Differential Effects of Two Period Genes on the Physiology and Proteomic Profiles of Mouse Anterior Tibialis Muscles

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The molecular components that generate and maintain circadian rhythms of physiology and behavior in mammals are present both in the brain (suprachiasmatic nucleus; SCN) and in peripheral tissues. Examination of mice with targeted disruptions of either mPer1 or mPer2 has shown that these two genes have key roles in the SCN circadian clock. Here we show that loss of the clock gene mPer2 affects forced locomotor performance in mice without altering muscle contractility. A proteomic analysis revealed that the anterior tibialis muscles of the mPer2 knockout mice had higher levels of glycolytic enzymes such as triose phosphate isomerase and enolase than those of either the wild type or mPer1 knockout mice. In addition, the level of expression of HSP90 in the mPer2 mutant mice was also significantly higher than in wildtype mice. These results suggest that the reduced locomotor endurance of the mPer2 knockout mice reflects a greater dependence on anaerobic metabolism under stress conditions, and that the two canonical clock genes, mPer1 and mPer2, play distinct roles in the physiology of skeletal muscle.

Keywords: Circadian Clock; mPer Mutant Mice; Muscle Contraction; Treadmill Running; Two-dimensional Gel Electrophoresis.

Introduction

Most animals have a circadian rhythm of locomotor activity with periods similar to the earth’s daily rotation about its axis. Circadian rhythms in mammals are controlled by free-running and self-sustaining pacemaker neurons located in the anterior hypothalamic region, the suprachiasmatic nucleus (SCN). It is widely accepted that the cellular mechanism underlying circadian rhythms is composed of interacting transcriptional and translational feedback loops in which the expression of so-called “circadian clock genes” is feedback regulated by their protein products (Lowrey and Takahashi, 2004; Reppert and Weaver, 2001).

In mammals, two basic helix-loop-helix (bHLH) domain-containing transcription factors called mCLOCK and BMAL1 form dimers that bind to the promoter regions of clock genes. They activate the expression of clock genes including three Drosophila period (per) homologs named mPer1, mPer2, and mPer3 and two Cryptochrome (Cry) genes, mCry1 and mCry2. The mPERs and mCRYs form multimeric protein complexes and enter the nucleus. Once in the nucleus, they function as negative regulators of mCLOCK-BMAL1, which results in the decline of their own expression and lets mCLOCK-BMAL1, reinitiate a new round of transcription (Gekakis et al., 1998; Shearman et al., 2000; Ueda et al., 2005).

Peripheral tissues such as liver and skeletal muscles also express clock genes. Many workers have suggested that the rhythmicity in peripheral tissues is under the control of the central SCN clock. Neuronal synaptic circuits and/or humoral factors might mediate communication between central and peripheral clocks (Balsalobre et al., 2000; Silver et al., 1997; Stokkan et al., 2001). Therefore, circadian rhythms in locomotion may be a result of clock gene activity in both central clocks and cell-specific regulation in muscle cells.

The physiological roles of genes in the circadian clock are studied by generating mice with gene-specific knockouts (KO). The rhythmicity of locomotor activity is completely disrupted in Bmal1 single KO mice, mPer1 and mPer2 double KO mice, and mCry1 and mCry2 double KO mice as measured by wheel-running activity rhythms.

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in constant conditions (Bae et al., 2001; Bunger et al., 2000; van der Horst et al., 1999; Zheng et al., 2001). Previously, one of the present authors generated mPer1 and mPer2 single KO mice and showed that these two clock genes have distinct roles in the SCN circadian clock. Although the two KO mice had similar behavioral phenotypes as measured by wheel-running activity, their running endurance was different. Although a number of factors can influence locomotor performance (e.g., neural, circulatory, bone properties, etc.), the properties of skeletal muscle are crucial (Dowson et al., 1998; Payne et al., 2005). Muscle cells are packed with myofibrillar and metabolic proteins, and the contractile capacity and subcellular attributes of the cells are good predictors of whole-organism performance (Dawson et al., 1998; Hornberger and Esser, 2004).

It is still unclear to what extent the two canonical clock gene, mPer1 or mPer2, affect the functional properties of muscles. One possibility is that an impairment of the SCN clock gene significantly affects gene expression in skeletal muscle cells and that this causes changes in their physiological and biochemical attributes that result in altered locomotor capacity. However, our Northern blot analysis of skeletal muscle after a day in constant darkness showed that the level and the phase of clock gene expression were unaffected in either single KO mouse (Lee and Bae, 2004). Secondly, local expression of these two canonical clock genes may have effects unrelated to the circadian clock. In that case the physiological and biochemical attributes of the muscles could remain unchanged, or undergo moderate change as the tissues adjust to altered physiological activities e.g., locomotion.

In the present report, we examined the roles of mPer1 and mPer2 in peripheral tissue physiology. To this end, we examined the effect of loss of either mPer1 or mPer2 at three levels: whole-organism locomotor capacity, skeletal muscle contractility, protein expression in the anterior tibialis (AT) muscles. Our results show that there are differences in the tissue-specific physiology and the level of protein expressions in skeletal muscles between the single KO mice.

Eight to ten week-old male mice were housed in LD cycles at least two weeks prior to analysis. All mice studied were of the same isogenic 129/sv genetic background. This study was approved by the University Animal Care and Use Committee and the animals were treated in accordance with the NIH guidelines for the care and use of experimental animals.

**Treadmill running** To investigate inter-group differences in locomotor capacity, we attempted treadmill running trials on the mice. The general protocol used in this study was described previously (Baker and Gleeson, 1999). Each animal was subjected to random-blind tests to avoid experimental bias. Locomotor capacity was gauged from running distance, a typical endurance measure, conducted in the light cycle at ZT6-8 (Zeitgeber Time 6 [ZT6] means six hours after lights-on time in the LD cycle) and in the dark cycle at ZT18-20 (ZT18 means six hours after lights-off time in the LD cycle). The treadmill (l × w × h = 273 mm × 70 mm × 48 mm) was caged with 10 mm thick plexiglass plates in an air-tight seal for oxygen consumption measurements (Oxymax System, USA) (Lee et al., 2002). The speed of the treadmill was adjusted precisely by a Panasonic DVUX960Y digital speed controller (± 0.1 rpm).

Before the actual trial we let individuals accustomed to the treadmill run for 10 min every morning and evening over seven days. We chose the treadmill speed (30–32 m min⁻¹) at which the animals ran at about 70% of the maximum aerobic metabolic rate. At the time of trial, a mouse was weighed and exposed to a warm-up bout of 2 min during which the treadmill speed was gradually increased to the experimental level. The actual trial was then started, and video-recorded (30 frames per sec) for accurate analysis. During the ZT18 treadmill test, a red background was placed right behind the treadmill for the video recording. Running duration (min) was determined from the length of time over which the animal could sustain its speed on the treadmill with a set number of pull-offs (Huang et al., 2002). On the basis of preliminary experiments we defined a ‘pull-off’ as an incident in which the running animal pulled back its head position from a half of the treadmill runway. The trial was completed as soon as the animals reached 20 pull-offs. Video playbacks demonstrated that the rate of pull-off increased with running time as the animals became more exhausted. We obtained the duration of running of each individual from the video timer, and calculated running distances (= duration × treadmill speed) for the inter-group comparisons.

**Muscle contraction** The experiment was performed using a separate set of animals from those in the treadmill tests. Each animal was anesthetized with sodium pentobarbital (i.p., 50 mg · kg⁻¹) either at ZT6 or ZT18, and the anterior tibialis muscles of both right and left hindlimbs were quickly removed. The AT muscle is one of the dorsiflexors at the ankle of the hind legs. The left muscle was frozen immediately in liquid nitrogen for 2-dimensional gel electrophoresis (2-DE) (see below). The right tissue was soaked in 70 ml of cold oxygenated Ringer’s solution (115 mM NaCl, 5 mM KCl, 4 mM CaCl₂, 1 mM MgCl₂, 1 mM

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**Materials and Methods**

**Subjects** The animals used in the experiment have been previously described (Bae et al., 2001; Lee et al., 2004a). Breeding stocks used to establish our colony were kindly provided by Dr. Steven Reppert and Dr. David Weaver. Mice were maintained at 23 ± 1°C in 12-h light: 12-h dark (LD) cycles, with standard Purina chow and water provided ad libitum. Genotypes were determined by polymerase chain reaction (PCR) amplification of genomic DNA extracted from tail biopsies, as previously described (Bae et al., 2001). The animals were grouped as wild-type (W), mPer1 knockout (Per1) and mPer2 knockout (Per2).
NaH₂PO₄, 24 mM NaHCO₃, 11 mM glucose, pH 7.39) containing 0.02 g L⁻¹ tubocurarine chloride. One tendon was tied to the 300B-LR servomotor arm (Aurora Scientific, Canada), and the other tendon to a micrometer to adjust it to the optimal muscle length.

The servo system has the ability to make both force and length steps in 1.3 ms and to shift from length control to force control without any length transient. A custom software program (DMC, Aurora Scientific) was used to control both the servo system and a Grass S48 stimulator. The stimulator was connected to a pair of bright platinum electrodes, and supplied a 1.0-ms square wave pulse or pulse train. Data on force or length changes were stored in a computer and analyzed by custom software (DMA, Aurora Scientific).

All the experiments (n = 9 for each group) were conducted at 25°C. For each preparation we determined the optimum muscle length and supramaximal voltage that generated maximum twitch force. A stimulus frequency of 80 Hz was used to produce fully fused tetani for the three groups. Maximum tetanic force (Fₜ) was stabilized, usually after the first three trials. A rest period of 20 min was given between electrical stimulations.

The shortening velocity of the tissue was examined at 0.35 F₀ as a reference load at which a near maximum power (= shortening velocity x load) was generated, as in previous reports (Lee et al., 2004b; Rome, 1998). To find this load, we first measured the shortening velocity of the muscle from quick-release isometric contractions, with 10 preset loads ranging from 0.1–0.8 F₀. Length changes were measured over periods of 20–50 ms starting about 15 ms after the release. A hyperbola was fitted to the data using Hill’s equation, and force-velocity and power-velocity relationships were established. From these preliminary experiments (n = 3), we determined the load level, 0.35 F₀, that best represented the force generating near-maximal power (see Results). This load level was then used in the subsequent experiments to determine the shortening velocity of each muscle. We rechecked F₀ after shortening velocity measurements, and discarded the muscle and the data if this F₀ was less than 93% of the initial F₀.

After completion of each experiment we measured the optimal muscle length using a micrometer under a light microscope. The tissue was frozen in place with liquid nitrogen, and its mass was measured with a chemical balance after tendons and connective tissues were removed. The muscle was then sectioned (8 μm) in a Microm HM505E cryostat and stained by a routine haematoxylin and eosin procedure. The cross-sectional area of the section was determined as in a previous study.

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The solubilized proteins were incubated at 30°C for 30 min with endonuclease (Sigma Chemical, USA), and centrifuged for 15 min at 17,000 rpm. The protein suspension was mixed with a rehydration buffer [7 M urea, 2 M thiourea, 65 mM DTT, 4% CHAPS, 40 mM Tris, 0.5% immobilized pH gradients (IPG) buffer (pH 3–10), and protein inhibitor Complete Mini (Roche, Penzberg, Germany)]. The sample was then incubated for 1 hr at room temperature and centrifuged for 30 min at 17,000 rpm. The supernatant was collected, and its protein concentration was determined using the Bradford assay kit (Bio-Rad, USA). For each tissue, a protein sample of 1.2 mg was used for 2-DE.

**Protein extraction and sample preparation** To screen candidate proteins whose expression differed significantly between the genotypes, we conducted a 2-DE analysis on the AT muscles collected at ZT6 (n = 4 per group). Our preliminary experiments showed no obvious differences in protein expression profiles at two time points, ZT6 and ZT18. On the basis of this 2-DE outcome, we attempted immunoblotting to delineate the detailed temporal responses of the proteins (see below). After tendons and connective tissues were removed, each muscle sample was crushed in liquid nitrogen using a mortar and pestle. The powdered sample was solubilized in 1 ml lysis buffer composed of 7 M urea, 2 M thiourea, 65 mM DTT, 4% CHAPS, 40 mM Tris, 0.5% immobilized pH gradients (IPG) buffer (pH 3–10), and protein inhibitor Complete Mini (Roche, Penzberg, Germany). The sample was then incubated for 1 hr at room temperature and centrifuged for 30 min at 17,000 rpm. The supernatant was collected, and its protein concentration was determined using the Bradford assay kit (Bio-Rad, USA). For each tissue, a protein sample of 1.2 mg was used for 2-DE.

**Separation of proteins by 2-DE** The solubilized proteins were incubated at 30°C for 30 min with endonuclease (Sigma Chemical, USA), and centrifuged for 15 min at 17,000 rpm. The protein suspension was mixed with a rehydration buffer [7 M urea, 2 M thiourea, 65 mM DTT, 4% CHAPS, 40 mM Tris, 0.5% IPG buffer (pH 3–10)] to obtain a total volume of 450 μl. The first dimension isoelectric separation was made on IPG of 3–10 L (240 x 3 x 0.5 mm) dry strips (Amersham Pharmacia Biotech, UK) using an Ettan IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech, UK). The strips were rehydrated at 60 V for 12 h, and focusing was run for 91,420 Vhr using a gradually increasing voltage protocol implemented by a programmable high voltage power supply (final voltage 8000 V).

Following isoelectric focusing, the strips were equilibrated for 15 min in 10 mL tributyl phosphate (TBP) solution [6 M urea, 2% SDS, 50 mM Tris/HCl gel buffer, 30% (v/v) glycerol (87% v/v), 5 mM TBP].

The second dimension separation was conducted with an Ettan DALT/six Electrophoresis Unit (Amersham Pharmacia Biotech, UK) on 12% SDS polyacrylamide gels. The strips were embedded in a 0.5% (w/v) agarose stacking gel, and the proteins were separated for 12 h at 30 mA per gel until the bromophenol blue marker dye ran off the bottom of the gel.

After 2-DE, gels were fixed for 12 h in a mixture of 45% methanol and 5% phosphoric acid, and stained for six hours in a solution of 0.1% Coomassie brilliant blue G-250, 17% ammonium sulfate, 3.6% phosphoric acid, and 34% methanol. The gels were then destained for 2 h by diffusion in a mixture of 15% methanol and 1% acetic acid, followed by washing for 1 h in deionized distilled water.

**Quantitative analysis** The stained gel was scanned using a PowerLook 1100 scanner (UMAX Technology, USA). The scanned images were processed with ImageMaster Analysis software (Amersham Pharmacia Biotech, UK) to yield a list of position.
nates were centrifuged twice (10 min at 12,000 g) using a minihomogenizer (Kontes). Homogenates were incubated with 0.5% Triton X-100, 20 mM beta-Glycerophosphate, 1 mM Na$_3$VO$_4$, 10 mM NaF, 0.5 mM DTT, and protein inhibitor Complete Mini (Roche, Germany) using the manufacturer's instructions (Pierce). An equal volume of 2× SDS-sample buffer was added to the supernatant and the mixture boiled at 4°C for 5 min. Equal amounts of total protein (~50 μg total) were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membrane and the immunoblots exposed by chemiluminescence (ECL, Amersham) as previously described (Lee et al., 2004a). 8% to 10% SDS-polyacrylamide gels were used to visualize HSP90, whereas 10% to 12% gels were used to detect enolase and triose phosphate isomerase (TPI). The antibodies used in this study were purchased from Santa Cruz Biotechnol-

### Table 1. Morphological properties of the three groups of mice studied.

<table>
<thead>
<tr>
<th>Variables</th>
<th>W</th>
<th>Per1</th>
<th>Per2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>20.96±0.84</td>
<td>20.07±0.60</td>
<td>21.93±0.38</td>
</tr>
<tr>
<td>Muscle mass (mg)</td>
<td>29.97±0.11</td>
<td>27.01±0.96*</td>
<td>32.82±1.71*</td>
</tr>
<tr>
<td>Muscle mass/body mass (×10$^{-3}$)</td>
<td>1.43±0.03</td>
<td>1.35±0.004</td>
<td>1.50±0.09</td>
</tr>
<tr>
<td>Muscle length (mm)</td>
<td>10.12±0.18</td>
<td>10.54±0.25</td>
<td>10.89±0.25</td>
</tr>
<tr>
<td>Muscle cross-sectional area (mm$^2$)</td>
<td>2.96±0.12</td>
<td>2.56±0.04</td>
<td>3.04±0.19</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 9.

* Significant differences between groups by one-way ANOVA and Scheffe’s post hoc tests (P < 0.05).

Protein identification From the scanned gel images, we identified 22 protein spots that appeared to differ in spot volume and intensity between the three groups. These spots were excised from the Coomassie-stained gels. Peptide mass fingerprinting was performed at the Yonsei Proteome Research Center using a Perkin-Elmer/PerSeptive Biosystems Voyager-DE-PRO MALDI-TOF mass spectrometer, operating in a delayed reflector mode at an accelerating voltage of 20 kV.

Immunoblotting From each genotype, the AT muscles were collected at the indicated times during LD cycles and homogenized at 4°C in three volumes of tissue lysis buffer [20 mM Hepes (pH 7.4), 75 mM NaCl, 2.5 mM MgCl$_2$, 0.1 mM EDTA, 0.05% (v/v) Triton X-100, 20 mM beta-Glycerophosphate, 1 mM Na$_3$VO$_4$, 10 mM NaF, 0.5 mM DTT, and protein inhibitor Complete Mini (Roche, Germany)] using a minihomogenizer (Kontes). Homogenates were centrifuged twice (10 min at 12,000 × g) and supernatants saved to new tubes. Protein concentration was determined using a Commassie protein assay kit according to the manufacturer’s instructions (Pierce). An equal volume of 2× SDS-sample buffer was added to the supernatant and the mixture boiled at 95°C for 5 min. Equal amounts of total protein (~50 μg total) were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membrane and the immunoblots exposed by chemiluminescence (ECL, Amersham) as previously described (Lee et al., 2004a). 8% to 10% SDS-polyacrylamide gels were used to visualize HSP90, whereas 10% to 12% gels were used to detect enolase and triose phosphate isomerase (TPI). The antibodies used in this study were purchased from Santa Cruz Biotechnology (catalog no. sc-7455 for enolase, sc-22031 for TPI, sc-2922 for HRP-conjugated rabbit anti-goat IgG; Santa Cruz, USA), Stressgen (product # SPA-830 for HSP-90; Canada) or Lab Frontier (catalog no. LF-PA0146 for alpha-tubulin; Korea) and used at final concentrations of 1:3,000 to 1:10,000. Scanned images of autoradiographs were prepared using Adobe Photoshop 7.0 software.

Data analysis All data are presented as mean ± 1SE, unless otherwise noted. Differences in the means of morphological and functional variables between groups were examined with one-way analysis of variance (ANOVA) and Scheffe’s post hoc multiple comparison tests. Statistical procedures were performed using SPSS/PC+ (SPSS Inc.).

### Results

#### Muscle Physiology

**Morphology** The three morphological variables (body mass, muscle length and cross-sectional area) were not significantly different between the W, Per1 and Per2 groups (one-way ANOVA, P > 0.05; Table 1), and specific mass (relative to body mass) was the same. However muscle mass was greater in Per2 than in Per1 (P < 0.05).

**Locomotion** The running distances of the three groups were compared to see whether diurnal rhythm affects locomotory capacity (Figs. 1A and 1B). Within each group, running distance did not differ between the light and dark phases (paired t-test, P > 0.05). In both phases, running distance was 0.82- to 0.84-fold less in the Per2 group than in the W group (one-way ANOVA and Scheffe’s tests, F$^{2}_{2,31}$ = 3.67, P = 0.04 for light phase; F$^{2}_{2,31}$ = 4.36, P = 0.02 for dark phase). Running distance did not differ significantly between the W and Per1 groups and between the Per1 and Per2 groups (P > 0.05).

**Muscle contractile function** A typical tetanic force curve and force-velocity and power-velocity curves are shown in Figs. 2A and 2B for the AT muscle of wild-type mice. The contractile properties of the muscle from the three groups are summarized in Table 2. We found that the five contractile variables, maximum tetanic tension, tetanic rise time, half-relaxation time, rate of tension production, and shortening velocity, did not differ between the three groups (P > 0.05).
Table 2. Contractile properties of the anterior tibialis muscle of the three groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>W</th>
<th>Per1</th>
<th>Per2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum tetanic tension (mN mm⁻²)</td>
<td>185.02 ± 11.77</td>
<td>218.30 ± 16.39</td>
<td>206.93 ± 25.56</td>
</tr>
<tr>
<td>Tetanic rise time (msec)</td>
<td>73.33 ± 1.90</td>
<td>76.13 ± 3.69</td>
<td>80.56 ± 4.57</td>
</tr>
<tr>
<td>Half-relaxation time (msec)</td>
<td>12.76 ± 0.29</td>
<td>12.64 ± 0.62</td>
<td>12.66 ± 0.38</td>
</tr>
<tr>
<td>Rate of tetanic production (mN mm⁻² msec⁻¹)</td>
<td>1.50 ± 0.10</td>
<td>1.77 ± 0.15</td>
<td>1.59 ± 0.21</td>
</tr>
<tr>
<td>Shortening velocity (ML sec⁻¹)</td>
<td>2.15 ± 0.06</td>
<td>2.23 ± 0.05</td>
<td>2.26 ± 0.16</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 9.

Proteomic analysis
We initially identified 22 candidate proteins from the 2D gels and studied in detail four proteins that showed significant differences in spot volume and intensity. Protein identities and the corresponding gel spots are given in Table 3 and Fig. 3, respectively. Figure 4 presents comparisons of the expression levels of the four proteins in the three groups. The results are grouped as follows.

Contractile proteins
We identified on the 2D gels the seven proteins that are the major constituents of muscle contraction: actin, tropomyosin α- and β-chains, myosin essential light chain (skeletal isoform), myosin regulatory light chain (skeletal isoform), troponin T (slow skeletal isoform), and troponin I (fast skeletal isoform). None of these proteins, however, showed significant differences in expression level between the three groups (P > 0.05).

Metabolic proteins
Among seven metabolic enzymes (9 spots) identified, two glycolytic enzymes (TPI and beta enolase) showed significant inter-group differences in expression levels (Figs. 4A and 4B). The level of TPI, an enzyme catalyzing isomerization between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, was significantly greater in Per2 than in W and Per1 (P < 0.05), but was not different between W and Per1. The level of beta enolase, a skeletal muscle enzyme catalyzing the transformation of 2-phosphoglycerate to phosphoenolpyruvate, was also greater in Per2 than in W (Spot No. 27; P < 0.05). Another spot of beta enolase (No. 24), presumably an electrophoretic variant, showed a similar pattern although there were no significant inter-group differences.

Regulatory and plasma proteins
The level of carbonic anhydrase III, which catalyzes the reversible hydration of CO₂ and HCO₃⁻, was significantly lower in Per2 than in W (P < 0.05), while there were no significant differences in the other intergroup comparisons (Fig. 4C). The levels of both creatine kinase M chain and serum albumin precursor did not differ.

Stress proteins
The level of 90-kDa heat shock protein (HSP90), which stabilizes proteins before folding or activation (Srikakulam and Winkelmann, 2004), was 0.6-fold lower in Per1 and 1.7-fold higher in Per2 than in W (P < 0.05). The level of 78-kDa glucose regulated protein (GRP78), another chaperone protein (Lee et al., 2002), did not differ among the three groups.
**Table 3.** Summary of 22 identifiable proteins. The four proteins in boldfaced italic are those that showed significant differences in expression between groups.

<table>
<thead>
<tr>
<th>Protein function</th>
<th>Spot No.</th>
<th>Protein identification</th>
<th>Theoretical pl/MW using ExPASy*</th>
<th>Estimated pl/MW</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contractile proteins</strong></td>
<td>4</td>
<td>Actin</td>
<td>5.20/44.017</td>
<td>5.23</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Tropomyosin β-chain, skeletal muscle</td>
<td>4.52/34.900</td>
<td>4.72</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Tropomyosin α-chain, skeletal muscle</td>
<td>4.74/33.929</td>
<td>4.71</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Myosin essential light chain, skeletal isoform</td>
<td>5.04/23.161</td>
<td>5.08</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Myosin regulatory light chain, skeletal isoform</td>
<td>4.68/15.900</td>
<td>4.70</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Troponin T, slow skeletal muscle</td>
<td>5.85/31.196</td>
<td>5.46</td>
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<tr>
<td></td>
<td>43</td>
<td>Troponin I, fast skeletal muscle</td>
<td>8.34/24.560</td>
<td>8.86</td>
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<td><strong>Metabolic proteins</strong></td>
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<td>ATP synthase β-chain mitochondrial precursor</td>
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<td>5.04</td>
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<tr>
<td></td>
<td>12</td>
<td>Pyruvate dehydrogenase</td>
<td>5.9/38.848</td>
<td>5.41</td>
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<td>Lactate dehydrogenase B-chain</td>
<td>5.7/36.573</td>
<td>5.35</td>
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<td></td>
<td>24</td>
<td>Beta enolase</td>
<td>6.68/45.750</td>
<td>6.63</td>
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<td>27</td>
<td>Beta enolase</td>
<td>6.52/46.003</td>
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<tr>
<td></td>
<td>32</td>
<td>Phosphoglycerate kinase</td>
<td>7.57/42.000</td>
<td>7.64</td>
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<tr>
<td></td>
<td>33</td>
<td>Fructose bisphosphate aldolase A</td>
<td>8.03/39.728</td>
<td>8.00</td>
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<tr>
<td></td>
<td>34</td>
<td>Fructose bisphosphate aldolase A</td>
<td>7.69/39.728</td>
<td>7.67</td>
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<td></td>
<td>36</td>
<td>Triose phosphate isomerase</td>
<td>6.87/25.937</td>
<td>6.70</td>
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<td><strong>Regulatory and plasma proteins</strong></td>
<td>19</td>
<td>Serum albumin precursor</td>
<td>5.41/66.352</td>
<td>5.44</td>
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<tr>
<td></td>
<td>20</td>
<td>Serum albumin precursor</td>
<td>5.49/66.176</td>
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<tr>
<td></td>
<td>28</td>
<td>Creatine kinase M chain</td>
<td>6.75/40.352</td>
<td>6.60</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>Carbonic anhydrase III</td>
<td>6.9/27.220</td>
<td>6.90</td>
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<tr>
<td><strong>Stress proteins</strong></td>
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<td>Heat shock protein 90-kDa</td>
<td>5.04/85.913</td>
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<tr>
<td></td>
<td>2</td>
<td>78-kDa glucose regulated protein</td>
<td>5.01/72.255</td>
<td>5.04</td>
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* The abbreviations pl, MW, and ExPASy represent isoelectric point, molecular weight, and the Expert Protein Analysis System proteomics server (http://us.expasy.org), respectively.

**Immunoblotting** To determine the temporal expression profiles of HSP90 and two glycolytic enzymes (TPI and enolase), we performed immunoblotting using specific antibodies against these proteins. Both of the metabolic enzymes from the AT muscles were expressed constitutively in all three genetic backgrounds (data not shown). The expression profiles of the two glycolytic enzymes in W, Per1, and Per2 were similar overall throughout the daily cycle, with a higher amount in Per2 than in W and Per1 at ZT6, in accord with the 2-DE analysis. When the gel runs of the protein samples collected at ZT6 were compared side-by-side it was obvious that the levels of HSP90 and TPI were higher in the mPer2 KO mice than in wild-type mice ($P < 0.05$) (Figs. 5A and 5B).

**Discussion**

It is thought that the central SCN clock synchronizes peripheral tissues by means of hormones and the autonomic nervous system, but very little is known about the role of peripheral clocks in this process. The need for many pe-
Peripheral clocks may ensure local tissue-specific control of circadian functions. To decipher the individual roles of per homologs in the peripheral tissues of mammals we explored the locomotor and skeletal muscle responses of mice that had lost one or other of two clock genes, mPer1 or mPer2.

Our data demonstrate that the locomotor capacities of mPer2 KO mice were more affected than those of mPer1 KO mice; the running distance of the mPer2 mice was about 20% lower than that of their wildtype littermates in both light and dark phases (Fig. 1). The loss of mPer2 was not accompanied by significant alterations in the contractility and proteomic responses of the AT muscles except for the expression of a small number of proteins (Tables 2 and 3). There were essentially no significant differences between the tissue and subcellular responses of the mPer1 mutants and those of wild type mice. These results point to distinct roles of the two mPer genes in peripheral tissues and also indicate that the peripheral clock functions independently of the SCN clock. It is well established that the peripheral clock in the liver can be easily uncoupled from the SCN clock by restricted daytime feeding (Damiola et al., 2000). Recently a master-slave clock hierarchy was proposed and it was suggested that the SCN has the role of an orchestrator of several peripheral clocks in different phases with respect to the organismic clock (Yoo et al., 2004). It is noteworthy that our data indicate that the individual clock genes have different effects on noncycling gene expression in peripheral cells (Figs. 3 and 4). The differences in the expression of four proteins in the mPer2 mutant mice reflect an adjustment of the myofibers to the altered locomotor activity, but muscle contractile function did not differ from the wildtype in either of the clock gene mutants. This strongly suggests that the myofibrillar properties of the AT muscle (e.g., numbers of cross-bridges, size of sarcoplasmic reticulum) are unaffected by changes in the circadian clock genes in the brain. Our proteomic analysis is consistent with the contractile protein outcomes in that expression of most of the contractile proteins was the same in all three groups.

The reduced running endurance of the mPer2 mutant mice may be due to a greater dependence on energy production via glycolysis, as these mice expressed higher levels of glycolytic enzymes (TPI, enolase) (Figs. 4 and 5). Feeding activates glycolysis, which generates pyruvates as end product, and the reduction of pyruvate to lactate by the tissue-specific action of skeletal muscles results in a net change of cellular redox state. This redox change may entrain the peripheral clock. In this regard, it is worth mentioning that the transcription factor NPAS2, an mCLOCK homolog, interacts with BMAL1 to bind DNA and induces circadian gene expression outside the...
SCN. The DNA binding activity of NPAS2:BMAL1 dimer is dependent on cellular redox state (Kaasik and Lee, 2004; Rutter et al., 2001). Recently it has been shown that the mCLOCK-BMAL1 dimer participates in the regulation of glucose homeostasis (Rudic et al., 2004). Moreover, Dallmann et al. (2006) report that their Per1^B/E mutant mice are lighter than WT and Per2^B/E mice, and that the metabolic rate of the Per1^B/E mice is higher, with impaired daily glucocorticoid rhythms. Therefore it is likely that altered control in peripheral clocks, as in the case of loss of mPer2, alters mCLOCK:BMAL1-mediated gene expression, and this may be responsible for the greater dependence of these mice on anaerobic glucose metabolism.

The running endurance of mPer2 mice was further limited by the lower level of carbonic anhydrase III, because cellular acidosis should be enhanced due to the decreased CO2 and H+ buffering capacity during the 2–3 min of treadmill running. Indeed, it is known that carbonic anhydrase, blood glucose, pH, and pCO2 display significant diurnal changes in mammals (Challet et al., 2003) and that disruption of D site-binding protein (DBP) results in loss of circadian expression of other genes in the liver, many of which take part in energy metabolism. There is also evidence that clock genes regulate the expression of non-cycling genes (Damiola et al., 2000; Sehgal, 2004). Therefore, if DBP in liver, mPer2 mice may serve to perform tissue-specific functions in non-neural tissues including skeletal muscles by regulating the expression of genes and the activity of their protein products. Furthermore, the expression of HSP90 in AT muscle at ZT6 was significantly elevated in the mPer2 mice (Figs. 4 and 5). Interestingly, the expression of HSP90 in both mPer mice was higher than in wild type mice at ZT18 when measured by immunoblotting (data not shown). This could reflect a differential level of time-of-day-specific responses to stress in the two groups of mPer mice. We have obtained evidence for daily variation in the expression of HSP isoforms in the SCN of mPer mutant mice (Kim and Bae, 2006). Many researchers, including us, have noted that the expression of this protein is sensitive to the amount of unloading-reloading stress impinging on the myofibers (Kregel, 2002; Isfort et al., 2002; Seo et al., 2006). Hence the mPer2 mutant mice with their higher level of expression of this protein may be under greater stress on account of their greater activity and the resulting acidosis.

We also analyzed the detailed temporal expression profiles of two glycolytic enzymes, TPI and enolase, by immunoblotting. Neither enzyme was rhythmic but instead constitutively expressed in all genetic backgrounds, ruling out the possibility that we are simply detecting a phase difference between the expression of various proteins in the different genotypes (data not shown). Although the overall level of enolase in the mPer2 mutant mice was higher than in the mPer1 mutant and wild type mice, this was not statistically significant (Fig. 5). The discrepancy between the 2-DE and immunoblotting data may reflect the sensitivity of experimental methods (Hirano et al., 2006). Of the three isoenzymes of enolase, alpha, beta, and gamma, ββ homodimers and αβ heterodimers are the predominant forms in muscle tissues (Merkulova et al., 2000). For immunoblotting, we used polyclonal antibody raised against a peptide near the C-terminus of the alpha enolase. The antibody detects both isoforms of enolase that are abundantly expressed in muscle, and possibly a variant of beta enolase (Spot No. 24 in 2-DE gel), all of which makes it difficult to identify subtle differences between the genotypes. An increase in the level of the major muscle enolase subunit β is correlated with the regeneration of myofibers after injury (Merkulova et al., 2000). From the 2-DE analysis, the amount of another glycolytic enzyme, TPI, was significantly higher in the mPer2 mutant mice than in wild type mice. All together the differential modulation of the levels of these proteins may reflect the adjustment of peripheral effector muscles to altered physiological needs, particularly in the mPer2 mutant mice.

There is little other evidence pointing to distinct functions of the two mPER proteins in the circadian clock. Normally, the two mPERs are expressed in peripheral tissues as well as in the SCN itself in response to a variety of inducing signals (Balsalobre et al., 2000). Both mPer1 and mPer2 in the SCN respond to light pulses in a time-of-day specific manner (Yan and Silver, 2004). Others have shown that mPer2 mutant mice are prone to develop certain types of cancer (Fu et al., 2002). Our current results indicate that there are clear differences in the locomotor capacity and levels of protein expression in the AT muscles between mPer1 and mPer2 mutant mice, strengthening the view that the two mammalian per homologs have distinct functions in peripheral tissue physiology.

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References


