relaxing factors and contracting factors

Clinical Perspective p 2195
factors participates in pathogenesis of cardiovascular diseases. Research from the past decades has provided firm evidence for the vasoprotective role of endothelium-derived nitric oxide (NO), which is produced from L-arginine via endothelial NO synthase (eNOS). NO causes vasodilation and inhibits smooth muscle cell proliferation, platelet aggregation, and inflammatory responses. Therefore, endothelial dysfunction, reflected by impaired endothelium-dependent relaxations in response to various agonists such as acetylcholine, ATP, histamine, and ionomycin, plays an essential role in pathogenesis of cardiovascular diseases. The mechanisms underlying endothelial dysfunction are attributable to (1) a defect in eNOS gene expression; (2) eNOS enzymatic activation; or (3) an increase in oxidative stress. Besides (1) a defect in eNOS gene expression; (2) eNOS enzymatic activation; or (3) an increase in oxidative stress. Besides these mechanisms, increased production of endothelium-derived contracting factors from cyclooxygenase (COX) has also been implicated in endothelial dysfunction under various disease conditions by counteracting the vascular relaxing effect of NO.

Clinical studies have well documented the circadian pattern of physiological cardiovascular functions and pathological cardiovascular events. The onset of unstable angina, myocardial infarction, sudden cardiac death, and stroke occurs usually at specific moments of the day. It has been suggested that it is attributed primarily to the diurnal variations of cardiovascular parameters, such as sympathetic nerve activity, blood pressure, and heart rate, in addition to variations in plasma lipid, platelets, coagulation factors, or endothelial dysfunction. However, it is unknown whether the diurnal variation of cardiovascular parameters is controlled primarily by circadian clock genes or influenced secondarily by external factors. The aim of the present study is to investigate whether there are alterations in endothelial functions in a mouse model with Per2 mutation and whether the endothelial functional changes are associated with the metabolic cardiovascular risk factors, the phenotype observed in the CLOCK and BMAL1 mutant mice.

Methods

Materials

See the online-only Data Supplement.

Animals

The Per2 mutant mice were from Zheng et al. and propagated in our own facility. Adult 3-month-old male wild-type (WT) (C57Bl/6J) and Per2 mutant littermates produced from heterozygote-heterozygote crosses were fed ad libitum and euthanized by decapitation. Artificial light was provided daily from 6 AM (Zeitgeber time (ZT0)) to 6 PM (ZT12) (12 hours of light/12 hours of darkness) with room temperature and humidity kept constant (temperature, 22±1°C; humidity, 55±5%). Mice were euthanized at ZT3, ZT6, ZT9, or ZT15. All the animal experimental protocols were approved by the Ethical Committee of Veterinary Office of Fribourg, Switzerland.

Vasomotor Responses

The descending thoracic aortas with intact endothelium were isolated and dissected free from perivascular tissues and cut into rings (3 mm in length). The rings were suspended in a Multi-Myograph System (model 610M, Danish Myo Technology A/S, Denmark) filled with Krebs-Ringer bicarbonate buffer (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 0.026 EDTA, and 11.1 glucose) at 37°C, aerated with 95% O2 and 5% CO2. Changes in isometric tension were recorded with MyoData software as previously described.

Aortic rings were allowed to equilibrate for 45 minutes and were progressively stretched to a passive tension of 5.0 mN that gives the optimal length-tension relationship. To study the effects of Per2 on endothelium-dependent or -independent relaxations, aortic rings from WT and Per2 mutant mice were precontracted with norepinephrine (0.1 to 0.3 µmol/L) to match the precontraction. Acetylcholine, ATP, or ionomycin, which release NO from endothelial cells, or the NO donor sodium nitroprusside (0.1 mmol/L to 10 µmol/L) was added to the precontracted aortic rings. To study whether arginine and free radical formation are involved in the regulation of endothelial function, the arterial rings were treated with the arginine inhibitor l-nornaline (0.2 mmol/L, 60 minutes) in the presence of l-arginine (10 mmol/L) or with superoxide dismutase (150 U/mL) plus catalase (1000 U/mL; 15 minutes), respectively, followed by the response to acetylcholine (1 mmol/L to 10 µmol/L). To study the role of cyclooxygenase in regulation of vascular functions, the vascular rings were incubated with indomethacin (1 µmol/L) for 30 minutes. The endothelium-dependent responses to acetylcholine, ionomycin, or ATP were then performed.

Protein Expression of eNOS, COX, and Acetylcholine-M3 Receptor in Mouse Aortas

Aortic segments with intact endothelium were snap-frozen and kept at −80°C until use. Crude protein extracts were obtained by homogenizing the aortic tissues in protein extraction buffer as described previously. Protein concentrations were measured by the Lowry method; 30 µg of the extract was resolved by SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride membrane (Millipore). The protein of interest was detected by immunoblotting with the use of antibodies against eNOS (1:2500), COX-1 (1:500), COX-2 (1:500), or acetylcholine-M3 receptor (1:500) as primary antibodies followed by incubation with a corresponding secondary fluorophore-conjugated antibody. The signals were visualized with the use of the Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification of the signals was performed with the use of Odyssey Application Software 1.2. Protein levels were expressed as the ratio against tubulin.

In Vitro ECG

See the online-only Data Supplement.

Blood Pressure Measurement by Telemetry

All mice were instrumented with an arterial catheter connected to an implantable transducer (model TA11PA-C20, Data Sciences International) to monitor arterial pressure by telemetry. Through a paratracheal incision with the use of stereomicroscopy, the left carotid artery was exposed, briefly clamped, and punctured with a 26-gauge needle. The cannula was then inserted into the aortic arch against the flow and secured in place with tissue adhesive. The body of the implant was placed subcutaneously on the right flank. After completion of the surgery, the mice were housed in individual standard mouse cages (23×17×14 cm high, Indulab AG, Switzerland) for blood pressure measurement. At least 12 days were given to animals to recover from surgery and for blood pressure and heart rate to stabilize.

Continuous arterial pressure monitoring was performed in unrestrained conscious mice. Each animal cage was equipped with 1 RPC-1 plastic receiver (Data Sciences International) fixed on the bottom of the cage and connected to an analog output device (R11-CPA), allowing for the continuous recording of the analog pulsatile arterial pressure signal. The analog pressure signal was then fed to a 12-bit analog-to-digital converter (Dash-16, Metrabyte Corp), sampled at 1 kHz, and processed with customized algorithms for beat-to-beat analysis. To analyze only data from the undisturbed periods, the 2-hour period of animal maintenance and cage cleaning (8 to 10 AM) was excluded from the analysis.

Downloaded from circ.ahajournals.org by on April 24, 2007
Statistical Analysis

Relaxations were expressed as percentage of decrease in tension of the contraction to norepinephrine. Data are given as mean ± SEM. In all experiments, n equals the number of animals from which the blood vessels were dissected. Area under the curve or responses to agents at each corresponding concentrations among the different groups of animals were compared either by the Student t test for unpaired observations or by ANOVA for multiple comparisons followed by Bonferroni adjustment. A 2-tailed value of P < 0.05 is considered to indicate a statistical difference.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Endothelium-Dependent Relaxations to Acetylcholine in Per2 Mutant Mice

In aortic rings with intact endothelium precontracted with norepinephrine (0.1 or 0.3 μmol/L), acetylcholine (1 nmol/L to 10 μmol/L), which stimulates NO release from the endothelium via activation of endothelial acetylcholine-M3 receptor,35 evoked potent concentration-dependent relaxations in WT mice at ZT3, the beginning of the inactive phase for the rodents (Figure 1A; n = 9). The response was significantly decreased in the Per2 mutant mice (Figure 1A; n = 9; P < 0.05 versus WT). The smooth muscle sensitivity to NO as demonstrated by endothelium-independent relaxations to the NO donor sodium nitroprusside (0.1 nmol/L to 1 μmol/L) was comparable between the 2 groups of animals (Figure 1B; n = 9; P = NS).

Western blots showed no difference in protein levels of endothelial acetylcholine-M3 receptor or eNOS in the aortas of the WT and Per2 mutant mice (Figure 2; n = 5 to 6). Neither inhibition of oxidative stress by incubating aortic rings with superoxide dismutase (150 U/mL) plus catalase (1000 U/mL) nor inhibition of arginase by L-norvaline (20 mmol/L, 1 hour, to increase L-arginine substrate availability for eNOS) improved endothelium-dependent relaxations to acetylcholine in Per2 mutant mice (Figure 1A and 1B in the online-only Data Supplement; n = 6).

Furthermore, we showed that the decreased endothelium-dependent relaxations in response to acetylcholine in Per2 mutant mice were consistently present during the inactive phase as demonstrated in animals at ZT6 and ZT9 (Figure II in the online-only Data Supplement). Interestingly, the endothelium-dependent relaxations induced by acetylcholine were further enhanced in the WT mice at ZT15, the beginning of the active phase (Figure 1A; n = 7 to 9; P < 0.05 versus WT).
mice at ZT3), but decreased in the Per2 mutants (Figure 1A; n=7 to 9; P<0.05 versus Per2 mutants at ZT3).

Endothelium-Dependent Relaxations to Ionomycin in Per2 Mutant Mice

Moreover, the endothelium-dependent relaxations to ionomycin (10 nmol/L to 1 μmol/L), which stimulates eNOS enzymatic activity via non–receptor-mediated increase in intracellular Ca²⁺ concentration, were also reduced significantly in the Per2 mutant mice during the inactive phase (maximum effect: 46.7±12.1%) compared with the WT animals (maximum effect: 75.1±6.5%; P<0.05; Figure 3; n=7). During transition of the animals from the inactive to the active phase, the endothelium-dependent relaxations to ionomycin were significantly enhanced in both groups of animals (P<0.05 between ZT9 and ZT15 in the WT or Per2 mutants). However, the relaxations remained weaker in the Per2 mutants than the WT animals (Figure 3; n=7 to 9; P<0.05) also in the presence of indomethacin (Figure III in the online-only Data Supplement; n=7; P<0.05).

Role of COX in Endothelium-Dependent Responses in Per2 Mutant Mice

We further analyzed whether COX-derived vasoactive prostanoids play a role in the functional changes in endothelium-dependent responses. Interestingly, a significantly higher protein level of COX-1 in aortas was found in the Per2 mutant mice than in the WT animals by immunoblotting (1.8±0.3-fold increase; Figure 4A; n=9; P<0.05). No COX-2 signal could be detected in the aortas of the animals. Inhibition of COX by indomethacin (1 μmol/L, 30 minutes) did not affect the endothelium-dependent relaxations to acetylcholine in both WT and Per2 mutant mice (Figure 4B; n=5 to 6; ZT3), whereas it significantly enhanced the relaxations to ionomycin in the Per2 mutants (Figure 4C).

The enhanced production of COX-derived vasoconstrictor(s) in the Per2 mutants was further demonstrated in the quiescent aortic rings stimulated by ATP in the presence of N²-nitro-L-arginine methyl ester (L-NAME) (to eliminate the vasorelaxing effect of NO) (Figure 5A and 5B). ATP (0.1 to 10 μmol/L) evoked a transient but more pronounced vasoconstriction in the Per2 mutant (14.5±1.8% of KCl 100 mmol/L) than the WT mice (5.0±1.3%; n=4; P<0.05). The contraction was abolished by the COX inhibitor indomethacin (Figure 5B; 1 μmol/L, 30 minutes).

Further experiments in the precontracted aortic rings demonstrated that ATP at 0.1 μmol/L did not cause vascular relaxation in both WT and Per2 mutant mice (Figure 5C), whereas it caused an endothelium-dependent relaxation at 1 μmol/L, which was more pronounced in the WT (57.1±6.0%) than in the Per2 mutant mice (21.3±7.7%; n=6; P<0.05) (Figure 5C). Incubation of the aortas with the

Figure 3. Endothelium-dependent relaxations to ionomycin in Per2 mutant mice. Relaxation to ionomycin (10 nmol/L to 1 μmol/L) in the aortas of WT and Per2 mutant mice during the inactive phase (open symbol) and the active phase (solid symbol) is shown. n=7 to 9; ANOVA with Bonferroni adjustment for the area under the curve among the 4 groups. NE indicates norepinephrine.

Figure 4. COX and endothelium-dependent relaxations in the Per2 mutant mice. A, A representative immunoblotting shows increased COX-1 expression in the aortas of Per2 mutant mice. B, Effects of the COX inhibitor indomethacin (Indo) (1 μmol/L, 30 minutes) on endothelium-dependent relaxations to acetylcholine (ACh) and ionomycin (C). No COX-2 signal could be detected in the aortas of the animals. NE indicates norepinephrine. n=7 to 9; *P<0.05 and **P<0.01 vs WT or Per2+Indo. Student unpaired t test for COX-1 expression between WT and Per2 mutants and for the responses to acetylcholine or ionomycin in the presence or absence of indomethacin in WT or Per2 mutant mice.
COX inhibitor indomethacin (1 μmol/L, 30 minutes) significantly reduced the relaxation to ATP at 1 μmol/L in the WT mice (Figure 5C; 35.4±5.6%; n=7; \( P<0.05 \)) but was unable to affect the response in the Per2 mutants. These relaxations in both WT and Per2 mutant mice were fully inhibited by incubation of the aortas with the eNOS inhibitor L-NAME (0.1 mmol/L, 15 minutes; data not shown). Furthermore, the endothelium-dependent relaxation induced by the highest concentration of ATP (10 μmol/L) was also significantly reduced in the Per2 mutant mice, and indomethacin was unable to significantly modulate the response in both groups (Figure 5C). Approximately 30% of relaxations were still present in the aortas incubated with indomethacin plus L-NAME, which can be fully blocked by KCl 100 mmol/L (data not shown), indicating the involvement of the endothelium-derived hyperpolarizing factor in endothelium-dependent relaxation to ATP at the highest concentration.

**Metabolic and Hemodynamic Parameters**

Mean arterial pressure for the whole-day period (22 hours) in Per2 mutant animals (105.9±1.9 mm Hg; n=10) was significantly lower than that in their WT counterparts (116.5±2.1 mm Hg; n=9; \( P<0.05 \)). Whole-day values for heart rate were not different between Per2 mutant mice (530.9±5.7 bpm; n=10) and WT animals (535.7±11.3 bpm; n=9; \( P=NS \)). In vitro ECG showed no difference in changes of PP interval or PQ interval in response to increasing concentrations of acetylcholine (1 nmol/L to 0.1 mmol/L) between the 2 groups (Figure IV in the online-only Data Supplement; n=8).

There was no difference in plasma concentrations of total cholesterol and triglyceride between the 2 groups of animals (Figure V in the online-only Data Supplement; n=4). Notably, a previous study showed no difference in plasma glucose concentration between the WT and Per2 mutant mice and an increased insulin sensitivity in the latter.\(^{36}\)

**Discussion**

In the present study, we provided the first evidence for a role of the circadian clock gene Per2 in the regulation of endothelium-mediated vascular responses in a mouse model, which is not associated with phenotypes resembling cardiovascular metabolic risk factors.

**Endothelial Dysfunction in Per2 Mutant Mice**

The endothelium controls vascular functions by releasing NO from eNOS in response to various hormonal factors.\(^{37}\) In the present study, we showed decreased endothelium-dependent relaxations in the Per2 mutant mice on stimulation with acetylcholine but endothelium-independent relaxations to the NO donor sodium nitroprusside comparable to those in the WT animals. Notably, the decreased endothelium-dependent relaxation to acetylcholine persists throughout the daytime, the inactive phase of the rodents (Figure I in the online-only Data Supplement). This is not attributable to a differential eNOS gene expression or acetylcholine receptor expression between the 2 groups because the protein levels of eNOS and acetylcholine-M₄ receptor that mediate NO release from the endothelial cells\(^{35}\) are comparable between the Per2 mutants and WT littermates. Furthermore, impaired endothelium-dependent relaxations in the Per2 mutants are also demonstrated with ATP as well as ionomycin, which activates ENOS by non–receptor-mediated increase in intracellular Ca²⁺ con-
centration, demonstrating endothelial dysfunction in the mutants.

Studies in the past demonstrate that besides alterations in eNOS gene expression, defective eNOS enzymatic activity and increased oxidative stress are potential mechanisms for the impairment of endothelial NO-mediated vascular relaxations. It is unlikely that increased oxidative stress is responsible for the endothelial dysfunction in the Per2 mutant mice because the decreased endothelium-dependent relaxations could not be reversed by scavenging reactive oxygen species with superoxide dismutase and catalase. Furthermore, inhibition of arginase to enhance l-arginine availability for eNOS or supplementation of the eNOS cofactor BH4 had no effects on endothelium-dependent relaxations to acetylcholine in the Per2 mutant mice (data not shown). It is noteworthy that Per2 mutant mice have decreased calmodulin expression in the liver, as demonstrated in a previous study. Because calmodulin is essential for the Ca2+–dependent activation of eNOS by various agonists, it is conceivable that the decrease in calmodulin expression could also occur in the endothelial cells, which might be responsible for the impaired endothelium-dependent relaxations in the Per2 mutant mice in response to the various agonists. This hypothesis warrants further investigation.

**Diurnal Changes of Endothelial Function in Per2 Mutant Mice**

Interestingly, during the transition from the inactive to the active phase, the endothelium-dependent relaxation to acetylcholine is increased in the WT but decreased in the Per2 mutant mice. In contrast to acetylcholine, the endothelium-dependent relaxation to ionomycin is enhanced in both groups of the animals during the transition from the inactive to the active phase, whereby the response still remains weaker in the Per2 mutant mice. These results further demonstrate endothelial dysfunction in the mutant mice in the inactive as well as in the active phase. The increased response to ionomycin in both groups of animals in the active phase may be explained by the hemodynamic adaptation during this time period, ie, the increase in blood flow in the circulation, that increases NO release from the endothelium. The possibility of different physical activity between WT and Per2 mutant mice can be ruled out because a previous study showed normal behavioral activity of the Per2 mutant mice under normal light/dark cycles. The observation that the endothelium-dependent relaxations in response to acetylcholine are further decreased in the Per2 mutant mice in the active phase might be due to opposite changes in the signal transduction pathways mediated by acetylcholine receptor, which leads to a further decrease in eNOS activation in the active phase of the Per2 mutant mice. This conclusion is supported by the following evidence: First, ionomycin-induced endothelium-dependent relaxation is increased instead of decreased in the active phase, as shown in the present study; second, a study from another group showed that mouse aortic eNOS activity does not display 24-hour rhythmicity under a normal light/dark cycle. Increased endothelium-dependent relaxation in healthy subjects in the morning hours has been reported in humans, which is in agreement with the results from the WT mice, although the results are controversial in clinical studies. The inconsistent results among the clinical studies are not clear but might be related to the different vascular beds investigated, ie, resistance vessels and conduit blood vessels, or might be due to changes in sympathetic nerve activity in vivo. Whether our results can explain the increased incidence of cardiac events in the morning hours in certain patient populations remains speculative.

Notably, the heart seems not to be affected by the defective acetylcholine response in the Per2 mutant mice because the responses to acetylcholine in the heart, ie, increases in PP interval or PQ interval, that are mediated by acetylcholine-M1 receptor remain unchanged, as demonstrated by in vitro ECG. In addition, the blood pressure is significantly lower and heart rate is the same during 24 hours in the Per2 mice compared with the WT animals, suggesting that the parasympathetic nerve or acetylcholine-mediated signaling in the heart of the Per2 mutant mice remains intact.

**COX-1 Expression and COX-Derived Vasoactive Prostanoids in Per2 Mutant Mice**

Another important finding of our present study is the increased expression of COX-1 (no signal of COX-2 detectable) in the aortas of the Per2 mutant mice. The mechanism of the increased COX-1 expression in the Per2 mutants is unknown. Whether COX-1 is a target gene of Per2, ie, regulated directly by Per2, or regulated secondarily remains to be investigated. Accordingly, the endothelium-dependent relaxations induced by ionomycin (but not acetylcholine) are enhanced by the COX inhibitor indomethacin in the Per2 mutant mice, demonstrating that COX-1–derived vasoconstrictor(s) is stimulated by ionomycin but not by acetylcholine, which partly contributes to the endothelial dysfunction in the Per2 mutant mice. The lack of the effect of indomethacin on acetylcholine-induced response in Per2 mutants further indicates a defective acetylcholine receptor–mediated signaling in the aortic endothelial cells of these animals. It is possible that enhanced COX-1 expression could influence endothelial function in the Per2 mutant mice if the defect in endothelial acetylcholine signaling could be corrected.

The increased production of COX-1–derived vasoconstrictor(s) is also demonstrated in the quiescent rings incubated with L-NAME (to inhibit eNOS and to unmask the contracting effect of COX-derived vasoconstrictors). Under this condition, ATP causes a more pronounced vasoconstriction in the Per2 mutants than in WT mice, which is inhibited by indomethacin. However, indomethacin cannot improve endothelium-dependent relaxations to ATP (in contrast to ionomycin) in the Per2 mutants, but it reduces the relaxation response to ATP (1 μmol/L) in the WT mice. These results suggest that ATP releases a vasodilator produced from the COX pathway, most likely prostaglandin I2, another well-known endothelial vascular protective factor, in the WT mice but not in the Per2 mutants. Notably, the increased production of COX-1–derived vasoconstrictor(s) that was observed in the quiescent aortic rings of Per2 mutant mice is apparently not sufficient to counteract the effect of NO induced by ATP (1 μmol/L). Only 3% of the maximum contraction to KCl 100 mmol/L was observed under this condition. Notably,
ATP at a higher concentration of 10 μmol/L also releases the endothelium-derived hyperpolarizing factor, which in part compensates the vasodilatory response to the agonist. Although 15% contraction occurs in response to the higher concentration of ATP of 10 μmol/L, the relaxation effect exerted by both NO and endothelium-derived hyperpolarizing factor is dominant in the Per2 mutants. Nonetheless, the results in the quiescent rings demonstrate an increased production of COX-1–derived vasoconstrictor(s) in the aortas of the Per2 mutant mice.

Vascular functions, including endothelial functions, can be impaired by various risk factors that are clustered in the metabolic syndrome. Recent studies demonstrate that mice with disruption of the Clock gene are prone to develop a phenotype resembling metabolic syndrome. In contrast to the Clock mutant mice, our Per2 mutant mice have lower blood pressure and do not develop dyslipidemia. It has been shown recently that the Per2 mutant mice have faster glucose clearance than the WT animals. These results imply that the endothelial dysfunction in the Per2 mutant mice is not necessarily associated with metabolic risk factors but rather is linked directly to the Per2 gene. Whether the endothelial function is regulated directly by the peripheral circadian clock or indirectly by the master clock in suprachiasmatic nuclei remains to be investigated.

In conclusion, mutation of circadian clock genes is not necessarily associated with metabolic syndrome. Functional loss of the Per2 gene impairs endothelial function in mouse aortas involving decreased production of NO and a vasodilatory prostaglandin(s) as well as increased release of COX-1–derived vasoconstrictor(s) (Figure 6). The results suggest an important role of the Per2 gene in maintenance of normal cardiovascular functions.

**Sources of Funding**

This work was supported by the Swiss National Science Foundation (grants 3100A0-105917/1 to Dr Yang, 3100A0-104222 to Dr Albrecht, and 3200BO-105900 to Dr Antic), the Swiss Heart Foundation, the Swiss Cardiovascular Research and Training Network Program, and the European Union Research Program EU CLOCK.

**Disclosures**

None.

**References**


Figure 6. Alterations in endothelial function in the Per2 mutant mice. Per2 mutation is associated with decreased endothelial NO release and deficiency in vasodilatory prostaglandins, most likely prostacyclin (PGI2), but increased vasoconstrictive prostaglandins (PGs). EDHF indicates endothelium-derived hyperpolarizing factor.
Furthermore, an increase in cyclooxygenase-1 protein level and cyclooxygenase-derived vasoconstrictor production is observed in the mutant mouse aorta. The endothelial dysfunction in the Per2 mutant mice during transition from the inactive to the active phase, whereas it is increased in the wild-type littermates. Circ. 2003;9:RA1–RA8.


**CLINICAL PERSPECTIVE**

Clinical studies document an increased frequency of cardiovascular events such as unstable angina, myocardial infarction, sudden cardiac death, and thrombotic stroke during the morning hours, usually from 6 AM to noon. It is, however, not known whether this is primarily related to the diurnal variation of cardiovascular parameters and physical activities or controlled by the master clock that resides in the suprachiasmatic nuclei of the hypothalamus in mammals. In the present study, we show that mice with the mutation of the clock gene *Per2* exhibit impaired endothelium-dependent relaxations due to decreased endothelial nitric oxide synthase activity and a decreased production of vasodilatory prostaglandin(s) in the aorta. The endothelium-dependent relaxation in response to acetylcholine is further decreased in the *Per2* mutant mice during transition from the inactive to the active phase, whereas it is increased in the wild-type littermates. Furthermore, an increase in cyclooxygenase-1 protein level and cyclooxygenase-derived vasoconstrictor production is observed in the *Per2* mutant mouse aorta. The endothelial dysfunction in the *Per2* mutant mice is not associated with hypertension or dyslipidemia. The results of the present study demonstrate that mutation of the *Per2* gene in mice is associated with endothelial dysfunction involving decreased production of nitric oxide and vasodilatory prostaglandin(s) as well as increased release of cyclooxygenase-1–derived vasoconstrictor(s). The results suggest a potential role of the *Per2* gene in maintenance of normal cardiovascular functions and may provide certain clues at the molecular level for the circadian pattern of cardiac events in clinical settings.


