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A Circadian Clock Gene, Rev-erbα, Modulates the Inflammatory Function of Macrophages through the Negative Regulation of Ccl2 Expression

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Disruption of the circadian rhythm is a contributory factor to clinical and pathophysiological conditions, including cancer, the metabolic syndrome, and inflammation. Chronic and systemic inflammation are a potential trigger of type 2 diabetes and cardiovascular disease and are caused by the infiltration of large numbers of inflammatory macrophages into tissue. Although recent studies identified the circadian clock gene Rev-erbα, a member of the orphan nuclear receptors, as a key mediator between clockwork and inflammation, the molecular mechanism remains unknown. In this study, we demonstrate that Rev-erbα modulates the inflammatory function of macrophages through the direct regulation of Ccl2 expression. Clinical conditions associated with chronic and systemic inflammation, such as aging or obesity, dampened Rev-erbα gene expression in peritoneal macrophages from C57BL/6J mice. Rev-erbα agonists or overexpression of Rev-erbα in the murine macrophage cell line RAW264 suppressed the induction of Ccl2 following an LPS endotoxin challenge. We discovered that Rev-erbα represses Ccl2 expression directly through a Rev-erbα-binding motif in the Ccl2 promoter region. Rev-erbα also suppressed CCL2-activated signals, ERK and p38, which was recovered by the addition of exogenous CCL2. Further, Rev-erbα impaired cell adhesion and migration, which are inflammatory responses activated through the ERK- and p38-signaling pathways, respectively. Peritoneal macrophages from mice lacking Rev-erbα display increases in Ccl2 expression. These data suggest that Rev-erbα regulates the inflammatory infiltration of macrophages through the suppression of Ccl2 expression. Therefore, Rev-erbα may be a key link between aging- or obesity-associated impairment of clockwork and inflammation. The Journal of Immunology, 2014, 192: 407–417.

The impact of circadian rhythm on human health has attracted increasing attention in recent years, and circadian dysfunction is now regarded as a contributory factor to the incidence and severity of a wide range of pathophysiological and clinical conditions. People exposed to constant circadian disruption, as the result of long-term shift work, frequent air travel, or chronic restriction of sleep, show an increased incidence of chronic diseases, such as diabetes, obesity, depression, sleep disorders, and cancer (1–4). Chronic and systemic inflammation is one of the important pathogenic features of these diseases, which gives rise to the hypothesis that circadian clock molecules play a crucial role in the regulation of inflammation.

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Chronic and systemic inflammation is closely linked to aging and obesity and is a potent contributor to most age- or obesity-related diseases, such as metabolic disorders, cardiovascular diseases, neurodegenerative diseases, musculoskeletal disorders, and cancers (5–8). Chronically inflamed tissues are characterized by the presence of infiltrating inflammatory cells, such as macrophages (9). Increased caloric intake and physical inactivity causes white adipose tissue (WAT) hypertrophy, which initiates inflammation that is due to an influx of macrophages that secrete proinflammatory cytokines (6). Several lines of evidence indicate that CCL2 (also known as MCP-1), a member of the family of chemoattractant cytokines called chemokines, and its receptor CCR2 play crucial roles in the initiation of obesity-induced inflammation and insulin resistance by the recruitment of macrophages into WAT (10).

Recent evidence demonstrated that macrophages exhibiting a rhythmic expression of several clock genes, including Rev-erbα, are capable of cell-autonomous gene oscillation in culture and display a robust circadian gating in the responses of the cells to LPS endotoxin challenge (11–14). Rev-erbα, an orphan nuclear receptor encoded by NrlId, is part of the clock machinery and plays an important role in maintaining proper circadian timing (15). Rev-erbα binds to a monomeric response element, ROR response element (RORE), consisting of a 6-bp core motif, (A/G) GGTCGA, flanked by an AT-rich 5′ sequence or a dimeric site (Rev-erb direct repeat 2) composed of a direct repeat of the core motif separated by two nucleotides (16, 17). Rev-erbα represses transcription of the target genes through the binding to the sites, including a clock gene Bmal1, which partly contributes to the formation of feedback loops that fine-tune the circadian clock and maintain its oscillations (18). Moreover, Gibbs et al. (11) dem-
onstrated that Rev-erbα has control over several genes involved in human innate immunity, including Il16, Il19, Cxcl6, Cxcl11, and Ccl2, suggesting that Rev-erbα acts as a critical intermediary between the core clockwork and inflammatory pathways. However, the molecular mechanism that couples inflammatory function to Rev-erbα remains unknown.

In the current study, using pharmacological and genetic targeting in murine macrophages, we discovered that Rev-erbα is a direct repressor of the Ccl2 gene. We showed that Rev-erbα suppresses CCL2-activated intracellular signals and inflammatory functions of macrophages. Furthermore, mice lacking Rev-erbα display increases in Ccl2 gene expression in macrophages. Thus, regulation of Ccl2 by Rev-erbα represents a novel link between the impairment of clockwork and the resultant inflammation.

Materials and Methods

Animals

C57BL/6j mice, B6.Cg-Lep<+/−> (ob/ob) mice, and B6.Cg-Scr1d1< tml/Ven>+/− (Rev-erbα−/−) mice were obtained from Sankyo Labo Service, Tokyo, Japan. Peritoneal macrophages were collected from the peritoneal cavity of female mice at 8 to 12 weeks of age and cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-denatured FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) and cultured for 3 h to allow macrophages to adhere to the plate. After nonadherent cells were removed, adherent cells were used as peritoneal macrophages. To investigate the effects of aging or obesity on Rev-erbα gene expression in peritoneal macrophages, we used 12- to 15-month-old male C57BL/6j mice to investigate the effects of aging on Rev-erbα gene expression in peritoneal macrophages. To evaluate whether obesity influences Rev-erbα gene expression in peritoneal macrophages, male 10-week-old C57BL/6j mice were fed a high-fat diet (HFD) for 6 wk. The diet (HFD60; Oriental Yeast, Tokyo, Japan) contained 55% of its calories by weight and provided 62.2% of its calories from fat (19). Male 2- to 3-month-old genetically obese (ob/ob) mice were also used for the experiment. As control mice for each experiment, male 2- to 3-month-old C57BL/6j mice were fed a standard diet (CE-2; Japan Clea, Tokyo, Japan) that contained 4% of its calories by weight and provided 10.6% of its calories from fat. In some experiments, 2- to 3-month-old Rev-erbα−/− mice were obtained according to the supplier’s genotyping information, and their wild-type (+/+) counterparts were used as control.

Preparation and culture of peritoneal macrophages

Peritoneal macrophages were collected and cultured as described previously (19–21). Briefly, the mice were injected (i.p.) with 4 ml thioglycollate broth, respectively (12). In separate experiments, the cells were treated or not with 1 μg/ml LPS for 24 h. Each of the Rev-erbα agonists was dissolved with DMSO, and the control cells were treated using the same volume of DMSO. In some experiments, the peritoneal exudate cells from Rev-erbα−/− mice and their wild-type counterparts were harvested at ZT10 when Rev-erbα expression reached its peak (12). Adherent cells were used as peritoneal macrophages and were treated or not with 1 μg/ml LPS for 24 h.

Cell line culture

The murine macrophage cell line RAW264 (RCB0535) was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured as described previously (19, 20, 22). To study the effects of Rev-erbα agonists on Ccl2 gene expression, the cells were treated or not with 20 μM GSK4112, 50 μM Hemin, or 1 μg/ml LPS for 16 h. For further study of the effects of Rev-erbα agonists on CCL2-activated signals, the cells were treated with 1 μg/ml LPS for 6 h following pretreatment with 20 μM GSK4112 or 50 μM Hemin for 2 or 3 h. In separate experiments, cells were treated with 250 ng/ml mouse rCCL2 (R&D Systems, Minneapolis, MN) for 1 min or 10 μM CCR2 antagonist RS504393 (Tocris Bioscience, Minneapolis, MN) for 24 h.

Real-time quantitative PCR

Total cellular RNA was prepared from peritoneal macrophages using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and from RAW264 cells using RNAiso reagent (Takara Bio, Siga, Japan). Extracted RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random primers. The reaction mixture was amplified in Power SYBR Green Master Mix using a 7500 Real-Time PCR System (both from Applied Biosystems) with 200 nM oligonucleotide primers (forward and reverse). The oligonucleotide sequences used for real-time quantitative PCR (qPCR) were as follows: Rev-erbα, 5′-ACG ACC TGC CAC TCC AAT AA-3′ (forward), 5′-CCA TTG CAG CTG TCA CTG TAG A-3′ (reverse); Rora, 5′-GGG AAC AGC TCC AGC AGA TA-3′ (forward), 5′-ACA GCT GCC ACA TCA CCT CT-3′ (reverse); Ccl2, 5′-GCC TGC TGT CTA CAG TGT C-3′ (forward), 5′-CAG GTG AGT AGG GCG GTA TTA-3′ (reverse); Integrin β1 (Igbi), 5′-CAC AAC AGC TGC TGC TAA AAT TGG-3′ (forward), 5′-TCC AIA AAG TGG TAG AGA TCA ATG GGC-3′ (reverse); Cxcl6, 5′-GGT CAG CTC ATC AAG AAT GC-3′ (forward), 5′-GCT GTG GTG CAT GAA GAC TC-3′ (reverse); and βactin (Actb, internal control), 5′-AAG GCC AAC CGT GAA AAT AT-3′ (forward), and 5′-GTT GTA CGA CCA GCA GGA TAC-3′ (reverse). The expression of the target gene was normalized to the housekeeping gene Actb.

Western blot analysis

Nucleic protein was extracted as described previously (20, 23). Cytoplasmic protein was extracted with radio-immunoprecipitation assay buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Extracted proteins were separated by SDS-PAGE (8–10%) and then transferred to a polyvinylidene difluoride membrane (Millipore, Milford, MA). Membranes were blocked with 5% nonfat dried milk in TBST and then immunoblotted with rabbit anti-rep-erbα antibody (Abcam, Cambridge, MA) diluted 1:1000 and HRP-conjugated donkey anti-rabbit IgG (Bio-Rad, Hercules, CA) diluted 1:10000. Immunoblotting was visualized with an ECL reagent (Bio-Rad, Hercules, CA). The Western blot analysis was repeated three times, and the results were averaged.

Full-length murine Rev-erbα and Rora cDNAs were obtained by PCR using the primers 5′-CTG GAT GCC TTC TGC AGA ATC GC-3′ (forward) and 5′-GGT GAG GAC GAG AAC AGT GC-3′ (reverse) for Rev-erbα and 5′-GAT CCC AGC GAT GAA AGC ACC ATC TC-3′ (forward) and 5′-CTC GGC GGC GAC ATT TAC TC-3′ (reverse) for Rora. The amplified Rev-erbα and Rora fragments were subcloned into pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA), digested at BamHI/Xhol and KpnI/Apal sites, respectively, and then cloned into pcDNA4/TO/myc-His B vector or pcDNA4/TO/myc-His A vector at the corresponding sites (Invitrogen). The plasmid DNA used for transfection was prepared with an EndoFree Plasmid Kit (QIAGEN). RAW264 cells were transfected with the pcDNA4 vector, pcDNA4-Rev-erbα, or pcDNA4-Rora using a Lipofectamine reagent (Invitrogen). Selection was initiated in medium containing 500 μg/ml zeocin (Invitrogen). After selection, a stable Rev-erbα transfectant (RAWvecB), as well as a stable Rora transfectant (RAWror) and the control cell line (RAWvecA) were established.

ELISA

Cells were cultured at 37°C for 24 h in the absence or presence of 1 μg/ml LPS, and the supernatant was collected. CCL2 concentration was determined using an ELISA kit (eBioscience, San Diego, CA), according to the manufacturer’s instructions.
Luciferase reporter assay

The murine Ccl2 promoter (distal fragment, −6942 to +85; proximal fragment, −642 to +85) was amplified from mouse genomic DNA (Promega, Madison, WI) using a LA Taq polymerase (Takara Bio) and subcloned into pCR-XL-TOPO vector (Invitrogen). The subcloned distal fragment was digested at MluI and SmaI sites, the blunt end and cloned into MluI- and SmaI-digested pGL3-enhancer luciferase reporter vector (pGL3 vector; Promega). The subcloned proximal fragment was also digested at KpnI/Xhol sites and cloned into pGL3 vector (Promega) at the corresponding sites, RAW264.7 and RAW36B and RAW264 and RAW264C cells were transiently transfected using a Lipofectamine reagent (Invitrogen) with distal or proximal constructs containing the luciferase reporter gene, and luciferase activity was determined with a Dual Luciferase Assay System Kit (Promega). Activity was normalized relative to an internal cotransfected constitutive control (Renilla luciferase expression vector, pRL-TK vector; Promega), as described (20, 22).

Mutagenesis

The distal Ccl2 promoter mutant construct was made using a KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan), which is simple to mutate into a long plasmid sequence. The proximal Ccl2 promoter mutant construct was made using a QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), as described (22). The proximal RORE (−542 to −531) was mutated from ATCATACTCA to ATACATACTCA using the mutant primers 5′-AGA TCT CAA AGC TTC TTA GTG CTG CAA AAT ATC-3′ (forward) and 5′-TGA TAA GAT TAA CTT GTA GTG CAA AGC AAG-3′ (reverse) for the distal construct mutagenesis and 5′-TGG TTA CGG AAG TGC AGC AAG-3′ (forward) and 5′-CTA AGA AGC TTT GAG ATC TTG ATG TGA ACT G-3′ (reverse) for the proximal construct mutagenesis (underlining indicates mutant sequences). The mutant plasmid DNAs were confirmed by sequencing.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was carried out using an EpiQuik Chromatin Immunoprecipitation Kit (Epigentek, Brooklyn, NY). Normal mouse IgG (0.5 μg) and anti-Rev-erbα (AB10130; Millipore) or anti-ROREs (638302; BioLegend) Abs were used for each immunoprecipitation. Purified DNA was subjected to 40 cycles of PCR amplification. The oligonucleotide sequences of the Ccl2 gene were designed as follows: distal ROREs, 5′-GAG GTG CAC ATC GTC GTG CTG-3′ (forward), 5′-TGA GTG TAG TCT GGG CAA TG-3′ (reverse); and proximal RORE, 5′-CAG ATT ACC TAG GTG AG-3′ (forward), and 5′-CTT GGT TAT CAG GCC TTG TTG-3′ (reverse).

Adhesion assay

Adherent cells were determined using methods established by Ashida et al. (24) with a slight modification. Briefly, polystyrene 96-well flat-bottom plates were coated with 25 μl 5 μg/ml soluble mouse recombinant E-selectin, 31.25 μg/ml soluble mouse rICAM-1, or 25 μg/ml soluble mouse rVCAM-1 (all from R&D Systems) at room temperature for 1 h. After incubation, wells were blocked by incubation with 225 μl 10 mg/ml heat-denatured BSA at room temperature for 30 min. One hundred microliters of each cell, suspended at a concentration of 2 × 10⁶/ml in 0.1% BSA–RPMI 1640, was incubated at 37°C for 20 min. After incubation, nonadherent cells were removed, and attached cells were fixed with methanol. Attached cells were washed and then stained with a Giemsa stain solution (Wako Pure Chemical, Osaka, Japan) at room temperature for 30 min. Excess dye was removed by washing, and the bound dye was solubilized with 100 μl 10% acetic acid. The absorbance of each well at 590 nm was measured. Each sample was assayed in triplicate, and each assay was performed four times.

Flow cytometry

Flow cytometric analysis was carried out, as described previously (21), using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Prior to the immunofluorescence test, cells (1 × 10⁶) were incubated with rat anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA) in PBS at 4°C for 10 min to avoid nonspecific binding to FcR. Furthermore, the cells were treated with PE-conjugated anti-integrin β1 mAb or FITC-conjugated anti-integrin β2 polyclonal Ab (R&D Systems).

Chemotaxis assay

The migration of each cell was determined using a QCM Chemotaxis Assay (Millipore), according to the manufacturer’s instructions. Briefly, 250 μl cells, resuspended at a concentration of 1 × 10⁶/ml in 0.5% BSA–RPMI 1640, was added to the upper chamber of a 24 Transwell apparatus (5 μm pore size). Five hundred microliters of cell-free 0.5% BSA–RPMI 1640 was added to the lower chamber. Then the plate was incubated at 37°C for 24 h in an incubator with air containing 5% CO₂. After incubation, the upper chamber was dipped into 400 μl Cell Stain Buffer at room temperature for 20 min. Excess dye was washed by dipping three times into distilled water, and the nonmigratory cells were completely removed using a cotton swab. After air drying, the upper chamber was dipped into 200 μl Extraction Buffer, and the plate was shaken, to extract the stain from the membrane, at room temperature for 15 min. One hundred microliters of reactions was transferred to a 96-well plate, and the OD was measured at 560 nm. Each sample was assayed in duplicate, and each assay was performed three times.

Statistical analysis

The results are expressed as the mean ± SE. When two means were compared, a Student’s t test for unpaired samples was used. For more than two groups, the statistical significance of the data was assessed by one-way ANOVA. When significant differences were found, individual comparisons were made between groups using the t-statistic and adjusting the critical value according to the Tukey–Kramer method. Differences were considered significant at p < 0.05.

Results

Aging or obesity impairs Rev-erbα gene expression in murine peritoneal macrophages

Rev-erbα mRNA expression in peritoneal macrophages from control C57BL/6J mice at 10 h after the onset of light (ZT10) was significantly higher than that at ZT22 (Fig. 1). A similar trend was observed by Hayashi et al. (12). To clarify whether this temporal difference in Rev-erbα gene expression in peritoneal macrophages is susceptible to chronic and systemic inflammation, we used aged mice, HFD-induced obese mice, and genetic obese ob/ob mice as the models for chronic and systemic inflammation. We observed that Rev-erbα gene expression was dampened at ZT10 in the peritoneal macrophages of aged, diet-induced obese, and ob/ob mice compared with control mice (Fig. 1). These results suggest a link between Rev-erbα gene expression and the inflammatory response of macrophages.

FIGURE 1. Aging or obesity dampen Rev-erbα gene expression in peritoneal macrophages. Peritoneal exudate cells from 2–3-mo-old C57BL/6J mice (control), 12–15-mo-old C57BL/6J mice (aged), 2–3-mo-old C57BL/6J mice fed an HFD for 6 wk (HFD), and 2–3-mo-old genetic obese mice (ob/ob) were harvested at ZT10 (5 PM) or ZT22 (5 AM). After nonadherent cells were removed, adherent cells were used as peritoneal macrophages. The gene expression of Rev-erbα in peritoneal macrophages was analyzed by qPCR. For normalization, Actb mRNA was used. Data are mean ± SE (n = 4–5). *p < 0.01 versus control mice, #p < 0.01 versus ZT10, one-way ANOVA followed by the Tukey–Kramer test.
Rev-erba agonists suppress Ccl2 induction following LPS stimulation

To determine the role of Rev-erba in inflammatory responses, we analyzed the effects of Rev-erba agonists GSK4112 and Hemin on the gene expression of Ccl2 as a crucial inflammatory molecular element in macrophages. Ccl2 mRNA induction after LPS stimulation was dose dependently repressed by the addition of GSK4112 or Hemin (Fig. 2A). Because several studies (24, 25) revealed that CCL2 activates two distinct MAPKs, ERK and p38, we next performed Western blot analysis to investigate whether Rev-erba agonists suppress the phosphorylation of ERK and p38

in murine peritoneal macrophages. The phosphorylation of ERK and p38 following LPS stimulation was repressed by pretreatment with either GSK4112 or Hemin (Fig. 2B). Furthermore, as shown in Fig. 2C, qPCR analysis confirmed that either GSK4112 or Hemin treatment also decreased the induction of Ccl2 mRNA after LPS stimulation in murine macrophage RAW264 cells, as well as in peritoneal macrophages. As Fig. 2D shows, pretreatment with either GSK4112 or Hemin also suppressed the phosphorylation of ERK and p38 in response to LPS challenge in RAW264 cells, as well as in peritoneal macrophages. These data suggest that activation of Rev-erba leads to the suppression of Ccl2 gene induction and CCL2-mediated signals in macrophages.

Rev-erba overexpression represses Ccl2 expression

To investigate the potential role of Rev-erba in Ccl2 expression in macrophages, a stable Rev-erba transfectant (RAWrev) and a vector control (RAWvecB) were established. Western blot and qPCR analyses confirmed the increase in Rev-erba protein expression and Rev-erba mRNA expression, respectively (Fig. 3A). As seen in Fig. 3B, overexpression of Rev-erba repressed the gene

FIGURE 2. Rev-erba agonists repress Ccl2 gene induction and CCL2-activated signals following a LPS challenge in macrophages. (A) Ccl2 mRNA induction following an LPS challenge is dose dependently repressed by Rev-erba agonists in peritoneal macrophages. Peritoneal macrophages were harvested as adherent cells from 2–3-mo-old C57BL/6J mice and were either left untreated or treated with 1 μg/ml LPS, 2 or 20 μM GSK4112, or 5 or 50 μM Hemin for 16 h. The gene expression of Ccl2 was analyzed by qPCR. (B) Phosphorylation of ERK and p38 after LPS challenge is repressed by Rev-erba agonists in peritoneal macrophages. Peritoneal macrophages were preincubated or not with 20 μM GSK4112 or 50 μM Hemin for 2 or 3 h. After the incubation, cells were stimulated with 1 μg/ml LPS for 6 h, and total cell lysates were analyzed by Western blot for p-ERK, ERK, p-p38, and p38. The lower band was identified as p-p38 through its m.w. determined by m.w. marker. (C) Ccl2 mRNA induction following an LPS challenge is repressed by Rev-erba agonists in RAW264 cells. Murine macrophage RAW264 cells were either left untreated or treated with 1 μg/ml LPS, 20 μM GSK4112, or 50 μM Hemin for 16 h. The gene expression of Ccl2 was analyzed by qPCR. (D) Phosphorylation of ERK and p38 after LPS stimulation is repressed by Rev-erba agonists in RAW264 cells. RAW264 cells were preincubated or not with 20 μM GSK4112 or 50 μM Hemin for 2 or 3 h. After the incubation, cells were stimulated with 1 μg/ml LPS for 6 h, and total cell lysates were analyzed by Western blot for p-ERK, ERK, p-p38, and p38. For normalization, Actb mRNA was used. Data are mean ± SE (n = 3) and are representative of three separate experiments. *p < 0.05 versus LPS-stimulated cells without GSK4112 and Hemin, one-way ANOVA followed by Tukey–Kramer test.

FIGURE 3. Rev-erba represses Ccl2 expression in RAW264 cells. (A) Transfected Rev-erba expression in RAW264 cells transfected with a Rev-erba construct (RAWrev) or with vector alone (RAWvecB). Nuclei lysates were analyzed by Western blot (left panel) for Rev-erba and actin (loading control). The gene expression of Rev-erba was analyzed by qPCR (right panel). (B) Overexpression of Rev-erba in RAW264 cells represses the gene expression of Ccl2 and the secretion of CCL2. RAW264 cells transfected or not with Rev-erba were left untreated or treated with 1 μg/ml LPS for 24 h, and the gene expression of Ccl2 or the secretion of CCL2 was analyzed by qPCR (left panel) and ELISA (right panel), respectively. For normalization, Actb mRNA was used. Data are mean ± SE (n = 4–5) and are representative of two separate experiments. *p < 0.05 versus vector control, Student t test.
expression of Ccl2 and the secretion of CCL2 in either the absence or presence of LPS.

RORα overexpression enhances Ccl2 expression

Rev-erba is known to engage in cross-talk with RORα, an orphan nuclear receptor encoded by Nrf1f, which has a similar DNA-binding specificity to Rev-erba, acts as a constitutive transcriptional activator, and, thus, competes with the binding of Rev-erba (16, 17, 26–30). In a recent study (31), RORα directly trans-active- tivated Il6 gene expression in nonreactive astrocytes. From these findings, we hypothesized that RORα might positively regulate Ccl2 expression, and we established a stable RORα transfectant (RAWror) and a vector control (RAWvecA). The increase in the protein expression of RORα and the mRNA expression of Rora was confirmed by Western blot and qPCR analyses, respectively (Fig. 4A). Indeed, overexpression of Rora enhanced the gene expression of Ccl2 and the secretion of CCL2 in both the absence and presence of LPS (Fig. 4B), indicating that RORα positively regulates Ccl2 expression in macrophages.

Identification of putative ROREs in murine Ccl2 promoter

Next, we analyzed the murine Ccl2 promoter for the presence of putative ROREs using TFSEARCH. Examination of the murine Ccl2 promoter identified three putative ROREs within 10 kb up-stream from the transcription start site at −542 to −531, −6776 to −6790, and −6842 to −6830 (Fig. 5A).

Rev-erba represses the activity of murine Ccl2 promoter

To determine whether the Ccl2 promoter is sensitive to Rev-erba regulation, we cloned Ccl2 promoters with different lengths—a distal promoter that included two putative ROREs located in the distal region and one putative RORE located in the proximal region, as well as a proximal promoter that included one putative RORE located in the proximal region—into a luciferase reporter vector. Then these two constructs were transiently transfected into RAWrev and RAWrevB cells. The activities of each longitudinal promoter in RAWrev cells were considerably lower than those in RAWrevB cells in both the absence and presence of LPS (Fig. 5B). We next investigated whether the proximal RORE in the Ccl2 promoter was necessary for Rev-erba–mediated repression. As shown in Fig. 5C, the mutation of the proximal RORE abolished the repression of the promoter activities in RAWrev transfected with the distal construct, as well as the proximal construct. These findings suggest a critical role for the proximal RORE in Rev-erba–mediated repression of Ccl2 expression.

RORα enhances the activity of murine Ccl2 promoter

Because RORα activates target genes via ROREs in their promoters, we reasoned that RORα might be a positive regulator of the Ccl2 promoter. Therefore, we transiently transfected the distal and the proximal Ccl2 promoter constructs into RAWror and RAWvecA cells. The activity of each of the linear promoter in the RAWror cells was considerably higher than that in RAWvecA cells in the absence or presence of LPS (Fig. 5D). We also investigated whether the proximal RORE in the Ccl2 promoter was essential for RORα-mediated enhancement of Ccl2 expression. The mutation of a proximal RORE abrogated the enhancement of the promoter activities in RAWror cells transfected with either a distal or a proximal construct (Fig. 5E), suggesting that the positive regulatory effects of RORα on Ccl2 expression are mainly dependent on the proximal RORE in the Ccl2 promoter.

Rev-erba directly binds proximal RORE in murine Ccl2 promoter

We examined whether Rev-erba directly modulated Ccl2 promoter activity via binding to proximal RORE in its promoter sequence by ChIP analysis in these RAW264 cells. As illustrated in Fig. 5F, proximal RORE in the murine Ccl2 promoter was bound by Rev-erba and RORα. However, Rev-erba and RORα did not bind to two distal ROREs in the murine Ccl2 promoter. These results suggest that Rev-erba transrepresses murine Ccl2 promoter activity via direct binding to the RORE located in the proximal Ccl2 promoter region, whereas RORα transactivates the promoter activity.

Rev-erba suppresses the phosphorylation of ERK

CCL2 is known to activate ERK- and p38-signaling pathways, which regulate two independent signaling cascades, leading to integrin activation and chemotaxis, respectively (24, 25). To investigate whether negative or positive regulation of Ccl2 expression by Rev-erba or RORα influences the MAPK–signaling pathways, we first analyzed the phosphorylation of ERK in RAWrev and RAWror cells. As seen in Fig. 6A, the overexpression of Rev-erba dramatically repressed the phosphorylation of ERK in both the absence and presence of LPS. In contrast, overexpression of Rora considerably enhanced the phosphorylation of ERK in both the absence or absence of LPS (Fig. 6A). Furthermore, the addition of mouse rCCL2 rapidly abolished the decrease in the phosphorylation of ERK in RAWrev cells, whereas treatment with...
FIGURE 5. A proximal RORE in the murine Ccl2 promoter is targeted by Rev-erbα and RORα. (A) Schematic representation of the murine Ccl2 promoter sequence. The 10-kb-long Ccl2 promoter contains two ROREs in its distal region and one RORE in its proximal region. (B) Rev-erbα suppresses Ccl2-luciferase reporter activity in RAW264 cells. Cells were transiently transfected with a luciferase reporter construct containing either a distal or a proximal construct of the Ccl2 promoter. After no treatment or treatment with 1 μg/ml LPS for 24 h, luciferase activities were determined. (C) Mutation of ROREp abrogates repression. The ATGTCA half-site in the proximal RORE was changed to AGATCT by site-directed mutagenesis of nucleotides −531 (A to T), −534 (G to A), and −535 (T to G). (D) RORα enhances Ccl2-luciferase reporter activity in RAW264 cells. (F) (Figure legend continues)
the CCR2 antagonist RS504393 inhibited the increase in the phosphorylation of ERK in RAWror cells (Fig. 6B). These results suggest that Rev-erbα suppresses the phosphorylation of ERK, whereas RORα enhances the phosphorylation of ERK in macrophages, which relies on secreted CCL2 in an autocrine or a paracrine manner.

Rev-erbα impairs adhesion of RAW264 cells to VCAM1

In the development of inflammation, leukocytes roll on endothelial cells, interact with E-selectin, adhere to endothelial cells by firm adhesion to ICAM1 and VCAM1, and then migrate into the subendothelium. The process of leukocyte recruitment is tightly regulated by the expression and activation of specific adhesion molecules on the surface of leukocytes and endothelial cells. CCL2 is also known to play a key role in leukocyte recruitment by promoting both adhesion and migration to inflammatory tissues (32–35). Furthermore, the activation of ERK is reported to be involved in CCL2-dependent promotion of adhesion (24). Because of these findings, we hypothesized that adherent activity would be impaired in RAWrev cells in which Ccl2 induction is repressed, whereas it would be improved in RAWror cells in which Ccl2 induction is enhanced. Indeed, the adhesion of RAWrev cells to VCAM1, but not to either E-selectin or ICAM1, was significantly lower than the adhesion of RAWvecB cells (Fig. 6C). In contrast, the adhesion of RAWror cells to VCAM1, but not to either E-selectin or ICAM1, was significantly higher than was the adhesion of RAWvecA cells (Fig. 6C). These data indicate that Rev-erbα impairs the avidity of integrin molecules on macrophages, whereas RORα improves it.

Rev-erbα decreases integrin β1 expression but not the expression of integrin β2

Integrins consist of several subtypes, and each subtype is specific to its ligand. For instance, integrin β1 adheres to VCAM1, and integrin β2 adheres to ICAM1, suggesting that the impaired adhesion of RAWrev cells to VCAM1 is closely related to the decrease in integrin β1 expression. Therefore, we analyzed integrin β1 expression on the surface of RAWrev and RAWror cells by flow cytometry. As illustrated in Fig. 6D, integrin β1 expression on the surface of RAWrev cells was obviously lower than on RAWvecB cells, without a change in integrin β2. In contrast, increased expression of integrin β1, but not integrin β2, was observed on RAWror cells compared with RAWvecA cells (Fig. 6D).

In addition, qPCR analysis revealed that the gene expression of Itgb1 was significantly downregulated in RAWrev cells with no alteration in Itgb2, whereas it was upregulated in RAWror cells (Fig. 6E). These results suggest that Rev-erbα impairs the cell adherent activity and integrin expression of macrophages through the CCL2-dependent repression of the ERK-signaling pathway, which is completely opposite from the positive action of RORα.

Rev-erbα represses the phosphorylation of p38

To gather additional evidence about whether Rev-erbα and RORα control the inflammatory function of macrophages, we next focused on the role of Rev-erbα and RORα in the p38-signaling pathway. Overexpression of Rev-erbα decreased the phosphorylation of p38, whereas overexpression of RORα increased it (Fig. 7A). Moreover, the decrease in the phosphorylation of p38 in RAWrev cells was rapidly recovered by the addition of exogenous CCL2 (Fig. 7B). Meanwhile, the increase in the phosphorylation of p38 in RAWror cells was abolished by treatment with the CCR2 antagonist RS504393 (Fig. 7B). These results suggest that Rev-erbα represses the phosphorylation of p38, whereas RORα enhances it in macrophages, which depends on secreted CCL2 in an autocrine or a paracrine fashion.

Rev-erbα impairs the migratory activity of RAW264 cells

Several reports (24, 36) showed that p38 and its upstream Rho family of GTPases are responsible for CCL2-mediated chemotaxis. To examine whether the alteration in the phosphorylation of p38 influences the chemotaxis of RAWrev and RAWror cells, we performed a chemotaxis assay based on the Boyden chamber principle. The migratory activity of RAWrev cells was significantly lower than that of RAWvecB cells, whereas that of RAWror cells was significantly higher than that of RAWvecA cells (Fig. 7C), suggesting that Rev-erbα impairs chemotaxis through the inhibition of the CCL2-mediated p38-signaling pathway, whereas RORα activates the chemotaxis of macrophages.

Peritoneal macrophages from Rev-erbα−/− mice display increases in Ccl2 gene expression

To test whether the results observed in the in vitro study are physiologically relevant, we investigated the effects of Rev-erbα deficiency on Ccl2 expression in peritoneal macrophages from Rev-erbα−/− mice, which mimic, at least partially, the impairment of Rev-erbα mRNA expression at ZT10 observed in aged and obese mice. As shown in Fig. 8A, Ccl2 gene expression either in the absence or presence of LPS in peritoneal macrophages from Rev-erbα−/− mice was significantly higher than that in wild-type mice. These results demonstrate that Ccl2 expression is negatively regulated by Rev-erbα in vivo, as well as in vitro. Furthermore, Itgb1 gene expression of peritoneal macrophages cultured with or without LPS was significantly higher in Rev-erbα−/− mice than in wild-type mice, whereas Itgb2 was not significantly different between the two genotypes (Fig. 8B), corresponding with the in vitro results. Therefore, it seems likely that Rev-erbα functions as a transcriptional represor of Ccl2 for physiological control of the inflammatory responses of macrophages.

Discussion

Both epidemiological and clinical data suggest circadian involvement in the predisposition, etiology, and progression of immune-related morbidities, such as cancer and autoimmune diseases (37, 38). Immune diseases, in particular, exhibit strong time-of-day symptoms. For example, rheumatoid arthritis has a strong diurnal variation in disease expression, which is accompanied by fluctuations in circulating IL-6 concentration (39). LPS-induced endotoxin shock displays temporal dependency (40), and circadian disruption mimicking jet lag can greatly magnify LPS response (41). According to recent evidence, components of the circadian clock regulate the expression of innate immune molecules, such as proinflammatory cytokines (42) and pattern recognition receptors (14). Rev-erbα is a key clock gene that controls inflammatory cytokine genes, including Il6, in macrophages, indicating that it...
negatively regulates the inflammatory responses in macrophages (11). In the current study, we found that chronic and systemic inflammatory conditions, aging and/or obesity, dampen Rev-erbα gene expression in murine peritoneal macrophages, suggesting that Rev-erbα plays a potential role in the regulation of the inflammatory functions of macrophages. However, other factors, such as free radicals and metabolic disorders associated with aging, HFD treatment, or genetic obesity, cannot be excluded. Thus, further

FIGURE 6. Rev-erbα and RORα regulate cell adhesion through ERK phosphorylation induced by CCL2 in RAW264 cells. (A) Effects of overexpression of Rev-erbα or Rora on the phosphorylation of ERK in RAW264 cells. Cells were stimulated or not with 1 μg/ml LPS for 6 h, and total cell lysates were analyzed by Western blot for p-ERK and ERK. Data shown are representative of three separate experiments. (B) Effects of overexpression of Rev-erbα or RORα on the phosphorylation of ERK are dependent on CCL2. RAWrev cells were stimulated or not with 250 ng/ml mouse rCCL2 for 1 min (upper panel). RAWror cells were stimulated or not with 10 μM CCR2 antagonist RS504393 for 24 h (lower panel). Total cell lysates were analyzed by Western blot for p-ERK and ERK. Data shown are representative of three separate experiments. (C) Rev-erbα and RORα regulate cell adhesion to VCAM1, but not ICAM1 or E-selectin, in RAW264 cells. Cells were subjected to adhesion assays on heat-denatured BSA (as control), E-selectin, ICAM1, or VCAM1 for 15 min. Data are mean ± SE for four separate experiments. Each experiment was assayed in triplicate cultures. (D) Rev-erbα and RORα change the expression of integrin β1, but not integrin β2, on the surface of RAW264 cells. Expression of integrin β1 (upper panels) and integrin β2 (lower panels) on the cell surface was analyzed by flow cytometry (left panels) and quantified by triplicate measurement (right panels). Red line, RAWrev cells; blue line, RAWror cells; black line, vector control (RAWvecB or RAWvecA cells); dotted line, isotype control. Data shown are representative of three separate experiments. (E) Rev-erbα and RORα change the gene expression of Itgb1, but not Itgb2, in RAW264 cells. The gene expression of Itgb1 and Itgb2 was analyzed by qPCR. For normalization, Actb mRNA was used. Data are mean ± SE (n = 4–7). *p < 0.05 versus vector control, Student t test.
studies on the molecular mechanisms underlying the association between inflammatory function and clockwork are needed.

CCL2 is an important chemokine that binds to the CCR2 on monocytes/macrophages to stimulate their migration and initiate inflammation (32–35). The deletion of macrophage Ccr2 or adipose tissue Ccl2 can lead to a decrease in the numbers of macrophages infiltrating into WAT in obesity, reduce tissue markers of inflammation, and ameliorate insulin resistance (43). A decrease in atherosclerotic lesions was observed in mice deficient for Ccr2 crossed with ApoE-knockout mice (44). This decrease also was observed in Ccl2-deficient mice crossed with LDL receptor-knockout mice (45). Interestingly, serum CCL2 concentration and Ccl2 gene induction in macrophage-rich peritoneal exudate cells show significant circadian-dependent variation in the magnitude of the response to LPS in mice (11). Moreover, the expression of Ccl2 mRNA displays robust oscillation during the day in murine peritoneal macrophages, whereas Ccr2 is expressed constantly (12). Therefore, Ccl2, and not its receptor Ccr2, may be a direct output gene of circadian clocks. However, little is known about the regulation of Ccl2 expression by core circadian clock proteins.

We analyzed the role of Rev-erba in the production of the inflammatory chemokine, Ccl2, in murine macrophages. We con-
firmed that Rev-erbx agonists, GSK4112 and Hemin, inhibit the induction of the Ccl2 gene in murine peritoneal macrophages and in murine macrophage RAW264 cells following LPS stimulation. Our results are consistent with the recently published results of Gibbs et al. (11), who demonstrated, using primary human monocyte–derived macrophages, that GSK4112 abolishes the induction of inflammation-related genes, including Ccl2, following LPS challenge. In the current study, overexpression of Rev-erbx also revealed that Rev-erbx contributes to the negative regulation of Ccl2 expression in macrophages. By contrast, peritoneal macrophages from mice lacking Rev-erbx display increases in Ccl2 gene expression either in the absence or presence of LPS. These results strongly suggest that Rev-erbx functions as a repressor of Ccl2 expression in macrophages.

We next took note of three putative ROREs identified in the Ccl2 promoter within 10 kb upstream from the transcriptional start site and demonstrated that Rev-erbx actually functions as a potent repressor at a proximal RORE in the Ccl2 promoter region (at −542 to −531 from the transcriptional start site). In contrast, Ccl2 promoter activity was enhanced by RORα, a clock component and orphan nuclear receptor that also recognized the RORE. Cross-talk between the two nuclear receptors has been observed for a number of genes, including Bmal1 (30), ApoAI (46), ApoCIII (47), fibrinogen-β (48), Pae1 (49), and Il6 (31), which indicates that the two nuclear receptors play an important role in the regulation of metabolism, the cardiovascular system, and inflammation. In the current study, we discovered that this dual regulation also pertains to Ccl2 gene expression: Rev-erbx potently represses Ccl2 expression, whereas RORα potently enhances Ccl2 expression.

Of note, although Rev-erbx and RORα are both implicated in circadian gene regulation, Rora does not exhibit the robust diurnal oscillation of mRNA expression that Rev-erbx does (12, 30). These findings indicate that Ccl2 is a direct circadian output gene and that Rev-erbx potently affects Ccl2 rhythm through RORE in its promoter. In addition to Ccl2 expression, signaling activities, including the phosphorylation of ERK and p38, and inflammatory activities, including adherent and migratory activities, might exhibit temporal oscillation through the negative regulation of Ccl2 expression by Rev-erbx. However, further examination of whether Rev-erbx governs diurnal fluctuation of the Ccl2 gene, intracellular signaling, and inflammatory functions in macrophages is obviously needed.

Scheiermann et al. (50) demonstrated that adhesion and migration of leukocytes to tissues under homeostasis are regulated through signals from the β-adrenergic nerve system and that the peak recruitment occurs at night in rodents, during a period of activity, and the minimum occurs during the day during a period of rest. Circadian-oscillated leukocyte recruitment to tissues depends on the circadian fluctuation of the expression of adhesion molecules and CCL2 in endothelial cells (50). CCL2 can play a key role in leukocyte recruitment by both integrin activation and by promoting migration (32–35). Two distinct MAPks are known to be activated by CCL2 and regulate two independent signaling cascades, leading to integrin activation and migration: ERK is responsible for adhesion, and p38 is responsible for migration (24). The current study showed that Rev-erbx impairs adherent and migratory activities of macrophages through CCL2-dependent suppression of ERK and p38 signals. Moreover, integrin β1 expression, but not integrin β2, was decreased by Rev-erbx in macrophages, suggesting the impairment of specific adhesion to VCAM1 in RAW264 cells. Therefore, it seems likely that the circadian tissue recruitment of leukocytes is governed by the circadian oscillations of adherent molecules and chemokines in tissues mediated through signals from the autonomic nervous system (50), as well as by adherent and migratory activities of leukocytes mediated through peripheral leukocytic clockwork, including Rev-erbx and RORα in macrophages.

Leukocyte recruitment to tissues under inflammatory conditions was also shown to display circadian oscillation: higher at night (ZT13) than during the day (ZT5), as well as under homeostasis (50). In the current study, we demonstrated that Rev-erbx gene expression is lower at night (ZT22) than during the day (ZT10), which might influence macrophage infiltration into tissues; macrophage infiltration into tissues is suppressed during the day when Rev-erbx expression reaches its peak, whereas it is enhanced at night when Rev-erbx expression is at its minimum. Indeed, we observed that macrophage infiltration into WAT was higher at night (ZT22) than during the day (ZT10) in control, aged, and obese mice (S. Sato, T. Sakurai, J. Ogasawara, H. Ohno, T. Kizaki unpublished observations). Furthermore, we observed an increase in the gene expression of Il6, as well as Ccl2, in peritoneal macrophages from mice lacking Rev-erbx. The increase in F4/80 mRNA expression in WAT was observed in Rev-erbx−/− mice (S. Sato, T. Sakurai, J. Ogasawara, H. Ohno, T. Kizaki unpublished observations). These observations suggest that impairment or deficiency of Rev-erbx heightens macrophage adherent activity, leading to the acceleration of macrophage infiltration into WAT. Meanwhile, obesity increases macrophage infiltration into WAT (6, 10, 19). The current findings that expression of the inflammatory repressor Rev-erbx in macrophages from HFD-induced obese mice and ob/ob mice was markedly reduced during the day (ZT10) compared with control mice suggest that it contributes, at least in part, to the increased macrophage infiltration into WAT of obese mice.

In summary, as depicted in Fig. 8C, we provide new evidence that a circadian clock gene, Rev-erbx, mediates inflammatory functions, such as cell adhesion and migration of macrophages through the regulation of Ccl2 expression. In addition, we demonstrated that the gene expression of Ccl2 and its downstream Il6 in peritoneal macrophages from Rev-erbx−/− mice was higher than that from wild-type mice, suggesting in vivo physiological roles for Rev-erbx in the regulation of inflammatory function via the CCL2-activated signaling pathway. However, further studies are needed to clarify the precise role of Rev-erbx in the link between aging- or obesity-associated impairment of clockwork and inflammation. Thus, the current study highlights the orphan nuclear receptor Rev-erbx as an inflammatory repressor and a therapeutic target in inflammatory disease and strikes a note of warning about the impact of the disruption of clockwork induced by irregular lifestyles, such as long-term shift work, frequent air travel, and chronic restriction of sleep, on human health.

Disclosures
The authors have no financial conflicts of interest.

References


