Valproic Acid Suppresses Interleukin-1ß-induced Microsomal Prostaglandin E_2 Synthase-1 Expression in Chondrocytes Through Upregulation of NAB1

NADIA ZAYED, FATIMA EZZAHRA EL MANSOURI, NADIR CHABANE, MOHIT KAPOOR, JOHANNE MARTEL-PELLETIER, MOHAMED BENDERDOUR, JEAN-PIERRE PELLETIER, NICOLAS DUVAL, and HASSAN FAHMI

ABSTRACT. ***Objective.*** Microsomal prostaglandin E_2 synthase-1 (mPGES-1) catalyzes the terminal step in the biosynthesis of PGE_2. Early growth response factor-1 (Egr-1) is a key transcription factor in the regulation of mPGES-1, and its activity is negatively regulated by the corepressor NGF1-A-binding protein-1 (NAB1). We examined the effects of valproic acid (VA), a histone deacetylase inhibitor, on interleukin 1ß (IL-1ß)-induced mPGES-1 expression in human chondrocytes, and evaluated the roles of Egr-1 and NAB1 in these effects.

Methods. Chondrocytes were stimulated with IL-1 in the absence or presence of VA, and the level of mPGES-1 protein and mRNA expression were evaluated using Western blotting and real-time reverse-transcription polymerase chain reaction (PCR), respectively. mPGES-1 promoter activity was analyzed in transient transfection experiments. Egr-1 and NAB1 recruitment to the mPGES-1 promoter was evaluated using chromatin immunoprecipitation assays. Small interfering RNA (siRNA) approaches were used to silence NAB1 expression.

Results. VA dose-dependently suppressed IL-1-induced mPGES-1 protein and mRNA expression as well as its promoter activation. Treatment with VA did not alter IL-1-induced Egr-1 expression, or its recruitment to the mPGES-1 promoter, but prevented its transcriptional activity. The suppressive effect of VA requires *de novo* protein synthesis. VA induced the expression of NAB1, and its recruitment to the mPGES-1 promoter, suggesting that NAB1 may mediate the suppressive effect of VA. Indeed, NAB1 silencing with siRNA blocked VA-mediated suppression of IL-1-induced mPGES-1 expression.

Conclusion. VA inhibited IL-1-induced mPGES-1 expression in chondrocytes. The suppressive effect of VA was not due to reduced expression or recruitment of Egr-1 to the mPGES-1 promoter and involved upregulation of NAB1. (First Release Jan 15 2011; J Rheumatol 2011;38:492–502; doi:10.3899/jrheum.100907)

Key Indexing Terms:
MICROSOMAL PROSTAGLANDIN E SYNTHASE-1 CHONDROCYTES
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Prostaglandin E_2 (PGE_2) plays an important role in the pathophysiology of arthritis, and excessive levels have been reported in serum and synovial fluids from patients with osteoarthritis (OA) and rheumatoid arthritis (RA)[1]. PGE_2 contributes to the pathogenesis of arthritis by inducing cartilage proteoglycan degradation[2], enhancing the activation and production of matrix metalloproteases (MMP)[3], and by promoting chondrocyte apoptosis[4]. PGE_2 is also involved in neoangiogenesis and mediates pain responses[5].

The biosynthesis of PGE_2 from arachidonic acid requires 2 enzymatic activities. Cyclooxygenase (COX) enzymes convert arachidonic acid into PGH_2, which is in turn isomerized to PGE_2 by PGE synthase (PGES) enzymes. Two isoforms of the COX enzyme, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in most tissues, whereas COX-2 is inducible by various stimuli, including proinflammatory signals[6]. At least 3 distinct PGES isoforms have been cloned and characterized, including cytosolic PGES (cPGES), microsomal PGES-1 (mPGES-1), and mPGES-2[7]. cPGES is constitutively and ubiquitously expressed and is functionally coupled with COX-1, promoting immediate production of PGE_2[8]. In con-

From the Osteoarthritis Research Unit, University of Montreal Hospital Research Center (CRCHUM), Notre-Dame Hospital, Montréal, Québec, Canada.

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N. Zayed, MSc; F.E. El Mansouri, MSc; N. Chabane, MSc; J. Martel-Pelletier, PhD; M. Kapoor, PhD; J-P. Pelletier, MD; H. Fahmi, PhD, Osteoarthritis Research Unit, University of Montreal Hospital Research Center; M. Benderdour, PhD, Research Centre, Sacré-Coeur Hospital; N. Duval, MD, Duval Orthopaedic Clinic.

Address correspondence to H. Fahmi, Osteoarthritis Research Unit, Centre hospitalier de l'Université de Montréal, Hôpital Notre-Dame, 1560 rue Sherbrooke est, Montréal, Québec H2L 4M1.
E-mail: h.fahmi@umontreal.ca

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trast, mPGES-1 is markedly upregulated by inflammatory or mitogenic stimuli and is functionally coupled with COX-2, promoting delayed PGE_2 production[9]. mPGES-2 is constitutively expressed in various cells and tissues and can be coupled with both COX-1 and COX-2[10]. We and others have previously shown that the level of mPGES-1 is elevated in articular tissues from patients with OA and RA and in animal models of arthritis[11,12,13,14]. Moreover, mPGES-1 deficiency was protective in animal models of chronic inflammation, pain, and arthritis[15,16], which implicates mPGES-1 as a potential target for therapeutic intervention in arthritis.

The expression of mPGES-1 is upregulated in several cell types after treatment with proinflammatory stimuli such as interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) and is downregulated by antiinflammatory glucocorticoids[9,11,17]. Transcriptional induction of mPGES-1 is primarily controlled by the transcription factor early growth response factor-1 (Egr-1)[18,19]. Like most transcription factors, the activity of Egr-1 is negatively regulated by corepressor proteins. Two corepressors of Egr-1, NGF1-A-binding proteins (NAB)1 and NAB2, have been identified[20,21].

Acetylation and deacetylation of histone and nonhistone proteins play a critical role in the control of gene transcription[22,23]. The acetylation status is determined by interplay between histone acetyltransferases (HAT) and histone deacetylases (HDAC). In general, histone acetylation is associated with transcription activation through relaxed chromatin structure, whereas histone deacetylation is associated with transcription repression via chromatin condensation[22,23]. Recent studies, however, have revealed that transcription activation is not necessarily associated with histone acetylation and that HDAC activity can also activate transcription. For instance, global analysis of gene expression showed that inhibition of HDAC activity results in both induction and repression of gene expression[24,25,26]. In addition, genome-wide genetic studies with yeast demonstrated clearly that HDAC are required in both transcriptional activation and repression[27,28]. Finally, inhibition of HDAC activity was reported to repress transcription in several cell types including chondrocytes[29,30,31,32,33,34,35,36].

We examined the effect of valproic acid (VA), an HDAC inhibitor[37], on IL-1-induced mPGES-1 expression in human OA chondrocytes. We showed that VA suppressed IL-1-induced mPGES-1 expression without interfering with the expression or the recruitment of Egr-1 to the mPGES-1 promoter. Further, we provide evidence that the suppressive effect of VA on IL-1-induced mPGES-1 expression involves upregulation of NAB1.

MATERIALS AND METHODS

Reagents and antibodies. Human recombinant (rh) IL-1 was obtained from Genzyme (Cambridge, MA, USA). TNF-α and IL-17 were purchased from R&D Systems (Minneapolis, MN, USA). VA, cycloheximide (CHX) aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma-Aldrich Canada (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal calf serum (FCS), and Trizol reagent were supplied by Invitrogen (Burlington, ON, Canada). Plasmid DNA was prepared using a kit from Qiagen (Mississauga, ON, Canada). FuGene-6 transfection reagent was from Roche Applied Science (Laval, QC, Canada). The luciferase reporter assay system was from Promega (Madison, WI, USA). Anti-mPGES-1 and anti-cPGES antibodies were from Cayman Chemical (Ann Arbor, MI, USA). Antibodies against Egr-1, NAB1, NAB2, and ß-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit anti-mouse immunoglobulin G (IgG) coupled with horseradish peroxidase (HRP) and polyclonal goat anti-rabbit IgG with HRP were from Pierce (Rockford, IL, USA). All other chemicals were purchased from Fisher Scientific or Bio-Rad (Mississauga, ON, Canada).

Chondrocyte isolation and treatment. Articular cartilage samples from femoral condyles and tibial plateaus were obtained from OA patients undergoing total knee replacement (n = 49, mean age 67 ± SD 17 yrs). Informed consent had been obtained from patients with OA for the use of their tissues for research purposes. All OA patients were diagnosed according to the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA[38]. At the time of surgery, patients had symptomatic disease requiring medical treatment in the form of nonsteroidal antiinflammatory drugs or selective COX-2 inhibitors. Patients who had received intraarticular injection of steroids were excluded. The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the use of human articular tissues.

Chondrocytes were released from cartilage by sequential enzymatic digestion as described[33]. Briefly, small pieces of cartilage were incubated with 2 mg/ml pronase for 1 h followed by 1 mg/ml type IV collagenase (Sigma-Aldrich) for 6 h at 37°C in DMEM and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS.

Confluent chondrocytes were detached by trypsinization, seeded at 3.5×10^5 cells per well in 12-well culture plates (Costar, Corning, NY, USA) or at 7×10^5 cells per well in 6-well culture plates in DMEM supplemented with 10% FCS, and cultivated at 37°C for 48 h. Cells were washed and incubated for an additional 24 h in DMEM containing 0.5% FCS and pretreated with VA, trichostatin A (TSA), or BA for 30 min, before stimulation with IL-1, TNF-α, or IL-17. In another set of experiments chondrocytes were pretreated for 30 min with CHX, before stimulation with IL-1 or VA. The expression level of mPGES-1 protein was determined 24 h after stimulation, whereas the level of mPGES-1 messenger RNA (mRNA) was determined at 8 hours. Only first-passaged chondrocytes were used.

PGE_2 determination. Levels of PGE_2 were determined using a PGE_2 enzyme immunoassay (EIA; Cayman Chemical). The detection limit and sensitivity was 9 pg/ml. All assays were performed in duplicate.

Protein extraction and Western blot analysis. For histone extraction, chondrocytes were washed with phosphate buffered saline (PBS) and lysed in ice-cold lysis buffer containing 10 mM hydroxyethyl-piperazine ethanesulfonic acid potassium hydroxide (HEPES-KOH), pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1.5 mM PMSF, 1 mM Na_3VO_4, and 10 μg/ml aprotinin, leupeptin, and pepstatin. Sulfuric acid was added to a concentration of 0.2 N and the resultant supernatant was collected and dialyzed twice against 0.1 M acetic acid and 3 times against sterile water. For whole-cell lysate preparation, chondrocytes were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12,000 rpm for 15 min. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce). Then 20 μg of total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose

membrane (Bio-Rad). After blocking in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween 20, and 5% (wt/vol) nonfat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with Tris-buffered saline (TBS), pH 7.5, with 0.1% Tween 20. The blots were then incubated with HRP-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA).

RNA extraction and cDNA synthesis. Total RNA was isolated from chondrocytes using the TRIzol reagent, and dissolved in 20 μl of diethylpyrocarbonate-treated H_2O. One microgram of total RNA was treated with RNase-free DNase and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase. One-fifth of the reverse transcriptase reaction was analyzed by real-time PCR as described below. The following primers were used: mPGES-1: sense 5'-GAA GAA GGC CTT TGC CAA C-3' and antisense 5'-GGA AGA CCA GGA AGT GCA TC-3'; cPGES: sense 5'-GCA AAG TGG TAC GAT CGA AGG-3' and antisense 5'-TGT CCG TTC TTT TAT GCT TGG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5'-CAG AAC ATC ATC CCT GCC TCT-3' and antisense 5'-GCT TGA CAA AGT GGT CGT TGA G-3'.

Real-time PCR. Real-time PCR analysis was performed in a total volume of 50 μl containing cDNA template, 200 nM of sense and antisense primers, and 25 μl of SYBR® Green master mix (Qiagen). Incorporation of SYBR Green dye into PCR products was monitored in real time using a GeneAmp 5700 sequence detector (Applied Biosystems, Foster City, CA, USA) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After incubation at 95°C for 10 min to activate the AmpliTaq Gold enzyme, the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension). After PCR, dissociation curves were generated, with one peak indicating the specificity of the amplification. A C_T value was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems). Data were expressed as fold-changes relative to control conditions (unstimulated cells) using the $\Delta\Delta C_T$ method as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control from the ΔC_T value of each treatment. Fold-changes compared with the control (unstimulated cells) were then determined by raising 2 to the $\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon, as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on 2 separate occasions from at least 3 independent experiments.

Transient transfection. The mPGES-1 promoter construct (–538/–28) was provided by Dr. T. Smith (University of California, Los Angeles, CA, USA). Egr-1 expression vector was donated by Dr. Y. Chen (Morehouse School of Medicine, Atlanta, GA, USA). Expression plasmids for NAB1 and NAB2 were provided by Dr. J. Savren (University of Wisconsin, Madison, WI, USA). The ß-galactosidase reporter vector under the control of SV40 promoter (pSV40-ß-gal) was from Promega. Transient transfection experiments were performed using FuGene-6 transfection reagent according to the manufacturer's recommendation (Roche Applied Science). Briefly, chondrocytes were seeded 24 h prior to transfection at a density of 3×10^5 cells/well in 12-well plates and transiently transfected with 1 μg of the mPGES-1 promoter construct and 0.5 μg of the internal control pSV40-ß-gal. Six hours later, the medium was replaced with DMEM containing 1% FCS. At 1 day after transfection, the cells were treated with IL-1 in the absence or presence of VA for 18 h. In the overexpression experiments, the amount of transfected DNA was kept constant by using the corresponding empty vector. At the end of the indicated treatment, the cells were washed twice in ice-cold PBS and extracts were prepared for luciferase reporter assay. Luciferase activity was normalized for transfection efficiency using the corresponding ß-galactosidase activity.

RNA interference. Small interfering RNA (siRNA) specific for NAB1 and scrambled control were obtained from Dharmacon (Lafayette, CO, USA). Chondrocytes were seeded in 12-well plates at 3×10^5 cells/well and incubated 24 h. Cells were transfected with 100 nM siRNA using the HiPerFect Transfection Reagent (Qiagen) following the manufacturer's recommendations. The medium was changed 24 h later and the cells were incubated an additional 24 h before stimulation with IL-1 in the absence or presence of VA. Cell lysates were prepared and analyzed for mPGES-1 or NAB1 protein expression by Western blotting.

Chromatin immunoprecipitation (ChIP) assay. The ChIP experiments were performed according to the ChIP protocol provided by Upstate/Millipore Biotechnology and published protocols[39,40]. The primer sequences used were mPGES-1 promoter sense 5'-CCC GGA GAC TCT CTG CTT C-3' and antisense 5'-TCA ACT GTG GGT GTG ATC AGC-3'.

Statistical analysis. Data are expressed as the mean ± SD. Statistical significance was assessed by 2-tailed Student t test. P values < 0.05 were considered statistically significant.

RESULTS

VA suppressed IL-1-induced mPGES-1 protein expression. Blocking histone deacetylation with specific inhibitors modulated gene expression in several cell types[29,30,31,32,33,34,35,36]. To determine whether HDAC inhibitors can modulate PGE_2 production and mPGES-1 expression in chondrocytes, cells were stimulated with IL-1 in the absence or presence of increasing concentrations of VA, and the release of PGE_2 and the expression of mPGES-1 protein were evaluated by EIA and Western blotting, respectively. As shown in Figure 1A, stimulation with IL-1 dramatically increased PGE_2 production and mPGES-1 protein expression. Treatment with VA suppressed IL-1-induced PGE_2 release and mPGES-1 protein expression in a dose-dependent manner. In contrast, the expression of cPGES protein was not affected by these treatments. To determine whether VA inhibits HDAC activity in chondrocytes, we examined its effect on the acetylation of histone H3 protein. As shown in Figure 1B, treatment with VA increased histone H3 protein acetylation in a dose-dependent manner. Thus, VA suppressed IL-1-induced mPGES-1 expression and enhanced histone H3 acetylation in chondrocytes. Treatment of chondrocytes by 2 additional HDAC inhibitors, butyric acid (BA) and VA, also suppressed IL-1-induced mPGES-1 expression in a dose-dependent manner (Figure 1C).

To examine whether the inhibitory effect of VA on mPGES-1 expression was specific for IL-1, we assessed its effects on TNF-α- and IL-17-induced mPGES-1 protein expression. Interestingly, VA suppressed the induction of mPGES-1 expression by both TNF-α and IL-17 (Figure 1D), indicating that its effect was not restricted to IL-1. These data indicate that VA can downregulate the induction of mPGES-1 expression in human chondrocytes. The concentrations of VA utilized did not affect chondrocyte viability as judged by the trypan blue exclusion and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (data not shown).

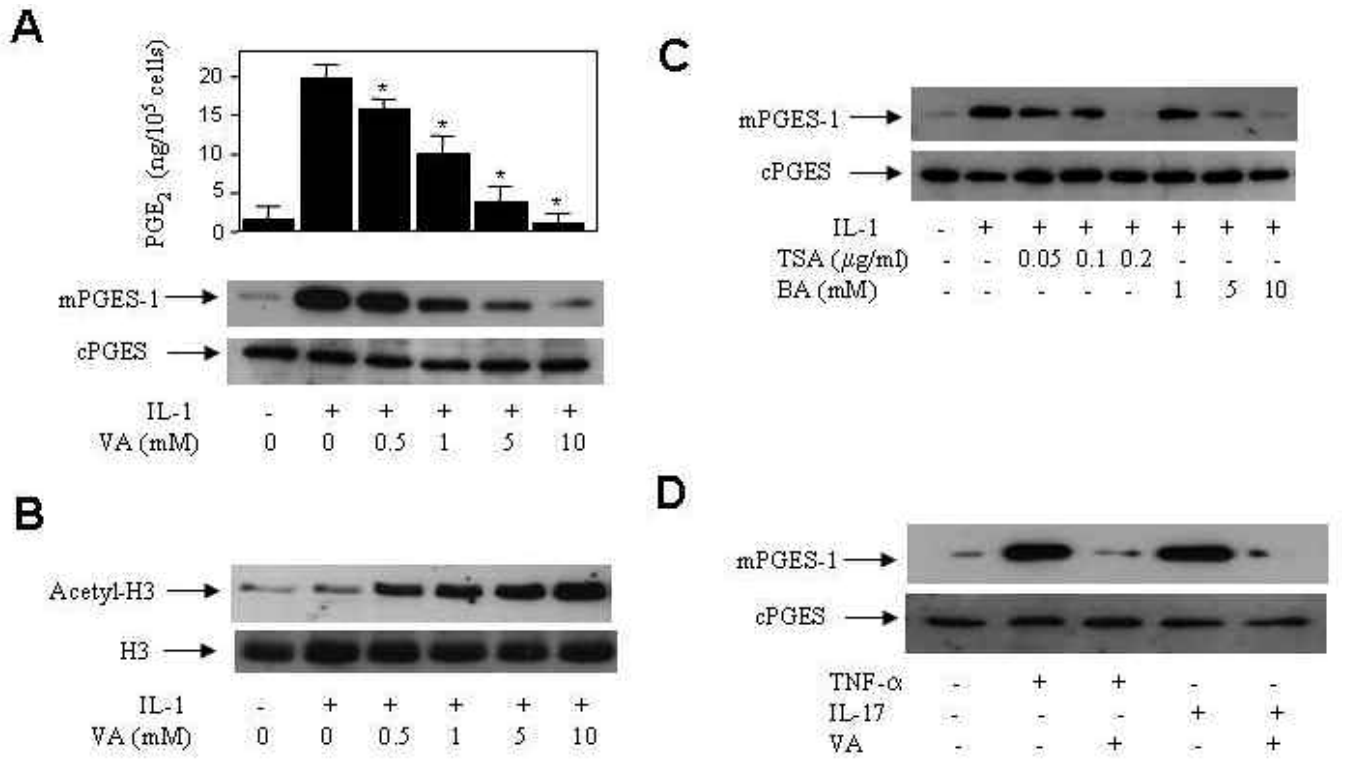

Figure 1. Valproic acid (VA) suppressed interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) expression in chondrocytes. A. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 20 hours. Conditioned media were collected and analyzed for PGE_2 release. Results are expressed as mean ± SEM from 4 independent experiments (*$p < 0.05$ compared with cells treated with IL-1 alone). Cell lysates were prepared and analyzed for mPGES-1 protein expression by Western blotting. B. Chondrocytes were treated as indicated, histones were extracted and immunoblotted for acetyl-H3. C, D. Chondrocytes were treated with IL-1 in the absence or presence of increasing concentrations of trichostatin A or butyric acid (C) or with 1 ng/ml TNF-α or 100 ng/ml IL-17 in the absence or presence of VA (10 mM) (D) for 20 hours. Cell lysates were prepared and analyzed for mPGES-1 protein expression by Western blotting. In the lower panels the blots were stripped and reprobed with specific anti-cPGES (A, C, D) or anti-histone H3 (B) antibodies. Blots are representative of similar results obtained from 4 independent experiments.

VA prevented IL-1-induced mPGES-1 expression at the transcriptional level. To determine whether the suppressive effect of VA was due to inhibition of mPGES-1 mRNA induction, chondrocytes were stimulated with IL-1 in the absence or presence of increasing concentrations of VA, and the level of mPGES-1 mRNA expression was determined using real-time PCR. The relative expression level of mPGES-1 mRNA was plotted as fold-change over control untreated cells. GAPDH gene expression was used for normalization. As expected, treatment with IL-1 resulted in a marked increase (~12-fold) of the mPGES-1 mRNA level, but this effect was dose-dependently attenuated in the presence of VA (Figure 2A), suggesting that VA exerts its effects at the transcriptional level. To confirm this, we carried out transient transfection experiments. Chondrocytes were transfected with the human mPGES-1 promoter-luciferase reporter gene and then stimulated with IL-1 in the absence or presence of VA. As shown in Figure 2B, IL-1 induced the luciferase activity of the mPGES-1 promoter and this activation was dose-dependently reduced by VA, consistent with its effect on mPGES-1 mRNA expression. Taken together, these data indicate that the suppressive effect of VA on IL-1-induced mPGES-1 takes place, at least in part, at the transcriptional level.

VA did not target Egr-1 expression and recruitment to the mPGES-1 promoter, but prevented its ability to transactivate the mPGES-1 promoter. The transcription factor Egr-1 plays a key role in the induction of mPGES-1 expression[18,19]; therefore, we considered whether the inhibition of IL-1-induced mPGES-1 expression by VA was due to prevention of Egr-1 expression. Chondrocytes were incubated with IL-1 in the absence or presence of VA, and cell extracts were prepared and analyzed by Western blotting. As shown in Figure 3A, IL-1 strongly induced the expression of Egr-1, and this effect was not altered in the presence of VA, indicating that the suppressive effect of VA is not due to reduced expression of Egr-1.

To determine whether VA affects the recruitment of Egr-1 to the endogenous mPGES-1 promoter, we performed ChIP assays. Chondrocytes were stimulated with IL-1 in the absence or presence of VA, and formaldehyde cross-linked DNA proteins were immunoprecipitated with an anti-Egr-1 antibody. No-antibody and non-immune serum were used as controls. DNA isolated from the immunoprecipitates was

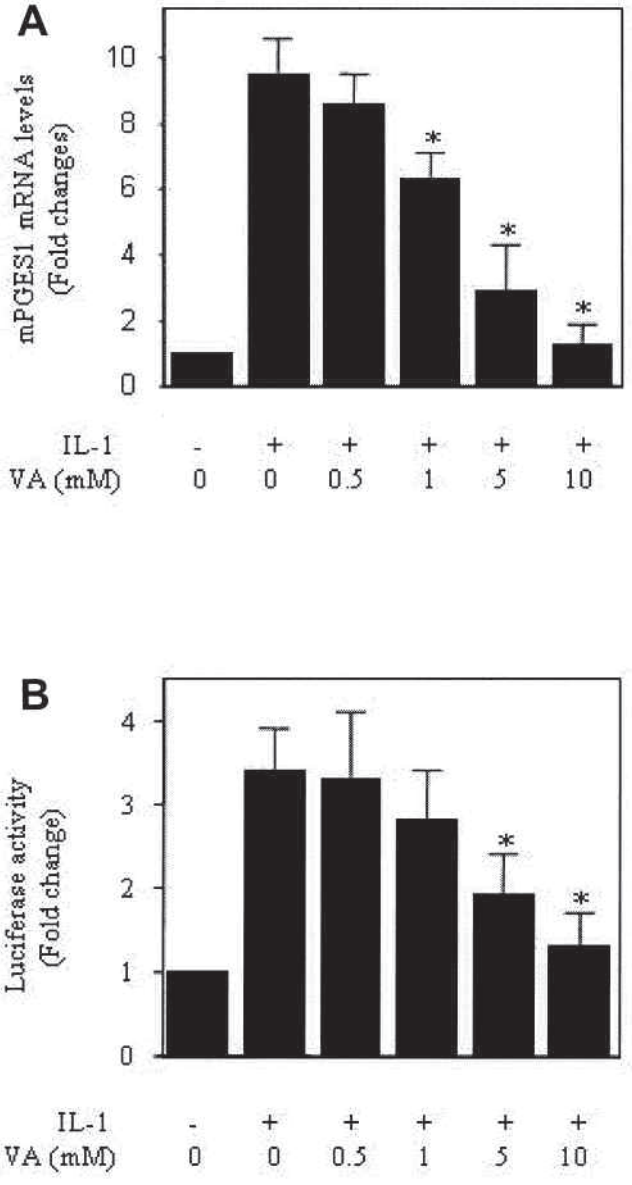

Figure 2. Valproic acid (VA) inhibited interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) expression at the transcriptional level. A. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 8 hours. Total RNA was isolated and reverse-transcribed into cDNA, and mPGES-1 levels were quantified using real-time PCR. GAPDH gene expression was used for normalization. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. B. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well) and the internal control pSV40-ß-gal (0.5 μg/well) using FuGene 6 transfection reagent. The next day, transfected cells were treated with IL-1 (100 pg/ml) in the absence or presence of increasing concentrations of VA for 18 hours. Luciferase activity values were determined and normalized to ß-galactosidase activity. Results are expressed as fold-changes, considering 1 as the value of untreated cells, and represent the mean ± SD of 4 independent experiments [*$p < 0.05$ compared with cells treated with IL-1 alone (control)].

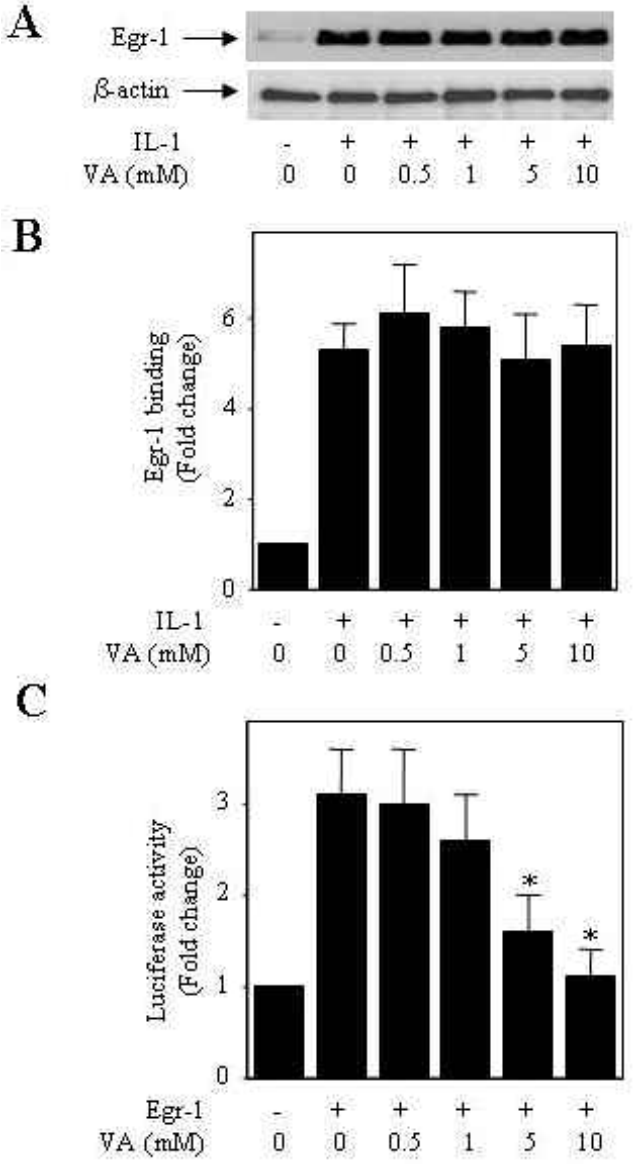

Figure 3. Valproic acid (VA) did not target Egr-1 expression and recruitment to the microsomal prostaglandin E_2 synthase-1 (mPGES-1) promoter, but prevented its transcriptional activity. A. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 2 hours. Cell lysates were prepared and analyzed for Egr-1 protein by Western blotting. In the lower panel the blots were stripped and reprobed with specific anti-ß-actin antibody. This blot is representative of similar results obtained from 4 independent experiments. B. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 1 hour. ChIP assays, coupled with real-time PCR, were performed using a specific anti-Egr-1 antibody. Results are expressed as fold-changes of Egr-1 binding to the mPGES-1 promoter relative to untreated cells and represent the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions ($p < 0.05$ compared with untreated cells). C. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well) and an expression vector for Egr-1 (100 ng/ml) together with the internal control pSV40-ß-gal (0.5 μg/well) using FuGene 6 transfection reagent. The next day, transfected cells were treated with the indicated concentrations of VA for 18 hours. Luciferase activity values were determined and normalized to ß-galactosidase activity. Results are expressed as fold-changes, considering 1 as the value of cells transfected with the reporter construct alone, and represent the mean ± SD of 4 independent experiments [*$p < 0.05$ compared with cells transfected with Egr-1 (control)].

analyzed by real-time PCR using primers amplifying the mPGES-1 promoter region (bp –142 to –37) that harbors Egr-1 binding sites. As shown in Figure 3B, treatment with IL-1 enhanced (3.2-fold) the binding of Egr-1 to the endogenous mPGES-1 promoter. However, IL-1-induced Egr-1 binding was not affected by VA. We failed to detect immunoprecipitable mPGES-1 promoter DNA with the no-antibody and non-immune serum controls (data not shown). Therefore, VA inhibited IL-1-induced mPGES-1 expression by mechanisms independent of, or in addition to, impaired expression or recruitment of Egr-1 to the mPGES-1 promoter.

Next, we investigated the effect of VA on the ability of Egr-1 to transactivate the mPGES-1 promoter. Chondrocytes were cotransfected with the mPGES-1 promoter and an expression vector for Egr-1 and then left untreated or treated with inceasing concentrations of VA. As shown in Figure 3C, overexpression of Egr-1 highly increased the mPGES-1 promoter activity. Interestingly, treatment with VA dose-dependently attenuated Egr-1-mediated activation of the mPGES-1 promoter. Together, these data suggest that VA inhibits mPGES-1 expression by interfering with the Egr-1 transcriptional activity.

VA-mediated inhibition of IL-1-induced mPGES-1 expression requires de novo protein synthesis. To determine whether the inhibitory effect of VA on IL-1-induced mPGES-1 expression requires *de novo* protein synthesis, we tested the effect of the protein synthesis inhibitor cycloheximide (CHX). Chondrocytes were pretreated with CHX for 30 minutes and stimulated with IL-1 in the absence or presence of VA for 8 hours. The levels of mPGES-1 mRNA were analyzed by real-time PCR. As shown in Figure 4, pretreatment with CHX blocked VA-mediated inhibition of IL-1-induced mPGES-1 expression, suggesting that the suppressive effect of VA was an indirect effect and was dependent on *de novo* protein synthesis.

NAB1 contributes to the suppression of IL-1-induced mPGES-1 by VA. The ability of CHX to block VA-mediated suppression of Egr-1-induced mPGES-1 expression suggests that VA induces the synthesis of one or more proteins that suppress Egr-1 activity. Possible candidates that may be involved in the suppressive effect of VA are NAB1 and NAB2. NAB1 and NAB2 negatively regulate the activity of Egr-1 and suppress the transcription of Egr-1-dependent target genes[20,21].

To determine whether NAB1 and/or NAB2 were involved in the suppressive effect of VA on IL-1-induced mPGES-1 expression, we first examined their ability to repress Egr-1-induced mPGES-1 promoter activation in chondrocytes. Cells were cotransfected with the mPGES-1 promoter and an expression vector for Egr-1 together with increasing concentrations of vectors encoding for NAB1 or NAB2. As shown in Figure 5A, overexpression of Egr-1 greatly increased the mPGES-1 promoter activity. Interestingly, cotransfection with NAB1 or NAB2

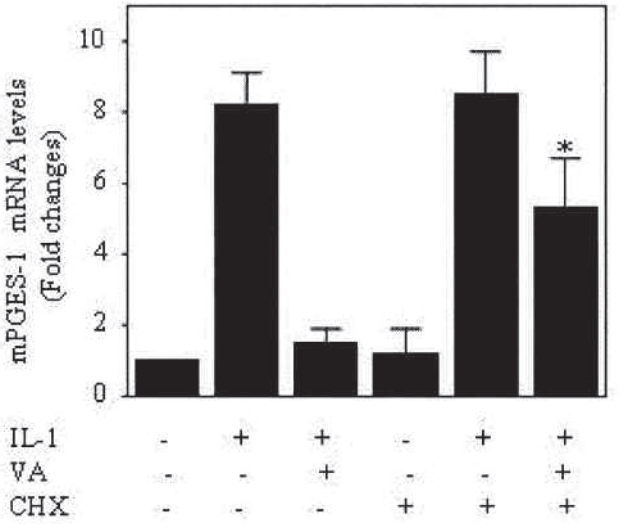

Figure 4. Downregulation of interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) expression by valproic acid (VA) requires *de novo* protein synthesis. Chondrocytes were pretreated with control vehicle dimethylsulfoxide or cycloheximide (10 μg/ml) for 30 minutes prior to stimulation with 100 pg/ml IL-1 in the absence or presence of 10 mM VA for 8 hours. Total RNA was isolated, reverse-transcribed into cDNA, and mPGES-1 mRNA was quantified using real-time PCR. The housekeeping gene GAPDH was used for normalization. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. Results are expressed as fold-changes, considering 1 as the value of untreated cells, and represent the mean ± SD of 3 independent experiments (*$p < 0.05$ compared with cells treated with IL-1 and VA).

dose-dependently reduced Egr-1-mediated activation of the mPGES-1 promoter. Similarly, overexpression of NAB1 or NAB2 dose-dependently abrogated IL-1-mediated activation of the mPGES-1 promoter (Figure 5B). These experiments demonstrated that NAB1 and NAB2 proteins inhibit both Egr-1- and IL-1-mediated activation of the mPGES-1 promoter in chondrocytes.

Next we analyzed the effect of VA on NAB1 and NAB2 expression in chondrocytes. Cells were treated with VA for different time periods, and the expression of NAB1 and NAB2 proteins was evaluated by Western blotting. As illustrated in Figure 6A, VA enhanced NAB1 expression in a time-dependent manner. NAB1 protein expression started to increase 0.5 hours post-stimulation, reached the maximum at 1 hour, and remained elevated until 12 hours. In contrast, VA had no significant effect on the expression levels of NAB2 (Figure 6A).

Transcriptional repression by NAB proteins requires their recruitment to target promoters through interaction with Egr-1[41,42]. Therefore, we examined whether VA promotes NAB1 recruitment to the endogenous mPGES-1 promoter. As shown in Figure 6B, treatment with either VA or IL-1 alone had no effect on the binding of NAB1 to the mPGES-1 promoter. However, the combined treatment of IL-1 and VA induced the recruitment of NAB1 to the mPGES-1 promoter. We did not detect obvious recruitment

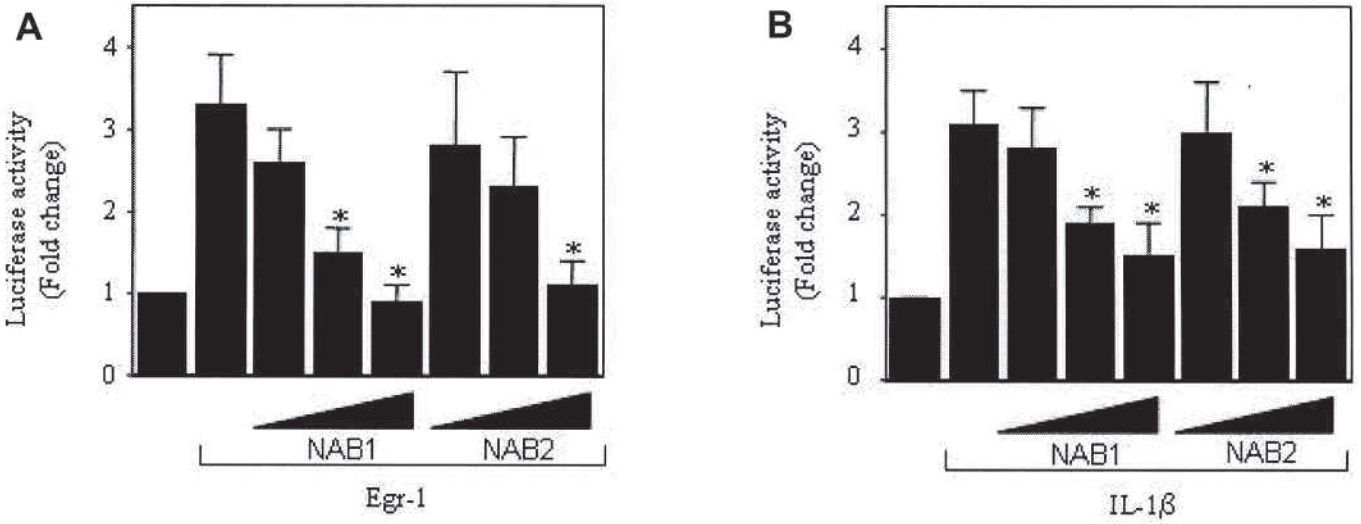

Figure 5. NAB1 and NAB2 suppress Egr-1 and interleukin 1 (IL-1)-mediated activation of the microsomal prostaglandin E_2 synthase-1 (mPGES-1) promoter. A. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well), the internal control pSV40-ß-gal (0.5 μg/well), an expression vector for Egr-1 (100 ng/ml), and increasing concentrations (0.1, 0.5, and 1 μg/ml) of expression vectors for NAB1 (A) or NAB2 (B). The total amount of transfected DNA was kept constant by addition of the empty vector. Total cellular extracts were prepared 40 hours after transfection, and luciferase activity values were determined and normalized to ß-gal activity. Results are expressed as fold-changes, considering 1 as the value of cells transfected with the reporter construct alone, and represent mean ± SD of 4 independent experiments [*$p < 0.05$ compared with cells transfected with Egr-1 alone (control)]. B. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well) and the internal control pSV40-ß-gal (0.5 μg/well) together with increasing concentrations of an expression vector for NAB1 or NAB2. The total amount of transfected DNA was kept constant by addition of the empty vector. The next day, transfected cells were treated with IL-1 (100 pg/ml) for 18 hours. Luciferase activity values were determined and normalized to ß-galactosidase activity. Results are expressed as fold-changes, considering 1 as the value of untreated cells, and represent mean ± SD of 4 independent experiments [*$p < 0.05$ compared with cells treated with IL-1 alone (control)].

of NAB2 to the mPGES-1 promoter. Thus, VA induced NAB1 recruitment to the mPGES-1 promoter in the presence of IL-1, suggesting that NAB1 may contribute to the suppressive effect of VA on mPGES-1 expression.

NAB1 silencing with small interfering RNA (siRNA) blocked VA-mediated suppression of IL-1-induced mPGES-1 expression. To confirm the involvement of NAB1 in the observed effect of VA, we evaluated the effect of NAB1 silencing by siRNA on VA-mediated suppression of IL-1-induced mPGES-1 expression. Chondrocytes were transfected with the scrambled control siRNA or siRNA for NAB1 and after 24 hours of transfection, the cells were stimulated with IL-1 in the absence or presence of VA. As shown in Figure 6C, transfection with NAB1 siRNA antagonized the suppressive effect of VA on IL-1-induced mPGES-1 expression, whereas transfection with scrambled control siRNA had no effect. NAB1 protein levels were almost completely suppressed in chondrocytes transfected with NAB1 siRNA compared to cells transfected with scrambled siRNA, confirming NAB1 gene silencing (Figure 6, lower panel).

These results support the notion that upregulation of NAB1 contributes to the suppression of IL-1-induced mPGES-1 expression by VA.

DISCUSSION

In this study, we demonstrate that treatment of human chondrocytes with VA, an HDAC inhibitor, suppressed IL-1-induced mPGES-1 expression at the transcriptional level. The inhibitory effect of VA was not associated with reduced expression of Egr-1 or its recruitment to the mPGES-1 promoter, and requires *de novo* protein synthesis. In addition, we identify NAB1 as an essential factor that mediates the suppressive effect of VA. To our knowledge, this is the first study to demonstrate that VA suppresses IL-1-induced mPGES-1 expression by upregulating the expression of NAB1.

Recently, a number of studies have demonstrated that HDAC inhibitors modulate inflammatory responses. For instance, HDAC inhibitors including VA reduced lipopolysaccharide-induced production of IL-1, TNF-α, and interferon-γ (IFN-γ) in human peripheral blood mononuclear cells[29], and production of TNF-α, IL-6, and reactive oxygen species in neuroglia cultures and primary microglia[30,31]. HDAC inhibitors have also been reported to suppress IL-12 production in dendritic cells and macrophages[32]. Further, we and others have reported that they downregulate inducible nitric oxide synthase and COX-2 expression in several cell types, including chondrocytes[33]. *In vivo*, HDAC inhibitors dose-dependently reduced the circulating levels of the proinflammatory cytokines TNF-α, IL-1, and IL-6 in an endotoxemia model[34]. In addition to their antiinflammatory properties, HDAC inhibitors exhibit chondroprotective effects. Indeed, Young, *et al*[35] showed that HDAC inhibitors blocked the induction of several enzymes responsible for cartilage degradation, includ-

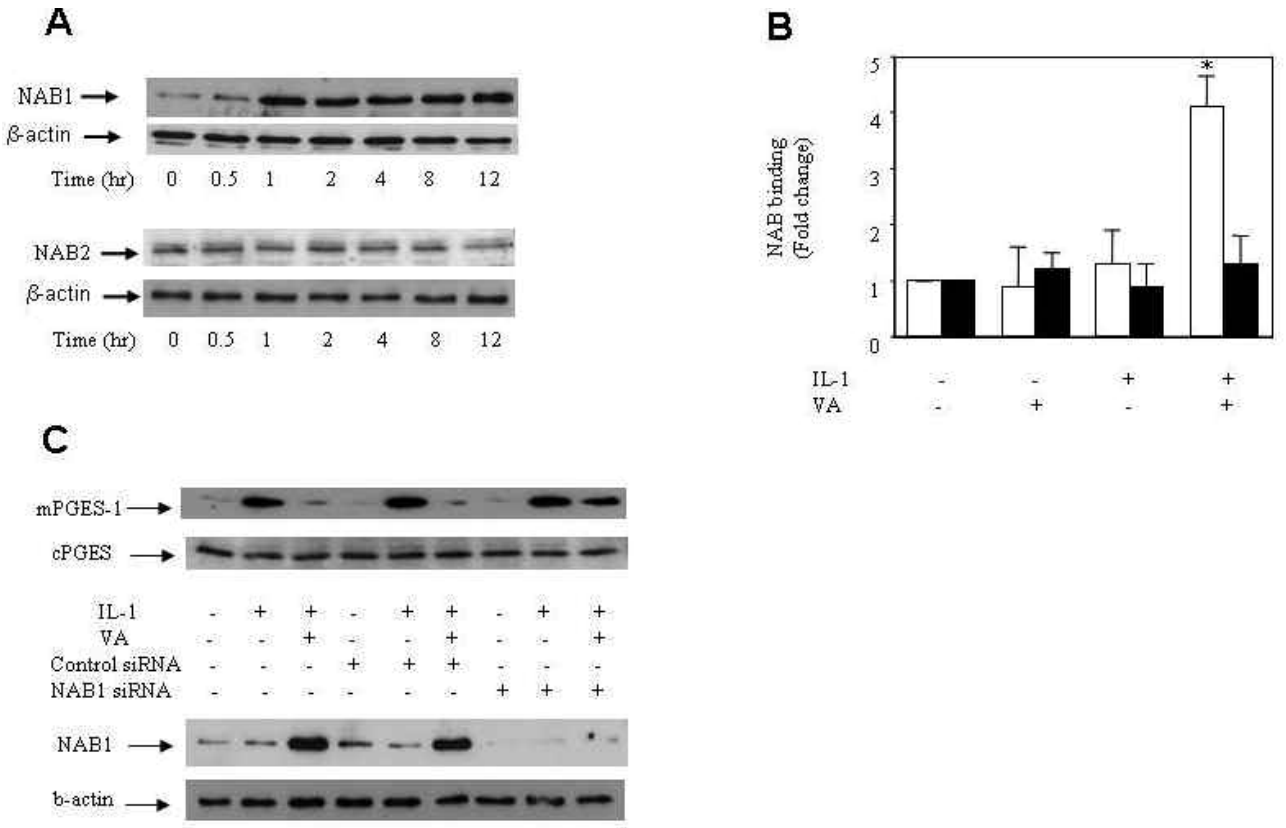

Figure 6. NAB1 contributes to the suppression of interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) by valproic acid (VA). A. VA upregulates NAB1 expression in human chondrocytes. Chondrocytes were treated with 10 mM VA for the indicated time periods. Cell lysates were prepared and analyzed for NAB1, NAB2, and ß-actin proteins by Western blotting. The blots are representative of similar results obtained from 4 independent experiments. B. VA promotes NAB1 recruitment to the microsomal prostaglandin E_2 synthase-1 (mPGES-1) promoter. Chondrocytes were treated with 10 mM VA for the indicated time periods. ChIP assays, coupled with real-time PCR, were performed using specific antibodies against NAB1 and NAB2. The results are expressed as fold-changes of NAB protein binding to the mPGES-1 promoter relative to untreated cells and represent the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions (*$p < 0.05$ compared with untreated cells). C. NAB1 is required for suppression of IL-1-induced mPGES-1 expression by VA. Chondrocytes were transfected with 200 nM of scrambled control siRNA or siRNA for NAB1. At 24 hours post-transfection, cells were washed and left untreated or treated with IL-1 in the absence or presence of VA (10 mM) for 20 hours. Cell lysates were prepared and analyzed for the expression level of mPGES-1 and cPGES (upper panel). Specific knockdown of NAB1 was confirmed by Western blotting using antibodies specific for NAB1 (lower panel). Blots are representative of similar results obtained from 4 independent experiments.

ing MMP-1, MMP-13, and a disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS)-4, -5 and -9, and prevented cartilage degradation in an explant assay[35]. Moreover, we recently showed that HDAC inhibitors downregulate IL-1-induced proteoglycan degradation in cartilage explants[33]. More recently, Grabiec, *et al*[36] reported that HDAC inhibitors suppressed the production of TNF-α and IL-6 by macrophages from patients with RA[36]. Together these data suggest that HDAC inhibitors may have protective effects in arthritis. Indeed, they inhibit joint swelling, synovial inflammation, and bone and cartilage destruction in autoantibody-mediated arthritis[43] and collagen-induced arthritis models[44,45]. We have extended these observations by showing that VA inhibits IL-1-induced mPGES-1 expression in human chondrocytes. Moreover, the induction of mPGES-1 expression by TNF and IL-17 was also inhibited by VA, suggesting that VA could repress mPGES-1 expression independently of the stimulus. The repressive effect of VA on IL-1-induced mPGES-1 expression occured at the transcriptional level, as determined by real-time RT-PCR analysis and transient transfection assays.

The transcriptional induction of mPGES-1 is primarily controlled by Egr-1 through 2 Egr-1 binding motifs located in the proximal region of the mPGES-1 promoter[18,19]. Therefore, we examined whether inhibition of Egr-1 expression and/or recruitment to the mPGES-1 promoter could be the mechanism by which VA prevents IL-1-induced mPGES-1 expression. Our results demonstrated that VA did not affect IL-1-induced Egr-1 expression and recruitment to the mPGES-1 promoter, indicating that VA acts at a step downstream of Egr-1 expression and recruitment to the mPGES-1 promoter. Indeed, VA inhibits both Egr-1 and IL-1-mediated activation of the mPGES-1 promoter.

We also demonstrated that inhibition of *de novo* protein synthesis blocked VA-mediated suppression of IL-1-induced mPGES-1 expression, suggesting that the sup-

pressive effect of VA is not direct, but rather indirect, through transcription of a target gene that suppresses mPGES-1 expression. Potential candidates that may mediate the suppressive effect of VA are the corepressors of Egr-1 activity, namely NAB1 and NAB2, known to suppress the expression of Egr-1-dependent genes[20,21]. We found that treatment with VA caused a rapid upregulation of NAB1 expression, whereas the level of NAB2 was not affected, suggesting that the upregulation of NAB1 contributes to the suppressive effect of VA on IL-1-induced mPGES-1 expression. Interestingly, NAB1 expression was reported to be upregulated by 2 antiinflammatory agents: glucocorticoids and aspirin-triggered lipoxin analog[46,47].

Further, we found using ChIP assays that NAB1 is recruited to the –142/–37 region (which contains 2 Egr-1 binding sites) of the mPGES-1 promoter when the cells are stimulated with the combination of IL-1 and VA, but not with IL-1 or VA alone. This suggests that NAB1 is recruited to the mPGES-1 promoter by Egr-1. Indeed, it has been shown that Egr-1 and NAB1 can directly interact *in vitro* and *in vivo* and that the association of Egr-1 with NAB1 is involved in the repression of several Egr-1-dependent genes[41,42]. Altogether, these results strongly suggest that upregulation of NAB1 expression and its recruitment to the mPGES-1 promoter mediates the suppressive effect of VA on IL-1-induced mPGES-1 expression. This is further supported by the fact that silencing of NAB1 by specific siRNA blocked the downregulation of IL-1-induced mPGES-1 expression by trichostatin A. This study is the first to our knowledge to show that NAB1 expression is upregulated by VA, and that NAB1 is important for the suppressive effect of VA on IL-1-induced mPGES-1 expression.

It should be noted that siRNA-mediated silencing of NAB1 did not completely block the suppressive effect of VA on IL-1-induced mPGES-1 expression, suggesting that additional NAB1-independent mechanisms also contribute to the suppressive effect of VA. Indeed, we recently showed that HDAC4 contributes to mPGES-1 expression in synovial fibroblasts, and that trichostatin A suppresses IL-1-induced mPGES-1 expression by interfering with this enzyme's activity[40]. Modulation of Egr-1 activity or expression by HDAC inhibitors is not mutually exclusive; both may operate concomitantly. Further investigation will be needed to elucidate whether VA modulates mPGES-1 expression in chondrocytes by targeting the activity of HDAC4 or other isoforms.

There are a number of potential mechanisms by which VA could increase NAB1 expression. One possibility is that VA activates NAB1 transcription through inhibition of HDAC activity and subsequent histone hyperacetylation at the NAB1 promoter. Indeed, several studies reported that VA can activate transcription by enhancing histone acetylation at target gene promoters[48,49,50]. Alternatively, VA may induce NAB1 expression through hyperacetylation of transcription factor or signaling molecules involved in NAB1 expression. Of note, HDAC inhibitors including VA have been shown to modulate the expression of a number of genes by increasing the acetylation levels of key transcription factors[51,52]. Finally, VA can upregulate NAB1 expression through NAB1 mRNA stabilization. Additional molecular and biochemical studies are needed to delineate the mechanisms by which VA modulates NAB1 expression.

Our data show that suppression of IL-1-induced mPGES-1 expression by VA was not due to altered expression or recruitment of Egr-1 to the mPGES-1 promoter, but instead, was likely due to the repression of Egr-1 transcriptional activity through upregulation of NAB1 expression.

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