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Title: Biochanin A enhances ROR γ activity through STAT3-mediated recruitment of NCOA1

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Abbreviations: AF: activation function, IL: interleukin, NCOA1: nuclear-receptor
coactivator 1, ROR: retinoic acid-related orphan receptor

Abstract

Interleukin (IL)-17-producing T cells play important roles in autoimmunity, chronic inflammation and host protection against extracellular bacteria and fungi. The retinoic acid receptor-related orphan receptors (ROR) α and γ are key regulators of the IL-17-producing phenotype. We previously showed that the isoflavone biochanin A enhanced ROR-mediated transcriptional activity. Here, we investigated the possible mechanisms underlying this ROR activation. Biochanin A-treated murine thymoma EL4 and primary splenocytes demonstrated enhanced induction of IL-17. Biochanin A also induced tyrosine-phosphorylation of signal transducer and activator of transcription 3 (STAT3) in these cells. Stable knockdown of either ROR γ or STAT3 in EL4 cells canceled biochanin A-induced upregulation of IL-17 expression. Importantly, biochanin A enhanced complex formation between ROR γ and STAT3 or nuclear-receptor coactivator 1 (NCOA1). Furthermore, the biochanin A-induced ROR γ -NCOA1 complex was disrupted by a dominant negative mutant of STAT3 or by the STAT3 specific inhibitor Stattic. These results suggest that biochanin A activates ROR γ -dependent IL-17 transcription through the enhancement of STAT3 phosphorylation and STAT3-mediated recruitment of NCOA1 to

ROR γ .

Keywords: Isoflavones, IL-17, STAT3, ROR γ

Introduction

Interleukin-17 (IL-17) is a proinflammatory cytokine produced by T cells and plays important roles in autoimmunity, chronic inflammation and host protection against extracellular bacteria and fungi [1, 2]. The transcriptional regulation of IL-17-producing T cells involves the crucial roles of the transcription factors signal transducer and activator of transcription 3 (STAT3) and retinoic acid receptor-related orphan receptor (ROR) γ t in their development and/or function. ROR γ t directs the differentiation program of proinflammatory IL-17⁺ T helper (Th17) cells [3]. STAT3 is also required for the differentiation and effector function of these cells [4]. Expression of IL-17 by Stat3-deficient T cells was greatly impaired, indicating an essential role for STAT3 in inflammatory helper T cell differentiation *in vitro* [5].

We recently identified various azole pesticides, including imibenconazole, as suppressors of IL-17 gene expression [6]. Additionally, we found that four isoflavones (daidzein, genistein, formononetin and biochanin A) enhance ROR α - and ROR γ -mediated transcriptional activity in CHO and Jurkat cell-based reporter assays [7]. Similar to other members of the nuclear receptor family, RORs interact with coactivators or corepressors to

positively or negatively regulate gene transcription [8]. Nuclear receptor coactivator 1 (NCOA1; also known as steroid receptor coactivator 1) [9] is a coactivator that can physically interact with ROR γ to enhance ROR γ -mediated transcriptional activation [10]. Immunoprecipitation and immunoblotting assays in our previous study [7] revealed that biochanin A enhanced the interaction between ROR γ t and NCOA1. This finding suggests that isoflavones enhance IL-17 gene expression by stabilizing the interactions between ROR α/γ and NCOA1. However, how this stabilization occurs remains unclear.

Here we show that biochanin A-induced activation of ROR γ -mediated IL-17 expression involves NCOA1 recruitment to the ROR γ complex by phosphorylated STAT3.

Material and methods

Reagents, cell culture and transfection

Biochanin A, genistein, formononetin, daidzein, apigenin, baicalein and resveratrol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The STAT3 inhibitor stattic (6-nitrobenzo[b]thiophene-1,1-dioxide) was purchased from Calbiochem (Gibbstown, NJ, USA).

Spleens of 12-week-old C57BL/6 female mice were harvested, and single-cell suspensions were obtained. All experiments were performed according to the guidelines of the institutional animal care and use committee of Hokkaido University. After red blood cells were lysed, splenocytes were washed and cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μ M 2-mercaptoethanol. Cells were treated with various concentrations of biochanin A (as indicated in Figure 1; ranging from 0.1 to 10 μ M) for the times indicated in the figure legends. EL4 cells were maintained in RPMI-1640 medium containing 10% FCS. Rorc- or Stat3-knockdown EL4 cell clones (EL4/shStat3#1, EL4/shStat3#2, EL4/shRorc#1 and EL4/shRorc#2) were established by electroporation of

EL4 cells with pGPU6/GFP/Neo vector (Shanghai GenePharma, Shanghai, China) bearing short hairpin RNA (shRNA) targeting Stat3 (5'-CAAATTCCTGAGTTGAAT-3') or Rorc (5'-GCTACCAGAGGAAGTCAATGT-3') with subsequent selection in G418-containing medium. Similarly, a control shRNA (nonsilencing; 5'-TTCTCCGAACGTGTCACGT-3') transfected EL4 cell line (EL4/shNC) was also established. The human cervix carcinoma cell line (HeLa) was maintained in DMEM containing 10% FCS and was transfected using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions.

Plasmid constructs

Mouse ROR γ t cDNA was kindly provided by Dr. M. Kubo (Tokyo University of Science, Chiba, Japan) and was amplified by PCR and subcloned into the HindIII/EcoRI site in the FLAG-tagged mammalian expression vector pSF1. FLAG-tagged ROR γ t deletion mutants were generated by PCR and subcloned into pSF1 vector. Primer sequences are available on request. STAT3 expression constructs were as described previously [11]. Expression vector for Il17-promoter luciferase was kindly provided by Dr. A. Yoshimura (Keio University, Tokyo, Japan).

Intracellular IL-17 staining

Splenocytes were stimulated with 5 ng/mL phorbol 12-myristate 13-acetate (PMA, purchased from Sigma-Aldrich) and 1 μ M ionomycin (LKT Laboratories, St. Paul, MN, USA) for 5 hours. One μ M brefeldin A (Wako Purechemicals, Osaka, Japan) was added to the culture during the last 2 hours. Cells were stained with FITC-conjugated anti-CD4 antibody (clone L3T4, BD Pharmingen, Palo Alto, CA, USA) and then fixed in 2% paraformaldehyde. Next, cells were permeabilized with PBS supplemented with 0.5% saponin, and then stained with phycoerythrin (PE)-conjugated anti-IL-17 (clone TC11-18H10, BD Pharmingen) antibodies. Data were acquired on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA).

RNA isolation, reverse-transcription and quantitative real-time PCR

Splenocytes and EL4 cells were cultured with 5 ng/mL PMA and 1 μ M ionomycin, without or with biochanin A. After cell harvesting, total RNA samples were extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 μ g of total RNA with ReverTra Ace reverse transcriptase (TOYOBO,

Osaka, Japan) using random nonamers. Quantitative real-time PCR (qRT-PCR) analysis was carried out using KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA, USA) and a Mx3005P real-time PCR system (Stratagene, Santa Clara, CA, USA). Primers used for qRT-PCR were: *Il17a*: 5'-ACCAGAAGGCCCTCAGACTA-3' (sense), 5'-AGCATCTTCTCGACCCTGAA-3' (antisense), *Actb*: 5'-TGTTACCAACTGGGACGACA-3' (sense), 5'-GGGGTGTGTAAGGTCTCAAA-3' (antisense).

Measurement of Il17-promoter activity

HeLa cells were transfected with control, STAT3, RORC or Src siRNA followed by transfection with Il17-promoter Luc (firefly luciferase expression construct) together with Renilla luciferase expression construct. At 24 h after siRNA treatment, cells were treated with 1 μ M biochanin A and incubated for additional 24 hours. Luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

siRNAs used in this study were as follows: siSTAT3, 5' - CCGUCAACAAUUAAGAAATT-3' ; siRORC, 5' - GCCAGAAUGACCAGAUCAUTT-3' ; siSrc, 5' -

CCAAGGGCCUCAACGUGAATT-3' . Control siRNA was obtained from Qiagen (nonsilencing; catalog# 1022076).

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting assays were performed as described previously [12]. Immunoprecipitates from cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA, USA). The membranes were then immunoblotted with the different primary antibodies as indicated in the figure legends. The following primary antibodies were obtained commercially: anti-STAT3, anti-ROR γ , anti-NCOA1 and anti-GST antibodies (Santa Cruz Biotechnology, Dallas, TX, USA); anti-phospho-STAT3 antibody (Cell Signaling Technology, Beverly, MA, USA); and anti-Myc (9E10), anti-FLAG (M2) and anti-actin antibodies (Sigma-Aldrich). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were purchased from GE Healthcare (Little Chalfont, United Kingdom). An enhanced chemiluminescence detection system was purchased from Millipore (Bedford, MA, USA).

Statistical analysis

Results shown are the means and standard deviations. Statistical evaluation of differences between populations was performed using Student's *t* tests. *P* values less than 0.05 were considered statistically significant.

Results

Biochanin A stimulates IL-17 expression in primary splenocytes

To determine whether isoflavones affect the production of IL-17 in primary cells, splenocytes were cultured for 48 h in the presence of biochanin A, and IL-17-expressing cells were detected by intracellular cytokine staining. Biochanin A significantly increased IL-17 production by the CD4⁺ population, but not by the CD4⁻ cells (Figures 1A and B). The percentage of IL-17⁺ cells in the CD4⁺ population was increased by biochanin A in a time-dependent manner (Figure 1C). Biochanin A also increased *Il17a* mRNA levels in splenocytes (Figure 1D). These results demonstrate that biochanin A upregulates IL-17 expression in primary splenocytes.

Knockdown of ROR γ or STAT3 cancels biochanin A-induced enhancement of *Il17a* expression

Our previous study [7] and its confirmatory experiment (Figure 1E) showed that biochanin A enhanced *Il17a* expression in the PMA/ionomycin-stimulated EL4 murine tumor cell line, which constitutively expresses ROR γ [13]. To investigate the role of

transcription factors in biochanin A-induced activation of *Il17a* induction, we used EL4 cells and established stable Rorc- or Stat3-knockdown EL4 cell clones (EL4/shRorc#1 and #2, EL4/shStat3#1 and #2) by introducing shRNA vectors targeting either Rorc or Stat3. Similarly, nonsilencing control (NC) shRNA-transfected EL4 cell lines (EL4/shNC) were also established. Reduction of endogenous ROR γ and STAT3 proteins were confirmed by western blotting analysis as shown in Figures 1F and H. These cell lines were treated with or without biochanin A in the presence of PMA/ionomycin. EL4/shRorc#1 and #2 cells, as well as EL4/shStat3#1 and #2 cells, showed reduced levels of *Il17a* mRNA (Figures 1G and I). Importantly, treatment of EL4/shNC cells with biochanin A resulted in an upregulation of *Il17a* mRNA, whereas this enhancing effect of biochanin A was not observed in EL4/shRorc or EL4/shStat3 cells, indicating that biochanin A enhancement of *Il17a* mRNA expression is mediated by ROR γ and STAT3. In addition, HeLa cells transfected with a luciferase reporter construct containing Il17-promoter showed an increased promoter activity in response to biochanin A in a manner dependent on STAT3 and ROR γ (supplemental Figures S1A and S1B), indicating that the biochanin A-induced activation of Il17-promoter can be reconstituted in non-lymphoid HeLa cells.

Biochanin A activates STAT3 by enhancing its phosphorylation

We tested the effect of biochanin A on the activation of STAT3. As shown in Figure 2A, splenocytes treated with biochanin A showed an enhanced phosphorylation of STAT3 on tyrosine 705, which is the hallmark of STAT3 transcriptional activation. Not only biochanin A, but also other isoflavones stimulated phosphorylation of STAT3 in splenocytes (Figure 2B). Biochanin A also increased the basal (at 0 h treatment with PMA/ionomycin) and PMA/ionomycin-induced phosphorylation of STAT3, as well as ROR γ protein levels, in EL4 cells (Figure 2C). For specificity control, the effects of plant-derived flavonoids (apigenin and baicalein) and stilbenoid (resveratrol) on phosphorylation of STAT3 in EL4 cells were tested. While biochanin A induced phosphorylation of STAT3 in EL4 cells, apigenin, baicalein and resveratrol did not show such effect (supplemental Figure S2).

Biochanin A-induced activation of STAT3 is mediated by Src kinase.

The non-receptor tyrosine kinase Src is a known STAT3-phosphorylating kinase [14]. We next investigated whether Src tyrosine kinase could be involved in the biochanin A-induced phosphorylation of STAT3. Treatment of EL4 cells with biochanin A induced a transient activation of Src kinase (Figure 2D), suggesting the possible involvement of Src kinase

activity in biochanin A-induced phosphorylation of STAT3. To test this possibility, EL4 cells were treated with biochanin A in the presence or absence of a Src kinase specific inhibitor, SU6656 [15]. Src kinase inhibition by SU6656 effectively blocked biochanin A-induced phosphorylation of STAT3 (Figure 2E). In HeLa cells transfected with Src-specific siRNA, biochanin A-induced phosphorylation of STAT3 was suppressed compared with that in control siRNA-treated cells (supplemental Figure S1C). Src knockdown also significantly suppressed biochanin A-induced activation of Il17-promoter reporter in HeLa cells (supplemental Figure S1C). These results suggest that biochanin A-induced STAT3 phosphorylation is mediated by Src kinase.

Biochanin A enhances protein interaction between STAT3 and ROR γ t in a STAT3 phosphorylation-dependent manner

We carried out immunoprecipitation experiments using HeLa cells transiently transfected with FLAG-tagged ROR γ t, and revealed that endogenous STAT3 protein can form a complex with ROR γ t protein and that biochanin A enhances STAT3 interaction with ROR γ t (Figure 3A). Next, we evaluated whether the biochanin A-induced changes in the phosphorylation status of STAT3 affects STAT3-ROR γ t interaction, using the

dominant-negative mutants of STAT3, STAT3D (point mutation at the position important for DNA binding) and STAT3F (phenylalanine substitution at Tyr705) [16] (Figure 3B). HA-tagged STAT3 mutants were co-expressed with FLAG-tagged ROR γ t. Cell lysates were immunoprecipitated with an anti-HA Ab (for STAT3), and immunoblotted with anti-FLAG Ab (for ROR γ t). As shown in Figure 3C, wild type STAT3 interacted with ROR γ t and its binding was enhanced after biochanin A addition. In contrast, STAT3D and STAT3F demonstrated clearly reduced binding to ROR γ t. Next, we determined the ROR γ -interacting domain in STAT3 using a series of GST-fused STAT3 deletion mutants (Figure 3B). We found that the deletion mutant of STAT3 (320–493), which contains the DNA-binding domain of STAT3, showed relatively strong interaction with ROR γ t even in the absence of biochanin A stimulation, although full-length STAT3 required biochanin A stimulation for efficient interaction with ROR γ t (Figure 3D). In addition, all other deletion mutants of STAT3 failed to interact with ROR γ t (Figure 3E).

We also attempted to determine the STAT3-interacting domain on ROR γ t using a series of ROR γ t deletion mutants (Figure 3F). The full length ROR γ t showed interaction with STAT3, whereas ROR γ t mutants lacking the C-terminal activation function 2 (AF2)-region or N-terminal half containing both AF1 and DNA-binding domain failed to bind to STAT3

(Figure 3G). Therefore, STAT3-ROR γ t interaction requires both the N-terminal region and AF2 region of ROR γ t.

Biochanin A-activated STAT3 mediates coactivator binding to ROR γ t

NCOA1 is a transcriptional coactivator that has been reported to interact with both ROR γ [10] and phosphorylated STAT3 [17] (Figure 4A). Previously we demonstrated that NCOA1 interacts with wild type ROR γ but not with the Δ AF2 mutant in a biochanin A-stimulation dependent manner [7]. We examined the role of biochanin A-induced phosphorylation of STAT3 for the complex formation between ROR γ t and NCOA1. When HeLa cells were transfected with expression plasmids for wild type STAT3 and ROR γ t, western blot analysis of the immunoprecipitates for endogenous NCOA1 showed that NCOA1 interacts with ROR γ t as well as STAT3. Of note, their interactions increased after biochanin A stimulation. In contrast, in HeLa cells transfected with phosphorylation-defective STAT3F mutant and ROR γ t, biochanin A did not stimulate ROR γ t-NCOA1 interaction, suggesting that phosphorylated STAT3 may enhance ROR γ t-NCOA1 complex formation (Figure 4B). In accordance with this hypothesis, the enhanced ROR γ t-NCOA1 interaction induced by biochanin A was clearly canceled by a

small-molecule inhibitor of STAT3, Stattic [18], in a dose-dependent manner (Figure 4C).

Therefore, enhancement of complex formation between ROR γ t and NCOA1 by phosphorylated STAT3 is a possible mechanism to upregulate IL-17a expression in biochanin A-treated cells.

Discussion

We previously reported that isoflavones can enhance ROR-mediated IL-17 gene expression, the mechanism of which has not been clarified. In this study, we showed that biochanin A stimulates Il17a mRNA expression as well as STAT3 phosphorylation in primary splenocytes and EL4 cells. We also demonstrated that biochanin A-induced IL-17 expression requires STAT3 and ROR γ in EL4 cells. In addition, transient overexpression assays revealed that phospho-STAT3 is required for the enhancement of ROR γ -NCOA1 complex formation. These results indicate that biochanin A indirectly activates ROR γ -dependent IL-17 transcription through the enhancement of STAT3 phosphorylation and STAT3-mediated recruitment of NCOA1 to ROR γ . To the best of our knowledge, this study is the first demonstrating the mechanism of isoflavone-induced IL-17 upregulation. Both STAT3 and ROR γ are important for IL-17 expression [19], while whether and how STAT3 functionally associates with ROR γ was unclear. This study provides a possible mechanism of action for phospho-STAT3-mediated ROR γ activation, which substantially enhances Il17a gene expression.

Isoflavones are naturally occurring phytochemicals found particularly in soybean and

clover [20], and their dietary intake may play a beneficial role in the treatment/prevention of obesity, cancer, osteoporosis, and cardiovascular disease [21]. Our finding that isoflavones influence IL-17 gene expression in T cells may add another beneficial role to isoflavone intake, as IL-17 is important for anti-fungal and anti-bacterial immunity [1]. Thus, efficient intake of isoflavones may support the immune system's ability to fight against fungi, although further studies are needed.

Isoflavones, especially genistein, have been reported to be nonspecific inhibitors of protein tyrosine kinases [22]. In our experiment, biochanin A as well as genistein did not show an inhibitory effect on the level of tyrosine phosphorylation of STAT3. Interestingly, Chau et al. observed that physiological concentrations of genistein can also activate STAT3 both *in vitro* and *in vivo* and increased STAT3 binding to the telomerase reverse transcriptase promoter in human prostate cancer cells [23].

There are several reports of ROR γ activity-modulating agents. Hydroxyl cholesterol is a natural ligand for ROR γ [24]. 7-oxygenated sterols function as high affinity ligands for both ROR α and ROR γ by directly binding to their ligand-binding domains, modulating coactivator binding, and suppressing the transcriptional activity of the receptors [25]. T0901317 was reported to be an inverse agonist of ROR α and ROR γ [26]. Recent studies

have also provided evidence that the synthetic chemical SR1001 [27], digoxin derivatives [28, 29], and ursolic acid [30] act as ROR inverse agonists. These chemicals appear to modulate ROR activity through direct binding to its ligand binding pocket, while biochanin A activates ROR in a STAT3-dependent manner, providing a previously unidentified and indirect mechanism of ROR γ activation in cells.

In summary, we demonstrated the mechanism of biochanin A-induced ROR γ activation, which involves phospho-STAT3-mediated NCOA1 recruitment to ROR γ . Our findings suggest that dietary biomolecules, such as isoflavones, may regulate the function of IL-17-producing cells, leading to potential beneficial effects on the immune system.

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Conflicts of interest

The authors declare no financial or commercial conflicts of interest.

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Figure legends

Figure 1 Biochanin A stimulates IL-17 expression in a STAT3- and ROR γ -dependent manner.

(A) Representative histograms of intracellular IL-17 levels in CD4 positive cells (CD4⁺, left) or CD4 negative cells (CD4⁻, right), stimulated with vehicle (gray filled histograms) or biochanin A (10 μ M, black lines) for 48 h. (B) IL-17 mean fluorescence intensities (MFIs) in cells treated as in A are indicated. (C) Percentage of IL-17⁺ cells of CD4⁺ T cells in biochanin A-treated splenocytes. (D) qRT-PCR confirmation of *Il17a* expression in vehicle- (open) or biochanin A-treated (filled) splenocytes. Data represent the levels of mRNA normalized to that of the ACTB internal control. (E) EL4 cells were stimulated with vehicle or biochanin A (0.1, 1, 10 μ M) in the presence of PMA (1 μ M) plus ionomycin (5 ng/ml). After 24 h, *Il17a* mRNA levels were examined by qRT-PCR. (F) Expression of endogenous ROR γ protein was examined by immunoblotting in EL4 cells stably expressing non-silencing control (NC) or Rorc shRNA. (G) EL4 cells stably expressing NC or Rorc shRNA were stimulated with vehicle or biochanin A in the presence of PMA plus ionomycin for 24 h. *Il17a* mRNA levels were assessed by qRT-PCR. (H) Expression of

endogenous STAT3 protein was examined by immunoblotting in EL4 cells stably expressing NC or Stat3 shRNA. (I) EL4 cells stably expressing NC or Stat3 shRNA were stimulated with vehicle or biochanin A in the presence of PMA plus ionomycin for 24 h. *Ili7a* mRNA levels were assessed by qRT-PCR. Data are the means and SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 2 Biochanin A stimulates tyrosine phosphorylation of STAT3. (A) Splenocytes were stimulated with biochanin A (10 μ M) for the indicated periods. The cells were lysed, and total cell lysates were immunoblotted with the indicated antibodies. (B) Splenocytes were stimulated with biochanin A (10 μ M), genistein (10 μ M), formononetin (10 μ M) or daidzein (10 μ M) for 48 h. The cells were lysed, and total cell lysates were immunoblotted for phosphorylated and total STAT3. (C) EL4 cells were pretreated with DMSO or biochanin A (10 μ M) for 6 h, then stimulated with PMA plus ionomycin for the indicated periods. The cells were lysed, and total cell lysates were immunoblotted with indicated antibodies. (D) Effect of biochanin A on Src kinase activation. EL4 cells were stimulated with biochanin A (10 μ M) for the indicated periods. The cells were lysed, and total cell lysates were immunoblotted for phosphorylated and total Src kinase. (E) Src kinase

inhibitor SU6656 ameliorated biochanin A-induced STAT3 phosphorylation. EL4 cells were pretreated with SU6656 (5 μ M) for 30 min and subsequently stimulated with biochanin A for 24 h. The cells were lysed, and total cell lysates were immunoblotted with indicated antibodies.

Figure 3 Interactions between STAT3 and ROR γ . (A) HeLa cells were transiently transfected with FLAG-tagged ROR γ t. Biochanin A (10 μ M) was added as indicated before cell lysis and incubated for 30 min. The cell lysates were immunoprecipitated with anti-STAT3 antibody or control Ig and immunoblotted with anti-FLAG (for ROR γ t) or anti-STAT3 antibody (for endogenous STAT3). Total cell lysates (TCL) were also immunoblotted as indicated. (B) Domain structure of STAT3 and mutant construct fragments are shown. (C) HeLa cells were transfected with FLAG-tagged ROR γ t in combination with either HA-tagged full-length (FL) STAT3, STAT3D or STAT3F. Biochanin A (10 μ M) was added as indicated before cell lysis and incubated for 30 min. The cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG or anti-HA antibody. (D) HeLa cells were transfected with FLAG-tagged ROR γ t in combination with either GST alone or GST-fused STAT3 deletion mutant.

Biochanin A (10 μ M) was added as indicated before cell lysis and the cell lysates were pulled down with glutathione-sepharose and immunoblotted. (E) HeLa cells were transfected with FLAG-tagged ROR γ t in combination with either GST alone or GST-fused STAT3 deletion mutant. The cells were lysed, pulled down with glutathione-sepharose and immunoblotted. (F) Domain structure of ROR γ t and FLAG-tagged mutant fragments (ROR γ t Δ AF2 and ligand binding domain (LBD)) are shown schematically. (G) HeLa cells were transfected with Myc-tagged STAT3 in combination with FLAG-tagged ROR γ t constructs. The cells were lysed and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc and anti-FLAG antibody.

Figure 4 Biochanin A-activated STAT3 mediates coactivator binding to ROR γ t. (A) The domain structure of NCOA1 with reported STAT3-binding region [17] and nuclear receptor-binding region [10]. (B) HeLa cells were transfected with FLAG-tagged ROR γ t and Myc-tagged STAT3 constructs as indicated. Biochanin A (10 μ M) was added as indicated before cell lysis and incubated for 30 min. The cell lysates were immunoprecipitated with control or anti-NCOA1 antibody and immunoblotted with anti-FLAG (for ROR γ t), anti-Myc (for STAT3) or anti-NCOA1 (for endogenous NCOA1)

antibody. (C) HeLa cells were transfected with FLAG-tagged ROR γ t. Biochanin A (10 μ M) was added as indicated before cell lysis and incubated for 30 min. The STAT3 inhibitor Stattic (10 or 100 nM) was added as indicated 1 h prior to biochanin A stimulation. The cell lysates were immunoprecipitated with control Ig or anti-NCOA1 antibody and immunoblotted with anti-FLAG (for ROR γ t), anti-STAT3 (for endogenous STAT3) or anti-NCOA1 antibody.

Figure 1

Figure 2

Figure 3

Figure 4