HHS Public Access

Author manuscript

FASEB J. Author manuscript; available in PMC 2023 May 09.

Published in final edited form as:

FASEB J. 2021 November ; 35(11): e21968. doi:10.1096/fj.202001098R.

St. John's Wort alleviates dextran sodium sulfate-induced colitis through pregnane X receptor-dependent NFκB antagonism

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Abstract

St. John's wort (SJW), from traditional herbs, activates the pregnane X receptor (PXR), a potential drug target for treating inflammatory bowel disease (IBD). However, how SJW alleviates dextran sodium sulfate (DSS)-induced experimental IBD by activating PXR is unknown. To test this, *PXR*-humanized, wild-type (WT) and *Pxr*-null mice, primary intestinal organoids cultures, and the luciferase reporter gene assays were employed. In vivo, a diet supplemented with SJW was found to activate intestinal PXR both in WT and *PXR*-humanized mice, but not in *Pxr*-null mice. SJW prevented DSS-induced IBD in *PXR*-humanized and WT mice, but not in *Pxr*-null mice. In vitro, hyperforin, a major component of SJW, activated PXR and suppressed tumor necrosis factor (TNF)α-induced nuclear factor (NF) κB translocation in primary intestinal organoids from *PXR*-humanized mice, but not *Pxr*-null mice. Luciferase reporter gene assays showed that hyperforin dose-dependently alleviated TNFα-induced NFκB transactivation by activating human PXR in Caco2 cells. Furthermore, SJW therapeutically attenuated DSS-induced IBD in *PXR*-humanized mice. These data indicate the therapeutic potential of SJW in alleviating DSS-induced IBD in vivo, and TNFα-induced NFκB activation in vitro, dependent on PXR activation, which may have clinical implications for using SJW as a herbal drug anti-IBD treatment.

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AUTHOR CONTRIBUTIONS

Yan T and Gonzalez FJ designed the research; Yan T, Luo Y, Wang P, Xia Y, Hamada K, Wang Q and Yan N performed the experiments research; Krausz WK contributed to obtain reagents or analytic tools; Yan T, Luo Y, and Wang P analyzed the data; Yan T wrote the manuscript; Gonzalez FJ and Hao H revised the manuscript and supervised this project. Yan T and Luo Y contributed equally to this study.

Tingting Yan and Yuhong Luo are co-first authors.

DISCLOSURES

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of the article at the publisher's website.

Keywords

DSS; IBD; natural products; NFκB; PXR; small intestine

1 | INTRODUCTION

Inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, a chronic immunologically mediated disease with various risk factors, has traditionally been found in North America and Western Europe but, in recent years, is increasing globally.[1] Clinical outcomes for IBD have improved, as evidenced by decreased intestinal resection rates in Crohn's disease.[2] However, IBD is still regarded as an incurable chronic and debilitating intestinal disease with limited available therapies, which causes an increased risk for colon cancer, depression, and morbidity.[3] Developing novel anti-IBD therapies is needed to improve the life quality of IBD patients.

Pregnane X receptor (PXR), a member of the nuclear receptor superfamily, is distributed in the small intestine and colon, and is considered a potential pharmacological target for IBD treatment, due in part to the attenuation of nuclear factor (NF) κB signaling and inflammation by both ligand-dependent and ligand-independent mechanisms.[4,5] Rifaximin (RIF), a potent gut-specific human PXR activator,[6] is used for alleviating the IBD symptoms in the clinic,[7] while RIF was revealed to decrease the symptoms of experimental IBD via human PXR-dependent inhibition of NFκB signaling in preclinical rodent models.[8] However, RIF as an antibiotic could cause antibiotic resistance of bacteria possibly leading to pathogenic bacterial overgrowth,[9] which is an unwanted and possibly deadly side effect. Therefore, safer non-antibiotic PXR agonists as novel anti-IBD pharmacological therapies deserve study.

Traditional herbs have been shown to harbor therapeutic effects for treating the IBD[10] or other diseases.[11,12] St. John's wort (SJW), a traditional herb, is widely avail- able as an FDA-classified dietary supplement and has been used as an over-the-counter antidepressant for many years in Europe and the United States, with few reported side effects.[13,14] Irritable bowel syndrome (IBD) is frequently accompanied by anxiety and depression in clinical patients.[15] Until now, the therapeutic effect of the anti-depressant SJW in treating IBD has not been explored in clinical trials, which is at least partially due to the lack of preclinical studies to support the potential beneficial effect of SJW in treating IBD.

Crohn's disease and ulcerative colitis are the two major forms of IBD, with Crohn's disease characterized by inflammation located in the whole digestive tract, particularly in the distal small intestine (ileum), while ulcerative colitis is mainly in the large intestine (colon) and rectum.[16] Each of the various IBD rodent models has unique advantages for characterizing specific mechanisms of gut inflammation.[17–19] Among these models, the dextran sodium sulfate (DSS)-induced IBD model, characterized by extensive inflammation both in the small intestine and colon, is widely used for drug discovery in treating both Crohn's disease and ulcerative colitis.[17,18,20–23] In preclinical studies, SJW was revealed to improve intestinal diseases, as revealed by ameliorating 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats[24,25] and preventing colon cancer in mice.[26] SJW extract

activates PXR, among which hyperforin, was revealed as the most potent PXR agonist[27] that could form a crystal structure in complex with the ligand binding domain of human PXR.[28] However, until now, how dietary SJW supplement activates PXR, particularly human PXR, to alleviate DSS-induced experimental colitis, is unknown.

PXR ligand specificity differs between mice and humans,[4,29] and thus *PXR*-humanized mouse was generated to better mimic human PXR signaling.[6,8,30,31] Immune dysregulation in IBD depends on the over release of diverse proinflammatory cytokines that orchestrate intestinal inflammation, among which TNFα is known to promote IBD progression since TNFα neutralization is firmly established as an effective anti-IBD approach[32] and various anti-IBD treatments produce the therapeutic effects via directly blocking TNFα-induced intestinal stress.[33–35] TNFα-exposed Caco2 cells is an in vitro model of acute intestinal inflammation,[36] while this in vitro model in combination with the use of NFκB luciferase reporter constructs was established for testing the effects of compounds in antagonizing TNFα-induced NFκB activation.[8,37,38] In addition, Caco2 cells have negligible levels of PXR and consequently fail to respond to the PXR agonist rifampin,[39] and thus co-transfection of Caco2 cells with PXR expression vectors and PXR target gene promoter luciferase reporter constructs or NFκB luciferase reporter vectors could be used for evaluating whether a compound activates PXR or antagonizes NFκB activation, dependent on the presence of PXR.[5,8,37,38] In this study, *PXR*-humanized mice that better mimic human PXR signaling in combination with wild-type (WT) mice and *Pxr*-null mice were used to examine the hypothesis whether SJW improved DSS-induced IBD via PXR activation in vivo and in primary intestinal organoids in culture. Luciferase reporter gene studies were used to examine how SJW extract or hyperforin directly antagonized NFκB activation through modulation of PXR.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Hyperforin, RIF, and pregnenolone 16α-carbonitrile (PCN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DSS was purchased from MP Biomedicals (Solon, OH, USA). SJW powder was a generous gift from Euromed USA, Inc. (Presto, PA, USA) that met European formulation standards for supplements certified for human use as described previously.[26] The extract was shipped and stored at 4°C in light-resistant containers. Primer oligonucleotides for qPCR were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Recombinant mouse TNFα protein was purchased from Peprotech (Rocky Hill, NJ, USA). EvaGreen master mix was from Applied Biological Materials Inc. (Richmond, BC, Canada). Gentle Cell Dissociation Reagent (Catalog, #07174) and IntestiCult™ organoid growth medium (mouse) (Catalog, #06005) were purchased from STEMCELL Technologies (Cambridge, MA, USA). Corning Matrigel Growth Factor Reduced Basement Membrane Matrix (Catalog, #354230) was purchased from Corning Inc. (Corning, NY, USA). Plasmids for pCMV-hPXR, pGL4TK(ER6)3 and renilla were gifted by Grace L. Guo as described previously,[40] while NFκB luciferase reporter plasmid and NFκB inhibitor α (IκBα) plasmid were provided by Yatrik M. Shah.[5]

2.2 | Diet preparation

Control purified diet (AIN-93G) and AIN-93G powder were purchased from Dyets Inc. (Bethlehem, PA, USA). AIN-93G powder was mixed with SJW powder to produce 2.5% SJW mixture by using a dough mixer and dried at 50°C. SJW diets were freshly prepared right before use and all diets were stored at 4°C for no more than 2 weeks. Evaluation of stability and integrity of SJW constituents in the diet was performed as described previously.[26] Similarly, RIF was mixed with food at 62.5 mg RIF per kg food, which was slightly modified based on a previous study.[8]

2.3 | Animal experiment designs

Age and weight-matched *PXR*-humanized, WT and *Pxr*-null male mice on a C57BL/6N background were housed in temperature- and light-controlled rooms. The *PXR*-humanized mice transgenic expressed the complete human PXR gene on the *Pxr*-null background as described previously.[6,30] All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals under a protocol LM061 approved by the National Cancer Institute Animal Care and Use Committee.

Two- to 3-month-old *PXR*-humanized, WT, and *Pxr*-null male mice were subjected to the DSS-induced IBD model. The age and body-weight matched mice of each mouse strain were divided into different groups based on body weights so that <10% intergroup variation of average body weight was found on the first dosing day. For testing the preventive effect of SJW, *PXR*-humanized, WT and *Pxr*-null mice were fed control diet or 2.5% SJW diet for 5 days, followed by control water or 1.5% DSS treatment for 7 days, during which the mice were maintained on control or 2.5% SJW diet, respectively. For testing the therapeutic effect, *PXR*-humanized mice were treated with 2% DSS for 5 days, and then fed control diet or 2.5% SJW diet for additional 7 days, during which the 2% DSS-containing water was replaced with control blank water. In *PXR*-humanized mice, RIF was used as the positive control for human PXR activation. RIF at 62.5 mg per kg food was mixed in the food for oral administration as described in *Diet preparation*. In WT and *Pxr*-null mice, PCN was used as the positive control of mouse PXR activation. PCN was dosed at 10 mg/kg via intraperitoneal injection once daily as described previously, while the other groups were dosed with matched control vehicle accordingly.[5] For all animal experiments, body weight was monitored daily and disease activity index (DAI) at the final day was calculated as described in Table S1. The mice were observed once or twice every day and killed if either the diarrhea score or rectal bleeding score was as high as 4 per the animal protocol approved by National Cancer Institute Animal Care and Use Committee. Pilot studies (2.5% DSS, 2% DSS and 1.5% DSS) were performed to determine the sensitivity of mice to DSS, and 1.5% DSS was chosen for experiments on the preventive effects of SJW and 2% DSS was chosen for the therapeutic effects of SJW. All mice survived until the end of the experiments when tissues were collected and analyzed. All mice were killed by CO_2 inhalation, colon lengths measured, and blood and tissues collected for further analysis.

2.4 | Nuclear and cytoplasm protein extraction and western blot analyses

Nuclear and cytoplasm proteins freshly isolated from distal ileum tissues, were collected from mice by using the commercial kit NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific, Rockford, USA, Catalog #78835). The protein extracts were subjected to western blot analysis. In brief, protein concentrations were determined with the BCA protein assay kit (Pierce Chemical, Rockford, IL). The samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylfluoride membranes, and incubated overnight at 4 °C with primary antibodies, followed by secondary antibody incubation at room temperature for 1–2 h. Proteins were visualized using the SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA) with an image analyzer (Alpha Innotech Corp., San Leandro, CA). The phospho-NFκB p65 (Ser536) antibody (Catalog, # 3033; RRID: AB_331284), Lamin B1 (LMNB1) antibody (Catalog, 13435; RRID: AB_2737428), and β-actin (13E5) (ACTB) antibody (Catalog, #4970; RRID: AB_2223172) were purchased from Cell Signaling Technology (Danvers, MA, USA). And NFκB p65 antibody (Catalog, #GTX102090; RRID: AB_10630493) was obtained from GeneTex (CA, USA). Anti-rabbit IgG, HRP-linked antibody (Catalog, #7074; RRID: AB_2099233) was used as the secondary antibody. ACTB was used as the internal standard loading control for cytoplasmic proteins, and LMNB1 was used as the internal standard loading control for nuclear proteins.

2.5 | Primary intestinal organoids culture

Isolation of primary intestinal organoids was slightly modified per the protocol provided for "intestinal epithelial organoid culture with intestiCult™ organoid growth medium (mouse)" by STEMCELL Technologies (Cambridge, MA, USA) as described previously.[41–43] In brief, the adult male mice (*PXR*-humanized and *Pxr*-null) were killed, and small intestines isolated in the cell culture hood. The small intestines were gently flushed with cold phosphorylated buffer saline (PBS), and the distal ileum cut into pieces using sterile small scissors, and then digested with the Gentle Cell Dissociation Reagent in ice for 15 min. The tissue pieces were resuspended in ice-cold PBS containing 0.1% bovine serum albumin and then filtered through a 70 μm filter in a fresh 50 ml conical tube. The filtered fractions were centrifuged at 290 *g* for 5 min at 4°C to collect the pellets and then washed with PBS containing 0.1% bovine serum albumin. The washed cells were centrifuged at 600 rpm for 3 min at 4 °C to pellet the intestinal crypts. The crypt fraction cells were resuspended in cold Dulbecco's Modified Eagle Medium /F-12 (Thermo Fisher Scientific, Waltham, MA, USA) and put on ice. Then, the resuspended cells were equally mixed with Matrigel Matrix (1:1). Fifty microliter of about 500-crypt suspension was equally seeded into the center of each of four wells of the prewarmed 24-well plate and then kept at 37 °C incubator for 10 min to allow the Matrigel to clot. Five hundred microliters of complete intestiCult™ organoid growth medium was then added to each well, while sterile PBS was added to the unused wells to ensure proper hydration of the cultures. The culture medium was changed twice per week. The cells were used for experiments at 7 days after seeding or further passages (no more than three generations).

For testing the effect of SJW extract or hyperforin in activating intestinal PXR signaling, the primary organoid cells were treated with SJW ethanol extract at 0.8 and 8.0 μg/ml or

hyperforin at 0.2 and 1.0 μM for 18 h. The doses of SJW extract and hyperforin were designed as described in an earlier report.[27] The 0.1% ethanol and 0.1% dimethyl sulfoxide (DMSO)-containing culture medium were used as the match blank vehicle. Accordingly, 0.1% DMSO was added to SJW extract-treated culture medium and 0.1% ethanol was added to hyperforin-treated culture medium, respectively, so that all the groups had exactly-matched vehicle, 0.1% ethanol and 0.1% DMSO. For testing the effect of SJW extract or hyperforin in inhibiting TNFα-induced NFκB activation, the organoid cells were pretreated with hyperforin at 0.2 or 1 μM for 2 h, followed by 20 ng/ml of TNFα for an additional 18 h. All organoid cells were washed with PBS three times, and then collected for further western blot analyses.

2.6 | Luciferase reporter assays

Caco2 cells were purchased from ATCC (Manassas, VA, USA), cultured in Dulbecco's Modified Eagle Medium (Corning Inc., Corning, NY, USA) containing 10% fetal bovine serum (BenchMark, Gemini Bio-Products, West Sacramento, CA, USA) and passaged for no more than 10 generations. Cells were seeded in 24-well plates at 90% confluency and were transfected with plasmids using Lipo3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA). For testing the efficacy of hyperforin in transactivating human PXR, Caco2 cells were transfected with or without 150 ng of human PXR expression plasmid, 150 ng of ER6-luciferase reporter (this plasmid construct is a pGL4-TK vector with three copies of ER6), 150 ng of human RXR expression plasmid, and 50 ng of renilla expression plasmid for each well for 24 h, and then treated with hyperforin at doses of 0.05, 0.20, and 1.0 μM or RIF at 10 μM for an additional 18 h.

For testing how hyperforin affected TNFα-induced NFκB activity, Caco2 cells were transfected with or without 150 ng of human PXR plasmid, with 150 ng of human RXR plasmid together with 150 ng of NFκB luciferase plasmid and 50 ng of renilla plasmid for 24 h, followed by hyperforin (0, 0.05, 0.20, and 1.0 μM) treatment for 2 h and then treated with 20 ng/ml of recombinant human TNFα protein for an additional 18 h to detect luciferase activities. IκBα plasmid transfection (50 ng/well) was used as a negative control for NFκB activation. At the end of each experiment, the cells were collected, washed with PBS twice and then luciferase activity measured by the dual-luciferase reporter assay system (Promega, Madison, WI). Renilla luciferase activity was used to normalize the transfection efficiency.

2.7 | Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from frozen ileum and colon tissues using TRIzol reagent. cDNA was synthesized from the extracted RNA using qScript™ cDNA SuperMix. qPCR assays were performed using EvaGreen master mix (Applied Biological Materials Inc., Richmond, BC, Canada) with Applied Biosystems 7500 (Fisher Scientific, Ottawa, Ontario, Canada). Primer sequences are provided in Table S2. The mRNA expression levels were calculated and normalized to their corresponding glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA.

2.8 | Histology analysis

Mice were killed and a part of the distal colons were collected after washing the tissues with cold PBS, immediately fixed in 10% neutral formalin, embedded in paraffin, and then stained with hematoxylin and eosin (H&E) at VitroVivo Biotech (Rockville, MD, USA). Histological analyses were performed using microscopic examination and slide digital images were collected using Pannoramic Viewer software. Images shown were representative of 3–5 biological replicates.

2.9 | Colitis scoring method

Colon histology was scored from 0 to 4 depending on the severity. In detail, 0—no lesions or normal; 1—minimal colitis: the epithelium is intact, and a few inflammatory cells present; 2—mild colitis: the epithelium is intact, and inflammation is diffuse; 3—moderate colitis: the epithelium is intact or focally lost, and inflammation is diffuse; 4—severe colitis:there is diffuse or multifocal loss of epithelium and marked diffuse inflammation, and focal hyperplasia may be seen.

2.10 | Statistical analysis

Statistical analysis was performed on Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Two-tailed Student's *t*-test was used to compare the statistic difference between two groups, while One-way ANOVA analysis was used among multiple comparisons. The values are present as mean ± SEM. A value of $p < .05$ was considered as statistically significant.

3 | RESULTS

3.1 | SJW activated ileum PXR and prevented DSS-induced IBD in *PXR*-humanized mice

To test whether SJW activated intestinal human PXR, age-matched *PXR*-humanized mice were divided into six groups. All mice were fed control diet, 2.5% SJW diet or RIF diet, respectively, for 5 consecutive days before the mice were put on 1.5% DSS or the matched control water treatment for additional 7 days (Figure 1A). Compared with control diet feeding, SJW diet significantly induced the mRNA levels of *Cyp3a11*, a PXR target gene, without changing levels of *PXR* mRNA in the mouse ileum (Figure 1B), while the mRNA levels of both *PXR* and *Cyp3a11* in the colon remained unchanged (Figure S1A). These data demonstrate that SJW diet feeding activated ileum but not colon PXR in *PXR*-humanized mice.

In DSS-treated *PXR*-humanized mice, 2.5% SJW diet significantly rescued the body weight loss and decreased DAI scores at the end of experiment, with RIF diet used as a positive control (Figure 1C,D). Consistently, 2.5% SJW diet reduced the DSS-induced decrease of colon length (Figure 1E) and small intestine length (Figure 1F) at the end of the experiment. With RIF as the positive control, further analyses showed that SJW diet supplementation significantly induced *Cyp3a11* mRNA, in the distal ileum (Figure 1G). In addition, 2.5% SJW diet significantly decreased the DSS-induced proinflammatory cytokine *Ccl2*, *Cox2*, and *Lcn2* mRNAs (Figure 1G). Thus, 2.5% SJW diet consistently rescued the DSS-induced damage markers including body weight loss, decreased colon length and

intestinal proinflammatory markers. Further histological analyses demonstrated that 2.5% SJW significantly attenuated gut damage, including crypt distortion, loss of goblet cells and mucosal damage in the colon of DSS-treated mice (Figure 1H). Given that PXR activation is known to antagonize NFκB activation, which is thought to decrease IBD symptoms, intestinal NFκB signaling was analyzed by western blot. SJW significantly decreased the protein levels of p-p65, but not total p65 levels (T-p65), in the ileum cytoplasm of DSS-treated mice (Figure 1I). DSS treatment induced significant nuclear accumulation of p-p65 and T-p65, both of which were significantly reduced by SJW in the distal ileum of DSS-treated mice (Figure 1J). In addition, under water treatment, both SJW diet and RIF diet treatment alone showed no significant effect on body weights (Figure 1C) and histological morphology of colon (Figure S1B), indicating that SJW and RIF are safe and non-toxic at the does employed. These data demonstrate that SJW activates intestinal PXR, prevents DSS-induced colitis in *PXR*-humanized mice, and reduces DSS-induced intestinal NFκB activation.

3.2 | SJW prevented DSS-induced colitis in WT mice, while this effect was lost in *Pxr*-null mice

To test whether SJW alleviates DSS-induced IBD in WT C57BL/6N mice, age and body weight-matched C57BL/6N mice were divided into 6 groups and fed with control diet, 2.5% SJW diet or dosed with PCN, respectively, following the experiment scheme described above (Figure 2A). In WT C57BL/6N mice, 2.5% SJW diet markedly induced *Cyp3a11* mRNA expression without changing levels of *Pxr* mRNA, with PCN as a positive control (Figure 2B), while not changing *Pxr* and *Cyp3a11* mRNA expression in the colon (Figure S1C). Consistent with the phenotype found with DSS-treated *PXR*-humanized mice, SJW decreased the loss of body weight (Figure 2C), reduced the DAI scores (Figure 2D), and rescued the colon length (Figure 2E) and small intestine length (Figure 2F) in DSS-treated WT mice. Further analyses of mRNA levels demonstrated that a 2.5% SJW diet significantly induced ileum *Cyp3a11* mRNA, while ileum *Pxr* mRNA remained unchanged (Figure 2G). In addition, 2.5% SJW sharply alleviated DSS-induced upregulation of proinflammatory markers, *Lcn2* and *Ccl2* mRNAs in the ileum (Figure 2G). SJW also markedly improved DSS-induced histological damage in the colon (Figure 2H). Under water treatment, both SJW diet and RIF diet alone showed no significant effects on body weights (Figure 2C) and histological morphology of the colon (Figure S1D), indicating that SJW and PCN are not toxic at the doeses employed. Accordingly, with PCN as the positive control, SJW markedly alleviated DSS-induced NFκB activation (Figure 2I,J) similar to that noted with the *PXR*-humanized mice. These data demonstrate that 2.5% SJW diet activates intestinal PXR, prevents DSS-induced IBD, and reduces DSS-induced NFκB activation in WT mice.

The role of PXR in protecting against IBD was revealed by studies using IBD models with PXR agonists in combination with the use of *PXR*-humanized mice, *Pxr*-null mice, and WT mice.[4,5,8] To further investigate whether SJW prevented DSS-induced IBD depending on the presence of PXR, *Pxr*-null mice were dosed with SJW (Figure 3A). As expected, 2.5% SJW diet feeding and PCN dosing failed to induce *Cyp3a11* mRNA in *Pxr*-null mice (Figure 3B). Accordingly, SJW failed to reverse DSS-induced body weight loss, DAI scores, colon and intestine length decreases in *Pxr*-null mice (Figure 3C,D). SJW diet feeding did not

affect levels of *Cyp3a11, Cox2, Lcn2* and *Ccl2* mRNAs in the ileum (Figure S1E). SJW diet also failed to induce *Cyp3a11* mRNA and reverse the increase in *Ccl2*, *Lcn2* and *Cox2* mRNAs in the distal ileum of DSS-treated *Pxr*-null mice (Figure 3G). Further histological analysis revealed that 2.5% SJW did not reverse DSS-induced colon damage (Figure 3H). In addition, both 2.5% SJW diet alone and PCN treatment alone showed no significant effect on body weights and the intestinal histological morphology of *Pxr*-null mice under water treatment (Figure S1F), indicating that both SJW and PCN have no toxic effects in *Pxr*-null mice at the doses used. Consistently, both SJW and PCN failed to rescue DSS-induced NFκB activation in the distal ileum of *Pxr*-null mice (Figure 3I,J). These data revealed that the preventive effects of SJW on DSS-induced IBD and intestinal NFκB activation were lost in *Pxr*-null mice with PCN as the positive control for the mouse PXR activator, thus indicating a PXR-dependent effect of SJW diet in treating IBD.

3.3 | SJW had a therapeutic effect on DSS-induced colitis in *PXR*-humanized mice

To further test the therapeutic effect of SJW in DSS-induced IBD, *PXR*-humanized mice were divided into four groups—control group, DSS group, DSS+SJW group, and DSS+RIF group. Mice in the control group were treated with water and control diet while all other mice were treated with 2% DSS for 5 days, followed by control water for additional 7 days, while the mice were fed with the 2.5% SJW diet or RIF diet for the last 7 days after the emergence of IBD symptoms (Figure 4A). With RIF as a positive control, SJW diet significantly rescued DSS-induced body weight loss, reduced DAI scores, rescued the DSS-induced decrease of colon length and small intestine length (Figure 4B–E). SJW diet significantly induced the expression of *Cyp3a11* mRNA and decreased the levels of *Cox2*, *Lcn2*, and *Ccl2* mRNAs in the ileum (Figure 4F). Further histological analyses revealed that 2.5% SJW diet markedly improved DSS-induced colon damage in *PXR*-humanized mice (Figure 4G,H). With RIF as the positive control, SJW markedly reversed DSS-induced NFκB activation in the distal ileum of DSS-treated *PXR*-humanized mice (Figure 4I,J). These data demonstrate that SJW de- creases DSS-induced IBD and intestinal NFκB activation in *PXR*-humanized mice, indicating a potential therapeutic role for SJW in treating IBD by activating human PXR.

3.4 | Hyperforin activation of PXR attenuates TNFα-induction of NFκB in primary organoids

SJW has both preventive and therapeutic effect in DSS-induced IBD, dependent on the presence of PXR. To further determine the mechanism, in vitro cell culture experiments were performed to examine whether SJW directly activated intestinal PXR to attenuate insult-induced inflammation. SJW diet only activated PXR expressed in the ileum, not in the colon, and thus primary organoids were isolated from the distal small intestines of *PXR*-humanized or *Pxr*-null mice and cultured for further experiments. In primary intestinal organoids, in the absence of TNFα, both SJW extract at a dose of 0.8 and 8.0 μg/ml and its active component hyperforin, one of the most potent PXR agonist in SJW extracts, at 0.2 and 1 μM, dose-dependently induced *Cyp3a11* mRNA, while *PXR* mRNA was unchanged in organoid cultures isolated from *PXR*-humanized mice (Figure 5A,B), and this *Cyp3a11*-induction effect by hyperforin or SJW extracts was lost in organoids isolated from *Pxr*-null mice (Figure 5C). DSS treatment is not practical to use on cultured cells,

while overactivation of TNFα signaling is known to mediate IBD that led to the clinical application of TNFα blockers for treating IBD.[44–46] Thus, recombinant TNFα protein was used to stimulate the intestinal organoid cells to mimic the proinflammatory condition of intestines from DSS-treated mice. Recombinant TNFα protein at 20 ng/ml significantly decreased *PXR* and *Cyp3a11* mRNAs, both of which were dose-dependently rescued by SJW extract or hyperforin, in primary intestinal organoids isolated from *PXR*-humanized mice (Figure 5D,E), while this effect was lost in organoids isolated from *Pxr*-null mice (Figure 5F). Given that 1.0 μM of hyperforin induced *Cyp3a11* mRNA to the greatest extent, 1.0 μM of hyperforin was chosen for further in vitro analysis. With RIF as the positive control, both hyperforin and SJW extract significantly attenuated TNFα-induced NFκB activation in primary intestinal organoids isolated from the distal ileum of *PXR*-humanized mice (Figure 5G), but not in primary intestinal organoids from *Pxr*-null mice (Figure 5H). These data demonstrate that SJW extract and hyperforin dose-dependently activate PXR in the absence of TNFα, normalize TNFα-downregulated PXR signaling, and alleviate TNFα-induced NFκB activation in primary intestinal organoid cultures in vitro.

3.5 | Hyperforin-activated PXR antagonizes TNFα-induced NFκB transactivation in Caco2 cells

To further examine whether hyperforin directly activated PXR, Caco2 cells were used for luciferase reporter gene analysis. Toxicity of drugs used were tested using the cell counting kit-8 assay (Figure 6A) and non-toxic drug doses were chosen for further study. The results demonstrated that hyperforin dose-dependently activated PXR only under the condition of forced PXR overexpression by co-transfection with a PXR expression plasmid, using RIF as the positive control as a known human PXR agonist (Figure 6B). These data suggest that hyperforin directly activates PXR in vitro dependent on the presence of PXR. Then, the possibility whether hyperforin directly attenuated TNFα-induced NFκB transactivation via PXR activation was investigated in Caco2 cells. Caco2 cells were transfected with or without the human PXR expression plasmid, human RXRα expression plasmid, and NFκB luciferase reporter plasmid for 24 h and then treated with hyperforin at the indicated doses for 2 h, followed by 20 ng/ml of TNFα treatment for an additional 18 h. An IκBα expression plasmid was co-transfected as a positive control. TNFα treatment sharply induced NFκB luciferase activities, which was reduced by PXR transfection (Figure 6C), indicating a role for PXR in antagonizing NFκB activation. Hyperforin dose-dependently alleviated TNFα-induced NFκB luciferase activity only with forced human PXR expression, while IκBα co-transfection totally abolished TNFα-induced NFκB activation as a positive control (Figure 6C). These data demonstrate that with RIF as the positive control, hyperforin directly alleviated TNFα-induced NFκB transactivation, dependent on the presence of PXR, providing a possible mechanistic explanation for the anti-IBD effect of SJW in mice.

4 | DISCUSSION

The current clinical anti-IBD drugs are not curable and may have adverse effects, and thus, herbal drug supplements for supportive anti-IBD treatment could be attractive alternatives. Among various IBD models, both rectally dosed TNBS and water-fed DSS induce colitis as a result of intestinal inflammation.[17,19,22,47] SJW via gavage was found to prevent the

colorectal carcinogenesis in mice[26] and intestinal inflammatory disease in the rat TNBS-induced colitis model.[24,25] However, the role of SJW in DSS-induced IBD has not been reported and the potential anti-IBD mechanism of SJW has never been explored. In this study, a potential therapeutic role for SJW dietary supplement was demonstrated in DSS-induced IBD. Mechanistically, the anti-IBD effect of SJW is dependent on PXR-mediated NFκB antagonism. The study using *PXR*-humanized mice and matched *Pxr*-null mice in this study demonstrate both prophylactic and therapeutic treatment of SJW alleviates DSS-induced IBD depending on human PXR activation, while the use of primary intestinal organoids and PXR/NFκB luciferase reporter assays supports the role of hyperforin in directly inhibiting TNFα-induced NFκB activation depending on the presence of PXR. The major findings of the current study are summarized in Figure 7.

Since the mutual repression between PXR and NFκB signaling was demonstrated in 2006,[4] pharmacologically activating PXR to antagonize NFκB activity was suggested for treating various diseases,[48] particularly the IBD.[5,8,49] In contrast to the adverse effects of the clinical PXR activator RIF, the herbal PXR activator SJW as a readily available anti-depressant and an FDA-classified dietary supplement, has a long history of long-term use and is generally believed to be safe. Whether SJW as a natural PXR activator alleviates experimental colitis depending on its activity of PXR activation is largely unknown, while the present data support the translational potential of SJW supplementation for possiblely repurposing as a supportive anti-IBD treatment. In line with earlier studies on the protective roles of pharmacotherapeutics on DSS-induced IBD via PXR-mediated antagonism of NFκB activation,[5,8,37,38,50–53] the present study further supports the therapeutic potential of this pathway in treating IBD.

In the current study, SJW or hyperforin, significantly induced the ileum *Cyp3a11* mRNA expression, which is a typical marker of PXR activation, in *PXR*-humanized mice but not *Pxr*-null mice in vivo, as well as in primary *PXR*-humanized organoids but not in *Pxr*-null organoids in vitro. sBoth SJW extracts and hyperforin induced PXR activation, which was further confirmed using PXR-driven luciferase reporter gene assays in Caco2 cells. In line with these data, previous reports found a similar ef- fect of SJW extracts or hyperforin on PXR activation of human hepatocytes.[27,28] The current study not only used the intestine-derived cell lines to further extend the effect of SJW extracts/hyperforin in activating intestinal PXR to antagonize TNFα-induced NFκB activation, but also employed primary intestinal organoids to demonstrate that SJW extracts or hyperforin could efficiently activate PXR to inhibit TNFα-induced NFκB activation in normal tissue-derived cells.

The crosstalk between the intestinal NFκB signaling and colitis remains elusive. The current study suggests a potential crosstalk between small bowl and colon. Intestinal NFκB activation at least partially contributes to colitis progression, thus pharmacologically targeting small intestinal NFκB signaling could potentially be used to treat IBD. In line with this, *Pxr*-null mice spontaneously developed greater inflammation in the small bowel but not in the colon,[54] indicating that PXR expressed in the small bowel plays a particularly important pathophysiological role in preventing the small ileum from inflammation. A recent study found a strict inverse correlation between colonic epithelial PXR levels and NFκB activation in colonic biopsies from Crohn's disease patients, and they also showed

that PXR activation by rifampicin, a human PXR activator, reduced mucosal NFκB activity as revealed by biopsies collected from IBD patients and in primary human intestinal organoids.[55] Thus, it's possible that small bowel PXR activation-mediated anti-inflammation could be pharmacologically targeted for treating IBD. The DSS-induced IBD model is manifest by severe inflammation in both the small bowel and the colon. In comparison to the previous finding that investigated whether PXR agonists alleviated DSS-induced IBD via activation of colon PXR,[8,37,51] SJW was found to activate the PXR expressed only in the small intestine and not in the colon, and notably SJW still alleviated DSS-induced both intestinal NFκB activation and colitis depending on the presence of PXR, suggesting that SJW dietary supplement targets PXR expressed in the small intestine to relieve the related colitis. However, to better validate the role of each tissue-specific PXR in modulating the progression of IBD and the therapeutic effect of SJW, tissue-specific PXR knockout mice, such as intestine-specific PXR and colon-specific PXR knockout mice, are still needed to distinguish the contribution of each tissue-specific PXR to IBD progression and the therapeutic effect of SJW. Given that PXR was found to be almost equally and ubiquitously expressed in the small intestine and colon at the mRNA levels,[4,8] the reason that SJW was only able to activate PXR expressed in the small intestine but not in the colon may be due to less distribution of hyperforin in the colon compared with that in the ileum or less response of colorectal PXR to hyperforin than ileum PXR.

While the present data demonstrate hyperforin as a potent PXR agonist, consistent with the previous publications,[13,27,28] it is unlikely that minor components of the extract have significant PXR activation potential since hyperforin has potent PXR agonist activity and is an abundant constituent of SJW [56] accounting for a marked percent (~3%) of all SJW components higher than that even hypericin (~0.1%–0.3%), another major active component of SJW.[56] Nevertheless, multiple components of traditional herbs may still synergize to produce the pharmacological effect, and the current study cannot exclude the possible involvement of other SJW components that have not yet been tested for PXR agonist activity in contributing to the anti-IBD effect of SJW. Whether hyperforin has anti-IBD effects in vivo were not examined in the current study due to the unavailability of sufficient amounts of hyperforin for animal experimentation. On the other hand, cystic fibrosis transmembrane conductance regulator (CFTR) mutations were found to be associated with increased bowel inflammation in patients,[57] and disturbed immune response in the distal small intestine of *Cftr*-null mice caused intestinal inflammation accompanied by a strong down-regulation of PXR target gene expression[58] as well as a reduction of intestinal farnesoid x receptor activity.[59] These studies indicate that other targets beyond PXR located in the distal ileum may also mediate the progression of IBD. However, whether SJW alleviated IBD via modulating the other targets was not explored in this study.

In summary, by the use of DSS treatment in *PXR*-humanized mice, *Pxr*-null mice and WT mice in vivo, TNFα treatment in the primary intestinal organoids, and luciferase assays in Caco2 cells in vitro, the current study demonstrates that SJW PXR-dependently decreases the susceptibility of mice to DSS-induced colitis via PXR activation-mediated suppression of NFκB activation in vivo and in vitro. These findings suggest that the widely used food supplement SJW has therapeutic potential in the management of human IBD. With SJW as a chemical probe that could only activate ileum but not colon PXR, this study suggests

that targeting PXR in the small intestine to antagonize intestinal NFκB activation could potentially relieve IBD. Hyperforin, a potent PXR agonist from SJW, may be regarded as a lead compound for future anti-IBD drug discovery. In light of the deleterious adverse effects associated with the clinical anti-IBD drugs, the current study supports the potential to explore safer PXR activator from herbal supplement for IBD treatment. SJW may be recommended for use in IBD patients and deserves further proof-of-concept clinical trials. Notably, it is still difficult to translate the current findings to improved patient care and thus clinical trials in IBD patients[60] are needed to repurpose SJW as an anti-IBD drug.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This project was supported by the National Cancer Institute Intramural Research Program, Center of Cancer Research, and the Office of Dietary Supplements, National Institutes of Health. Yangliu Xia was supported by the Chinese Scholarship Council (201806065070) and Ping Wang was supported by the Natural Science Foundation of Shanghai (18ZR1436500). We thank Linda G. Byrd for preparing the animal protocols, Lulu Sun for expert advice on isolation of primary intestinal organoids, and Fei Li and Grace L. Guo for advice on data interpretation. SJW was provided by Euromed USA, Inc.

Funding information

HHS | NIH | National Cancer Institute (NCI); Office of Dietary Supplements (ODS); Chinese Scholarship Council, Grant/Award Number: 201806065070; National Science Foundation of Shanghai, Grant/Award Number: 18ZR1436500

Abbreviations:

ACTB	β-actin
CFTR	cystic fibrosis transmembrane conductance regulator
DAI	disease activity index
DMSO	dimethyl sulfoxide
DSS	dextran sodium sulfate
H&E	hematoxylin and eosin
IBD	inflammatory bowel disease
IκB	NFκB inhibitor
LMNB1	Lamin B1
NFκB	nuclear factor kappa B
PBS	phosphorylated buffer saline
PCN	pregnenolone 16 α-carbonitrile
PXR	pregnane X receptor

qPCR	quantitative polymerase chain reaction
RIF	rifaximin
SJW	St. John's wort
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	tumor necrosis factor
WT	wild-type

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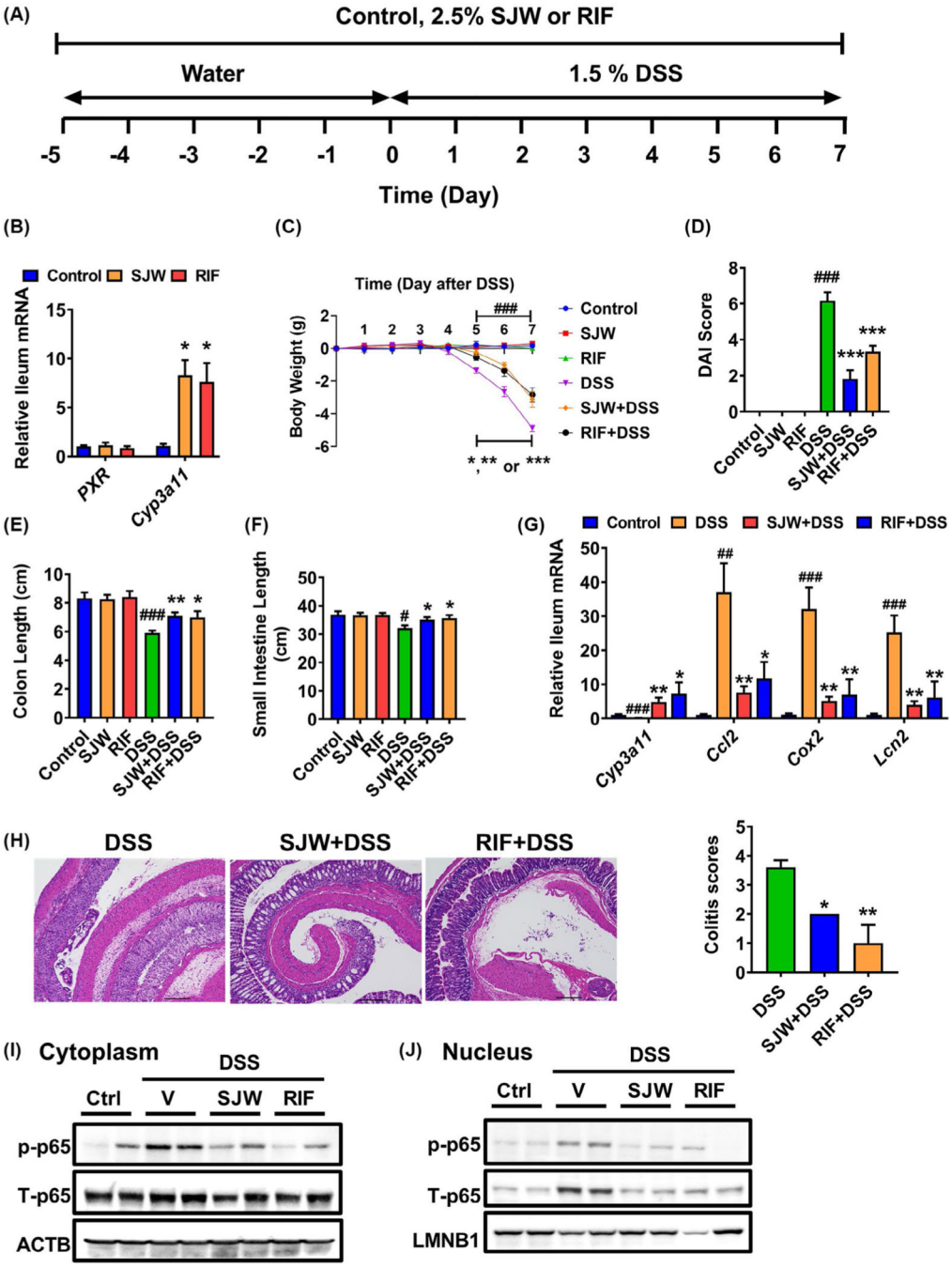

FIGURE 1.

St. John's wort (SJW) activated ileum PXR and prevented DSS-induced IBD in *PXR*-humanized mice. (A) Experimental scheme. (B) mRNA of ileum *PXR* and *Cyp3a11* in water-treated *PXR*-humanized mice. (C) Body weight change. (D) Disease activity index scores. (E) Colon length. (F) Small intestine length. (G) Relative ileum *Cyp3a11*, *Ccl2*, *Cox2*, and *Lcn2* mRNAs in ileums of DSS-treated *PXR*-humanized mice. (H) H&E staining and histological scores for colons, scale bar 100 μm. (I,J) Western bolt analyses of cytoplasm protein (I) and nuclear protein (J) isolated from the distal ileum of *PXR*-humanized mice.

Group legends for I and J: Ctrl, control group; V, DSS group; SJW, SJW+DSS group; RIF, RIF+DSS group. Group legends for the other groups: Control, mice treated with control diet and water ($N = 5$); SJW, mice treated with 2.5% SJW diet and water ($N = 5$); RIF, mice treated with RIF diet and water ($N = 5$); DSS, mice treated with 1.5% DSS and control diet ($N = 6$); SJW+DSS, mice treated with 1.5% DSS and 2.5% SJW diet ($N = 6$). RIF+DSS, mice treated with 1.5% DSS and RIF diet ($N = 6$). Data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA. #$p < .05$, ##$p < .01$ and ###$p < .005$ versus Control group. *$p < .05$, **$p < .01$ and ***$p < .005$ versus DSS group

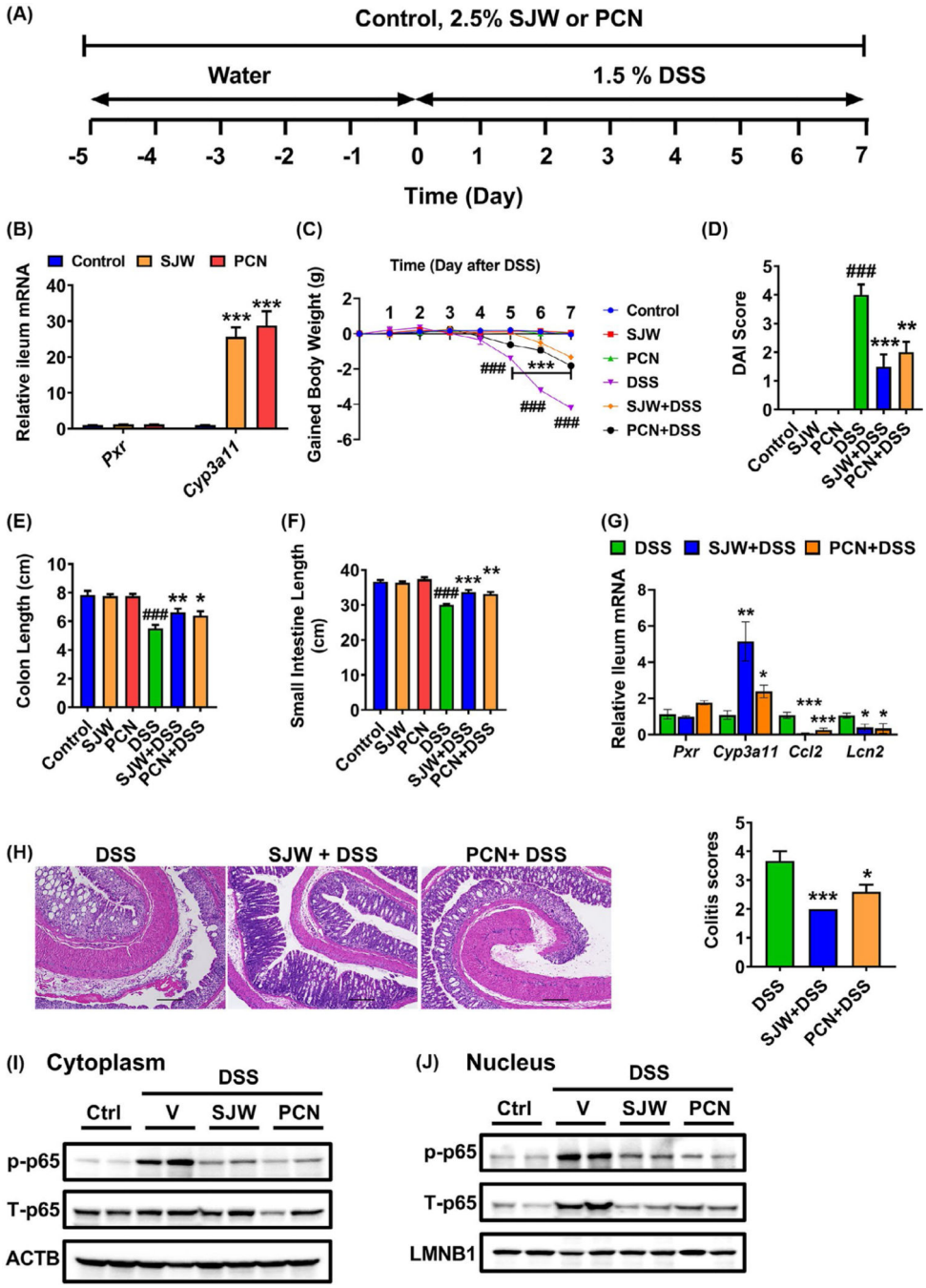

FIGURE 2.

St. John's wort activated ileum PXR and prevented DSS-induced IBD in C57BL/6N WT mice. (A) Experimental scheme. (B) mRNA of ileum *PXR* and *Cyp3a11* in water-treated WT mice. (C) Body weight change. (D) Disease activity index scores. (E) Colon length. (F) Small intestine length. (G) Relative mRNA expression of ileum *Cyp3a11*, *Ccl2*, *Cox2*, and *Lcn2* in the ileums of DSS-treated WT mice. (H) H&E staining and histological scores for colons, scale bar 100 μm. (I,J) Western bolt analyses of cytoplasmic (I) and nuclear proteins (J) from the distal ileum of WT mice. Group legends were similarly described as

in Figure 1, except PCN, mice treated with PCN and water; PCN+DSS, mice treated with 1.5% DSS and PCN. $N = 5–6$ for water-fed groups, and $N = 6$ for DSS-fed groups. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA. Data were presented as mean ± SEM. #$p < .05$, ##$p < .01$ and ###$p < .005$ versus Control group. *$p < .05$, **$p < .01$ and ***$p < .005$ versus DSS group

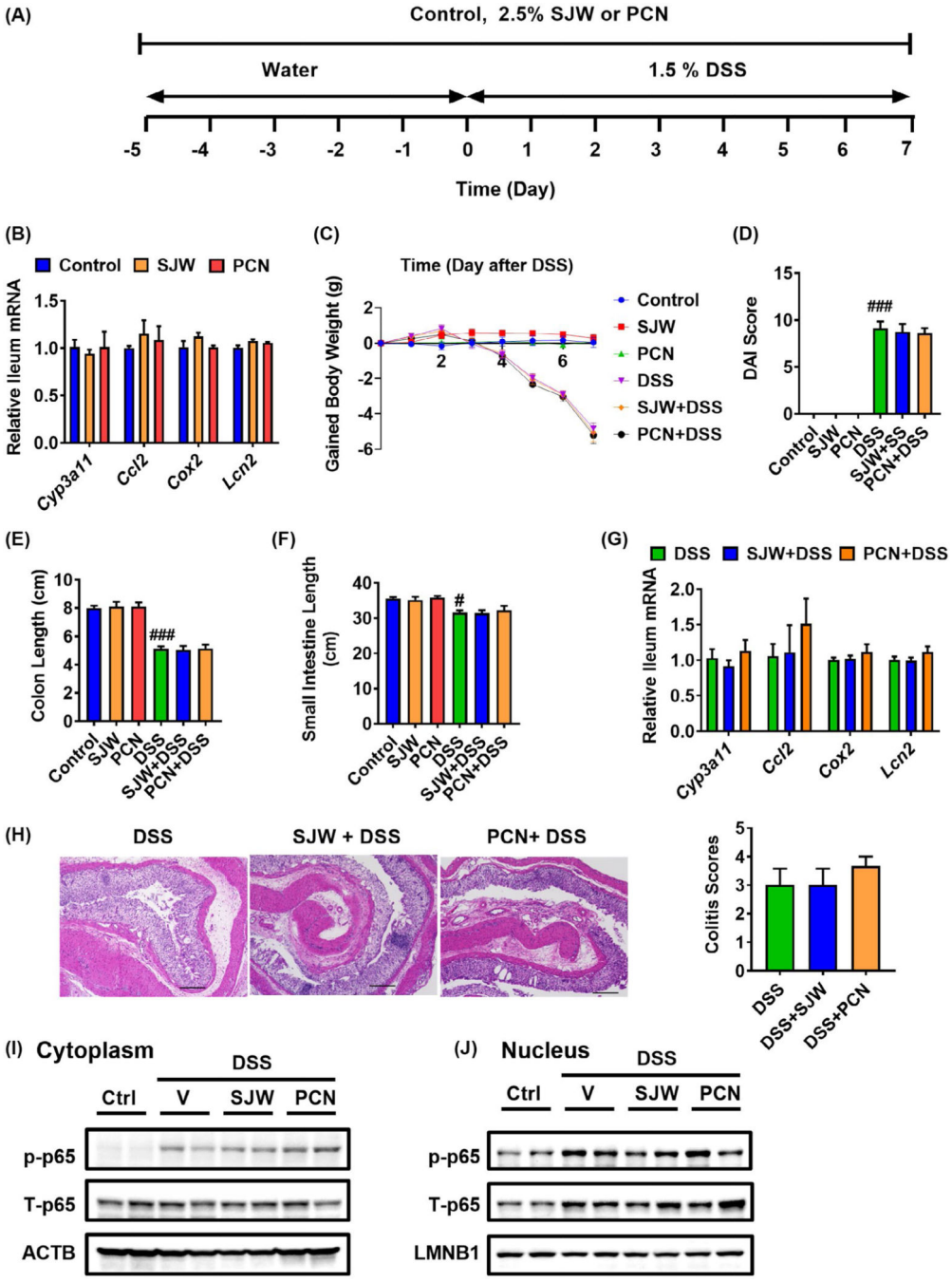

FIGURE 3.

St. John's wort failed to reduce DSS-induced IBD in *Pxr*-null mice. (A) Experimental scheme. (B) Expression of ileum *Cyp3a11* mRNA and proinflammatory gene mRNAs in water-treated *Pxr*-null mice. (C) Body weight change. (D) Disease activity index scores. (E) Colon length. (F) Small intestine length. (G) Relative levels of ileum *Cyp3a11*, *Ccl2*, *Cox2*, and *Lcn2* mRNAs in ileums of DSS-treated *Pxr*-null mice. (H) H&E staining and histological scores for colons, scale bar 100 μm. (I,J) Western bolt analyses of cytoplasm (I) and nuclear proteins (J) isolated from the distal ileum of *Pxr*-null mice. Group legends are

similarly described as in the Figure 2 legend. $N = 5$ for all groups. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA. Data are presented as mean ± SEM. Statistical analyses was performed using one-way ANOVA. #$p < .05$, ##$p < .01$ and ###$p < .005$ versus Control group. *$p < .05$, **$p < .01$ and ***$p < .005$ versus DSS group

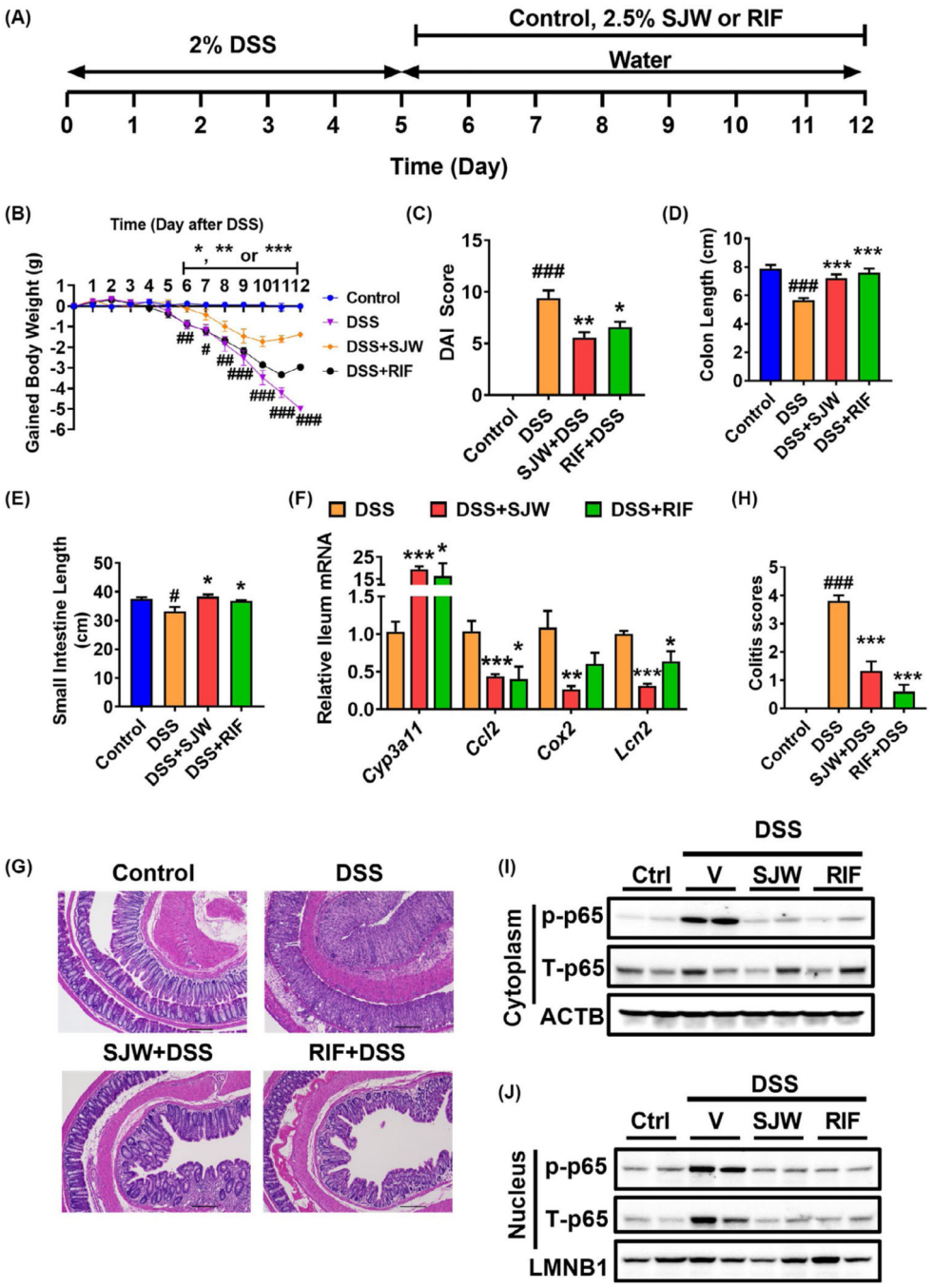

FIGURE 4.

St. John's wort (SJW) ileum PXR and prevented DSS-induced IBD in *PXR*-humanized mice. (A) Experimental scheme. (B) Body weight change. (C) Disease activity index scores. (D) Colon length. (E) Small intestine length. (F) Relative levels of ileum *Cyp3a11*, *Ccl2*, *Cox2*, and *Lcn2* mRNAs in the ileum of DSS-treated *PXR*-humanized mice. (G) H&E staining for colons, scale bar 100 μm. (H) Histological scores of colons. (I,J) Western blot analyses of cytoplasmic (I) and nuclear proteins (J) isolated from the distal ileum of *PXR*-humanized mice. Group legends for I and J: Ctrl, control group; V, DSS group; SJW,

SJW+DSS group; RIF, RIF+DSS group. Group legends for the other groups: Control, mice treated with control diet and water; DSS, mice treated with 2% DSS and control diet; SJW+DSS, mice treated with 2% DSS and 2.5% SJW diet. RIF+DSS, mice treated with 2% DSS and RIF diet. $N = 5$. Data are presented as mean ± SEM. Statistical analyses was performed using one-way ANOVA. #$p < .05$, ##$p < .01$ and ###$p < .005$ versus Control group. *$p < .05$, **$p < .01$ and ***$p < .005$ versus DSS group

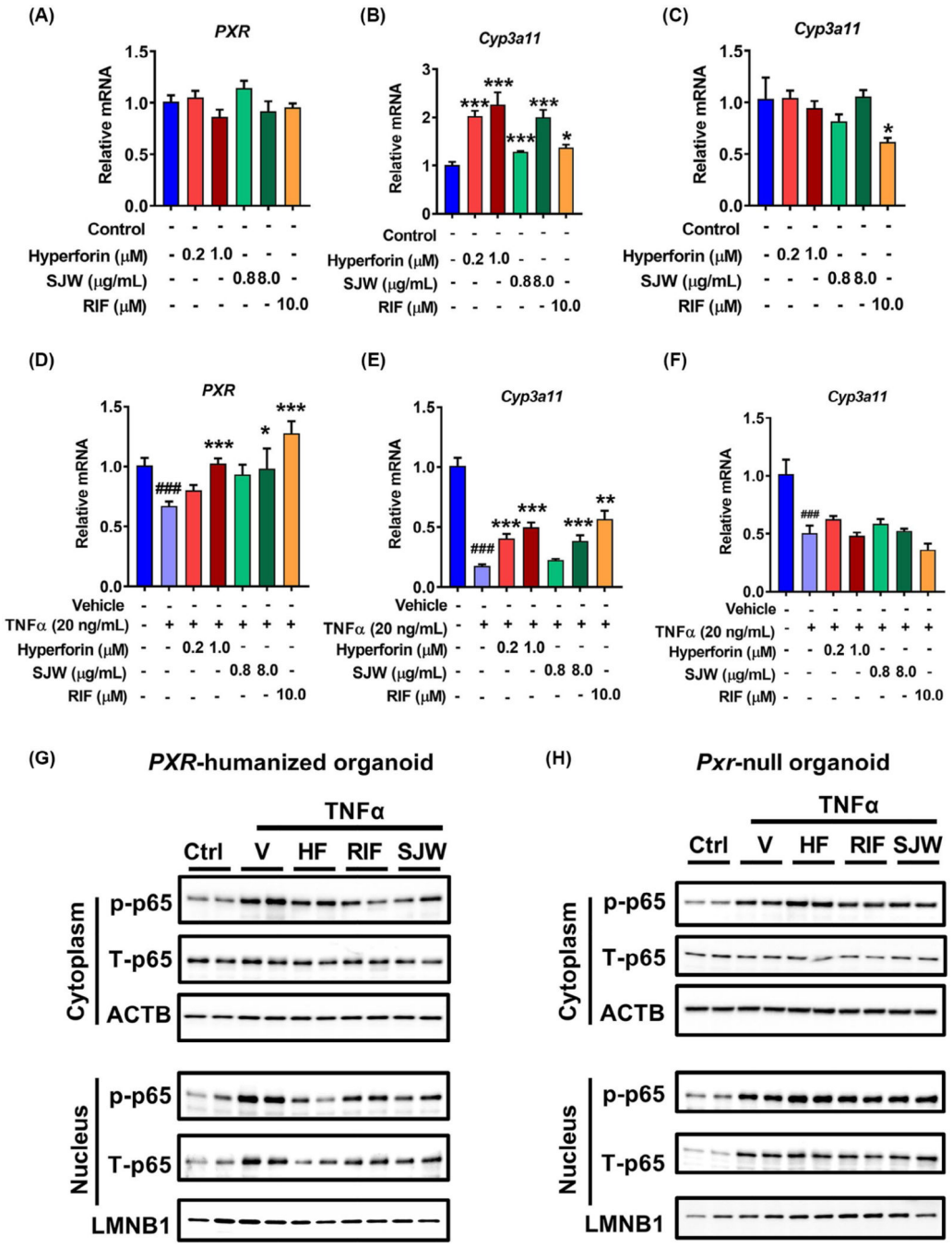

FIGURE 5.

Both St. John's wort (SJW) extracts and hyperforin activate PXR and alleviate TNFα-induced NFκB activation in primary ileum organoids isolated from *PXR*-humanized mice, but not *Pxr*-null mice. (A,B) *PXR* (A) and *Cyp3a11* (B) mRNA levels in hyperforin or SJW extract-treated primary organoids isolated from *PXR*-humanized mice. (C) *Cyp3a11* mRNA in hyperforin or SJW extracts-treated primary organoids isolated from *Pxr*-null mice. (D,E) *PXR* (D) and *Cyp3a11* (E) mRNA levels in hyperforin or SJW extract-pretreated and TNFα-treated primary organoids isolated from *PXR*-humanized mice. (F) *Cyp3a11* mRNA

levels in hyperforin or SJW extracts-pretreated and TNFα-treated primary organoids isolated from *Pxr*-null mice. (G,H) Effects of hyperforin or SJW extracts on TNFα-induced NFκB activation in primary ileum organoid cells isolated from *PXR*-humanized mice (G) or from *Pxr*-null mice (H). RIF at 10 μM was used as the positive control. Data are presented as mean ± SEM. $N = 3$. Ctrl, organoids treated with control vehicle; V, organoids treated with control vehicle and 20 ng/ml of TNFα. HF, organoids treated with 1 μM of hyperforin and 20 ng/ml of TNFα; RIF, organoids treated with 10 μM of RIF and 20 ng/ml of TNFα; SJW, organoids treated with 8.0 μg/ml of SJW extracts and 20 ng/ml of TNFα. Statistical analysis was performed using one-way ANOVA. #$p < .05$ and ###$p < .005$ versus Control group; *$p < .05$, **$p < .01$ and ***$p < .005$ versus TNFα group

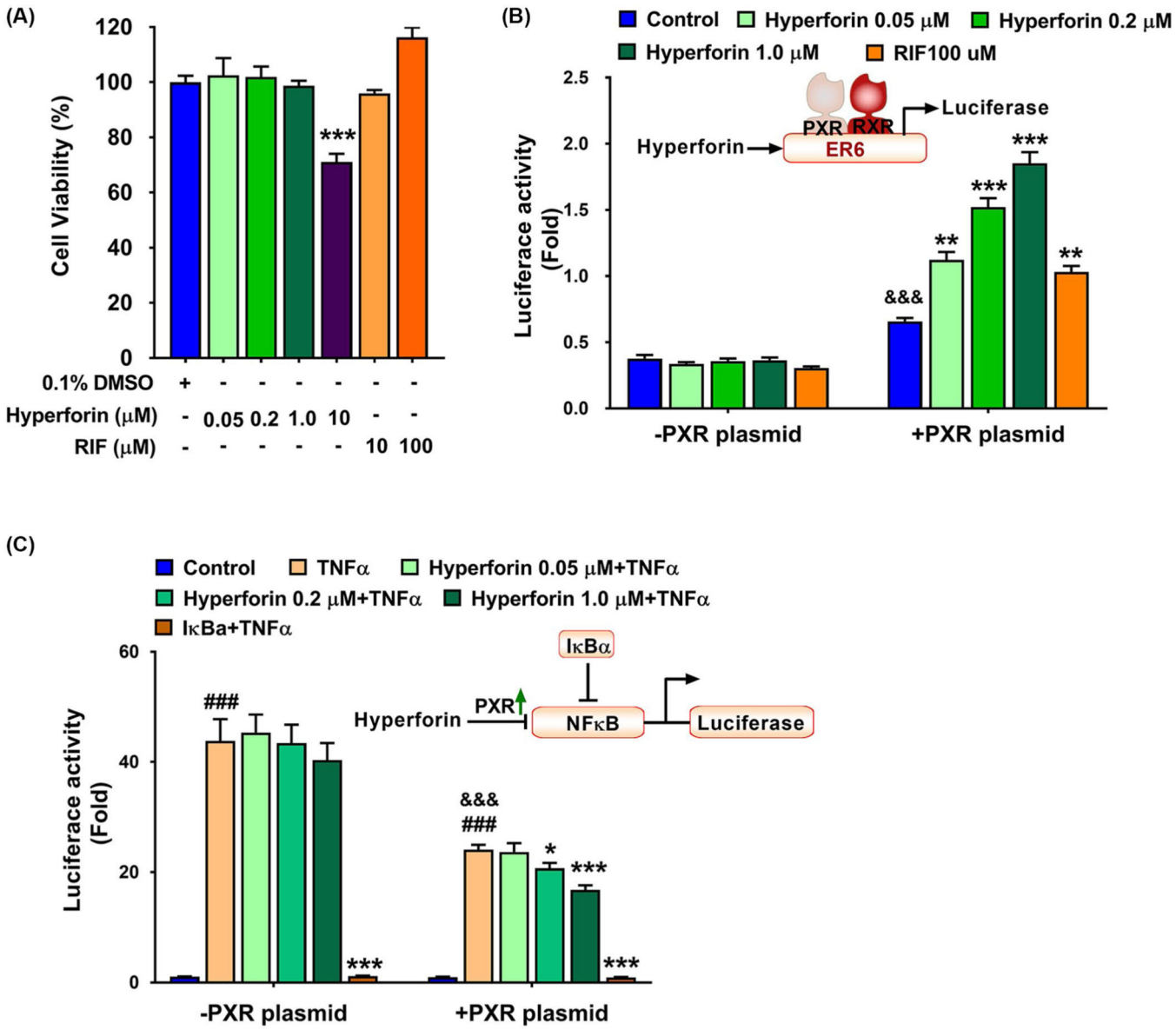

FIGURE 6.

Luciferase assays showed that hyperforin activated PXR to antagonize TNFα-induced NFκB transactivation in Caco2 cells. (A) Cell viability of hyperforin or RIF-treated Caco2 cells. (B) Luciferase activities in hyperforin or RIF-treated Caco2 cells transfected with or without PXR plasmid, while all cells were transfected with RXR plasmid and ER6-luciferase reporter plasmid. (C) Luciferase activities in hyperforin or IκBα plasmid co-transfected Caco2 cells transfected with or without human PXR plasmid, while all cells were transfected with NFκB luciferase reporter plasmid. $N = 4$. Data are presented as mean ± SEM. Statistical analyses was performed using one-way ANOVA. $^{**}p < .01$ and $^{***}p < .005$ versus Control group for Figure 6A,B; $^{*}p < .05$ and $^{***}p < .005$ versus TNFα group for Figure 6C. $^{###}p < .005$ versus Control group. $^{\&\&\&}p < .005$ represents comparison between Control groups with or without PXR plasmid transfection

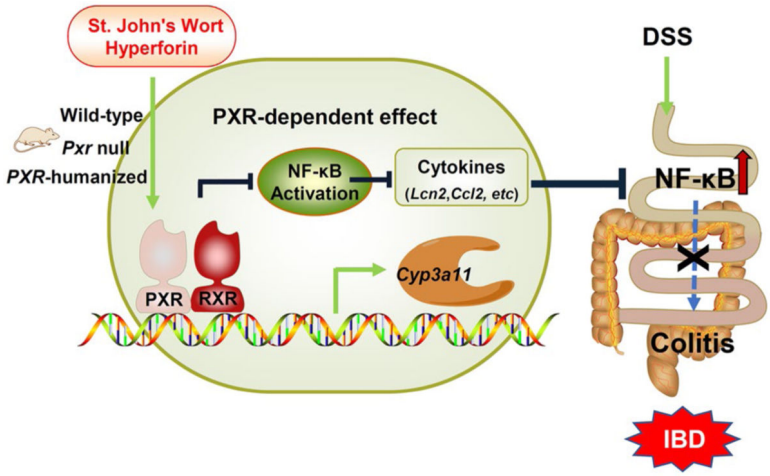

FIGURE 7.

Proposed model. In DSS-induced IBD model, St. John's Wort (SJW) was found to activate PXR in the ileum, but not in the colon, and to PXR-dependently prevent DSS-induced IBD in vivo. Mechanistically, hyperforin, a potent PXR agonist and major constituent of SJW, inhibits TNFα-induced inflammation in primary intestinal organoids and antagonizes TNFα-induced NFκB transactivation by activating PXR in Caco2 cells. A model is proposed whereby SJW dietary supplementation, via hyperforin, activates PXR in the small intestine to antagonize NFκB signaling, which in turn improves IBD.