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Stimulation of nuclear receptor REV-ERBs regulates tumor necrosis factor-induced expression of proinflammatory molecules in C6 astroglial cells

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ABSTRACT

Under physiological conditions, astrocytes maintain homeostasis in the CNS. Following inflammation and injury to the CNS, however, activated astrocytes produce neurotoxic molecules such as cytokines and chemokines, amplifying the initial molecular-cellular events evoked by inflammation and injury. Nuclear receptors REV-ERBα and REV-ERBβ (REV-ERBs) are crucial in the regulation of inflammation- and metabolism-related gene transcription. The current study sought to elucidate a role of REV-ERBs in rat C6 astroglial cells on the expression of inflammatory molecules following stimulation with the neuroinflammatory cytokine tumor necrosis factor (TNF). Stimulation of C6 cells with TNF (10 ng/ml) significantly increased the mRNA expression of CCL2, interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), and matrix metalloprotease (MMP)-9, but not fibroblast growth factor-2 (FGF-2), cyclooxygenase-2 (COX-2) and MMP-2. Treatment with either REV-ERB agonists GSK4112 or SR9009 significantly blocked TNF-induced upregulation of CCL2 mRNA and MMP-9 mRNA, but not IL-6 mRNA and iNOS mRNA expression. Furthermore, treatment with RGFP966, a selective histone deacetylase 3 (HDAC3) inhibitor, potently reversed the inhibitory effects of GSK4112 on TNF-induced expression of MMP-9 mRNA, but not CCL2 mRNA. Expression of *Rev-erbs* mRNA in C6 astroglial cells, primary cultured rat cortical and spinal astrocytes was confirmed by reverse transcription polymerase chain reaction. Together, the findings demonstrate an anti-inflammatory effect, downregulating of MMP-9 and CCL2 transcription, of astroglial REV-ERBs activation through HDAC3-dependent and HDAC3-independent mechanisms.

1. Introduction

Over-activation of astrocytes contributes to the induction of various neurological disorders [1–4]. Proinflammatory molecules such as cytokines, chemokines, prostanoids and neurotrophic factors, and activated enzymes, such as cyclooxygenases (COXs) and inducible nitric oxide synthase (iNOS), involved in the inflammatory response to CNS injury have been shown to be upregulated in activated astrocytes. Therefore, modulation of astrocytic activity could be an effective therapeutic strategy in treating inflammatory and neurodegenerative disorders [5].

Identified in various cell types, REV-ERBα and REV-ERBβ (REV-ERBs) are orphan nuclear receptors encoded by *NR1D1* and *NR1D2*, respectively, which exhibit rhythmic expression similar to that of clock genes [6]. In addition, REV-ERBs play critical roles in the regulation of metabolism, inflammation and cancer [7–9]. Like other nuclear receptors, REV-ERBs demonstrate a ligand-dependent inhibitory effect on gene transcription, including clock gene *Bmal1*, *apolipoprotein AI* and *fibrinogen-β* [6,10]. Recent studies have shown that REV-ERBα contributes to the suppression of the expression of proinflammatory molecules such as the cytokine interlukin-6 (IL-6) and the chemokine CCL2 in macrophages [8,11,12]. Although it is not entirely clear what mechanism might be involved in REV-ERBα-mediated modulation of gene transcription, recent studies have suggested that REV-ERBα could repress gene transcription through recruitment of nuclear receptor corepressor-

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1 (NCoR) or histone deacetylase 3 (HDAC3) [10,13]. Heme is reportedly an endogenous ligand for REV-ERBs [14]. However, since heme is nonspecific and easily metabolized, it is not a useful tool to elucidate the function of REV-ERBs. Recently, synthetic REV-ERB-specific ligands GSK4112 and SR9009 have been shown to repress the production of various proinflammatory molecules in activated macrophages [8,15]. Although it appears that the activity of REV-ERBs might be crucial in regulating macrophage activation, the role of REV-ERBs in regulating astrocytic function in particular has yet to be elaborated.

Tumor necrosis factor (TNF) is well known to be involved in the cellular response to injury and inflammation. Incubation of cultured astrocytes with TNF induces the production of a number of cytokines and chemokines [1]. Thus, cultured astrocytes were incubated in TNF to induce an inflammation-like state in order to test the hypothesis that REV-ERBs regulate the inflammatory response in astrocytes [16].

2. Materials and methods

2.1. Materials

Recombinant rat TNF was obtained from WAKO Pure Chemical Industries (Osaka, Japan). GSK4112 and RGFP966 were purchased from Cayman Chemical (Ann Arbor, MI). GSK4112 is a synthetic REV-ERBs agonist. (The 50% effective concentration (EC_{50}) to REV-ERBα is 0.4 μM. The EC_{50} to REV-ERBβ is not known.) [17]. SR9009, also a REV-ERBs agonist (REV-ERBα EC_{50} = 0.67 μM, REV-ERBβ EC_{50} = 0.8 μM), was obtained from Merck Millipore (Darmastadt, Germany) [7]. RGFP966 is a selective histone deacetylase 3 (HDAC3) inhibitor (50% inhibitory concentration (IC_{50}) = 0.08 μM), and is reported to have no affinity to other types of HDAC (IC_{50} > 15 μM) [18]. TNF was dissolved in distilled H_2O. GSK4112, SR9009 and RGFP966 were dissolved in DMSO. The final concentration of all solvents used in the experiments was maintained at 0.2%.

2.2. Cell culture

Rat C6 astroglial cells were acquired from the American Type Tissue Collection (CCL-107) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively) in an atmosphere of 5% CO_2/95% air at 37 °C. Twenty four hours before each experiment, the medium was exchanged with serum-free medium to minimize the potential for the effects of serums on the cells. C6 cells between passage numbers 40 and 60 were used for all experiments. Primary cultured astrocytes of rat cortex and spinal cord were prepared by the methods described previously [1,19].

To induce expression of inflammation-related genes in rat C6 cells, cells were incubated in TNF (10 ng/ml) and then harvested 1, 3, 6, 9 h of incubation for real-time PCR analysis. Significant expression of inflammation-related mRNA was observed at least 6 h after incubation in TNF (see Results). Thus, cells were harvested 6 h after TNF incubation in subsequent pharmacological experiments.

To assess a role of REV-ERBs in the expression of inflammation-related genes in C6 cells, cells were incubated for 30 min with either GSK4112 (3, 10, 20 μM) or SR9009 (3, 5, 10 μM). TNF was then added to the media and cells were harvested 6 h after TNF incubation.

Histone deacetylase 3 recruitment could be involved as an intermediary in REV-ERBs-mediated transcriptional inhibition. To determine if HDAC3 is crucial in the inhibitory effect of REV-ERBs, C6 cells were incubated in RGFP966 (2, 5 μM) for 30 min, followed by GSK4112 (20 μM) for 30 min. C6 cells were harvested after incubation in TNF for 6 h.

2.3. RT-PCR analysis

cDNA synthesized using 1 μg of total RNA in C6 cells, cultured rat cortical and spinal astrocytes and rat whole brain (used as a positive control) was subjected to PCRs for Rev-erbα, Rev-erbβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the specific primers and AmpliTag Gold™ (Applied Biosystems) at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, the annealing temperature (Rev-erbα; 60 °C, GAPDH; 50 °C) for 30 s, and 72 °C for 2 min with a final extension at 72 °C for 5 min. The sequences of primers were Rev-erbα, 5′-AGGGCCCATCGAGAAATC-3′ (forward) and 5′-GCGTAGACCATTCAGTGC-3′ (reverse), Rev-erbβ, 5′-AGAAGTGTCTGTCCGTGG-3′ (forward) and 5′-TCATGCGGCTCTGC-TAAG-3′ (reverse), and GAPDH, 5′-GAGCGAGATCCCGTCAA-GATCAAA-3′ (forward) and 5′-CACAGTCTTCTGAGTGGCAGTGAT-3′ (reverse). The resulting PCR products were analyzed on a 1.5% agarose gel and had the size (Rev-erbα; 208 bp, Rev-erbβ; 166 bp, GAPDH; 330 bp) expected from the known cDNA sequence.

2.4. Real-time PCR analysis

The effect of TNF over time on expression of inflammatory molecules CCL2, IL-6, fibroblast growth factor-2 (FGF-2), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), matrix metalloproteinase (MMP)-2 and MMP-9 in C6 cells was investigated by real-time PCR.

Total RNA in C6 cells was prepared by a previously described method [20] and used to synthesize cDNA with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and a random hexamer primer. cDNA synthesized using 1 μg of total RNA in each sample was subjected to real-time PCR assays with specific primers and EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen). The sequences of primers are as follows: CCL2, 5′-ACGCTTCTGGGCCTGTTGTT-3′ (forward) and 5′-CCTGCTGCTG GTGATTCTCT-3′ (reverse), IL-6, 5′-CTGCTCTGGTCTTCTGGAGT-3′ (forward) and 5′-GCATTGGAAGTTGGGGTAGG-3′ (reverse), FGF-2, 5′-ATCACTTCGCTTCCCGCA-3′ (forward) and 5′-TTTGACGTGTGG GTCGCT-3′ (reverse), COX-2, 5′-CTACCATCTGGCTTCGG-3′ (forward) and 5′-GTCTGGGTCGAACTTG-3′ (reverse), iNOS, 5′-CACA-CAGCCTCAGAGTCCTT-3′ (forward) and 5′-CAGGGCTCGATCTGG-TAGTA-3′ (reverse), MMP-2, 5′-AAGTTCCCGTTCCGCTTC-3′ (forward) and 5′-ACATGGGGCACCTTCTGA-3′ (reverse), MMP-9, 5′-CTAAAGGTCGCTCGGATG-3′ (forward) and 5′-CTTGCCCAGGAA-GACGAA-3′ (reverse), GAPDH, 5′-AGCCCAGAACATCATCCCTG-3′ (forward) and 5′-CACCACCTTCTTGATGTCATC-3′ (reverse). Real-time PCR assays were conducted using a DNA engine Opticon 2 real-time PCR detection system (Bio-Rad). The three-step amplification protocol consisted of 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. RNA quantities of target genes were calculated using the C_t method. The C_t values of CCL2, IL-6, FGF-2, COX-2, iNOS, MMP-2, and MMP-9 amplification were normalized to those of GAPDH amplification.

2.5. Statistical analysis

Data are expressed as the mean ± SEM of at least three independent determinations. Differences between means were determined using a one-way analysis of variance (ANOVA) with a pairwise comparison by the Tukey–Kramer method. Differences were considered to be significant when the P value was less than 0.05.

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