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Hyperforin, the Active Component of St. John's Wort, Induces IL-8 Expression in Human Intestinal Epithelial Cells *Via* a MAPK-Dependent, NF-κB-Independent Pathway

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St. John's wort is widely used as an herbal antidepressant and is among the top-selling botanical products in the United States. Although St. John's wort has been reported to have minimal side effects compared with other antidepressants, here we show that hyperforin, the active component of St. John's wort, can stimulate interleukin-8 (IL-8) expression in human intestinal epithelia cells (IEC) and primary hepatocytes. Hyperforin is also able to induce expression of mRNA, encoding another major inflammatory mediator—intercellular adhesion molecule-1 (ICAM-1). IEC participate in the intestinal inflammatory process and serve as a first line of defense through bidirectional communication between host and infectious pathogens. Although hyperforin is a potent ligand for the steroid and xenobiotic receptor (SXR), we found that hyperforin induced IL-8 mRNA through an SXR-independent transcriptional activation pathway. IL-8 induction by hyperforin required the activation of AP-1 but not the NF-κB transcription factor, thereby distinguishing it from the NF-κB-dependent IL-8 induction mediated by tumor necrosis factor α (TNFα). Further study revealed that extracellular signal-regulated kinase 1 and 2 (ERK1/2) were required for the hyperforin-induced expression of IL-8. Our results suggest a previously unsuspected effect of St. John's wort in modulating the immune and inflammatory responses.

KEY WORDS: Interleukin-8; St. John's wort; hyperforin; extracellular signal-regulated kinase; AP-1.

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INTRODUCTION

St. John's wort (*Hypericum perforatum*) is a long-lived, wild-growing herb that has been used for centuries to treat a variety of ailments including bruises, dysentery, jaundice, diarrhea, and a wide range of other complaints (1). In recent years, St. John's wort has become increasingly used as an herbal alternative to antidepressant drugs for the treatment of mild to moderate clinical depression. It also is used to treat anxiety, seasonal affective disorder, and sleep disorders (2, 3).

The use of St. John's wort is growing rapidly in the United States with sales of $210 million in 2001, up from $140 million in 1998 (4), placing it among the top-selling botanical products (5). St. John's wort contains a dozen major components; however, hyperforin is the key compound responsible for the herb's antidepressant effects (6, 7). The antidepressant activity of hyperforin is believed to reside in its ability to inhibit the synaptic reuptake of a variety of neurotransmitters in the brain (8). Hyperforin is unique among all known antidepressants for being a potent reuptake inhibitor of three of the most important neurotransmitters: serotonin, noradrenaline, and dopamine (9, 10). Moreover, it is also an effective inhibitor of L-glutamate and gamma-amino-*n*-butyric acid (GABA) uptake (11). The molecular mechanism of hyperforin's broad-spectrum reuptake inhibition of neurotransmitters is still unclear.

In addition to its antidepressant action, there is accumulating evidence that St. John's wort interacts with a variety of drugs. Hyperforin was shown to be able to induce drug metabolism through activation of the steroid and xenobiotic receptor (SXR) (also known as PXR (12), PAR (13), and NR1I2 (14)), an orphan nuclear receptor that regulates expression of the key xenobiotic metabolizing

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enzymes such as cytochrome P450 (CYP) 3A4 (15, 16). This provides a molecular mechanism for the interaction of St. John's wort with various drugs. In addition to its effects on neurotransmitter reuptake and drug metabolism, hyperforin was recently shown to inhibit the proliferation of several tumor cell lines (17). It was suggested that hyperforin activates a mitochondria-mediated apoptosis pathway by leading to the release of cytochrome *c*.

Although St. John's wort has been reported to have an improved side-effect profile comparing to other conventional antidepressants (3, 4), the majority of side effects are gastrointestinal symptoms, especially nausea and abdominal discomfort (18, 19). Here we show that hyperforin can induce expression of interleukin-8 (IL-8) in intestinal epithelial cells (IEC) in a time and dose-dependent manner. Hyperforin was also able to induce IL-8 expression in human primary hepatocytes and THP-1 monocytes. IL-8 is a member of the CXC family of chemokines and acts as an important chemoattractant and neutrophil activator (20). IL-8 can be produced by a wide variety of cell types and is believed to play a significant role in the initiation and maintenance of the inflammatory response (21). IEC are an integral component of the enteric immune system and exhibit a substantial inflammatory response to various extracellular stimuli including cytokines and bacterial products (22). IEC can produce large amounts of IL-8 and are thought to act as a first line of defense *via* bidirectional communication between host and infectious pathogens (23, 24). We show that IL-8 mRNA induction by hyperforin results from increased transcription through an SXR-independent pathway. Our data also show that hyperforin-induced expression of IL-8 in IEC requires the activation of AP-1 but not the NF-κB transcription factor. This induction is thus distinct from the tumor necrosis factor α (TNFα)-mediated pathway that requires NF-κB. Experiments using specific inhibitors of phosphorylation demonstrated that the mitogen activated protein kinase (MAPK) ERK1/2 was required for the hyperforin-induced expression of IL-8. Finally, we found that hyperforin was also able to induce another major inflammatory mediator—intercellular adhesion molecule-1 (ICAM-1; CD54) gene expression. Our demonstration that hyperforin modulates the expression of IL-8 in IEC and other cell lines including primary hepatocytes and THP-1 monocytes suggests a previously unsuspected role for St. John's wort in modulating the immune and inflammatory responses.

MATERIALS AND METHODS

Cells and Reagents

Human IEC lines, LS180 and CaCo-2 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagles's medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were seeded into 6-well plates and grown in DMEM-10% FBS until 70–80% of confluence. Twenty-four hours before treatment, the medium was replaced with DMEM containing 10% resin-charcoal stripped FBS. Immediately before treatment, the medium was removed; the cells were washed once with PBS and then treated with different reagents or methanol (MeOH) vehicle for appropriate times. The THP-1 monocytic cell line was also obtained from the American Type Culture Collection and were cultured in RPMI 1640 medium containing 10% FBS. Human primary hepatocytes were obtained from LTPADS (Liver Tissue Procurement and Distribution System, Pittsburgh, Pennsylvania). This material was provided in the form of attached cells in 6-well plates. The hepatocytes were maintained in Hepatocyte Medium (Sigma, St. Louis, MO) for at least 24 h before treatment. Hyperforin was purchased from AApin Chemicals (UK) and its purity was confirmed by mass spectrometry. TNFα, rifampicin (RIF), mifepristone (RU486), actinomycin D (Act D), and cycloheximide (CHX) were purchased from Sigma. PD98059, SB203580, and pyrrolidinedithiocarbamate (PDTC) were purchased from Calbiochem (San Diego, CA). U0126 was purchased from Promega (Madison, WI).

Northern Blot Analysis

Total RNA was isolated from LS180 cells, using TRIzol regent (InVitrogen Life Technology, CA) according to the manufacturer-supplied protocol. Fifteen micrograms of total RNA were loaded into each lane of a denaturing formaldehyde-agarose gel. After electrophoresis, the RNA was transferred to Nytran supercharge membrane (Schleicher and Schuell). Northern blots were performed using IL-8 (accession #NM_000584, nucleotide 474-892); ICAM-1 (accession #X06990, nucleotide 781-1491), and GAPDH (#NM_002046, nucleotide 625-1189) probes following standard methods (25).

Quantitative Real-Time RT-PCR

For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase according to the manufacturer-supplied protocol (InVitrogen Life Technology, CA). Quantitative real time (QRT-PCR) was performed using the following primer sets: IL-8 (5′-TCTGGCAACCCTAGTCTGCT-3′) (5′-AAACCAAGGCACAGTGGAAC-3′), GAPDH (5′-GGC CTCCAAGGAGTAAGACC-3′) (5′-AGGGGAGATTCA GTGTGGTG-3′), and the SYBR green PCR kit (Applied Biosystems) in a DNA Engine Opticon–Continuous

Fluorescence Detection System (MJ Research). All samples were quantitated using the comparative Ct method for relative quantitation of gene expression, normalized to GAPDH (26).

IL-8 ELISA

LS180 cells were cultured in 12-well plates until confluence and THP-1 cells were incubated at 2×10^6 cells/mL. Cells were then treated with hyperforin (1–10 μM) for 8 h. Medium was collected for quantification of IL-8 protein levels by commercial Human IL-8 ELISA Kit II following the manufacturer's instruction (BD Biosciences Pharmingen, CA). All assays were performed in duplicate using serial dilution.

Nuclear Protein Isolation and Electrophoretic Mobility Shift Assay (EMSA)

LS180 cells growing in T75 flasks were treated with DMEM containing hyperforin, TNFα, or MeOH vehicle for various times. Nuclear proteins were prepared by standard methods and aliquots were stored at −80°C until use (27). Protein concentrations were determined using the Bradford method (Bio-Rad, CA). The IL-8 NF-κB binding site was created by annealing the oligonucleotide 5′-ATCGTGGAATTTCCTCTGACA-3′ to its complementary strand. Other binding sites were as follows (point mutations are underlined): IL-8 mutant NF-κB (mNF-κB), 5′-ATCGTTAACTTTCCTCTGACA-3′; IL-8 AP-1, 5′-AGTGTGATGACTCAGGTTTG-3′; IL-8 mutant AP-1 (mAP-1), 5′-AGTGTGATATCTCAGGTTTG-3′; IL-8 C/EBP, 5′-CATCAGTTGCAAATCGTGGA-3′; IL-8 mutant C/EBP, 5′-CATAGCTTGCAAATCGTGGA-3′ (28). Double-stranded oligonucleotides were end-labeled using T4 DNA polynucleotide kinase (InVitrogen Life Technology, CA) and γ-^{32}P-ATP (Perkin Elmer Life Sciences, MA). Ten micrograms of nuclear extracts were incubated with 2 μg of poly (dI-dC) (Promega, WI), 2 μL of bandshift buffer (50-mM $MgCl_2$, 340-mM KCl), and 6 μL of delta buffer (0.1-mM EDTA, 40-mM KCl, 25-mM Hepes (pH 7.6), 8% Ficoll 400, 1-mM dithiothreitol) on ice for 10 min. ^{32}P-labeled double-stranded oligonucleotide probe (100,000 cpm) was then added, and the reaction was incubated for another 20 min on ice (29). For supershift assays, the appropriate amount of antibody was added to the nuclear extract and the reaction incubated on ice for 30 min before addition of the probe. The c-Jun antibody, anti-NF-κB p65 and p50 antibodies were purchased from Santa Cruz Biotechnology (CA). The binding complexes were subjected to electrophoresis in a 6% nondenaturing polyacrylamide gel containing 0.5 × TBE. The gels were dried, and the complexes were visualized on a Phosphorimager (Molecular Dynamics-Amersham BioSciences, NJ.

Transient Transfection and Luciferase Assay

VP16-SXR was constructed by fusing the potent transactivation domain from the Herpes virus VP16 protein (30) to the amino terminus of full-length SXR (31). LS180 cells were seeded into 6-well plates and transfected using Lipofectamine 2000 (InVitrogen Life Technology, CA) with either VP16-SXR or VP16 alone as a control for 48 h. Total RNA was isolated from transfected cells and used for real-time RT-PCR analysis.

BAC clone RP11-447E20 (GENBANK accession #: AC112518) containing the whole IL-8 gene and promoter region was used as the template to amplify the 7 kb (−7020 to +40) and 4 kb (−3973 to +40) IL-8 promoter region. Primers were as follows with regions corresponding to vector sequence and enzyme digestion sites underlined: −7kb: 5′-ACCCGGGAGGTACCCAGTATCTCAAAAC TCATACACAA-3′; −4 kb: 5′-ACCCGGGAGGTACCTT GCTCAAATCCAATGTGTTCAG-3′; +40 5′-CGGAAT GCCAAGCTTTGTGTGCTCTGCTGTCTCTG-3′. The PCR products were subcloned into *Asp718-Hind*III sites of the vector pGL2-basic (Promega, WI) using Exonuclease III-mediated ligation independent cloning (32).

IL-8 reporter constructs containing 1521 bp of the promoter region (nucleotides −1481 to +40) of the human IL-8 gene fused to luciferase, or the same construct harboring mutations in the NF-κB, AP-1, or C/EBP sites were provided by Dr C. Pothoulakis (Harvard Medical School, MA) (29). The substitution mutants were based on the sequences described by Wu *et al*. (33). The mutated NF-κB sequence was cgTTAACTTTCCtct; the mutated AP-1 sequence was gaTATCTCAgg; and the mutated C/EBP sequence was tcAGCTACGAGTcg. The AP-1-dependent reporter $(cAP\text{-}1)_3$-LUC and NF-κB-dependent reporter $(cNF\text{-}\kappa B)_3$-LUC constructs were previously described (34). The dominate negative MEK (MEK-KA) construct, encoding a mutant MEK in which the ATP binding site has been mutated, rendering it catalytically inactive, was kindly provided by Dr L. Bardwell (UC, Irvine) (35). The identity of each construct was confirmed by DNA sequence analysis.

To determine the response of the IL-8 promoter constructs to hyperforin or TNFα, LS180 cells were seeded into 12-well plates overnight and transiently transfected with the IL-8 reporters plus CMX-β-galactosidase transfection control using Lipofectamine 2000 in serum-free DMEM. Twenty-four hour posttransfection, cells were treated with hyperforin, TNFα or vehicle for 8 h. The cells were then lysed *in situ* and extracts prepared and assayed for β-galactosidase and luciferase activity as

described (31). Reporter gene activity was normalized to the β-galactosidase transfection controls and the results expressed as normalized RLU per OD β-galactosidase per minute to facilitate comparisons between plates. Fold induction was calculated relative to solvent controls. Each data point represents the average of triplicate experiments ± standard error and was replicated in independent experiments.

Western Blotting

After treatment with hyperforin, LS180 cells were washed and lysed in 150 μL of RIPA buffer (137-mM NaCl, 20-mM Tris-HCL, PH 7.5, 1% Triton X-100, 0.5% NP-40, 10% glycerol, 2-mM EDTA, pH 8.0; all from Sigma). One tablet of complete protease inhibitor cocktail (Roche Molecular Biochemicals) was added to 50-mL RIPA buffer immediately before use. Lysates were recovered, protein concentrations were determined using the Bradford method (Bio-Rad, CA), and lysates were stored at −80°C. Equal amounts of protein were loaded and separated by SDS-PAGE, then electroblotted to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with Tris-buffered saline containing 5% nonfat dry milk for 1 h at room temperature. The blots were probed with rabbit anti-human polyclonal antibodies for phosphorylated-ERK (1:1000; kindly provided by Dr L. Bardwell, UC, Irvine) overnight at 4°C. Blots were also probed with ERK2 antibodies to ensure the equal ERK protein loading. Blots were then washed and exposed to goat anti-rabbit HRP conjugated antibodies (1:10,000, Santa Cruz Biotechnology, CA) for 1 h at room temperature. Chemiluminescence was detected by using the Amersham ECL kit (Amersham Biosciences, Little Chalfont, UK).

RESULTS

Hyperforin Stimulates IL-8 Gene Expression and Protein Secretion in LS180 Human IEC

Hyperforin, the major antidepressant constituent of St. John's wort, is known to inhibit the neuronal uptake of serotonin, norepinephrine, dopamine, GABA, and L-glutamate. It is also an effective ligand for the nuclear receptor SXR (16). Treatment of the IEC line, LS180, with hyperforin and other SXR ligands has been shown to induce the expression of SXR target genes such as CYP3A4 and Mdr1 (36, 37) (and data not shown). While testing the ability of SXR ligands to induce target gene expression in a cell-type specific manner, we observed that hyperforin was able to induce IL-8 gene expression whereas

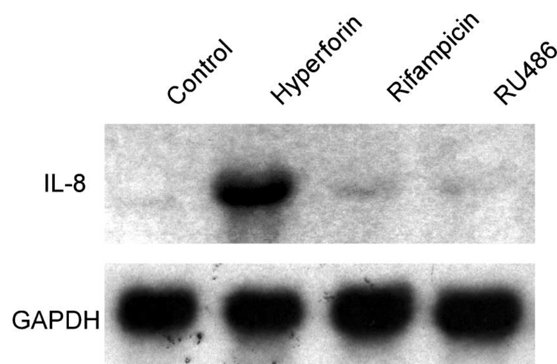

Fig. 1. Hyperforin induces IL-8 mRNA expression. LS180 cells were treated with 10-μM hyperforin, rifampicin or RU486 for 24 h. Total RNA was isolated and analyzed for IL-8 mRNA by Northern blot hybridization. Hybridization with GAPDH probes was used to verify the RNA input in the Northern blot membrane.

other SXR ligands such as RIF or RU486 did not (Fig. 1). Steady-state levels of IL-8 mRNA were upregulated by as little as 2.5-μM hyperforin and increased with hyperforin concentration (Fig. 2A). The time course of IL-8 mRNA expression in response to hyperforin was also investigated (Fig. 2B). IL-8 transcripts were induced as early as 1 h after hyperforin treatment, and reached maximum levels after 16 h of treatment (Fig. 2B). Exposure of LS180 cells to hyperforin also strongly stimulated IL-8 protein secretion (Fig. 2C). IL-8 secretion induced by hyperforin was dose-dependent at concentrations ranging between 1 and 10 μM, and significant induction was obtained at 2.5 μM. Taken together, these results demonstrate that hyperforin stimulates IL-8 gene expression as well as protein secretion in human IEC-LS180 cells.

IL-8 is an Immediate Early Target of Hyperforin

The IL-8 gene is regulated at both the transcriptional and posttranscriptional levels (38–41). To investigate the nature of the IL-8 induction by hyperforin, we employed several standard methods to distinguish between transcriptional and posttranscriptional modes of regulation. Actinomycin D treatment was employed to determine whether the hyperforin-mediated induction of steady-state IL-8 mRNA levels resulted from increased transcription of the IL-8 gene. After 2 h of treatment, increased levels of IL-8 mRNA were observed in LS180 cells treated with 10-μM hyperforin or 10 ng/mL TNFα, a known inducer of IL-8 transcription (Fig. 3A) (38). Pretreatment with actinomycin D inhibited both hyperforin- and TNFα-induced increases in IL-8 mRNA levels, suggesting that these increases are dependent on new transcription (Fig. 3A).

To determine whether ongoing protein synthesis is required for hyperforin induction of IL-8 expression, LS180

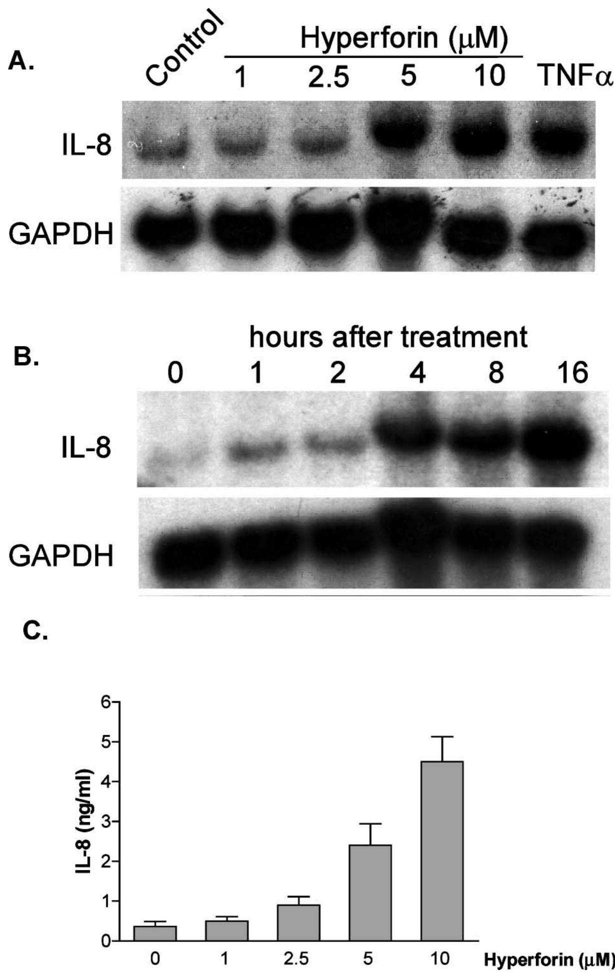

Fig. 2. Dose- and time-dependent accumulation of IL-8 mRNA and protein secretion by hyperforin. (A) LS180 cells were treated with 10-μM hyperforin for various times as indicated or (B) with different concentrations of hyperforin for 24 h. IL-8 mRNA levels were examined by Northern blot analysis. (C) LS180 cells show increased IL-8 protein secretion after 8 h exposure to hyperforin with indicated concentration. The medium was collected and IL-8 was measured by ELISA.

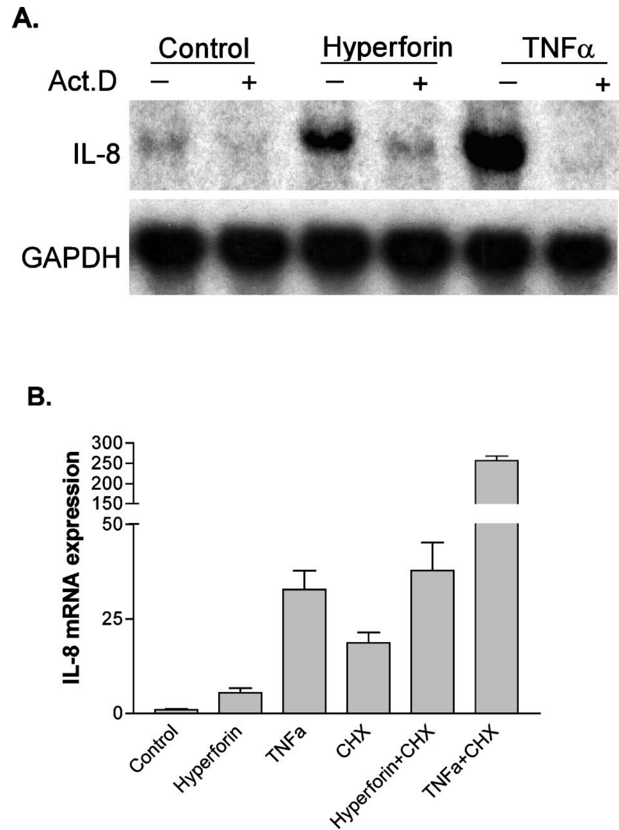

Fig. 3. IL-8 induction by hyperforin is due to new transcription and new protein synthesis is not required for hyperforin stimulated IL-8 gene expression. (A) Actinomycin D was added to the LS180 culture medium at 10 μg/mL for 30 min to inhibit transcription. LS180 cells were then treated with 10-μM hyperforin or 10 ng/mL TNFα for 2 h. IL-8 mRNA levels were examined by Northern blot analysis. (B) LS180 cells were treated with 10-μM hyperforin or 10 ng/mL TNFα after 30-min pretreatment with 10 μg/mL cycloheximide (CHX) or solvent vehicle. After 8 h, total RNA was isolated and QRT-PCR was performed to quantitate IL-8 mRNA levels.

cells were treated with the protein synthesis inhibitor, cycloheximide (CHX), prior to hyperforin treatment. Total RNA was isolated and QRT-PCR was performed after 8 h of treatment. In the absence of CHX, hyperforin and TNFα induced IL-8 mRNA approximately 5- and 32-fold respectively over vehicle (Fig. 3B). CHX treatment led to a superinduction of IL-8 mRNA in untreated cells, suggesting the presence of either an unstable inhibitor of IL-8 expression or a stabilization of IL-8 mRNA (Fig. 3B). Induction of IL-8 by hyperforin and TNFα compared with CHX alone (Fig. 3B) suggests that new protein synthesis is not required for hyperforin to upregulate IL-8 mRNA.

Hyperforin Stimulates IL-8 Expression in Caco-2 Cells and Human Primary Hepatocytes

To confirm that induction of IL-8 by was not restricted to LS-180 cells, we examined the effects of hyperforin on IL-8 in another widely used human IEC line—Caco-2. Similar to LS180 cells, hyperforin was also able to induce IL-8 gene expression in Caco-2 cells as detected by QRT-PCR (Fig. 4A). The steady-state levels of IL-8 mRNA were upregulated by as little as 1-μM hyperforin and increased with hyperforin concentration. In addition to intestine, liver is the major organ involved in the first-pass metabolism of drugs. Since IL-8 can induce acute-phase protein production and may have profound effects during an inflammatory response in human hepatocytes (42), we tested the effects of hyperforin on IL-8 gene expression in

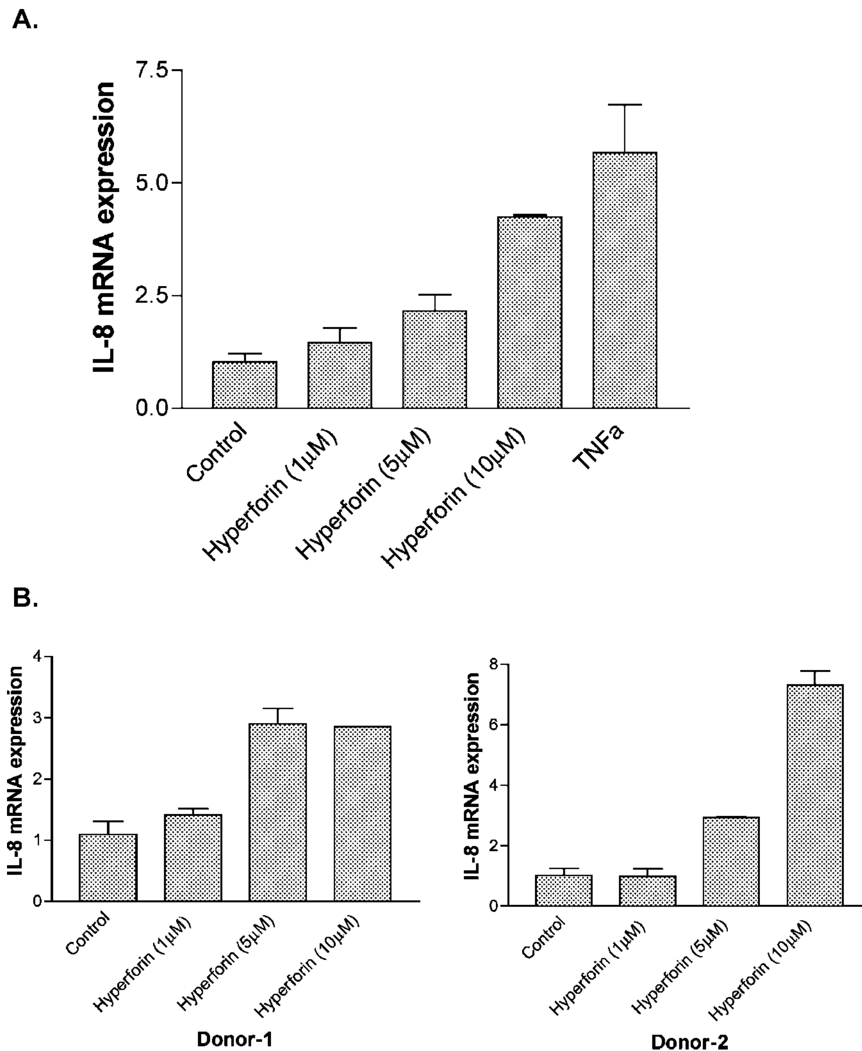

Fig. 4. Hyperforin induces IL-8 gene expression in Caco-2 cells and primary hepatocytes. (A) Caco- 2 cells and (B) primary hepatocytes from two different donors were treated with different concentrations of hyperforin for 8 h. Total RNA was isolated and IL-8 mRNA levels were examined by QRT-PCR.

human primary hepatocytes. We found that hyperforin induced the expression of IL-8 mRNA in a dose-dependent manner in primary hepatocytes from two different donors tested (Fig. 4B).

SXR Activation is not Required for Hyperforin Induction of IL-8 mRNA in IEC

The observation that hyperforin, but not RIF or RU486, induced IL-8 in LS180 cells (Fig. 1) suggested that hyperforin was not acting through SXR. To confirm this possibility, we transfected LS180 cells with a mutant form of SXR (VP16-SXR) that constitutively activates SXR target genes *in vitro* and *in vivo* (43). Total RNA from VP16-SXR transfected cells was isolated and QRT-PCR was performed. VP16-SXR transfection successfully induced expression of the known SXR target gene CYP3A4 (data not shown). However, as shown in Fig. 5, VP16-SXR transfection did not increase IL-8 mRNA levels compared with the control VP16 alone, suggesting that hyperforin induction of IL-8 mRNA is not mediated through activation of SXR.

The AP-1 Binding Site in the IL-8 Promoter is Required for Transcriptional Activity of the IL-8 Promoter in Response to Hyperforin

The IL-8 promoter has been characterized between −1481 and +40 bp of the transcription start site (44–47). We first tested whether the hyperforin-responsive region of

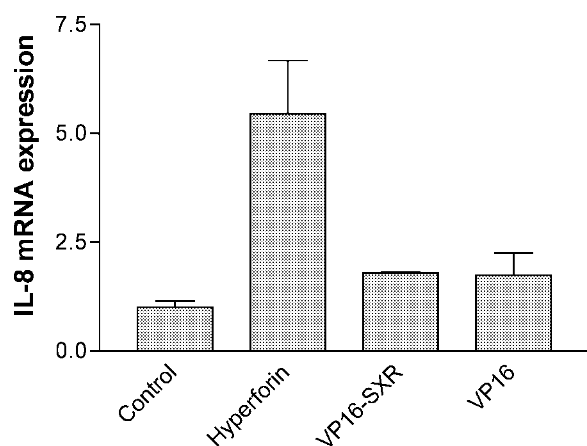

Fig. 5. VP16-SXR does not induce IL-8 expression. LS180 cells were transfected with VP16-SXR or VP16 expression plasmid or treated with 10-μM hyperforin. Total RNA from was isolated and IL-8 mRNA level was examined by QRT-PCR.

the promoter was in this region, or elsewhere in the promoter. Several promoter reporter constructs were made by inserting up to 7 kb of the IL-8 promoter into the pGL2-basic luciferase reporter plasmid to identify potential hyperforin response elements in the IL-8 promoter region. Transfection with these reporter plasmids showed that all of these reporters could be induced to similar levels (4-fold) by hyperforin (Fig. 6A). This confirms that hyperforin acts to increase transcription of the IL-8 promoter and suggests that hyperforin responsiveness resided in the previously characterized 1.5-kb promoter. Several important transcription factor binding sites had been identified within this region, including NF-κB, AP-1 and C/EBP. These elements were previously shown to be important for IL-1β, TNFα, and other stimuli to induce IL-8 gene expression (39). Constructs containing the wild-type IL-8 promoter or containing mutations of binding sites for either NF-κB, AP-1 or C/EBP in the IL-8 promoter were cotransfected into LS180 cells. As shown in Fig. 6B, luciferase activity was increased over 4-fold in cells transfected with the wild-type 1.5-kb IL-8 promoter after 10-μM hyperforin stimulation. The NF-κB and C/EBP mutant constructs were also induced to similar levels by hyperforin treatment suggesting that hyperforin does not act through these elements. In contrast, the AP-1 mutant construct did not respond to hyperforin treatment at all which indicates that AP-1 element is required for IL-8 induction by hyperforin (Fig. 6B). As expected, all three mutants showed reduced TNFα induction.

To further confirm that hyperforin was acting through the AP-1 element, we tested AP-1 or NF-κB-dependent reporter constructs containing three copies of either consensus AP-1 or NF-κB elements upstream of the luciferase reporter in transfection experiments (34). As expected, only AP-1 reporter activity could be induced by hyperforin (Fig. 6C), suggesting that hyperforin acts through this element to increase transcription of the IL-8 gene.

Hyperforin Treatment Enhances AP-1 but not NF-κB Binding Activity

We next determined whether hyperforin could enhance NF-κB or AP-1-binding activity for the IL-8 promoter by testing whether hyperforin could stimulate the nuclear translocation and DNA binding activity of NF-κB and AP-1 in LS180 cells. Consistent with the transfection analysis, EMSA results showed that hyperforin did not increase the DNA binding activity of NF-κB whereas TNFα treatment did (Fig. 7A). Competition experiments showed that excess wild type probe disrupted the complex (Fig. 7A, lanes 4–6) whereas probes containing mutations of the NF-κB element did not (Fig. 7A, lane 7). Since the members of the NF-κB/Rel family can form various complexes with each other, supershift experiments utilizing subunit-specific antibodies were performed. Preincubation with antibodies against two NF-κB subunits, RelA (p65) and NFκB1 (p50) resulted in different effects on the DNA-protein complex. Anti-p65 antibody was able to abolish the formation of the DNA-protein complex and resulted in a faint super-shifted band indicating the presence of p65 in this complex. In contrast, anti-p50 antibodies did not supershift or disrupt the complex, suggesting that p50 is not a component of this complex.

In contrast to NF-κB, hyperforin induced a time-dependent increase in the amount of the AP-1 complex in treated LS180 cells as measured by EMSA (Fig. 7B). The specificity of the shifted band was confirmed by competition-binding experiments. Excess unlabeled wild-type probe reduced the intensity of the shifted band (Fig. 7B lanes 6–8) whereas a mutant probe harboring point mutations that abolished AP-1 binding did not (Fig. 8B lane 9). In addition, an anti-c-Jun antibody specifically super-shifted and disrupted the protein-DNA complex, suggesting that AP-1 is a component of the protein complex that binds to the AP-1 element of the IL-8 promoter (Fig. 7B lane 14).

The EMSA results support the transfection results (Fig. 6) implicating AP-1 activation in the hyperforin-induced IL-8 gene expression.

ERK1/2 Activation is Required for Hyperforin-Induced IL-8 Expression

Since the activation of the AP-1 transcription factor is involved in hyperforin-induced IL-8 gene expression, we

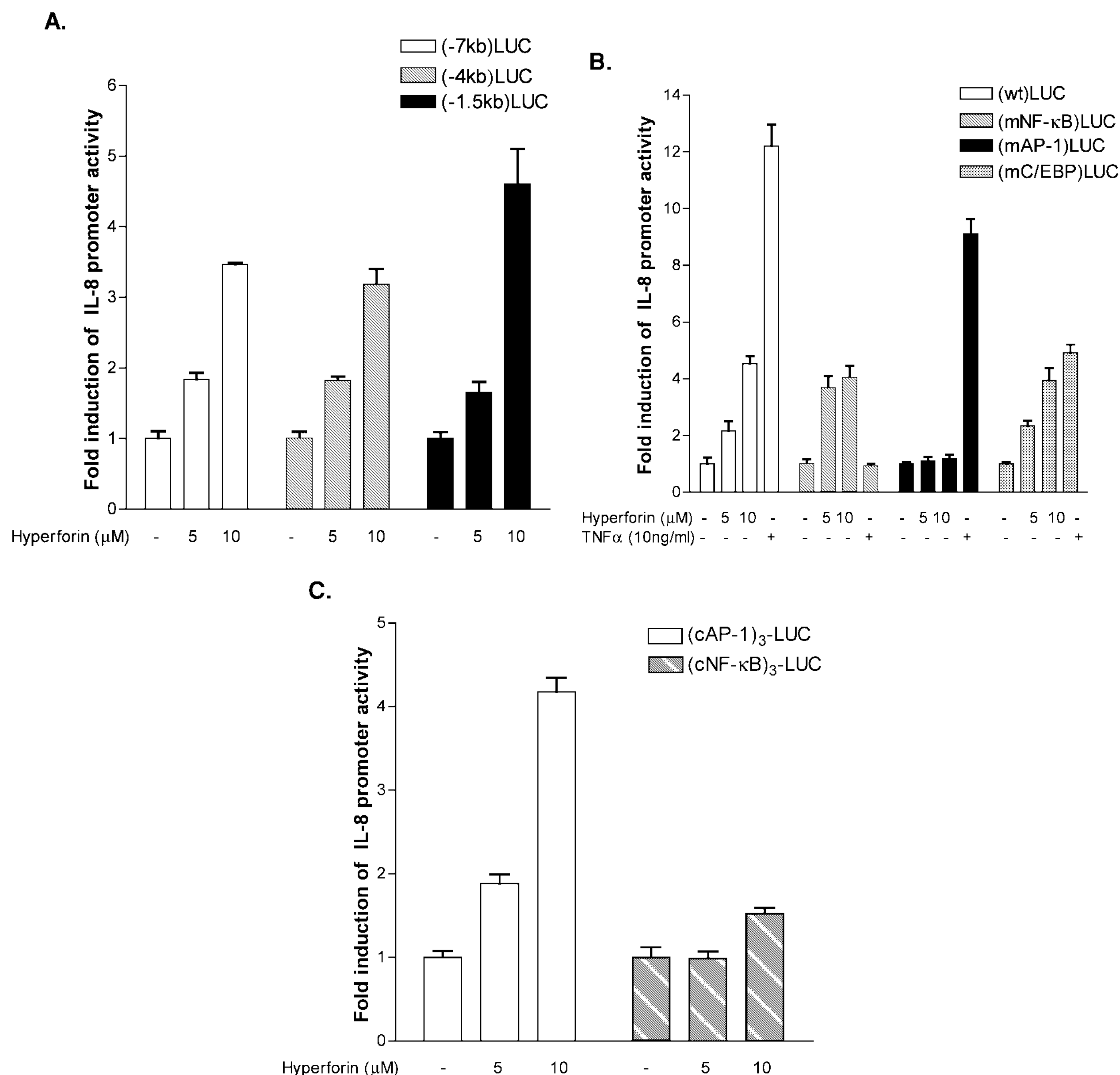

Fig. 6. AP-1-binding site of the IL-8 gene is required for transcriptional activity of the IL-8 promoter in response to hyperforin. LS180 cells were transiently transfected with different promoter constructs together with CMX-β-galactosidase as a transfection control. The transfected cells were incubated with media for 24 h and then treated with solvent controls, 5- or 10-μM hyperforin or 10 ng/mL TNFα for 8 h. Cells were harvested and assayed for luciferase and β-galactosidase activity. Data were normalized to β-galactosidase activity and expressed as relative promoter activity. Fold activations were obtained by comparing to the control values. All values represent the mean of triplicates ± S.E. (A) Sequences within −1481 to +40 bp of IL-8 promoter are sufficient to mediate hyperforin-induced gene activation. Three IL-8 promoter constructs containing IL-8 promoter sequence from −7 to −1.5 kb were transfected into LS180 cells and treated with 5- or 10-μM hyperforin. (B) Functional effect of site-directed mutations on hyperforin induced IL-8 promoter activity. LS180 cells were transiently transfected with wild-type (wt), AP-1 mutant (mAP1), NF-κB mutant (mNF-κB) or C/EBP mutant (mC/EBP) promoter constructs and treated with either hyperforin or TNFα. (C) LS180 cells were transfected with the (cAP-1)$_3$-LUC or (cNF-κB)$_3$-LUC reporter constructs and treated with 5- or 10-μM hyperforin.

next investigated the upstream signaling mechanism. A pharmacological approach using specific kinase inhibitors was employed to test the role of ERK1/2, p38 MAP kinase, and NF-κB in the hyperforin-induced expression of IL-8 in IEC. Pretreatment of LS180 cells with 25-μM U0126 or 50-μM PD98509 (ERK 1/2 inhibitors) for 30 min caused a significant inhibition in the hyperforin-induced increase in IL-8 mRNA detected by QRT-PCR (Fig. 8A). In contrast,

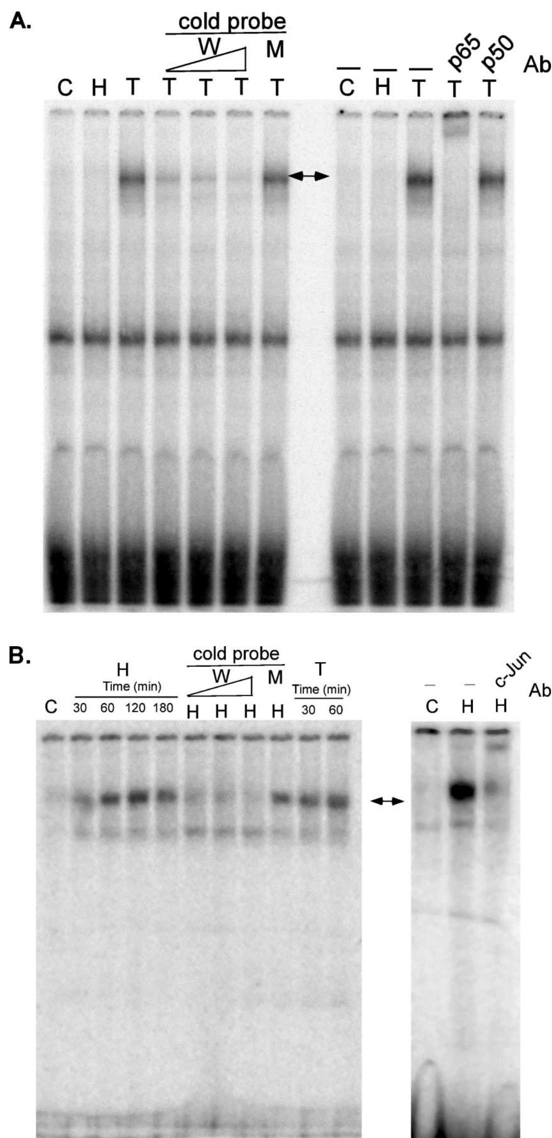

Fig. 7. Hyperforin enhances AP-1 but not NF-κB binding activity for the IL-8 gene. LS180 cells were treated with 10-μM hyperforin (H), 10 ng/mL TNFα (T) or MeOH control (C) for various time, and nuclear extracts were prepared for EMSA as described in Experimental Procedures section. (A) Hyperforin-induced IL-8 expression does not require NF-κB activation. NF-κB binding activity was determined by EMSA after 45-min treatment with hyperforin or TNFα. Competition experiments used 10-, 25-, or 50-fold excess of unlabeled wild-type (W) or 50-fold excess of unlabeled mutant (M) NF-κB probes. For supershift assays, 2 μg of anti-p65 or anti-p50 antibodies were preincubated with nuclear extracts for 30 min on ice prior to the addition of radiolabeled probes. (B) Hyperforin enhances AP-1-binding activity. Nuclear extracts from different time points and ^{32}P-labeled AP-1 probes were used to examine AP-1-binding activity. Competition experiments used 10-, 25-, or 50-fold excess of unlabeled wild-type (W) or 50-fold of unlabeled mutant (M) AP-1 probes for nuclear extracts from 180-min treatment. For supershift assays, 2 μg of anti-c-Jun antibodies were preincubated with nuclear extracts (120 min treatment) for 30 min on ice prior to the addition of radiolabeled probes.

pretreatment of cells with 20-μM SB203580 (p38 MAPK inhibitor) or 100-μM PDTC (NF-κB inhibitor) did not reduce the expression of IL-8 mRNA.

Since the inhibition of ERK1/2 inhibited the expression of IL-8 in IEC, we next tested whether hyperforin was able to induce the phosphorylation of ERK1/2. In accord with the transfection and inhibitor experiments, treatment of LS180 cells with 10-μM hyperforin resulted in increased phosphorylation of the ERK1/2 (Fig. 8B). The role of ERK1/2 in activation of IL-8 transcription in response to hyperforin was also shown using a construct encoding dominant negative MEK (DN-MEK). MEK is an immediate upstream signal of ERK1/2 and expression of DN-MEK has been shown to be able to significantly inhibit ERK1/2 activity (35). Cotransfection of LS180 cells with IL-8 reporter and DN-MEK suppressed hyperforin-induced IL-8 reporter activity (Fig. 8C). These data suggest that ERK1/2 is involved in the pathway leading to the enhanced expression of IL-8 in response to hyperforin.

Consistent with the QRT-PCR results (Fig. 8A), the pretreatment of LS180 cells with ERK1/2 inhibitors also blocked hyperforin stimulated IL-8 protein secretion (Fig. 8D). In contrast, the p38 MAPK inhibitor SB203580 had no significant effect on hyperforin induced IL-8 production. Similar results were also obtained with the human monocytic cell line THP-1 (Fig. 8E). Hyperforin was also able to stimulate IL-8 secretion and pretreatment with ERK1/2 inhibitors inhibited IL-8 production. These results indicate that hyperforin is able to induce IL-8 production in other cells in addition to IEC *via* the ERK1/2 MAPK pathway.

Finally, we investigated the expression of another major inflammatory mediator, ICAM-1, in LS180 cells. ICAM-1 is also a downstream target gene of ERK1/2 MAPK and is often coregulated with IL-8 because these genes share similar cis-acting elements (NF-κB, AP-1 and C/EBP) (48, 49). As expected, ICAM-1 mRNA levels were also upregulated by treatment with as little as 2.5-μM hyperforin (Fig. 8F).

DISCUSSION

IEC serve as a barrier between the body and the bacteria present in the intestinal lumen. There is increasing evidence that IEC participate in the intestinal inflammatory process (50). IEC have the capacity to express antigens to T cells, and produce cytokines and chemokines in response to bacterial invasion. IEC are also involved in mounting an immune response against harmful pathogens (51, 52). Here we showed that hyperforin, the active component of St. John's wort, induces IL-8 expression in human IEC, primary hepatocytes and THP-1 cells. IL-8 is

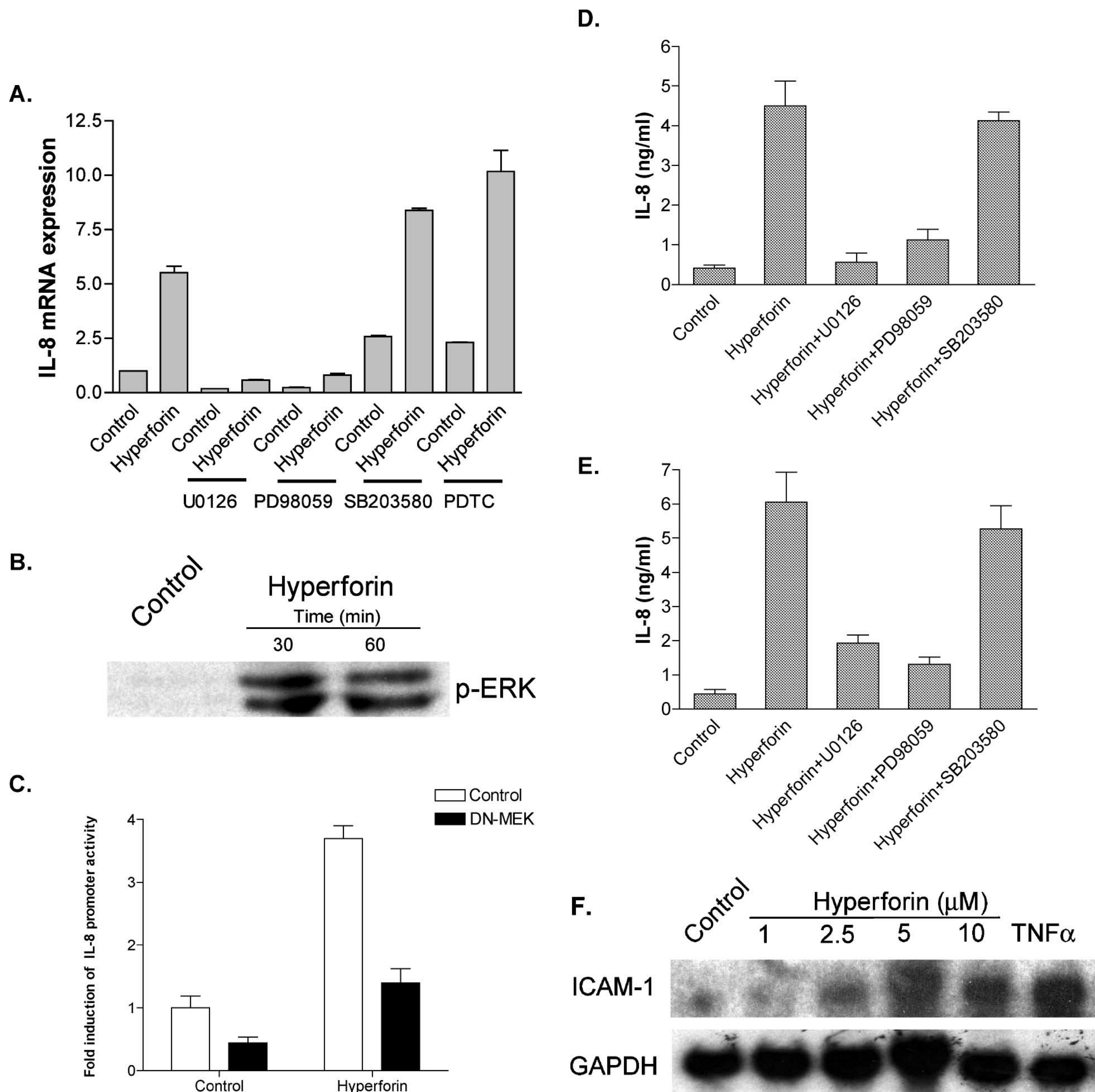

Fig. 8. ERK1/2 activation is required for hyperforin-induced IL-8 expression. (A) LS180 cells were pretreated with 25-μM U0126, 50-μM PD98509 (ERK1/2 inhibitors), 20-μM SB203580 (p38 MAPK inhibitor), or 100-μM PDTC (NF-κB inhibitor) for 30 min and treated with 10-μM hyperforin for 8 h. Total RNA was isolated and IL-8 mRNA levels were examined by QRT-PCR. (B) LS180 cells were treated with 10-μM hyperforin for up to 60 min and cells were lysed, subjected to SDS-PAGE, blotted onto nitrocellulose membranes. Immunoblots were obtained using polyclonal antibody against activated ERK1/2. Equal protein loading was confirmed by probing for nonphosphorylated ERK2 (data not shown). The results are representative of three independent experiments. (C) LS180 cells were transfected with 1.5-kb IL-8 promoter construct together with control or DN-MEK expression vector. The transfected cells were incubated with media for 24 h and then treated with solvent controls or 10-μM hyperforin for 8 h. (D) LS180 cells and (E) THP-1 cells were pretreated with U0126, PD98509 or SB203580 for 30 min and then treated with 10-μM hyperforin for 8 h. The medium was collected and IL-8 secretion was measured by ELISA. (F) LS180 cells were treated with different concentrations of hyperforin as indicated for 24 h. ICAM-1 mRNA levels were examined by Northern blot analysis.

a member of the CXC chemokine family and is one of the best described chemokines. IL-8 functions primarily as an activator of neutrophils and has been described as a neutrophil-activating peptide or a monocyte-derived neutrophil chemotactic factor (20, 53). IL-8 can be produced by various cell types, including IEC, during inflammatory responses and is thought to play a major role in intestinal inflammation (23, 54). Pharmacokinetic studies show that the blood levels of hyperforin range from 100 to 250 ng/mL (0.19–0.46 μM) following a single 900-mg dose of St. John's wort (55). This concentration may be higher with the higher dose usage and also higher in IEC. We have shown that low micromolar concentrations of hyperforin are able to induce IL-8 mRNA and protein secretion in IEC, suggesting that induction of IL-8 by hyperforin may be pharmacologically relevant. The majority of side effects of St. John's wort have been reported to be gastrointestinal symptoms, especially nausea and abdominal discomfort (18, 19). Our demonstration that hyperforin may induce immune responses in intestinal and liver cells could therefore be a concern for users of St. John's wort.

IL-8 gene expression in IEC is an immediate early transcriptional response to hyperforin stimulation (Figs. 3 and 4). Hyperforin is an SXR ligand and induces the expression of SXR target genes such as CYP3A4 and Mdr1 in LS180 cells; therefore, we next tested whether hyperforin induced IL-8 expression results from activation of SXR. SXR ligands other than hyperforin were unable to induce IL-8 gene expression (Fig. 1) and transfection with a constitutively active form of SXR (VP16-SXR) did not increase IL-8 mRNA levels (Fig. 6). Hence, we concluded that the hyperforin induction of IL-8 transcription is SXR-independent.

Since hyperforin induces IL-8 gene expression at the transcriptional level, IL-8 promoter constructs were utilized to investigate the mechanism through which hyperforin induces IL-8 gene expression. Transfection of LS180 cells with a 7-kb fragment of the IL-8 promoter did not show further enhancement in hyperforin inducibility compared with the 1.5-kb promoter construct (Fig. 6A). Therefore, we concluded that the potential hyperforin response element is within the well-characterized 1.5-kb IL-8 promoter region. Three transcription factor binding sites within this region have been described to be important for TNFα, IL-1β and PMA induced simulation of IL-8 transcription: AP-1, NF-κB and C/EBP (38, 39, 45). NF-κB signaling has been shown to be the main regulator of IL-8 in response to inflammatory mediator such as TNFα, IL-1β and endotoxin in many epithelial and endothelial cells (56–58). However, we found that only the AP-1 element was required for hyperforin-induced IL-8 promoter activity (Fig. 6B,C). The NF-κB and C/EBP elements are not necessary, although both are required for TNFα-induced IL-8 promoter activity (Fig. 6B). EMSA analysis supported our conclusion that hyperforin induction of IL-8 gene expression in IEC occurred through an AP-1-dependent but NF-κB-independent mechanism (Fig. 7).

We employed specific protein kinase inhibitors to further elucidate the mechanism through which hyperforin induces the expression of IL-8 mRNA. The results showed that inhibition of ERK1/2 but not p38 MAP kinase or NF-κB inhibited hyperforin-induced IL-8 expression (Fig. 8A). Consistent with these findings, hyperforin was able to induce the phosphorylation of ERK1/2 in IEC and expression of DN-MEK suppressed hyperforin-induced IL-8 promoter activity (Fig. 8B,C). Activation of AP-1 is known to be downstream of ERK1/2 signaling in other pathways and ERK1/2 signaling has been shown to control IL-8 production in many cell types including IEC (59, 60). Although MAP kinase pathways are among the best studied signaling pathways that are activated in response to various stimuli, it was unclear whether ERK1/2 or p38 MAP kinase were required for IL-8 gene expression in different cell types (61, 62). Our results established that ERK1/2 rather than p38 MAP kinase regulates IL-8 induction by hyperforin in LS180 cells and THP-1 monocytes. Furthermore, we found that hyperforin is also able to induce another ERK1/2 target gene—ICAM-1 expression. ICAM-1 is another major inflammatory mediator and is known to play important roles in the recruitment of circulating inflammatory cells (63). ICAM-1 has similar cis-acting elements to those in IL-8 and is often coregulated with IL-8 *via* the same pathway. Our conclusion that hyperforin activates ERK1/2 in IEC is also consistent with a previous report which showed that St. John's wort is able to stimulate ERK activity in glial and neuronal cells (64). Since ERK is a key component of a signal transduction pathway involved in gene expression, this may also provide a possible molecular mechanism related to its broad-spectrum reuptake inhibition of neurotransmitters.

In addition to its role in the immune and inflammatory responses, IL-8 expression also has effects on tumor cell growth and metastasis. In colon tumor tissues, IL-8 protein was found at high levels in patients with poorly differentiated tumors and was found to be higher yet in patients with liver or lung metastases (65). However, there is currently no evidence showing whether IL-8 stimulates or inhibits the development of colon tumors, *in vivo*. Transfection of IL-8 expression plasmids into cells leads to both tumor inhibition and promotion, depending on the cell type (66). Transfection of murine colon carcinoma CT-26 cells with a human IL-8 expression vector resulted in decreased tumor growth by increasing neutrophil and mononuclear cell

infiltration (67). In contrast, treatment of human colon carcinoma cell lines including HCT116A and HT29 with high concentrations of recombinant human IL-8 (1 μg/mL) led to stimulation of cell growth (68). Hyperforin itself was reported to have the ability to inhibit the growth of various human and rat tumor cell lines *in vivo*, with IC_{50} values between 3 and 15 μM. One possibility is that hyperforin may cause mitochondrial permeabilization (17) although other possible pathways such as IL-8 induction cannot be ruled out. Cell proliferation assays showed that hyperforin could also inhibit the growth of LS180 cells (data not shown) but the mechanism of this inhibition remains to be described. Further studies to test whether induction of IL-8 expression has an affect on proliferation of LS180 cells will contribute to our understanding of hyperforin-mediated inhibition of tumor cell line proliferation.

In summary, we found that hyperforin induced IL-8 expression in IEC and other cell types including primary hepatocytes and THP-1 monocytes through an SXR-independent mechanism. The induction of IL-8 in IEC occurs through a pathway requiring ERK1/2 signaling through the AP-1 transcription factor. The modulation of IL-8 expression as well as ICAM-1 by hyperforin suggests a previously unsuspected role for St. John's wort in modulating the immune and inflammatory responses in the intestine and liver. Since IL-8 has been shown to be able to induce acute-phase protein production in the liver and plays a major role in intestinal inflammation, the unexpected induction of IL-8 expression by hyperforin may be detrimental for some users of St. John's Wort. Taken together with the already known propensity for hyperforin to induce drug interactions, our results suggest that further caution is indicated in the use of potent botanical preparations for the treatment of human disease.

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